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Excision of the Staphylococcal Cassette Chromosome mec (SCCmec) and its crucial role in the horizontal transfer of methicillin resistance in staphylococci

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Excision of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) and its crucial role in the horizontal transfer of methicillin resistance in staphylococci

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Prof. Tadeusz Kawecki

PREAMBLE

This thesis dissertation aims at understanding the fascinating mechanisms involved in the spread of β-lactam resistance in staphylococci, and more specifically of the spread of the gene (*mecA*), which confers en-bloc intrinsic resistance to the whole family of β-lactam drugs, and is carried by a large mobile genomic island, called Staphylococcal Cassette Chromosome *mec* (SCC*mec*).

The present thesis is constructed around 6 chapters, which are intended to be self-explanatory and contain enough information to be read independently of each other. The first chapter is an introductory review that is meant to be both pedagogic and specific enough to bring the non-expert readers as well as the expert readers to the state of the art of the topic.

The 4 following chapters (chapters 2-5) describe new researches performed in the context of this thesis. They follow a logical order of experimental questions, starting with the early events driving mobilization and transmission of the *mecA* gene and the SCC*mec* island, and ending with the presentation of new molecular tools proposed to study the phenomenon on an epidemiological level rather than in selected bacterial isolates.

The last chapter summarizes the work and sets new questions and experimental perspectives.

Altogether the 6 chapters present a coherent development of scientific questions, answers and perspectives about as yet only partially solved questions regarding both fundamental mechanisms of genome evolution and practical healthcare issues – related to infectious diseases and antibiotic resistance. I hope that they will fascinate the readers as much as they have fascinated my scientific curiosity and myself.

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RÉSUMÉ

Staphylococcus aureus est un pathogène humain majeur ayant développé des résistances contre la quasi totalité des antibiotiques disponibles, incluant la très importante famille des β-lactamines. La résistance à cette classe d'antibiotiques est conférée par la « Staphylococcal Cassette Chromosome *mec* » (SCC*mec*), qui est un élément génétique mobile capable de s'insérer dans le chromosome bactérien et capable d'être transféré horizontalement chez d'autres staphylocoques. Le mécanisme moléculaire impliqué dans ce transfert horizontal demeure largement inconnu.

L'une des premières étapes du transfert est l'excision du SCC*mec* du chromosome bactérien. Cette excision est promue par des enzymes codées par l'élément SCCmec lui-même et appelées de ce fait « Cassette Chromosome Recombinases » (Ccr). L'un des buts de ce travail de thèse a été de comprendre la régulation de l'expression des gènes codant pour les Ccr recombinases. En utilisant des outils moléculaires originaux, nous avons été en mesure de démontrer en premier lieu que les Ccr recombinases étaient exprimées de façon « bistable », c'est à dire qu'uniquement quelques pourcents de cellules dans une population exprimaient ces gènes à un temps donné. Dans un deuxième temps, nous avons également démontré que l'expression de ces gènes était régulée par des facteurs étrangers au SCC*mec*.

L'expression bistable des recombinases est un concept important. Effectivement, cela permet à la majorité des cellules d'une population de conserver l'élément SCC*mec*, alors que seulement une petite fraction le perd afin de le rendre disponible pour un transfert. Ainsi, alors que l'élément SCC*mec* continue de se propager avec la multiplication des bactéries Staphylococcus aureus résistant à la méticilline (SARM), il peut être simultanément transmis à des souches susceptibles (Staphylococcus aureus susceptible à la méticilline, SASM), entraînant l'apparition de nouveaux SARM. De façon très intéressante, le fait que cette bistabilité est contrôlée par les bactéries, et non le SCC*mec* lui-même, montre que la décision

de transférer ou non la cassette SCC*mec* appartient à la bactérie. En conséquence, il doit exister dans la nature des souches qui sont plus ou moins aptes à effectuer ce transfert.

En nous appuyant sur ces observations, nous avons montré que l'excision du SCC*mec* était effectivement régulée de façon très étroite au cours de la division cellulaire, et ne se passait que pendant un temps limité au début de la croissance. Ce résultat est compatible avec une régulation génétique commandée par la densité cellulaire, qui pourrait être dépendante de la production de signaux extracellulaires, du type que l'on rencontre dans le *quorum sensing*. Les signaux hypothétiques entraînant l'excision du SCC*mec* restent inconnus à l'heure actuelle. La connaissance de ces signaux pourrait se révéler très importante afin de développer des stratégies pour interférer avec la dissémination de la résistance au β-lactamines.

Deux sujets additionnels ont été logiquement investigués au vu de ces premiers résultats. Premièrement, si certaines souches de SARM sont plus ou moins aptes à déclencher l'excision du SCC*mec*, de même certaines souches de SASM devraient être plus ou moins aptes à acquérir cet élément. Deuxièmement, afin d'étudier ces mécanismes de transfert au niveau épidémiologique, il nous a été nécessaire de développer des outils nous permettant d'explorer le phénomène à une plus large échelle. Concernant le premier point, il a été postulé que certains SASM seraient réfractaires à l'intégration génomique d'un SCC*mec* en raison de polymorphismes particuliers à proximité du site d'insertion chromosomique (*attB*). En étudiant plus de 40 isolats de *S. aureus*, provenant de porteurs sains, nous avons confirmé ce polymorphisme dans l'environnement d'*attB*. De plus, nous avons pu montrer que ces régions polymorphiques ont évolué parallèlement à des groupes phylogénétiques bien connus. Ainsi, si des telles régions réfractaires à l'intégration de SCC*mec* existent, celles-ci devraient ségréger dans des complexes clonaux bien définis qui devraient être facilement identifiables au niveau épidémiologique.

Concernant le second point, nous avons été capables de construire un système rapporteur de l'excision du SCCmec, en utilisant un plasmide à faible copie. Ce système consistait en un promoteur fort et un gène codant pour une protéine verte fluorescente (GFP) sous le contrôle d'un promoteur fort séparés à l'aide d'un élément SCC artificiel portant trois terminateurs de transcription. Ainsi, la fluorescence ne s'exprime que si l'élément SCC est excisé du plasmide. Ce système a été testé avec succès dans plusieurs types de staphylocoques, et est actuellement évalué dans d'autres souches et conditions stimulant ou inhibant l'excision.

De manière générale, cette dissertation représente parcours scientifique à travers plusieurs aspects d'un problème de santé publique majeur en rapport avec la résistance bactérienne aux antibiotiques. Ce travail s'attaque à des problèmes fondamentaux concernant le transfert horizontal de l'élément SCC*mec*. De plus, il s'intéresse à des aspects plus généraux de cet élément génétique mobile qui pourraient se révéler très importants en terme de mouvement de gènes au sein des staphylocoques, voir d'autres bactéries gram-positives. Finalement ce travail de thèse met en place le fondamentaux requis pour des recherches futures visant à interférer avec le transfert horizontal de la résistance aux β-lactamines.

SUMMARY

Staphylococcus aureus is a major human pathogen. Moreover, *S. aureus* have developed resistance to almost all available antibiotics, including the important family of β-lactam molecules. Intrinsic resistance to β-lactams is conferred by the Staphylococcal Cassette Chromosome *mec* (SCC*mec*), which is a mobile genomic island that inserts into the staphylococcal chromosome and can be horizontally transferred into other staphylococci. However, little is known about the molecular mechanisms involved in this horizontal transfer into naïve strains.

One of the first steps in SCC*mec* horizontal transfer is its excision from the chromosome. Excision is mediated by recombinase enzymes that are encoded by SCC*mec* itself, and named accordingly Ccr recombinases – for Cassette Chromosome recombinases. One goal of this thesis was to understand the regulation these recombinase genes. By using original molecular tools we could demonstrate ***first*** that the Ccr recombinases were expressed in a "bistable" manner, i.e. in only few percentages of the bacterial cells at a given time, and ***second*** that they were regulated by determinants that were not encoded on the SCC*mec* element, but elsewhere on the staphylococcal genome.

"Bistable" expression Ccr recombinases is an important concept. It allows SCC*mec* to be excised and thus available for horizontal transfer, while ensuring that only some cells, but not the whole population, loose their valuable SCC*mec* genes. Thus, while the SCC*mec* element expands with the multiplication of the MRSA colony, it can simultaneously be transmitted into methicillin-susceptible *S. aureus* (MSSA), which convert into new MRSA. Most interestingly, the fact that bistability was regulated by the cells, rather than by SCC*mec*, indicates that it was the choice of the bacteria to trigger or not SCC*mec* transfer. As a consequence, there must be, in nature, staphylococcal strains that are more or less prone to sustain SCC*mec* transfer.

Following these seminal observations we found that excision was indeed tightly regulated during bacterial division, and occurred only during a limited period of time at the beginning of bacterial growth. This is compatible with cell-density mediated gene regulation, and may depend on the production of extracellular signal molecules that transmit appropriate orders to neighboring cells, such as in *quorum sensing*. The potential signal triggering SCC*mec* excision is as yet unknown. However, it could be critical in promoting the horizontal transfer of methicillin resistance, or for the possible development of means to interfere with it.

Two additional hypothesis were logically investigated in the view of these first results. First, if some strains of MRSA might be more prone than others to promote SCC*mec* excision, then some strains of MSSA might be more or less prone to acquire the element as well. Second, to investigate these multiple mechanisms at an epidemiological level, one would need to develop tools amenable to explore *S. aureus* strains at a larger scale. Regarding the first issue, it was postulated by others that some MSSA might be refractory to SCC*mec* integration because they had peculiar DNA polymorphisms in the vicinity of the site-specific chromosomal entry point (*attB*) of SCC*mec*. By studying >40 *S. aureus* isolates from healthy carriers, we confirmed the polymorphism of the *attB* environment. Moreover, we could show that these polymorphic regions co-evolved with well-known phylogenic clonal clusters. Therefore, if SCC*mec*-refractory *attB* environments exist, then they would segregate in well-defined *S. aureus* clonal clusters that would be easy to identify at the epidemiological level.

Regarding the second issue, we were able to construct a new excision reporter system in a low copy number *S. aureus* plasmid. The reporter system consists in a strong promoter driving a green fluorescent protein (*gfp*) gene, separated by an artificial SCC-like element carrying three transcriptional terminators. Thus, fluorescence is not expressed unless the SCC-like element is excised. The system has been successfully tested in several *aureus* and

non- *aureus* staphylococci, and is now being applied to more strains and various excision-triggering or inhibiting conditions.

Altogether the dissertation is a scientific journey through various aspects of a salient medical problem with regard to antibiotic resistance and public health threat. The research work tackles fundamental issues about the mechanisms of horizontal transfer of the SCC*mec* element. Moreover, it also addresses more general features of this mobile element, which could be of larger importance with regard to gene trafficking in staphylococci, and maybe other gram-positive bacteria. Finally, the dissertation sets the fundamentals for future work and possible new ways to interfere with the horizontal transfer of methicillin resistance.

CHAPTER 1

General Introduction

1. Bacterial genome evolution

The striking bacterial diversity known today is the result of about 3.8 billion years of evolution. Bacteria are found in almost all niches and adapted even to the most extreme environmental conditions on earth, like high temperatures or drastic pH values: this enormous heterogeneity in lifestyles arose via heritable modification of the genetic material through different mechanisms that, acting in parallel, contributed to increase genetic diversity in microbial populations.

Compared to higher organisms, bacterial genomes are small, ranging between 500 kb and 10'000 kb, simply organized and have a high gene density. This apparent simplicity, combined with the lack of sexual reproduction, thought to be the major mechanism of genetic innovation, led to a vision of bacterial chromosome as a relatively static entity. Analysis of gene orders in *Escherichia coli* and *Salmonella typhimurium* carried out in the mid-1960s by mutation on primary pathways for nutrient biosynthesis, produced genetic maps showing an impressive collinearity of genes. The interpretation of this result was that chromosomes of the two organisms had not changed significantly since the separation of their lineages and was in agreement with the concept of clonality of bacterial reproduction, with minor changes provoked by point mutations and others genetic processes [1]. This idea remained widespread for many years and culminated in the periodic selection model proposed by Levin [2], although it was known for decades that bacteria could exchange genes via plasmid-mediated conjugation or bacteriophage-mediated transduction. Only in late 1980s and early 1990s, with the acquisition of completely sequenced genomes, this concept has changed: growing number of evidences gave a totally different view of the bacterial chromosomes. It became clear that a typical prokaryotic genome could be divided into two parts: a core gene pool and a flexible gene pool. The first component, referred to as "core genome", consists of genes needed for the basic cellular functions, such as DNA replication, transcription, translation, cell

architecture etc. The second component, referred to as “accessory genome”, encodes functions that are not essential for bacterial growth, but can confer selective advantage under particular conditions (e.g. additional metabolic pathways or antibiotic resistance) [3]. The idea of bacterial chromosome as a static entity was consequently replaced by a new view accentuating its plasticity and emphasizing the importance of lateral gene transfer in the evolution of bacterial genomes.

2. Genetic alterations and variation

The evolution of bacterial chromosomes depends on different types of genetic forces that continuously shape genome content. Heritable modifications at the DNA level can lead to modification, inactivation or differential regulation of existing genes. A first and most frequent source of genetic variation is due to mutations, which are small local DNA alterations, like nucleotide substitution, deletion or insertion that can arise either spontaneously or be provoked by chemicals or genetic mechanisms. Most of such mutations produce a high rate of lethal phenotypes and changes giving a selective advantage are usually very rare. This is the reason why bacteria possess enzymatic machineries able to correct in some extent this type of DNA alterations and restore the genome integrity, thus belonging to the evolutionary genes class that modulate the frequency of genetic variation.

A second source of genetic variation is due to enzymatic systems that mediate DNA homologous recombination, a mechanism that allows repair of certain types of DNA damage, like those generated by UV light exposure. In some cases these machineries can provoke recombination of two segments of high homology and cause chromosomal rearrangements, leading to duplications, deletions or DNA shuffling. Gene duplications can bring raw material on which evolutionary processes can act without compromising the original function of genes and avoiding lethal phenotypes. Rearrangements can modify the gene order, leading, in some

rare cases, to a genetic configuration that increases the fitness of the organism or give rise to a new combination of already existing functional domains, as many of them share sequence similarity required for recombination.

A third source of genetic variation is the horizontal gene transfer (HGT), defined as intercellular movement of DNA, which, together with genetic mechanisms mentioned above, plays a major role in prokaryotic evolution. Genetic "parasites" like plasmids, insertion sequences (IS), transposons, or prophages are involved in this process [4]. These mobile elements are collectively referred to as mobile genetic elements or MGEs.

In the following we concentrate our study on *Staphylococcus* spp. and the mobilization of a peculiar MGE referred to as "Staphylococcal Cassette Chromosome *mec*" SCC*mec*. This cassette confers intrinsic resistance to virtually all β-lactam drugs and thus is of great public health concern [5].

3. The peculiar case of *Staphylococcus aureus*

3.1 *General features*

The phylogenic characteristics of the genus *Staphylococcus* have been reviewed [6, 7]. *Staphylococcus* spp. contains 36 species, or which 16 are found in humans and only a few are pathogenic in the absence of predisposing immunosuppression or implanted foreign material. The most virulent ones in human are *Staphylococcus aureus* and *Staphylococcus lugdunensis*. *S. aureus* typically produce coagulase (an enzyme capable of triggering fibrinogen polymerization in the absence of thrombin) and are often referred to as coagulase-positive staphylococci. Both *S. aureus* and *S. lugdunensis* also avidly bind to fibrinogen, and thus can clump (clumping-positive) in the presence of plasma [8]. Besides, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus saprophyticus*, which are

coagulase-negative staphylococci (or CoNS), produce substantially less severe diseases than *S. aureus*, but may infect prosthetic devices or produce urinary tract infections [7].

S. aureus colonizes the skin and mucous membranes of humans and other mammals. In humans the main site of colonization is the nasal cavity, which is permanently colonized in approximately 20% of the population and intermittently colonized by another 30% [9]. Although the colonization is usually asymptomatic, *S. aureus* can occasionally cause a large variety of diseases, ranging from relatively benign skin infections to life-threatening infections such as endocarditis or osteomyelitis [7]. Antimicrobial treatment of these infections is greatly complicated if they are caused by methicillin-resistant *S. aureus* strains (MRSA), which have acquired the SCC*mec* cassette.

3.2 *Genomic features*

To date, at least 64 strains of *S. aureus* have been fully sequenced – many of which are MRSA –, and there are many more ongoing projects [10]. The genome size is comprised between 2.7 and 3.1 Mb and approximately 78% of the genes are conserved among strains [11]. *S. aureus* populations have typically a clonal structure. Sequence-based typing methods such as multilocus sequence typing (MLST, partial sequencing of 7 housekeeping genes) showed that the great majority of *S. aureus* strains cluster within 11 lineages, referred to as clonal complexes (CC) [11]. Comparative studies of available genomes showed that strains within the same lineage display an extreme conservation of the "core genome". For example, only 285 single nucleotide polymorphisms (SNPs) differences were found between strain MW2 (MRSA from the USA) and methicillin susceptible (MSSA) strain 476 (originating from the UK) among the core genome genes [12]. DNA microarray analyses indicated that 78% of the 2,817 open reading frames (ORFs) present in the *S. aureus* COL genome were common to all thirty-six *S. aureus* strains included in the study, thus representing the core

genome [13]. The remaining 22% corresponded to strain-specific ORFs that encode functions related to pathogenesis, antibiotic resistance and secondary metabolic functions [13, 14]. Many of these non-essential genes (of the "accessory genome") are found within MGEs, indicating that HGT plays a major role in the evolution of *S. aureus*, especially regarding to virulence, antibiotic resistance and adaptation to ecological niches. Genomic comparisons with other staphylococci have shown that the backbone of the *Staphylococcus* genus genome is relatively well conserved, while the MGEs are found predominantly in *S. aureus*, suggesting again their importance for pathogenicity and antibiotic resistance [11].

3.3 *S. aureus MGEs*

3.3.1 Bacteriophages

S. aureus displays a great intraspecies diversity in MGE content, which was hypothesized to result in different virulence potentials [15]. For example, 71 prophages of the *Siphoviridae* family, the largest in *S. aureus*, have been characterized so far [16]. Many bacteriophages carry virulence factors (for example PV-luk, the Panton-Valentine leukocidin, or enterotoxin A, the food poisoning superantigen) and can integrate into the genome as prophages. At least one prophage is present in each known *S. aureus* genomes, but some strains can carry up to four [16]. Some bacteriophages can act as vectors for DNA exchange and it is believed that generalized transduction is the major way of HGT in *S. aureus*, due to the absence of genes required for competence and the infrequent transfer by conjugation. Interspecies transduction of toxin genes from *S. aureus* to other staphylococci and, strikingly, even intergenic transduction to *Listeria monocytogenes* have been observed to occur with considerable efficiency, indicating the efficacy of this process [17, 18].

3.3.2 Plasmids

Most *S. aureus* plasmids carry mainly genes involved in antibiotic or inorganic ion resistance, while only few are associated with pathogenesis, encoding different types of toxins [19-22]. They have been classified in three major families. Class I plasmids are usually smaller than 5 kb, encode one or two antibiotic resistances and have the highest copy number. They do not possess genes for conjugation, but are efficiently transferred to new hosts via generalized transduction. Plasmids of class II are similar, but are larger in size (up to 40 kb) and often carry resistance genes located in integrated transposons. Class III plasmids are the largest (up to 60 kb) and possess *tra* genes, which encode for the conjugative transfer machinery [14].

3.3.3 Transposons

Many transposons are found in *S. aureus* genome. The most studied carry antibiotic resistance genes, as exemplified by Tn*552* (encoding penicillinase) and Tn*554* (encoding erythromycin methyl transferase). Moreover, the *vanA* gene cluster, encoding resistance to vancomycin, is found on a transposon (Tn*1546*) that was acquired from *Enterococcus* spp., and its acquisition resulted in high-level vancomycin-resistant *S. aureus*, which is of great medical concern [23]. Transposons can be present at multiple sites in the genome and are frequently carried by other MGE, favoring their spread between strains [12].

3.3.4 Pathogenicity and genomic islands

S. aureus pathogenicity islands (SaPIs) are the best-characterized class of genomic islands in *S. aureus*. At least one SaPI is found in each of the sequenced genomes, with the exception of MSSA476, and their integration occurs at specific sites in the chromosome. The first identified SaPIs (SaPIs 1, 2 and 3) carried genes for toxic shock syndrome toxin-1 (TSST-1),

along with other superantigens. Analysis of 20 sequenced SaPIs indicated a conserved gene organization and a size of 14-17 kb, with few exceptions [24].

Other important genomic islands of *S. aureus* comprise υSaα and υSaβ. The first one carries a gene cluster for staphylococcal superantigen-like protein and lipoproteins, while the second (υSaβ) encodes for a cluster of serine proteases and a cluster of enterotoxins. In both islands, the gene clusters form tandem repeats, which favor recombination and deletion processes, and result in variation in gene content between different isolates [11].

4. The Staphylococcal Cassette Chromosome

Staphylococcal cassette chromosomes (SCCs) are large genomic islands inserted at the 3' end of *orfX*, a conserved gene located near the origin of replication in *S. aureus* [25]. SCCs operate like integrons in Gram-negative bacteria, and capture exogenous genes conferring specific selective advantages to the recipient bacteria [26]. For instance, SCC*mec* has integrated the *mecA* gene, which is responsible for intrinsic β-lactam resistance in staphylococci. *S. aureus* strains carrying SCC*mec* are defined as MRSA and represent a major public health problem. Therefore, SCC*mec* are currently the most studied elements of the SCC family. However, other non-*mecA* SCCs do exist and can possess other functions. The diversity of SCC elements is summarized on page 17 of this Introduction section.

5. The mechanisms of β-lactam resistance in *S. aureus*

5.1 *Peptidoglycan synthesis and inhibition*

Penicillin and methicillin are antibiotics of the β-lactams class, which target the synthesis of the cell wall peptidoglycan. Peptidoglycan is a polymer of disaccharide-peptide subunits composed of β-1-4-N-acetylglucosamines and N-acetylmuramic acids, decorated with pentapeptide (L-alanine-D-glugamine-L-lysine-D-alanine-D-alanine) stem peptides. In

addition, in *S. aureus* the diamino acid lysine of the stem peptide is decorated with a penta-glycine side-chain [27]. Peptidoglycan biosynthesis is accomplished by membrane-bound penicillin-binding proteins (PBPs), which catalyze a transglycosylation reaction that elongates the glycan chains by inter-connecting the disaccharide subunits, and a transpeptidase reaction, which cross-links the penultimate D-alanine of the stem peptide to an acceptor of the penta-glycine side chain of a neighboring stem peptide. Methicillin-susceptible *S. aureus* are equipped with a set four "normal" PBPs, named PBP1 to PBP4, which are bi-functional enzymes performing both reactions.

β-lactam drugs are steric analogs of the of the D-ala-D-ala termini of the disaccharide-peptide precursors of peptidoglycan. They act as mechanism-based inhibitors of the PBP transpeptidation reaction, which they block irreversibly. The consequent inhibition of peptidoglycan assembly results in immediate growth arrest and eventually bacterial death [28].

5.2 *The mechanisms of β-lactam resistance*

The principal mechanism of β-lactam resistance in *S. aureus* is penicillinase, a plasmid-encoded secreted enzyme that cleaves the core β-lactam ring of penicillin. Penicillinase-producing *S. aureus* emerged rapidly after the introduction of penicillin in the clinics, already in the late 1940s. Today they represent close to 90% of clinical isolates both in the hospital and the community [29].

As a response to this threat, scientists and pharmaceutical industries developed penicillinase-resistant derivatives of β-lactams, such as methicillin, since the early 1960s [30]. Methicillin can still block the normal staphylococcal PBPs, but is resistant to penicillinase-induced hydrolysis. Hence, it is active against penicillinase-producing strains. However, almost simultaneously staphylococci succeeded in circumventing inhibition by methicillin

(and by other penicillinase-resistant β-lactams as well) by acquiring en-block the SCC*mec* cassette, which carries the *mecA* gene. The *mecA* gene encodes for a new PBP, referred to as PBP2a because it migrates close to native PBP2 on SDS-gel electrophoresis, which has a very low affinity for virtually all β-lactam molecules. Therefore, PBP2a can ensure the transpeptidase activity in the presence of otherwise inhibitory concentrations of β-lactams, thus conferring intrinsic resistance to this whole class of compounds [31].

An interesting feature of PBP2a is that it is a mono-functional enzyme carrying only a transpeptidase domain [32]. Thus, it must cooperate with the transglycosylase domain of other PBPs to build the cell wall.

5.3 *The first MRSAs*

The first MRSAs were confined to Europe until the early 1980s, and isolated in hospitals. These so-called “archaic” MRSA became rapidly replaced by new successful MRSA lineages that spread in the health-care facilities worldwide. Moreover, since the early 1990s MRSA escaped the hospital milieu, and new lineages were identified in the community (community associated MRSA, CA-MRSA), infecting individuals with no apparent risk factors. Retrospective analyses of CA-MRSA showed that these strains were genotypically unrelated to endemic hospital strains (healthcare-associated MRSA, HA-MRSA), and both clinical and experimental evidence indicated they had peculiar antibiotic-resistance and virulence characteristics that differentiated them from HA-MRSA strains [33, 34].

In general the *mecA* gene is a highly conserved in clinical isolates of MRSA. However, some exceptions to this rule have recently emerged, as a *mec* gene with poor *mecA* homology was described in cattle and a few hospital isolates [35].

5.4 *The origin and evolution of mecA*

The origin of *mecA* has been first linked with *pbpD*, its homolog found in coagulase-negative *Staphylococcus sciuri*. Indeed, upregulation *pbpD* through mutations in the promoter region resulted in methicillin resistant *S. sciuri* [36, 37]. More recently, the full class A *mec* complex (see section 6 of this introduction) has been discovered in the chromosome of *Staphylococcus fleurettii*, an animal-associated bacterium [38]. Analysis of the genes surrounding *mecA* strongly suggests that the *mec* locus of this strain might have been incorporated by a SCC element to generate SCC*mec*, which subsequently disseminated in the *Staphylococcus* genus.

As mentioned above, a homologue of *mecA* has been characterized in several strains from bovine and human origin in 2011. This novel PBP, encoded by the $mecA_{LGA251}$ gene, has only 70% similarity at the DNA level compared to "classical" PBP2a, thus giving negative results in *mecA* PCR screening using conventional primers [35]. Interestingly, the $mecA_{LGA251}$ locus was also comprised within a SCC, supporting the hypothesis that SCC*mec* arose from non-*mecA* SCCs that incorporated the *mecA* gene and disseminated throughout staphylococci.

5.5 *The regulation of mecA*

In the prototypic *mec* complex, the *mecA* gene is found in combination with *mecRI* and *mecI* regulators, which are homologues of the *blaRI* and *blaI* regulators of the penicillinase gene (*bla*). The products or *mecRI* and *blaRI* are membrane receptors, and the products of *mecI* and *blaI* are gene repressors. In the presence of β-lactams the extracellular domains of the *mecRI* or *blaRI* receptors trigger autocatalytic cleavage of their intracytoplasmic portions. The liberated intracytoplasmic peptides are metalloproteases that further hydrolyze the *mecI* or *blaI* repressors, thus derepressing expression of *mecA* or *bla* [7, 39]. *mecA* is flanked by one or two copies of IS431, which are believed to promote the capture of exogenous genes [40].

Strains carrying the fully functional locus *mecR1-mecI* are defined as pre-MRSA, as

they have a relatively low oxacillin MIC. Indeed, MecI represses so strongly the transcription of *mecA* that induction of resistance is not optimal, taking more than 60 minutes in strain N315 [41]. To bypass this limitation most of the clinical isolates of MRSA have a non-functional *mecR1-mecI* regulatory system, through accumulation of mutations in the *mecI* gene or *mecA* promoter or insertion of mobile element such as IS431 or IS1272 [42-45]. Nevertheless, a constitutive expression of the *mecA* gene could have a negative effect on the bacterial fitness when no antibiotic pressure is present, thus requiring a regulatory system. In addition to *mecA*, most clinical MRSA strain carry the *blaZ* gene encoding penicillinase, a narrow spectrum β-lactamase.

As mentioned, the *bla* locus has the same genetic organization as the *mec* locus. In the absence of a functional MecI, BlaI can bind to the *mecA* promoter and modulate its expression. Several studies have demonstrated that the *blaR1-blaI* regulon has a better induction efficiency than *mecR1-mecI*, as complete expression of *mecA* could be reached within 15 minutes, instead of 60 minutes, following exposure to β-lactams [41].

Nevertheless, recent reports indicate that some clinical isolates expressing high-level methicillin resistance do carry an intact *mecR1-mecI* regulatory system, and that even overexpression of *mecI* did not significantly alter their resistance phenotype [41]. This observation strongly suggests that other elements, presumably located on the chromosome, play a role in the modulation of *mecA* expression, a hypothesis that is supported by the fact that the genetic background of the host bacterium greatly influences the expression of methicillin resistance [46]. For instance, the deletion of the chromosomal gene encoding the DNA-binding protein SA1665, resulted in the increase of oxacillin resistance levels in some MRSA [47].

6. The classification of SCC*mec*

Although SCC*mec* form a class of very heterogeneous elements in size (21-67 kb) and gene content, there are some common characteristics allowing their classification [48]. Figure 1 depicts their general structure. It can be seen all contain a *mec* complex, a *ccr* complex and "J" (for junkyard) regions that may carry variable genetic materials.

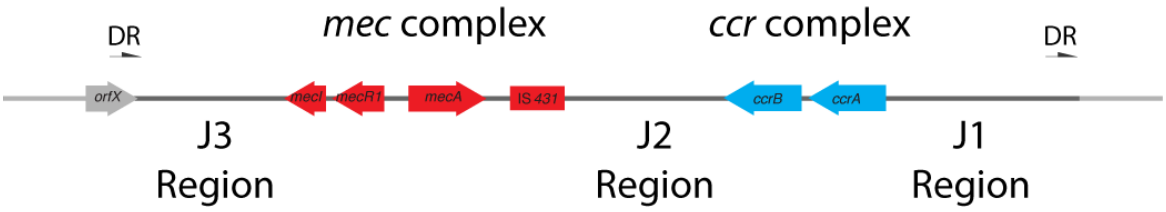
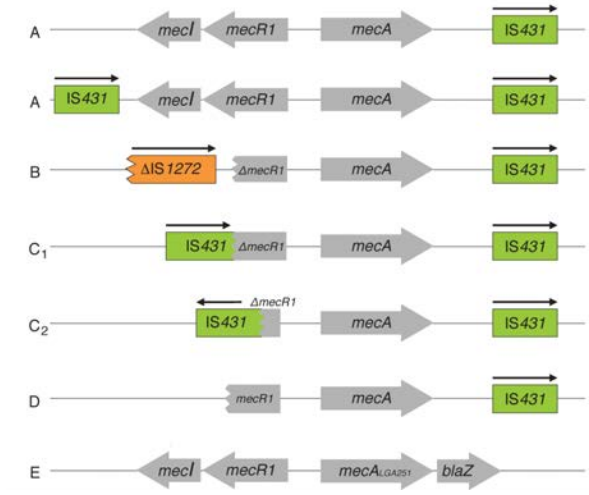


Figure 1 – Schematic structure of SCC*mec*. Common characteristics include: 1) the site-specific insertion at the end of *orfX*, 2) two 15-bp direct repeats (DR) flanking the element, 3) the *mec* complex, 4) the *ccr* complex and 5) the J1, J2 and J3 regions, which are highly variable and may contain other mobile genetic elements, like transposon or insertion sequences.

The configuration of *ccr* and *mec* complexes is the key feature of SCC*mec* classification. So far, in addition to the prototypic *mec* complex configuration (IS431-*mecA*-*mecR1*-*mecI*, discussed later in section 5.5), six different *mec* complexes have been described (Figure 2). The *ccr* complex comprises the Cassette Chromosome Recombinase genes *ccrAB* or *ccrC* discussed below (see section 8.2). Four major alleles have been identified for *ccrAB* (*ccrAB*1-4), while only one allele of *ccrC* has been found so far.

In addition to methicillin resistance, many types of SCC*mec* have been associated with resistance to other antibiotic classes, mainly by the acquisition of MGE in the joining regions, thus resulting in multidrug-resistant phenotypes (Table 1).

A.



B.

SCC*mec* type	*ccr* complex	*mec* complex	Strains
I	1 (A1B1)	B	NCTC10442, COL
II	2 (A2B2)	A	N315, Mu50, Mu3, MRSA252, JH1, JH9
III	3 (A3B3)	A	85/2082
IV	2 (A2B2)	B	CA05, MW2, 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, E-MRSA-15, JCSC6668, JCSC6670
V	5 (C1)	C2	WIS(WBG8318), TSGH17, PM1
VI	4 (A4B4)	B	HDE288
VII	5 (C1)	C1	JCSC6082
VIII	4 (A4AB)	A	C10682, BK20781
IX	1 (A1B1)	C2	JCSC6943
X	7 (A1B7)	C1	JCSC6945
XI	8 (A1B3)	E	LGA251

Figure 2 – Classification of SCC*mec*

A. Schematic organization of most common *mec* complexes. Type A is the prototypic complex with functional regulatory components *mecR1* (encoding the signal transducer protein MecR1) and *mecI* (encoding the repressor protein MecI). Types B, C1 and C2 carry truncated regulatory elements, which are disrupted by insertion sequences (IS431 and IS1272).

B. SCC*mec* types are assigned by the combination of *ccr* complexes (*ccr* alleles are indicated in parentheses) and *mec* complexes.

Table 1 – Antibiotic resistance determinants linked with SCC*mec*[1]

MGE	SCC*mec*	Gene	Resistance
pUB110	I, II, IVA	*ble*	Bleomycin
		ant4'	Tobramycin
Tn554	II, SCCHg, VIII	*ermA*	Erythromycin
		aad9/spc	Streptomycin/Spectinomycin
SCCHg		*mer*	Mercury
pT181	III	*tet*	Tetracycline
ΨTn554	III	*cad*	Cadmium
Tn4001	IV	*aacA-aphD*	Aminoglycosides

[1]Adapted from Turlej *et al.* [49].

6.1 *The molecular detection of SCCmec*

The growing diversity of SCC*mec* variants has led to the creation in 2009 of the "International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements" (IWG-SCC), which established a standardized protocol of SCC*mec* classification and assignment of novel SCC*mec* types [48]. This has helped develop rapid detection methods allowing to better trace of MRSA clones and SCC*mec* types, which is crucial for epidemiological studies during epidemic outbreaks. Several PCR-based SCC*mec* typing methods have been described so far. Most reliable results were obtained with the method developed by Kondo et al. [50], which involves six multiplex PCRs reactions (M-PCRs) targeting the key structures of SCC*mec* according to the scheme:

M-PCR 1: *mecA*, *ccr* (types 1 – 4)
M-PCR 2: *mec* class
M-PCR 3: J1 region of SCC*mec* types I and IV
M-PCR 4: J1 region of SCC*mec* types II, III and V
M-PCR 5: gene alleles located in the J2 region
M-PCR 6: gene alleles located in the J3 region

However, these methods do have some limits in specificity and sensitivity. For instance, the repertoire does not amplify *mec* class C1, resulting in the non-detection of SCC*mec* types VII and X. However, reactions M-PCR 1 and 2, detecting the SCC*mec* type are usually enough for epidemiological purposes. Besides, other SCC*mec* typing methods involve real-time PCR. The most complete method is based two reactions using molecular beacon probes and allows the detection of SCC*mec* types I, II, III, IV, V, VI and VIII, but not the subtypes [51]. The main advantage of this method is the rapidity, allowing the SCC*mec* type detection from the biological sample within 4h.

Nevertheless, since the development of this classification in 2007, new SCC*mec* types have been discovered. Typically, the recent *mecA*$_{LGA251}$variant was not identified by standard PCR techniques [35].

6.2. *Non-mec SCC elements*

Other non-*mecA* SCCs have been described indicating that these elements are not only restricted to methicillin resistance, but can possess other functions. They are summarized in Table 2.

Table 2 – Diversity of SCC

Name	Characteristics and organism	Reference
SCC*mec*	Resistance to β-lactam antibiotics (*S. aureus* and other staphylococci)	[25]
SCCHg	Resistance to mercury (*S. aureus*)	[52, 53]
SCC CI	Composite SCC carrying pbp4 (homolog to PBP4) and tagF (encoding a teichoic acid biosynthesis protein) (*S. epidermidis*)	[54]
SCC476	Resistance to fusidic acid (*S. aureus*)	[55]
SCCcap1, SCC15305cap	Gene cluster for capsule biosynthesis (*S. aureus* and *S. saprophyticus*)	[56, 57]
SCC15305RM	No apparent function involved in pathogenicity or antibiotic resistance (*S. saprophyticus*)	[56]
SCC12263	Genes homologous to restriction-modification genes (*S. hominis*)	[58]
ΨSCCh1	Genes for potassium-transporting system (*S. haemolyticus*)	[59]
ΨSCC ACME	Arginine catabolic mobile element	[60]

Note that two pseudo-SCC (ΨSCCh1 and ΨSCC ACME) lack the *ccr* genes and could not be mobilized alone. The arginine catabolic mobile element (ACME) has been associated with a selective advantage in a rabbit model of infection [60]. ACME carries the *arc* and *opp3* gene clusters, which encode the arginine deiminase pathway and an oligopeptide permease system, respectively, and have been previously associated with an increase in virulence [61, 62]. Moreover, ACME contains the terminal repeats implicated in the insertion/excision mechanisms and can be consequently mobilized in *trans* by another SCC [60]. ACME has been associated with the major CA-MRSA clone USA300. In this strain, the deletion of ACME, but not SCC*mec*, resulted in a decreased competitive fitness in a rabbit model of bacteremia, suggesting an important role of this element in the spread of this epidemic clone [60].

The importance of non-*mecA* SCC is likely to be largely underestimated, because the majority of the studies focus on MRSA, and more generally on clinical isolates of MRSA, which represent a selection bias. Moreover, recent reports confirm that the presence of non-*mecA* SCC is not rare in CoNS causing infections.

In a collection of 45 nosocomial isolates of *Staphylococcus hominis*, 11 (24%) isolates were carrying *ccr* genes while being negative for *mecA* [63]. Similarly, 3/30 (10%) blood isolates of *Staphylococcus epidermidis* obtained from neonates were positive for *ccr* genes while they did not carry *mecA* [64]. Further characterization of genes present on non-*mecA* SCC could confirm their function and a potential selective advantage.

7. The evidences for horizontal transfer of SCC*mec*

7.1 *General features*

Although the genetic determinant of methicillin resistance (i.e. *mecA*) was known since the late 1980s, it is only in 2000 that it became clearly associated with a mobile element SCC*mec*

[25]. For years it had been speculated that worldwide spread of MRSA was solely due to clonal expansion of few successful strains and that *de novo* acquisition of SCC*mec* was an extremely rare event [65]. This was supported by the fact that out of the 11 major *S. aureus* lineages collected between 1961 and 2004, MRSA were mainly confined to 5 clonal complexes (CC8, CC5, CC30, CC45 and CC22) [33, 66]. However, the presence of almost identical cassettes in unrelated strains or different staphylococcal species and the presence of different types of SCC*mec* in closely related strains have made increasingly clear that horizontal transfer of SCC*mec* was playing an important role in MRSA evolution and dissemination [67, 68]. First estimates indicated that at least 20 independent cases of horizontal transfer of SCC*mec* took place during MRSA evolution [69], but more recent analyses confirmed that this value could be underestimated by at least one order of magnitude [70].

The evidence of horizontal mobility of SCC*mec* is even more striking in localized geographic areas. In western Switzerland (CantonVaud), despite the fact that each of the predominant MRSA clones was associated with a specific SCC*mec*, atypical MRSA-SCC*mec* pairs were found in 5, 3, 18 and 8% cases, respectively [71]. Whether these new MRSA clones will be able to outcompete the endemic clones is as yet unclear. Indeed, experimental evidences suggested that *mecA* might be deleterious for some strains. The introduction of plasmid-encoded *mecA* genes in MSSA-only lineages resulted in its instability and inactivation, as a consequence of mutations in the promoter region or premature stop codons [72].

7.2 *The occurrence of SCCmec in CoNS, a reservoir for S. aureus?*

Although SCC*mec* was primarily studied in MRSA, increasing evidences indicate that its main reservoir could be represented by methicillin-resistant CoNS, which are ubiquitous

colonizers of the skin and mucosa. The occurrence of SCC*mec* in CoNS was demonstrated both in the hospital and the community [68, 73, 74]. For instance, up to 75% of hospital isolates of *S. epidermidis* were shown to be resistant to methicillin (i.e. MRSE) and were carrying highly diverse SCC*mec* types (some of which remained non-typable by the method used for MRSA), probably as a result of genome plasticity [75-77]. Likewise, other CoNS such as *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *Staphylococcus hominis* and *Staphylococcus capitis*, were also shown to carry SCC*mec* at a high frequency [74, 77, 78].

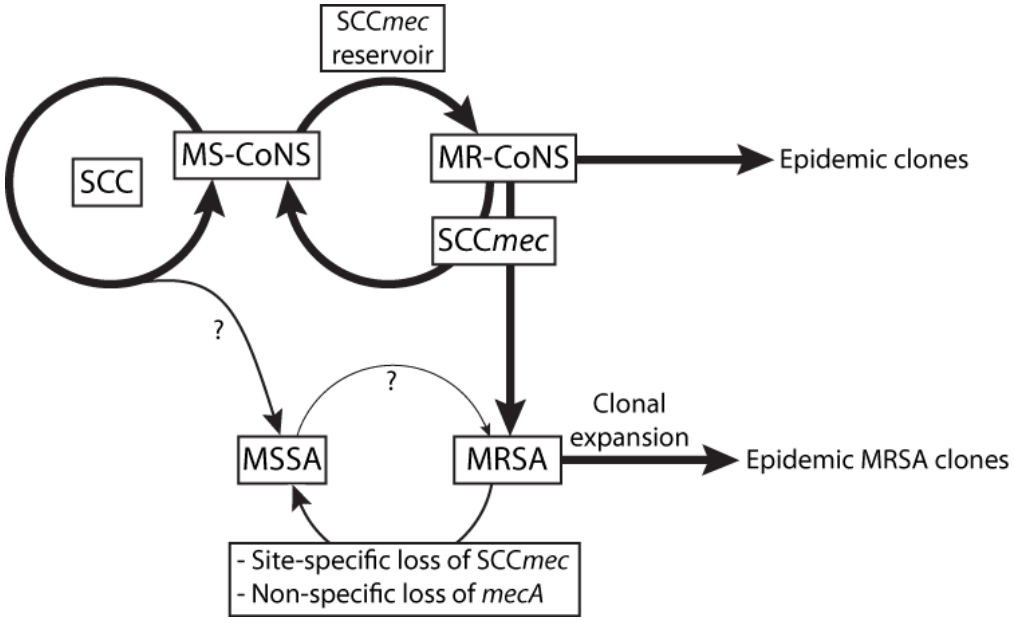


Figure 3 – Proposed dynamics between the reservoir of SCC, the generation of SCC*mec*, and the transfer of SCC*mec* to *S. aureus*.

The left side of the Figure shows that an occult reservoir of SCC is likely to exist in CoNS, as well as in *S. aureus* to some extent. The central part of the Figure shows the acquisition of the *mecA* element, which most likely occurred in the context of environmental and maybe human CoNS already containing an SCC cassette. The right part of the Figure shows the transfer of SCC*mec* from methicillin-resistant CoNS to *S. aureus*, and the clonal expansion of both types of organisms in humans, due to the selective pressure of antibiotics. Of note, there are as yet no clues regarding to the assembly line leading to the first SCC or to its environmental reservoir.

Since CoNS are so ubiquitous and often carry SCC*mec* elements, they could play an important role in the dissemination of methicillin resistance. Several reports indicate a possible transfer of SCC*mec* from MRSE to MSSA in a co-colonized host, resulting in *de novo* creation of MRSA [79, 80]. Another indirect clue is suggested by the fact that type IV SCC*mec* were highly prevalent in *S. epidermidis* isolates in the 1970s, whereas the first MRSA isolates carrying type IV SCC*mec* were reported only in the early 1980s [68]. Figure 3 proposes a global model for the trafficking of SCC*mec* between various staphylococci and the environment.

Please note that while it is postulated that CoNS might indeed represent one SCC*mec* reservoir, we do not have clues as to the existence of other potential reservoirs, nor to the assembly line that result in the eventual SCC and *mecA* coalescence.

8. The mechanisms of SCC*mec* excision and spread

8.1 *General features*

To be transferred from one donor strain to a new recipient, a process that will result in a new methicillin-resistant *Staphylococcus* sp., SCC*mec* must first be excised from the chromosome of the DNA donor. Site-specific excision is catalyzed by recombinases (described below) that are encoded on the cassette. Excision does occur spontaneously and has been studied in some details [25, 81]. However, there are as yet no clues as to which are the conditions and the exact mechanisms that drive the decision of excision, neither to the ways through which the excised SCC*mec* are transferred into recipient strains.

Because SCC*mec* and *S. aureus* do not possess conjugation or transfer genes, it is presumed that SCC*mec* is shuffled between bacteria via general transduction. Likewise, because the donor staphylococci loose their copy of SCC*mec*, and thus also the associated beneficial genes, it is probable that excision and transfer operate in an economics benefitting

the whole cell population rather than individual cells, in a way analogous to the recently described "bistable" expression of excision genes in the genomic island *clc* [82], and illustrated in Figure 4. Below we describe the mechanisms of recombinase-mediate excision of SCC*mec* and their postulated implication transfer.

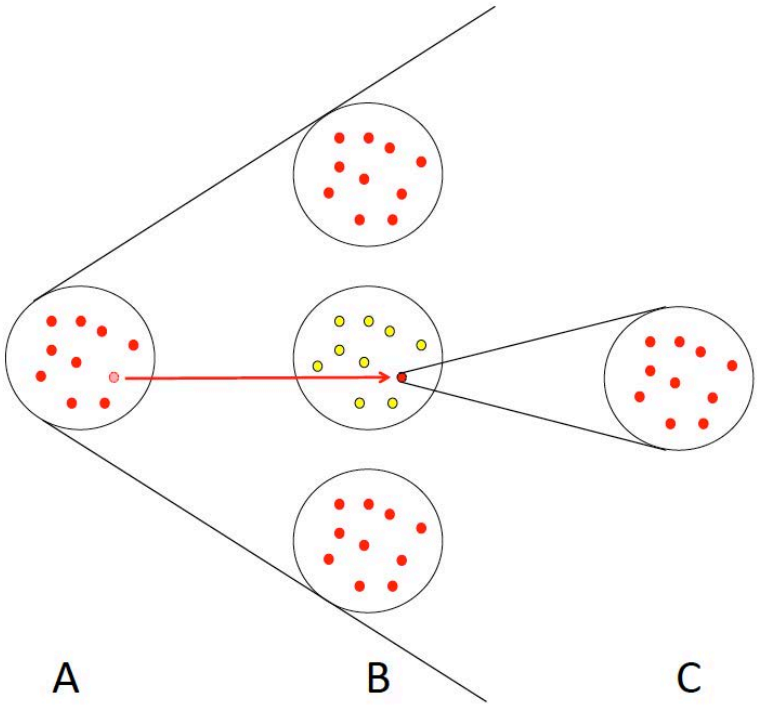


Figure 4 – Schematic representation of SCC*mec* transfer via the population-benefiting economics of "bistability".

(A) Within a population of MRSA (red dots), only a very small proportion of individuals (pink dot; accounting for 10^{-6} to 10^{-5}) express their recombinases genes and commit excision of SCC*mec*, whereas the rest of the population keeps the element. (B) During growth in the presence of methicillin or other β-lactams, the population of MRSA expands (red dots) while the excisant (pink dot) is killed. However, its excised SCC*mec* cassette could be transferred (most likely by generalized transduction) into a recipient from a neighboring population of MSSA (yellow dots). (C) Upon continuous growth in the presence of methicillin, the original MRSA keep growing, the MSSA population is killed, but the MSSA-recipient of SCC*mec* can grow and generate a new clone of MRSA. Thus, the disappearance of the few excisants of (A) barely affected the survival of their congener colony, whereas their "sacrifice" gave rise to entirely new MRSA population.

8.2 *The Ccr recombinases*

Site-specific recombination promotes mobilization of genetic materials at well-defined regions in the chromosome. Insertion and excision reactions are catalyzed by specific enzymes, named recombinases, which exchange DNA strands between the chromosome and the exogenous DNA. Recombinases are often encoded on the MGEs themselves. Although they quite diverse, recombinases fall in two major classes: tyrosine recombinases (also referred as integrases) and serine recombinases (known as resolvases/invertases).

This classification is based on the amino acid, at the enzyme's active site, which forms a transient covalent bond with DNA molecules during the integration/excision processes [83]. Among the serine recombinases, a subfamily, called the large serine recombinases, comprises the Cassette Chromosome Recombinases (Ccr), which catalyze the site-specific integration/excision of SCC*mec*. Large serine recombinases share conserved structures with other serine recombinases at their N-terminal domain, but have a large C-terminal extension of unknown function. Such extensions are also present in phage-related integrases of the serine recombinase family [84]. Three phylogenetically distinct *ccr* genes have been described so far: the *ccrA* and *ccrB* genes (*ccrAB*), being part of the same transcription unit, and the *ccrC* gene, which is encoded by a separate unit. The three *ccr* genes share less than 50% of sequence similarity [48]. Several allotypes (sequence similarity below 85%) have been described for *ccrA* and *ccrB*, while only one allotype was found for the *ccrC* gene [48]. Specific lineages of these various genes are presented in Figure 5.

8.3 *The mechanism of Ccr-mediated insertion and excision*

Site-specific integration into the chromosome occurs at the 5' end of *orfX*, a highly conserved gene, located near the origin of replication in *S. aureus*. It involves a core chromosome sequence (*attB*), which recombines with the cognate core sequence located on the SCC*mec*

(*attS*). Upon integration, recombination results in two direct repeats (renamed *attR* and *attL*) flanking the element. Accessory sequences located near the *attS* sites, which form imperfect inverted repeats of various sizes, seem also to play a role in the site-specific integration/excision process in many SCC*mec* [60, 85].

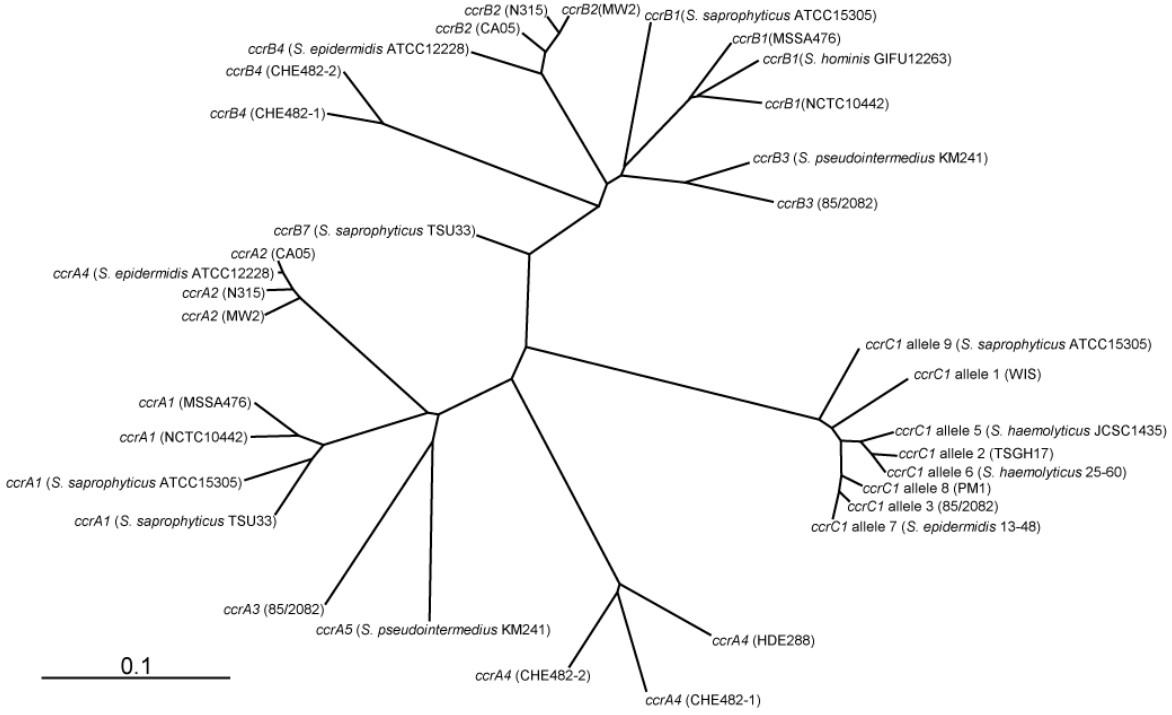


Figure 5 – Phylogenetic tree of *ccr* genes.
Nucleotide sequences of 14 *ccrA*, 14 *ccrB* and 9 *ccrC* were aligned by the ClustalW2 algorithm (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using the neighbor-joining method using. The tree was visualized with the TreeView software (http://taxonomy.zoology.gla.ac.uk/ ROD/treeview.html). The origin of the *ccr* genes is indicated in parenthesis (strain name for *S. aureus* and complete name for non-*S. aureus* strains). The branch lengths indicate the distances, expressed as the number of substitutions per 100 bases.

CcrAB proteins seem not to show any cassette-specificity, as all the allotypes are able to excise SCC*mec* of different types [86]. This is not the case for CcrC recombinases, which apparently show specificity for SCC*mec* carrying *ccrC* [85].

Spontaneous SCC*mec* excision can be detected using primers amplification of the newly formed chromosomal junction following excision [25, 72, 87-89]. This process is likely to be Ccr-dependent, as sequencing of the PCR product gives the predicted sequence lacking the SCC*mec*. Moreover, the frequency of excisants can be dramatically increased by introducing a multicopy-plasmid carrying *ccrAB* in the host MRSA [25, 81].

Most of the studies on SCC*mec* excision/integration were performed in strains carrying *ccrAB*. In this system, electrophoretic mobility shift assays showed that CcrB alone, which contains a C-terminal DNA-binding motif, directly interacted with the target DNA sequence and promoted SCC*mec* excision. However, CcrB alone tended to excise at alternative sites, and required CcrA for appropriate site-specific excision. Therefore, it was proposed that CcrA, which lacked the DNA-binding domain, interacted with the excision/integration complex to position CcrB for site-specific excision [81].

In vitro studies have shown that CcrB requires a minimal binding sequence CGTATCATAAGTAA, in which the central cytosine plays an essential role [90]. This sequence was found to be more extensive for optimal *in vivo* integration of SCC*mec*, requiring more than 100 bp at both ends of the core sequence [90].

8.4 *The regulation and conditions promoting excision*

Little is known about the regulation and signals triggering the decision of excision, which is a key step in horizontal transfer. Likewise, little is known about the timing of excision during bacterial growth, the amount of bacteria attempting excision versus the ones that succeeds, as well as the fate of excisant cells versus non-excisants in various conditions.

Recent work studying *ccrAB*-promoter expression in whole bacterial cultures – using β-galactosidase and quantitative RT-PCR assays – indicated that *ccrAB*-promoter activity and expression increased by 2-3 time in response to stressed like β-lactam or vancomycin

treatment [91]. Other studies sought to determine the proportion of excisants in MRSA cultures by amplifying the unoccupied *attB* sites, which represent potential excisants that have lost their SCC*mec* cassette and converted to MSSA [92]. They found that SCC*mec* excision occurs at low frequency, less than 1 event in 10^4 cells. However, these results do not provide information on the factors that trigger expression of *ccrA* or the timing of excision in a culture. Yet, this information is critical information in order to understand the dynamics of SCC*mec* transfer *in vitro* and in nature and maybe interfere with it.

It was the purpose of the research chapters of this thesis to tackle these questions (especially chapters 2, 3, and 5). Very briefly, they indicate that (i) *ccrAB* expression does indeed obey a bistable phenotype (chapter 1), (ii) *ccrAB* expression is commanded by determinants that are located outside of the SCC*mec* element in the bacterial genome, and thus is strain-dependent rather than SCC*mec*-dependent (chapter 2), (iii) excision occurs early during growth, maybe in a cell-concentration dependent manner (chapter 3 and 5). Additional studies will help understand the dynamics of SCC*mec* excision in more natural circumstances, as in *in vivo* models.

9. Non-integral excision of SCC*mec* leading to the loss of *mecA*

Site-specific excision of SCC*mec* is required for the en-block transfer of the SCC*mec* cassette from one cell to another. This assumes that the SCC*mec* element is beneficial for the bacterium. However, it is possible that in certain circumstances the *mecA* gene, or other elements of the cassette, become deleterious for the bacterium and thus must be deleted. Indeed, several studies have reported partial loss of SCC*mec*, involving the region containing the *mecA* gene [93-96]. The loss of *mecA* might represent a selective advantage for the host in the absence of β-lactam treatment, especially in challenging conditions for the pathogen, like the competition for an ecological niche or treatment with other chemotherapeutic agents. This

is the reason why this phenomenon is mainly observed for clinical strains, in opposition with the SCC*mec* integrity in laboratory strains.

Non-integral excision of SCC*mec* is of concern for molecular diagnosis of MRSA infections, because several commercially available kits are based on the detection of the *orfX*-SCC*mec* junction by quantitative real-time PCR [97, 98]. The result is the detection diagnosis of false-positive MRSA and falsely restricts the therapeutic choice especially for multidrug resistant isolates. Little is known about the molecular mechanisms involved in this process, with the exception of the specific case of MRSA ST398 carrying a type V (5C2&5) SCC*mec*. In this case, homologous recombination can occur between the two *ccrC* genes, which are located on both sides of *mecA*, thus resulting in a non-*mecA* SCC. Again, this has been primarily observed in the clinical setting [99]. The only case reported in the laboratory was during exposure to vancomycin, which resulted in the deletion of *mecA* accompanied with additional rearrangements of other parts of the chromosome [100]. Other studies suggest that IS elements might play a role in SCC*mec* rearrangements, since in two independent cases the deleted parts of SCC*mec* were terminating at the very same nucleotide after IS431 of the *mec* complex [94, 96].

Conversely, some molecular mechanisms also tend to stabilize the SCC*mec* cassette in the chromosome, for instance by inactivating the *ccrAB* genes or preventing excision through modification of sequences recognized by the recombinases [86]. Thus, while the advantages provided by SCC*mec* are intuitive in case of β-lactam treatment, the cassette or some of its gene may become undesirable in other circumstances, which may drive either loss or appropriation of the element by the bacterium through modification, or so-called “amelioration”, of it.

10. About the research chapters

The following research chapters concentrate on the initial events occurring during horizontal transfer of SCC*mec*, i.e. its excision from the chromosome of the SCC*mec*-donor strain. While research by others has focused on the mechanisms of action of CcrAB recombinases [81, 90] there have been virtually no studies examining the circumstances that trigger the decision of excision, and the mechanisms that regulate it. In this research we concentrated on some of these aspects, and constructed several molecular tools to study them.

In Chapter 2 we combined a green fluorescence (GFP) reporter system to epifluorescence microscopy and flow cytometry to determine what populations of cells were activating the promoter of the *ccrAB* recombinases genes, and to what extent. Indeed, while previous work have investigated activity of the *ccrAB* (in fact *ccrA*) promoter using different techniques [91], they did that only at the level of whole cell populations. However, also informative, studies on whole population do not provide information on single-cell behavior, which is known to be very heterogeneous.

The results of Chapter 2 clearly disclosed the implication of single-cells in the decision of SCC*mec* excision, and show that the decision obeys a bistable dynamics. Most importantly, the observations revealed that the determinants driving bistability and *ccrAB* promoter activation were not located on the SCC*mec* element itself, but elsewhere on the staphylococcal genome – at least in the strains studied herein. This has potentially fundamental implications regarding to the strain-dependency of SCC*mec* horizontal transfer. Indeed, staphylococcal strains might also be heterogeneous regarding to their proneness either to accept an heterologous SCC*mec* element, or to transmit it further. Understanding such specificities could help better follow the epidemiology and spread of methicillin resistance.

Chapter 3 aimed to follow the dynamics of actual SCC*mec* excision, by quantifying the proportion of chromosomal scar left by excised SCC*mec* as compared to chromosomes

with an inserted SCC*mec* in whole cell cultures. Measurements were done in time-course experiments. The results showed that *de novo* excision did take place in bacterial cultures, but occurred only during a restricted period of time, early during growth. This was reminiscent of cell density-driven gene regulation, such as in *quorum sensing*. Ongoing work with additional reporter tools (see Chapter 5 below) should help further clarify this issue.

Chapter 4 addressed the issue of the DNA sequence polymorphism around the chromosomal attachment (*attB*) site of SCC*mec*, and the chance for a MSSA strain to be a good SCC*mec* acceptor. Indeed, recent work has suggested that polymorphism downstream (at the 3') of *attB* could impede SCC*mec* integration, and that this might correlate with the quasi-absence of methicillin resistance in certain *S. aureus* CCs [101]. Our present results confirmed the polymorphism of the 3' *attB* environment in different strains. Moreover, we also observed a very close correlation between the polymorphism of the 3' *attB* environment and the MLST clustering of our strain collection [102]. This shows that while 3' *attB* polymorphism does exist, it is conserved among different clones and closely follows the standard phylogenic pattern, although this genetic region is not taken into account by MLST. Nevertheless, this observation does not conclude that this polymorphism is responsible for the apparent exclusion of certain *S. aureus* CCs from being MRSA. Indeed, other types of barriers to SCC*mec* acquisition could be operative, such as restriction systems, or fitness cost of the SCC*mec* cassette, which could select against it. Recent results suggest that this could be the case [90].

To cap these first studies, Chapter 5 describes the construction of an additional molecular tool, which purpose is to facilitate the exploration of the excision dynamics in multiple staphylococcal backgrounds. Indeed, Chapters 2 and 3 showed that excision was variable between strains, and Chapter 4 indicated that DNA polymorphism of the 3' *attB* environment was clone-specific. In Chapter 5 we constructed a fluorescence-based reporter

system that could capture the actual excision event by packing the important chromosomal or SCC*mec* sequences (including *attL* and *attR*), as well as a series of transcription terminators, between a strong promoter an a *gfp* gene. Thus, expression of GFP is blocked by the artificial construct, and restored in case of its excision. The plasmid in which the construct was tested is quite ubiquitous for staphylococci, and allows testing multiple strains. Moreover, important DNA sequences around *attL* and *attS* can be incorporated, thus allowing testing their implication in excision, and ultimately identifying the implication of regulatory proteins.

Altogether the four research chapters should lead the reader through a logical continuum, and provide openings on new questions and new perspectives to understand the fundamentals of SCC*mec* horizontal transfer.

CHAPTER 2

Expression of Staphylococcal Cassette Chromosome *mec* Recombinases in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*

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- 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco (USA), 12-15.09.2009
  Poster presentation: “Cassette Chromosome Recombinase expression in MRSA”.

- 68th Assembly of the Swiss Society for Microbiology, Lausanne (Switzerland), 04-05.06.2009
  Poster presentation: “Ccr recombinase expression in methicillin resistant *Staphylococcus aureus”*

1. Abstract

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 catalyzed by the Ccr recombinases, which are encoded on the cassette. Here we determined the *ccr* promoter activity of individual cells in methicillin-resistant *Staphylococcus aureus* (N315, COL, MW2) and *Staphylococcus epidermidis* (RP62A) carrying different SCC*mec* types and exposed to various stresses. One mutant cured of its SCC*mec* (N315EX) was also used. Methods included *ccr* promoter-*gfp* fusions, epifluorescence imaging and flow cytometry. Overall, *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 ca. 3% to 1% between logarithmic to stationary growth phases; $p < 0.05$). Thus, SCC*mec* excision operates through bistability, favoring a small fraction of cells to "sacrifice" their genomic islands for transfer, while the rest of the population remains intact. 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. This shows that the determinants responsible for the activity of *ccr* promoter were not located on SCC*mec*, but elsewhere on the genome. Thus, staphylococcal chromosome plays a key role in determining SCC*mec* stability and transferability.

2. 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 [28]. The *mecA* gene is carried by a genomic island named staphylococcal cassette chromosome *mec* (SCC*mec*), which is responsible both for vertical and horizontal transfer of methicillin resistance. It was originally believed that worldwide spread of MRSA was solely due to clonal expansion of a few successful strains, implying that *de novo* acquisition of SCC*mec* was extremely rare [65]. However, more recent studies indicate that lateral spread of SCC*mec* is more frequent than expected [13, 69]. 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 presence of different types of SCC*mec* in closely related strains [67, 70, 76].

To be transferred SCC*mec* must first be excised from the chromosome of the donor strain. Site-specific excision of SCC*mec* is catalyzed by the Ccr recombinases, which are large serine recombinases of the resolvase/invertase family and are encoded on the SCC*mec* cassette [25]. 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% of sequence similarity with *ccrAB*. Several allotypes (sequence similarity below 85%) have been described for *ccrA* and *ccrB* and are used for classification or their cognate SCC*mec* cassettes. In contrast, only one allotype was found for the *ccrC* gene [48].

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 chromosomal core sequence (*attB*), which recombines with the cognate core sequence located on the SCC*mec* (*attS*) resulting, upon integration, in two direct repeats (*attR* and *attL*)

flanking the element [25]. 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 excision in many SCC*mec* [60, 85]. Interestingly, CcrAB recombinases do not display cassette-specificity, as all the allotypes are able to excise SCC*mec* of different types [86]. Conversely, CcrC recombinases are specific to their own SCC*mec* [85].

Spontaneous SCC*mec* excision has been observed on several occasions both *in vivo* and *in vitro* [80, 86, 87, 103]. For instance, overexpression of plasmid-located *ccrAB* genes generally leads to SCC*mec* excision and results in conversion of MRSA to MSSA [25]. 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 [91]. This could favor the propagation of SCC*mec* via a two-step scenario. First, beta-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, scarifying themselves to transfer SCC*mec* into new recipient strains.

Here we tested this hypothesis by using promoter-*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 *S. epidermidis* strain RP62A, as more and more evidences suggest that coagulase-negative staphylococci (CoNS) are the reservoir of SCC*mec* for *S. aureus* [68, 79, 80].

3. Materials and methods

3.1 *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) 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) 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 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.

3.2 *DNA manipulations*

For *S. aureus* and *S. epidermidis*, genomic DNA was extracted using a protocol adapted from Bae et al. [104]. Briefly, 3 ml of an overnight culture were harvested and resuspended in 50 µl of TE supplemented with lysostaphin (final concentration 0.5 µg/ml). After 30 minutes of incubation at 37°C, 300 µl of “Nuclei lysis solution” (Promega Corporation, Madison WI, USA) were 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 minutes on ice. After centrifugation (4°C, 13’000 rpm), supernatants were collected and 300 µl of isopropanol were 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 EB (Promega AG).

Table 1 – Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Reference
Strains		
E. coli DH5α	Host for DNA cloning	Laboratory collection
S. aureus		
RN4220	Restriction-deficient derivative of RN450	[105]
N315	HA-MRSA carrying type II SCC*mec*	[106]
N315EX	Isogenic MSSA derivative of N315	This study
COL	MRSA carrying type I SCC*mec*	[107]
MW2	MRSA carrying type VI SCC*mec*	[107]
S. epidermidis RP62A	Methicillin-resistant *S. epidermidis* carrying type II SCC*mec*	[106]
Plasmids		
pCN36	*E. coli-S. aureus* shuttle vector; Tc^R; ± 22 copies/cell	[108]
pCN68	*E. coli-S. aureus* shuttle vector; source of *gfpmut2* gene	[108]
pSR3-1	Thermosensitive-replicon plasmid carrying the *ccrAB* genes of strain N315 (used for SCC*mec* excision in N315)	[25]
pPGFP-N315	P_{ccrAB}N315-*gfpmut2* fusion cloned in pCN36; *tet(M)*	This study
pPGFP-MW2	P_{ccrAB}MW2-*gfpmut2* fusion cloned in pCN36; *ermC*	This study
pPGFP-COL	P_{ccrAB}COL-*gfpmut2* fusion cloned in pCN36; *ermC*	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 *ermC*; 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 *ermC*; used as positive control	This study

Plasmids were isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). 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 "QIAquick Gel Extraction Kit" (Qiagen Inc.) according to manufacturer's protocols. Ligations were performed using 1 µl of T4 ligase (Promega Corp.) according to the manufacturer's specifications.

3.3 *Polymerase chain reaction (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 manufacturers' specifications. The primers used in this study are listed in Table 2.

3.4 *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 by 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 random 146-bp DNA fragment, and a positive control plasmid (pGFPS10) was constructed by replacing the same region with the constitutive promoter of ribosomal protein S10.

Table 2 – Primers used in the study

Primer	Sequence	Description
Prom fw	TTTTTTGGATCCTTGTCTTTATCATACAACTGTG *BamHI*	Amplification of the promoter fragment in strains N315, MW2 and RP62A
Prom rev	TTTTTTACTAGTATCGGCTCCTCCTTTCACAGT *SpeI*	
PromCOL fw	TTTTTTGGATCCTAACTTAAAGATGAAATCGTACAGG *BamHI*	Amplification of the promoter fragment in strain COL
PromCOL rev	TTTTTTACTAGTCGTATTTCCTCCTTCCAAAGT *SpeI*	
neg fw	TTTTTTGGATCCAAACGCTATGTCATCAAACAC *BamHI*	Amplification of the fragment used as a negative control
neg rev	TTTTTTACTAGTTTTCTATAGAAAATGGCGACCTTAC *SpeI*	
PS10 fw	TTTTTTGCATGCCATTCACCACCGTTCTTATGAC *SphI*	Amplification of the promoter region of the *rpsJ* gene (30S ribosomal protein S10 – SA2048 in N315)
PS10 rev	TTTTTTCTGCAGTCCCTCCTTATTCGTCTACATTT *PstI*	
GFP fw	TTTTTTACTAGTATGAGTAAAGGAGAAGAACTT *SpeI*	Amplification of the *gfpmut2* gene from pCN68
GFP rev	TTTTTTGGATCCTATTTGTATAGTTCATCCATG *BamHI*	

3.5 *Microscopy*

Epifluorescence microscopy was performed using an Axioskop2 epifluorescence microscope (Zeiss, Germany), with a 100X objective (Plan-NEOFLUAR 100x/1.30 oil, Zeiss). Bacteria from 3 ml aliquots from cultures in the exponential growth phase as described (ref ICAAC) were harvested by centrifugation and resuspended in 50 µl phosphate saline buffer, of which 10 µl were deposed on glass slides and analyzed.

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

3.6 *Flow cytometry*

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

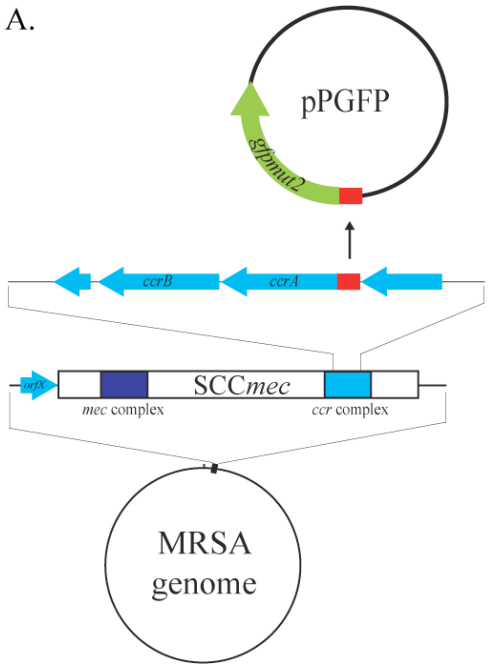
3.7 *Artificial SCCmec excision*

SCC*mec* was cured from *S. aureus* N315 by using a method from Katayama et al. [25]. Briefly, strain N315 was electroporated with the thermosensitive plasmid pSR3-1 (containing *ccrAB* genes) and transformants were cultured for 24 hours at 30°C in TSB supplemented with tetracycline, serially diluted and plated on TSA supplemented with tetracycline. Single colonies were picked, grown for 24 hours at 42°C TSB to 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 primers pair Excision fw and rev (Table 2). This isolate was named N315EX and used in further experiments.

4. Results

4.1 *ccrAB promoters and plasmid reporter construction*

The expression of *ccrAB* genes was analyzed using *gfp* translational fusions as depicted in Figure 1A. 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 analyzed for all the strains examined in this study (Table 3). Figure 1B 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 4, were well conserved, with a sequence similarity of 97% (Figure 1B).



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
        ********************************* ********************


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 red box represents the intergenic region containing the cloned *ccrAB* promoter regions.

B. Alignments of the intergenic regions containing the *ccrAB* promoter regions of the strains analyzed in the study. The start codon of the *ccrA* gene is highlighted in red.

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

In contrast, the promoter region of strain COL was shorter (i.e. 190 bp), missing the 5' part, and showing a sequence similarity of 77% when compared to N315 (Figure 1C).

The promoter regions were fused with the *gfpmut2* gene and cloned in the pCN36 vector, giving the reporter plasmids pPGFP (Figure 1A), which were introduced in the corresponding strain. Moreover, 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.

4.2 *Microscopy analysis*

The activity of the *ccrAB* promoters was first evaluated by epifluorescence microscopy during exponential phase of growth (i.e. samples taken after 3h of inoculation). Figure 2 exemplifies such results with strains N315 and RP62A. It can be seen that both strains expressed GFP uniformly form the positive control vector pGFPS10, while cells carrying the negative control pNEG were devoid of fluorescence. In sharp contrast, only a small proportion of the two cell populations showed GFP expression when they carried their specific pPGFP-N315 or pPGFP-RP62A.

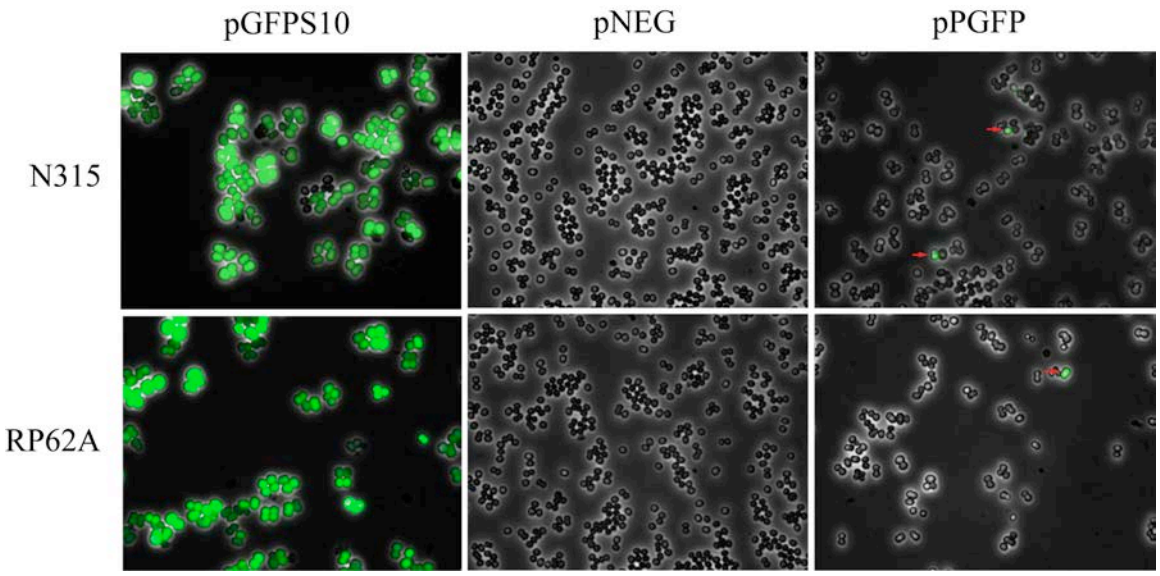

Figure 2 – GFP expression

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

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, which must have been located elsewhere in the genome. 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 rather qualitative and may be dependent on the observer, the experiments described above were repeated using FACS analysis as described below.

4.3 *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 with epifluorescence microscopy, only a minor proportion of the cell population expressed GFP. In order to determine the proportion of GFP-positive, we delineated a threshold using the GFP-negative control, as shown in Figure 3.

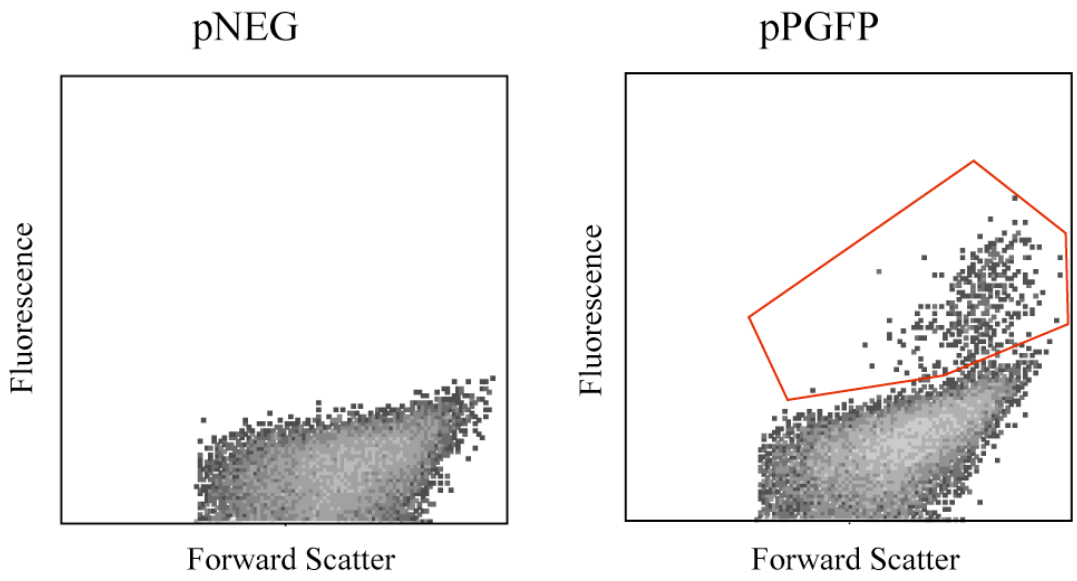

Figure 3 – Quantification of N315 cells expressing GFP from the *ccrAB* promoter
Fluorescence expression profiles of cells carrying pNEG (A) and pPGFP (B). Gated cells show the subpopulation expressing GFP. For each measurement, gates were arbitrarily determined with respect to the negative control.

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 in all test conditions, whereas COL (which had truncated promoter region of 190 bp versus 236 bp in the other strains) and MW2 (which had a N315-like promoter region) remained essentially below the limit of detection (Figure 1).

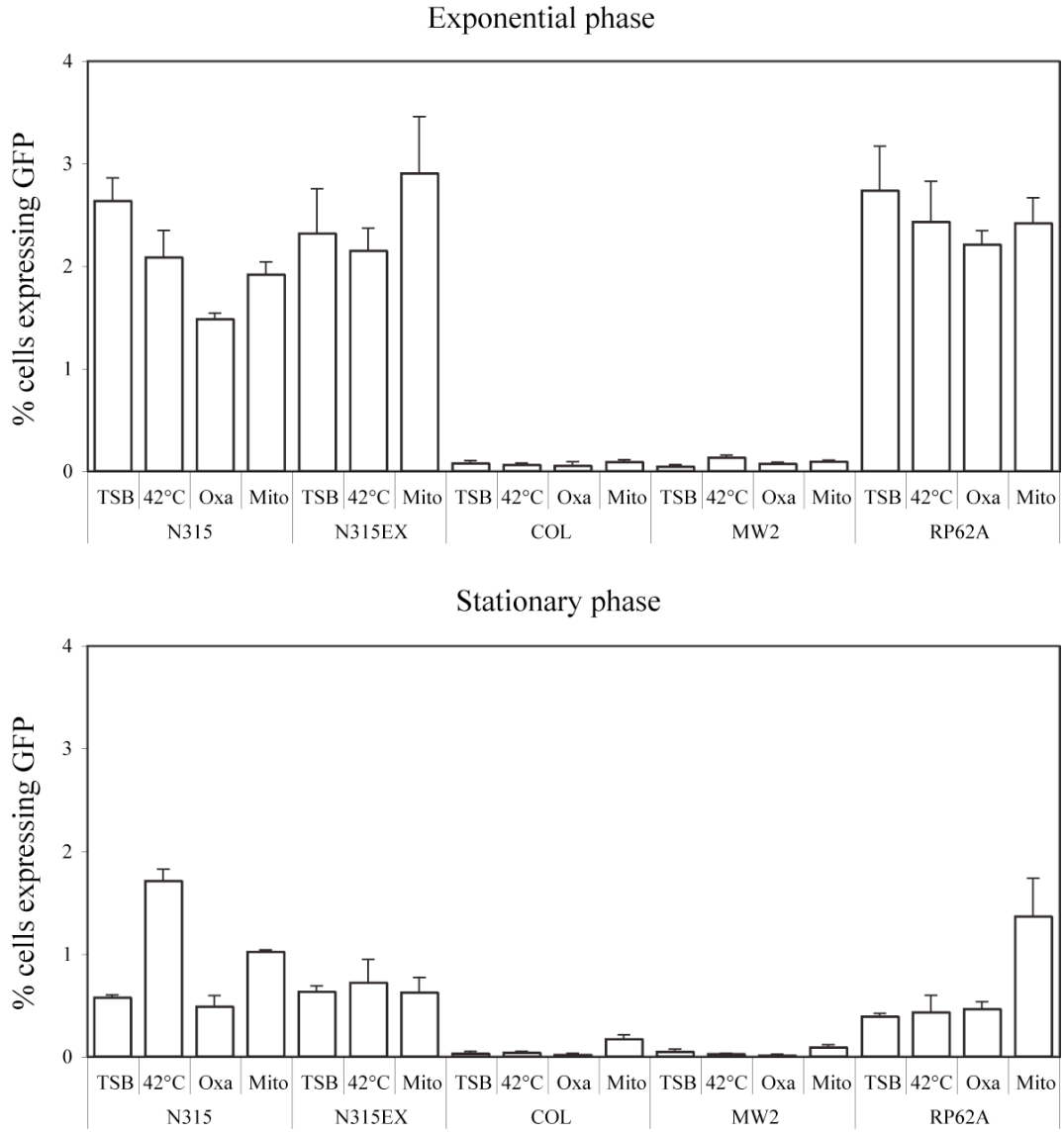

Figure 4 – Flow cytometry analysis of the effects of various stresses on *ccrAB* promoter activity.

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, oxacillin 4 mg/L (Oxa) or mitomycin C 0.5 mg/L (Mito)). Samples were removed from the cultures after 3h (exponential phase) or 24h (stationary phase) of growth, diluted in phosphate saline buffer in order not to exceed 800 FACS events per seconds, and fluorescence of 20,000 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 deviation.

In the exponential growth phase strains N315, N315EX and RP62A exhibited subpopulations expressing GFP at rates comprised between 2 and 3%, which is compatible with epifluorescence microscopy (Figure 2). Similar results were obtained in 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 in most conditions, with some exception for N315 at 42°C and RP62A with mitomycin C.

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 truncated 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 completely restored promoter activity. Thus, promoter activity was highly dependent on the bacterial background.

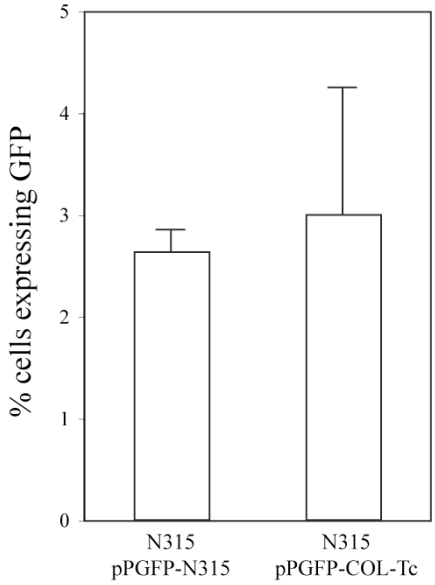

Figure 5 - FACS analysis of N315 transformed with either pPGFP-N315 or pPGFP-COL-Tc

The experimental protocol was as is Figure 4. The bars represent the averages of three independent measurements in three different cultures and error bars represent the standard deviation.

5. Discussion

The present experiments employed of a GFP expression system cloned into a low copy number plasmid in order to test the promoter activity of type I, II and IV *ccrAB* allotypes in different staphylococcal backgrounds. The results yielded several findings that my help better understand the excision and transmission 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 a 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” [110, 111] and was described in the transfer of other genomic islands, including the ICE*clc* in *Pseudomonas knackmussi* [82]. 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 24h and 96h of incubation. This suggested a dependency on cell concentration, and may be 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 II *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 truncated type I *ccrAB* promoter region of MRSA COL, which was totally inactive in its COL parent strain, regained full N315-like activity when transformed into MRSA N315. 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 truncated promoter region of the COL *ccrAB* was functional in N315 restricted critical parts of this region to the 5' end of ≤190 bp.

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 transacting determinants were located outside of the SCC*mec* cassette, the rate of bistability could vary as a function of growth phase. For instance, it decreased by >5 times (from ca. ≥2.5% to <0.5%; $p < 0.0001$, unpaired *t*-test) between the logarithmic and stationary growth phases in *S. aureus* N315, and CoNS 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.

The fact that frequency of bistability decreased in late growth was coherent 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 [89]. These results were obtained by determining the proportion of chromosomes from which the SCC*mec* cassette had been excised as compared to total bacterial chromosomes at different time points 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 RT-qPCR) after exposure to beta-lactams and vancomycin [91]. 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 ORF, whereas Higgins et al. [91] added the 21 first bp of the *ccrA* gene plus 20 bp of the preceding ORF 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. [91] 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. all along growth. Thus, one cannot exclude that *ccrAB* activity was punctually increased at the time of stress exposure, as in Higgins *et al.* study [91], but returned to baseline afterwards. Moreover, the present study determined the heterologous ON or OFF activity of the *ccrAB* promoter whereas Higgins et al. [91] measured the sum of all these individual activities. Although both studies used low copy number plasmids (with a range of ca.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 FACS 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 less 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* form one cell to another.

CHAPTER 3

Excision of Staphylococcal Cassette Chromosome *mec* (SCC*mec*) in methicillin-resistant *Staphylococcus aureus* (MRSA) assessed by quantitative PCR

Presented at:

- 50th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston (USA), 12-15.09.2010
  Poster presentation: "The dynamics of SCC*mec* excision in methicillin-resistant *Staphylococcus aureus* (MRSA)".

- 69th Assembly of the Swiss Society for Microbiology, Zurich (Switzerland), 24-25.06.2010Poster presentation: "The dynamics of SCC*mec* excision in methicillin-resistant *Staphylococcus aureus* (MRSA)"

- 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco (USA), 09-12.09.2012
  Oral presentation: "Dynamics of excision of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) in Methicillin-resistant *Staphylococcus aureus* (MRSA)"

1. Abstract

In *Staphylococcus* spp. methicillin-resistance is conferred by the *mecA* gene, which is carried by the genomic island Staphylococcal Cassette Chromosome *mec* (SCC*mec*). SCC*mec* comes in at least 11 chimeric forms. Epidemiological evidence indicates that its intra- and interspecies transfer among coagulase-negative and coagulase-positive staphylococci accounts for the spread of resistance. Transfer of SCC*mec* requires first its excision from the host chromosome, but the conditions and frequency of such events are not known. Here we sought to quantify the rate of SCC*mec* excision in two MRSA (N315 and Mu50) at various growth times (3h, 24h and 72h) in broth cultures. The effect of various stress conditions on excision (including growth at 42°C, exposure to oxacillin, vancomycin and mitomycin C) was assessed. The proportion of intact MRSA with chromosome-integrated SCC*mec* and newly formed excisants without SCC*mec* was evaluated by qPCR amplification of the chromosome-chromosome junctions and the SCC*mec* circular form resulting from excision, respectively. qPCR quantification was performed using a newly developed qPlasmid containing all amplification targets. SCC*mec* excision occurred at a rate of 10^{-5}-10^{-6} in both organisms, and most likely occurred transiently at an early stage of growth. The excision was barely affected by external stresses, except for mitomycin C, which increased early excision by tenfold in N315. The molecular tools presented herein will help better delineate the exact conditions of SCC*mec* excision and transfer in various staphylococcal backgrounds, and may provide new clues for how to avoid, and perhaps interfere with such transfer.

2. Introduction

Staphylococcus aureus is a successful human pathogen, causing a large variety of diseases that range from minor skin infections to life-threatening bloodstream infections and endocarditis [7]. Many of the virulence factors encoded by *S. aureus* are located within mobile genetic elements, which come in addition to the well conserved core genome, and account for the majority of genetic variation between the isolates [11, 12, 20]. This is also often the case for antibiotic resistance genes, which can be found on plasmids, transposons or genomic islands [20]. The ability of *S. aureus* to acquire such elements results in multiresistant strains and leads to failure of antimicrobial therapy. An example is the emergence of methicillin-resistant *S. aureus* (MRSA) during the 1960s, due to the acquisition of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). SCC*mec* is a staphylococcal genomic island carrying the *mecA* gene, which encodes for the low affinity penicillin-binding protein 2a (PBP2a) that is responsible for cross resistance to virtually all antibiotics of the β-lactam family [28, 33].

In nature, SCC*mec* come in a variety of forms that vary greatly in gene content and sizes. Eleven major types of SCC*mec* have been described so far [112]. These major types are defined according to the combination of two genetic complexes, i.e. the *mec* complex that comprises *mecA* and its upstream regulatory genes *mecR1* and *mecI* (intact or truncated), and the *ccr* complex (for Cassette Chromosome Recombinase) comprising the *ccrAB* genes or the *ccrC* gene alone.

The Ccr proteins are large serine recombinases that presumably play an important role in the horizontal mobility of SCC*mec*. They catalyze site-specific recombination at a unique location of the chromosome, between a 15-bp chromosomal sequence at the 5’ end of the *orfX* gene (*attB*), and a 15-bp homologous sequence on SCC*mec* (*attS*). Upon integration the *attS* and the *attB* sequences form two direct repeats (designated *attL* and *attR*, respectively)

flanking the cassette [25]. Recombination is catalyzed by the CcrB and CcrA enzymes. Interestingly, the association of CcrA and CcrB is critical for site-specific recombination, whereas CcrB alone is capable of excising SCC*mec* at alternative *att* sites. Thus, sequences flanking *attB* might be critical for targeting the CcrA-CcrB complex to its specific integration/excision site [81].

It had been speculated for years that worldwide spread of MRSA was solely due to clonal expansion of few successful parental strains, and that *de novo* acquisition of SCC*mec* was a quite rare event [65]. Early evaluations suggested that at least 20 independent cases of horizontal transfer of SCC*mec* took place during MRSA evolution, but more recent analyses confirmed that this value could be an underestimation of at least one order of magnitude [67, 70, 76]. This is exemplified by anecdotal cases of SCC*mec* transmission from skin or mucosal methicillin-resistant *Staphylococcus epidermidis* (MRSE) to methicillin-susceptible *S. aureus* (MSSA) of the same patient, thus resulting in a new MRSA [113]. Moreover, the presence of almost identical cassettes in unrelated *S. aureus* strains, as well as in different staphylococcal species, and the simultaneous presence of different types of SCC*mec* in closely related strains indicate that horizontal transfer of SCC*mec* is more common than originally thought [73, 114, 115]. This underscores the likely importance of SCC*mec* horizontal transfer for MRSA evolution and dissemination. Nevertheless, although it is now generally agreed that horizontal transfer of SCC*mec* might be a critical determinant of MRSA epidemiology, the mechanism of SCC*mec* transfer remains poorly understood.

The experiments presented herein describe a new qPCR system to quantify the rate of excision of the SCC*mec* cassette from the *S. aureus* chromosome and detect its resulting excised closed circular forms. The system was used to determine the dynamics of SCC*mec* excision in MRSA N315 and vancomycin-intermediate MRSA Mu50 grown either in rich medium or exposed to various stress conditions.

3. Materials and methods

3.1 *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) 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) in a rotating incubator (at 180 rpm) at 37°C. If required, tetracycline and kanamycin (AppliChem) were added at a final concentration of 10 mg/L. Oxacillin and vancomycin were commercially purchased. Oxacillin was used at 4 mg/L, i.e. its MIC for strain N315. This value was well below the MIC for strain Mu50 (512 mg/L, [105]). Vancomycin was added at 4 mg/L (MIC for strain Mu50). Mitomycin C (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added at 0.5 mg/L, corresponding to the MIC for both strains.

3.2 *DNA manipulations*

For *S. aureus*, genomic DNA was extracted using a protocol adapted from Bae et al. [109]. Cells were harvested by centrifugation and resuspended in 50 µl of TE supplemented with lysostaphin (final concentration 0.5 µg/ml). After 30 minutes of incubation at 37°C, 300 µl of "Nuclei lysis solution" (Promega AG, Dübendorf, Switzerland) were 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 AG) was followed by incubation for 5 minutes on ice. After centrifugation (4°C, 13'000 rpm), supernatants were collected and 300 µl of isopropanol were 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 EB (Promega AG). For *E. coli*, plasmids were isolated using the

QIAprep Spin Miniprep Kit (QIAGEN Inc., Hilden, Germany).

Table 1 – Bacterial strains and plasmids

Strain or plasmid	Relevant Characteristics	Reference
Strains		
E. coli DH5α	Host for DNA cloning	Laboratory collection
S. aureus		
RN4220	Restriction-deficient derivative of RN450	[104]
N315	Hospital acquired MRSA carrying type II SCC*mec*	[105]
N315EX	Isogenic MSSA derivative of N315	This study
Mu50	Hospital acquired vancomycin-intermediate MRSA carrying type II SCC*mec*	[105]
Plasmids		
pUC28	ColE1 replicon, high copy number vector for cloning, Amp^R	[116]
pSR3-1	Thermosensitive-replicon plasmid carrying the *ccrAB* genes of strain N315 (used for SCC*mec* excision in N315); Tc^R	[25]
qPlasmid	Plasmid used for qPCR analysis	This study

Digestions with restriction enzymes (Promega AG) 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 "QIAquick Gel Extraction Kit" (Qiagen Inc.) according to manufacturer's protocols. Ligations were performed using 1 µl of T4 ligase (Promega AG) according to the manufacturer's specifications.

3.3 *PCR and quantitative PCR (qPCR)*

GoTaq DNA polymerase (Promega AG) 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. End-point PCR, performed with GoTaq polymerase (Promega AG), for detection of circular SCC*mec* was carried out using primers qCirc fw and rev (Table 3) with the following run protocol: initial denaturation step at 95°C for 2 min then 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 20 sec.

Table 2 – qPCR reaction mix

Component	Final volume	Final concentration
KAPA SYBR® FAST qPCR Mix 2X	10.0 µl	1X
Forward primer (10 µM)	Variable	Variable
Reverse primer (10 µM)	Variable	Variable
Template DNA	5.0 µl	2 ng/µl
H_2O	Up to 20 µl	

Table 3 – Primers used in the study

Primer	Sequence	Position[1]
Screening primers		
Excision fw	GTTTAGGCCCATACACCAAGATAGA	33768 - 33792
Excision rev	TGTCCACCATTTAACACCCTCC	87194 - 87173
Cloning primers		
orfXcloning fw	TTTTTTGCATGCAAATCACCATTTTAGCTGTAGGG *SphI*	33696 - 33718
orfXcloning rev	TTTTTTTCTAGAATTGAATGAACGTGGATTTAATG *XbaI*	87215 - 87193
circCloning fw	TTTTTTGAATTCTTGTGGAAGGTTTGAAGACTACG *EcoRI*	86883 - 86905
circCloning rev	TTTTTTGCATGCGAAAGACTGCGGAGGCTAACTAT *SphI*	34316 - 34294
qPCR primers		
qControl fw	CGTTTAGGCCCATACACCAAGATAGAC	33767 - 33793
qControl rev	TGATACATTCAAAATCCCTTTATGAAGCG	34215 - 34187
qEx fw	CGCAGTAACTACGCACTATCATTCAGC	34049 - 34075
qEx rev	TGAATGAACGTGGATTTAATGTCCACC	87213 - 87187
qCirc fw	GGTTTAATTAATAGAGGAGTGGAGCCTTTGA	87037 - 87067
qCirc rev	CTTCTTAAAAACATAACAGCAATTCACATAAACC	34259 - 34226

[1]Position in the chromosome of *S. aureus* N315 (Accession number BA000018)

qPCR reactions were performed on DNA extracted from three independent cultures using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems). Cultures were initiated by inoculating 1ml of overnight culture (approximately 10^9 CFU) in 100 ml of broth. Reactions were carried out in MicroAmp optical tubes (Applied Biosystems, Foster City CA, USA) using an ABI7000 machine (Applied Biosystems). The composition of qPCR reactions is showed in Table 2. The following run protocol was used: initial denaturation step at 95°C for 2 min then 40 cycles of 95°C for 10 sec and 60°C for 40 sec. The primers used in this study are listed in Table 3.

3.4 *Designing a system to quantify site-specific SCCmec excision and SCCmec circular form*

The experimental system is depicted in Figure 1. qPCR was used to measure the amounts of excised chromosomes and SCC*mec* circular forms by specifically amplifying the unoccupied chromosomal insertion site remaining after SCC*mec* excision, or the junction formed upon SCC*mec* circularization. These were compared to the total chromosome numbers, which were determined by targeting a chromosomal location near to the SCC*mec* insertion site.

In order to standardize the assay we constructed qPlasmid (Figure 2), which is a vector carrying the three targets of the qPCR reactions, and used it to optimize the qPCR reactions efficiencies. It was constructed in pUC28 by cloning a 555-bp fragment containing the chromosomal junction formed upon excision, amplified from chromosomal DNA of *S. aureus* N315EX, using restrictions sites *SphI* and *XbaI*. A second fragment of 411 bp, containing the *attS* site from the SCC*mec* circular form, was amplified from chromosomal DNA of *S. aureus* N315 and cloned using *EcoRI* and *SphI* restriction sites (Figure 2).

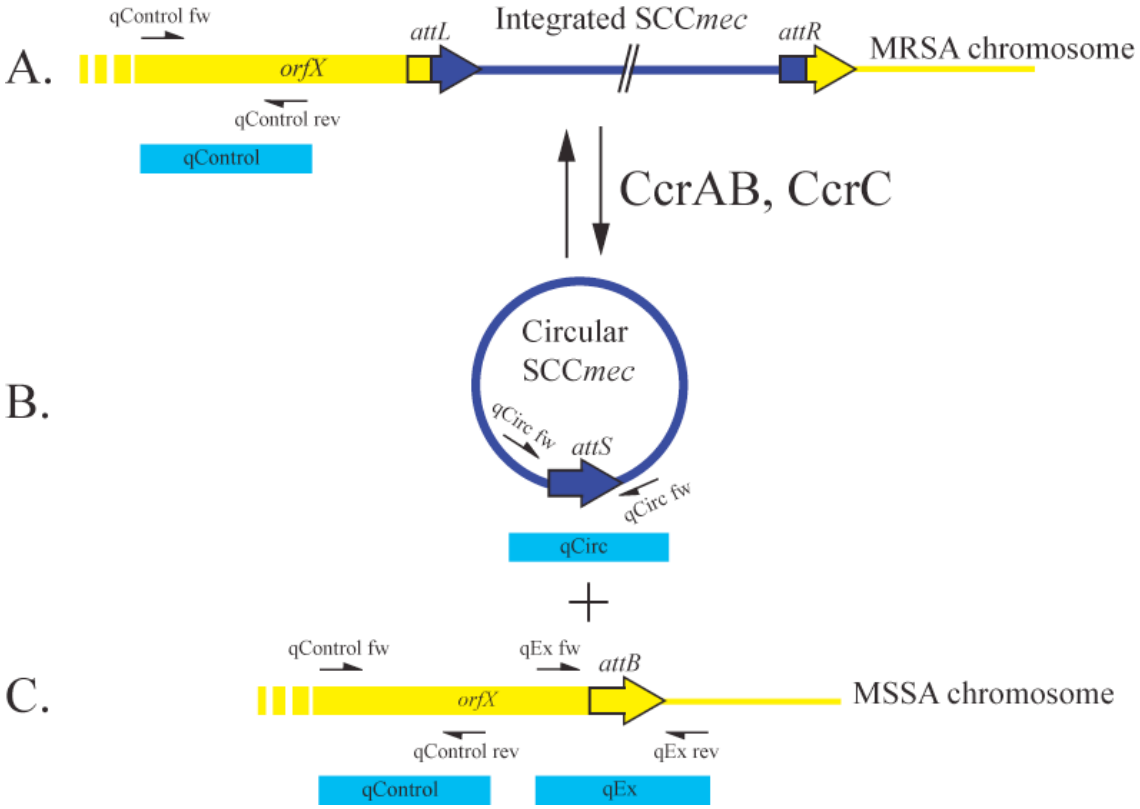

Figure 1 – Schematic representation SCC*mec* excision and the formation of its circular form, including the linear SCC*mec* integrated in the chromosome (A), the circularized SCC*mec* excised from the chromosome (B) and the religated chromosome after excision (C). Chromosomal DNA is shown in yellow and SCC*mec* DNA is shown in dark blue. Locations of primers used for qPCR are shown by arrows and their amplicons are highlighted in light blue. qControl amplicon is used to determine the number of chromosomes in the assay, while qEx and qCirc specifically detect excisants and circularized SCC*mec*, respectively.

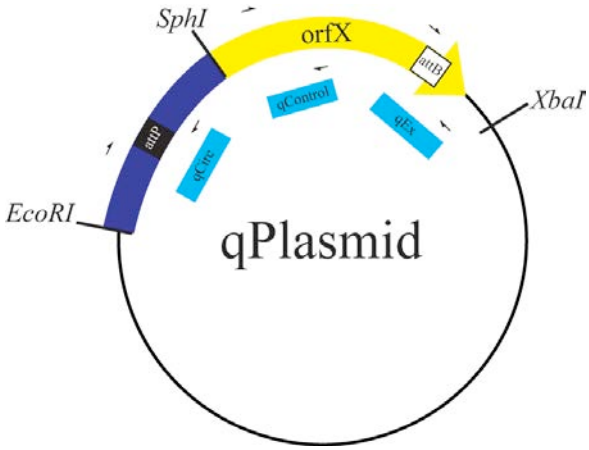

Figure 2 – Schematic representation of qPlasmid, used for primers optimization and absolute quantification. Details are as in the legend of Figure 1.

Different amounts ($4x10^2$, $4x10^4$, $4x10^6$, $4x10^8$ copies) of qPlasmid were used as template for different primer concentration combinations (Table 4).

Table 4 – qPCR reaction efficiencies

Primers concentrations (nM)	Amplicon		
	qControl	qEx	qCirc
50/50	E = 97.52% R^2 = 0.9925	E = 90.00% R^2 = 0.9951	—
75/75	E = 97.55% R^2 = 0.9957	E = 99.59% R^2 = 0.9983	—
100/100	E = 110.57% R^2 = 0.9966	E = 108.53% R^2 = 0.9972	E = 98.49% R^2 = 0.9969
300/300	E = 114.58% R^2 = 0.9988	E = 111.19% R^2 = 0.9996	E = 111.32% R^2 = 0.9993
900/900	—	—	E = 112.99% R^2 = 0.9997

Footnotes: 100-fold dilutions of qPlasmid were used as template for the three qPCR reactions in order to find the best primer concentrations (forward/reverse). "E" values represent the efficiency of the reaction and R^2 values represent the correlation factor between replicates. Horizontal lines indicate that the reaction was not performed. Primer concentrations used in this study (highlighted in grey) matched the best amplification efficiency as well as correlation factor higher than 0.99.

To determine excised SCC*mec*, circularized SCC*mec*, and total chromosome copies in live cells, total DNA was extracted and quantified using NanoDrop (Fisher Scientific AG, Switzerland). DNA samples were diluted in water at a concentration of 10 ng/µl and used as template for the assay. Quantification of qControl, qEx and qCirc amplicons was determined by extrapolating the results from the standard curve prepared with different dilutions of qPlasmid, using the following formula:

$$\text{Sample copy number} = 10\text{^}((Ct_{sample} - b)/a)$$

where a and b are the slope and the y-intercept of the standard curve, respectively. The dynamic range of the assay ranged from $4x10^8$ to 40 amplicon copies. Proportions of

excisants and circular form of SCC*mec* were calculated by dividing the absolute amount of the qEx and qCirc amplicons by the absolute amount of qControl amplicon, respectively.

3.5 *Artificial SCCmec excision*

SCC*mec* was cured from *S. aureus* N315 using a method from Katayama and al. [25]. Briefly, strain N315 was electroporated with thermosensitive plasmid pSR3-1 (containing *ccrAB* genes) and transformants were grown for 24 hours at 30°C in TSB supplemented with tetracycline, serially diluted and plated on TSA supplemented with tetracycline. Single colonies were picked, grown for 24 hours at 42°C TSB to promote cure 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 recovered and the absence of SCC*mec* in it was confirmed by PCR amplification of the chromosomal junction formed upon excision using primers pair Excision fw and rev (Table 3). This isolate was named N315EX and used in further experiments.

3.6 *Fitness competition assay*

Fitness competition assays were performed in triplicates. 10^3 CFU of overnight cultures of parent MRSA N315 and its excisant N315EX were washed with phosphate buffered saline and inoculated in 10 ml of TSB. The CFU counts of the two strains were determined by plating dilutions of the cultures at 0, 7, 24, 48 and 72 hours. Strain N315 was selected on TSA plates supplemented with kanamycin (the resistance to which is encoded by SCC*mec*) and total number of cells was determined by plating the competition mixture on plain TSA plates. Quantification of strain N315EX was calculated by subtracting the number of CFU of N315 (i.e. kanamycin-resistant) from the total number of cells.

4. Results

4.1 *Fitness comparison between the parent N315 and its excised mutant N315EX*

A prerequisite to following the proportion of parent and excisant cells along growth in the same culture was that they both shared a similar fitness. Therefore, it was critical to ensure that the parent MRSA N315 and its excised mutant N315EX grew at the same rate in our experimental conditions. Indeed, any fitness advantage of one of the strains over the other would bias the results because that strain would become overrepresented in the culture over time. Figure 3 depicts a fitness assay in which 10^3 CFU of N315 and N315EX were inoculated together in 10 ml TSB and their ratio followed over time. It can be seen that both strains grew at the same rate and did not interfere with each other for up to 72 h. Therefore, the loss of SCC*mec* did not confer a selective advantage or disadvantage in the present laboratory conditions.

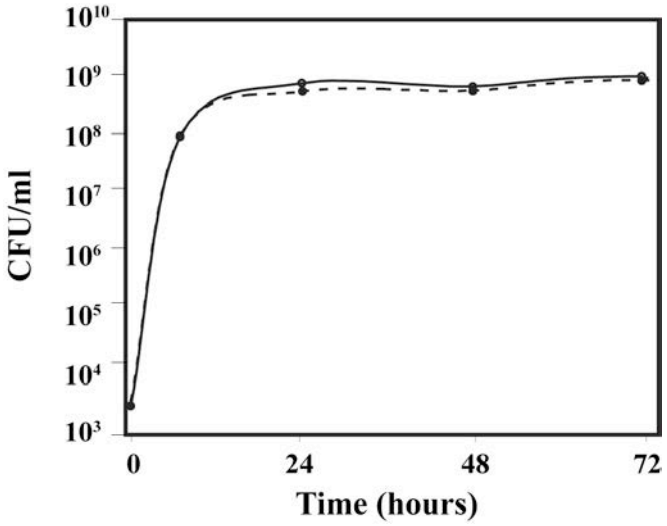

Figure 3 – Colony counts of the parent MRSA N315 (dotted line) and its excisant MSSA N315EX (black line) during growth in mixed culture. Samples were taken at various time points and plated on both plain agar and on agar containing kanamycin, which selected for the presence of SCC*mec*. The number or N315EX was calculated by subtracting the number of kanamycin-resistant colonies from the total number of colonies.

4.2 *Rate of SCCmec excision in standard experimental conditions*

The ratio of excised forms versus total chromosomes was first measured during growth at 37°C in plain TSB. Figure 4 indicates that this ratio was approximately 10^{-6} already at the first time point (i.e. 3 h), and remained constant in spite of further bacterial multiplication. This suggested at least two possibilities. One would be that excisants arose punctually at frequency of 10^{-6} and thus were already present in the 10^7 CFU inoculum; then, such excisants could have merely multiplied along during growth, without any additional excision. Another would be that continuous excision-reinsertion occurred all along growth, and respected a constant proportion of 10^{-6} excisants in the culture. To answer these questions we performed two specific experiments, which supported the hypothesis of punctual excision during early growth.

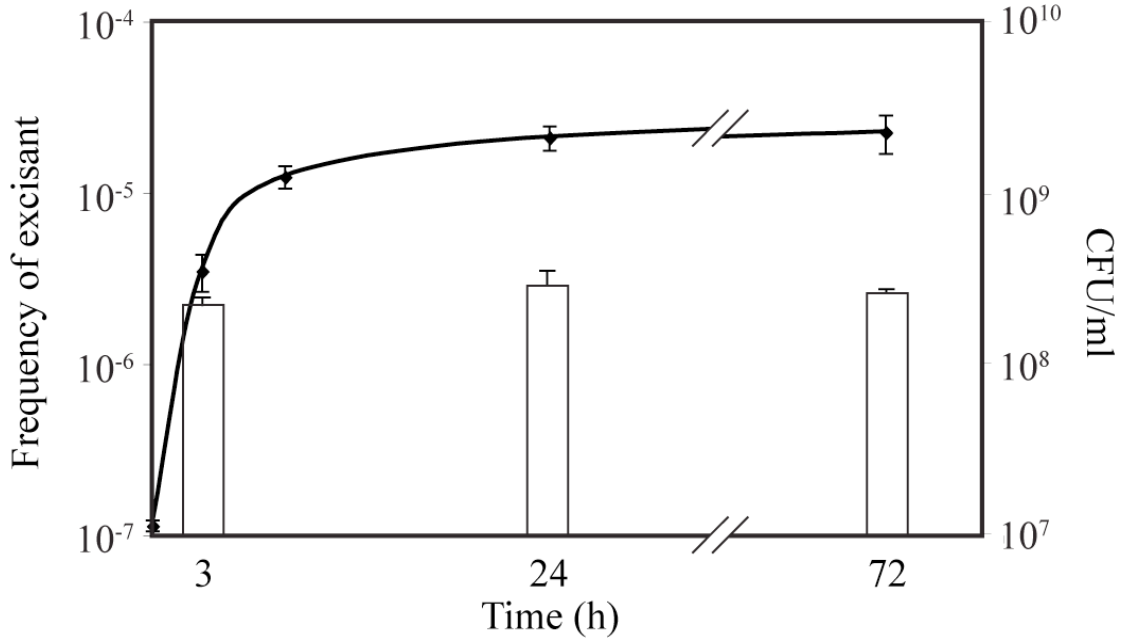

Figure 4 – Growth curve and frequency of excisants in MRSA N315 grown in TSB. Bacteria were grown at 37°C as described (closed diamonds) and sampled after 3, 24 and 72 h for DNA extraction and qPCR analysis. The open columns represent the ratio of excised versus chromosomes copies at each sampling time. Error bars represent the mean ± SD of three independent measurements performed in three independent cultures.

In the first experiment, the original culture presented in Figure 4 was serially passaged for 9 successive times in order to test whether the ratio of excised versus total chromosomes would increase over the successive passages. The experimental design is presented in Figure 5A.

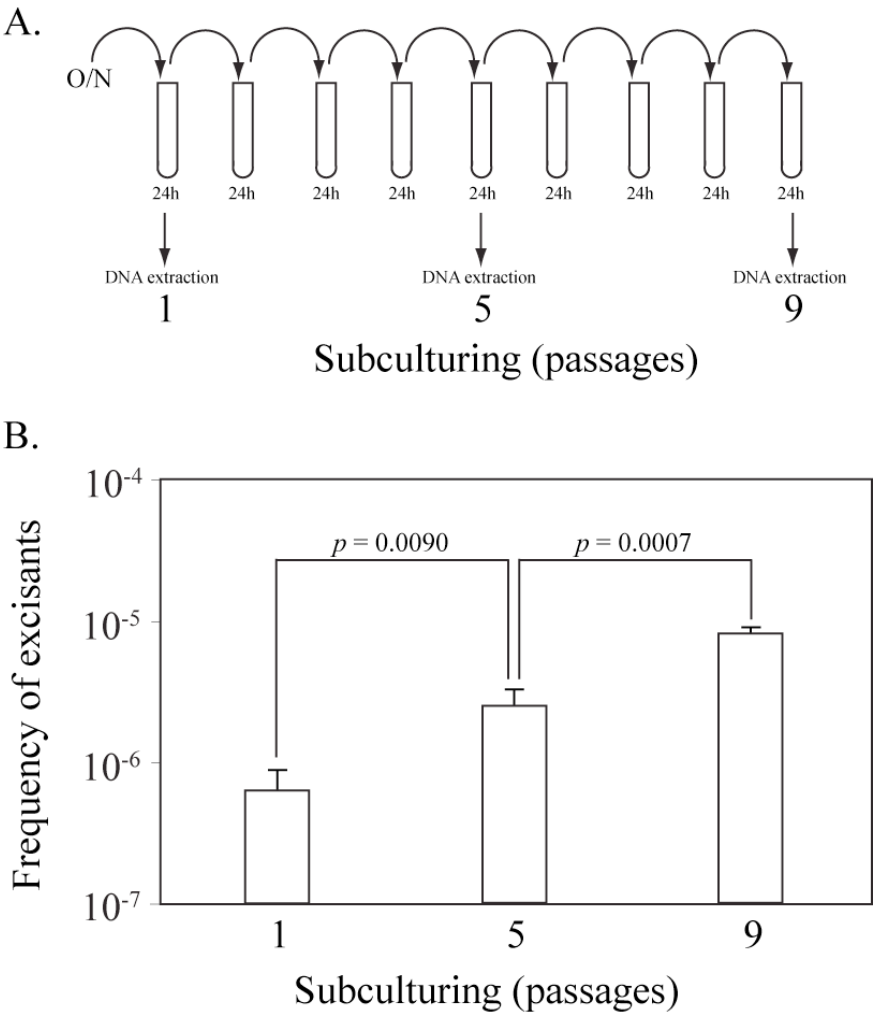

Figure 5 – Determination of rates of excisants during serial subculturing of MRSA N315. Panel A: an overnight culture grown in TSB at 37°C was used to inoculate a fresh 10 ml TSB culture with a dilution of 1/100. The new culture was allowed to grow overnight and used to reinoculate a new fresh culture, and this was repeated for 9 passages. DNA extractions and qPCR were performed at cycles 1, 5 and 9 as indicated. Panel B: evolution of the frequency of excisants over the passages. Error bars represent the mean ± SD of three independent measurements performed in three independent cultures. Statistical differences were assessed by the unpaired *t*-test.

In short, a 1/100 dilution (i.e. 0.1 ml in 10 ml) from the original culture in stationary phase (which contained 10^{-6} excisants) was inoculated in a new tube containing fresh broth and let to grow to the stationary phase. Then, the new culture was used to reinoculate a second fresh culture that was also allowed to grow to the stationary phase, and this was repeated for 9 consecutive times. This is formally identical to the fitness experiments described in Figure 3, but stopped at 24 h instead of 72 h.

Stationary phase cultures contained 10^{8}-10^{9} CFU/ml among which 10^{-6} (i.e. 100-1000 CFU/ml) were excisants (see Figure 4). Thus, re-inoculating 1/100 dilutions of a 10^{8}-10^{9} CFU/ml overnight culture accounted for a final concentration of 10^{6}-10^{7} CFU/ml in the fresh culture, which contained the same 10^{-6} ratio of excisants, i.e. a total of 1 to 10 excisants/ml in the fresh broth. If no additional excision occurred during the successive passages, then this 10^{-6} ratio should remain stable over time. In contrast, if additional excision occurred, then the ratio of excised to total chromosomes should progressively increase over time. Figure 5B shows that the ratio of excised forms to total chromosomes had indeed progressively increased by 10-folds over the 9 passages. This indicated that each passage had contributed for an increase of a little more than $1x10^{-6}$ excisants, which summed up to a 10-folds greater ratio of excisants (i.e. 10^{-5}) at the end of the experiment compared to the beginning. This also corresponded to the original 10^{-6} ratio of excisants observed in the single experiment of Figure 4.

In the second experiment we diluted out the inoculum from the overnight culture to completely abrogate the carry over of excisants. A dilution of 1/100 between cultures accounted for a final carry-over of 1-10 excisants/ml into the fresh cultures. To circumvent this bias we repeated the experiments by inoculating fresh cultures with 1/10,000 dilutions of the overnight culture. This would account for a carry-over of 0.01-0.1 excisants/ml of fresh broth, which was below the limit of detection of the subculturing system. Figure 6 (black

columns) shows that a ratio of approximately 10^{-6} excised forms was again present in the new culture, which could not result from carry-over. Moreover, this 10^{-6} corresponded again to the $1x10^{-6}$ *de novo* excision rate per culture cycle observed in the preceding experiments. Thus, *de novo* excision did occur in the present experimental settings. This observation further raised the additional question of when excision occurred along growth.

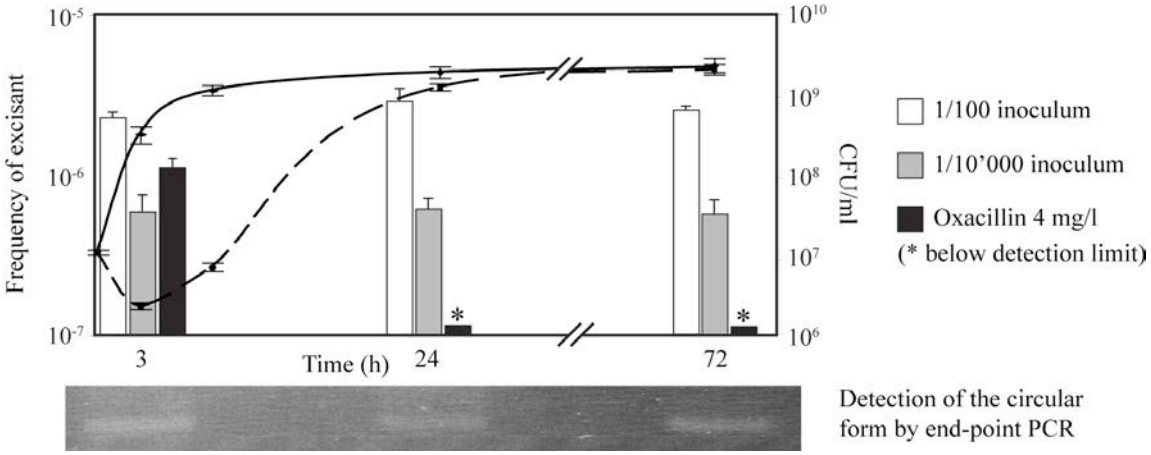

Figure 6 – Determination of rates of excisants and presence of circular forms of SCC*mec* during growth of MRSA N315 various growth conditions. In a first series of experiments, bacteria were grown at 37°C in plain TSB (continuous line), but inoculated with either a 1/100 or a 1/10,000 dilutions of an overnight culture. Open columns represent the rates of excisants following inoculation with the 1/100 dilution and grey columns represent the rates of excisants following inoculation with the 1/10,000 dilution. In a second series of experiments oxacillin (final concentration of 4 mg/l) was added at the time of inoculation and both growth (dotted line) and rates of excisants (black column) were followed over time. The presence of closed circular SCC*mec* during growth in plain TSB was detected by endpoint PCR (bottom of the Figure), but could not be quantified by qPCR because it remained below detection limits. Error bars represent the mean ± SD of three independent measurements performed in three independent cultures. Asterisks indicate that measurements were below the limit of detection.

4.3 *Dynamics of SCCmec excision*

The experiments described above show that 10^{-6} excisants were already present after 3 h of growth, and that their ratio remained stable thereafter. There are again at least two scenarios to account for this observation. First, excision could have occurred transiently at the beginning of incubation, at a rate of 10^{-6}, and then the excisants could have grown along with the non-excised forms. Second, excision-reinsertion could have occurred continuously during growth, at a rate ensuring a constant proportion of 10^{-6} excised forms versus total chromosome.

We tested the first possibility by supplementing the cultures with a final concentration of 4 mg/L oxacillin, in order to inhibit the growth of the *mecA*-negative excisants, while letting the parent cells to divide. We reasoned that if excision occurred only transiently at the beginning of incubation, then the oxacillin-susceptible excisants should be inhibited and become diluted off along growth. In contrast, if excisants would be continuously produced at a sizable rate of ca. 10^{-6} by the parent cells growing in the presence of oxacillin, then molecular signatures of excised chromosomes should be detectable even if the resulting excisants could not grow, due to the fact that *de novo* excision occurs constantly. Figure 6 shows that excisants rapidly disappeared after addition of oxacillin, and that no detectable excision occurred thereafter. This supports the hypothesis that excision most likely occurred early and only transiently during growth.

We tested the second possibility, i.e. equilibrium of continuous excision-reinsertion, by measuring the amounts of excised circular forms of SCC*mec* along growth. Circular forms cannot multiply autonomously and must be constantly generated *de novo* in order to be detectable. Thus if excision-reinsertion occurred at a constant rate of ca. 10^{-6} all along growth, then similar proportions of excised chromosomes and excised closed circular SCC*mec* should be detectable at any time. Conversely, if excision occurred only transiently at the beginning of growth, excisant cells should be able to grow along with the parents, and thus remain

detectable, whereas excised closed circular forms should become rapidly diluted off because they do not divide. The bottom of Figure 6 shows that only traces of closed circular SCC*mec* could be detected by endpoint PCR, but remained below quantification levels at any time of growth. Therefore, excision most likely occurred early and transiently during bacterial growth.

4.4 *Influence of external factors on SCCmec excision*

Next, we sought whether growth in various conditions could affect the rate of excision in *S. aureus* strains N315 as well as in *S. aureus* Mu50. We tested *S. aureus* Mu50 in parallel because it carries a similar SCC*mec* cassette as N315, but nevertheless has a different antibiotic resistance profile, i.e. it demonstrates homogeneous resistance to methicillin and intermediate resistance to vancomycin [105, 117]. Figure 7 compares the ratios of excised versus total chromosomes during growth at 37°C in plain TSB, or TSB supplemented with 4 μg/ml of oxacillin, 0.5 μg/ml of mitomycin C, or 4 mg/l of vancomycin (for Mu50 only). Moreover, the experiment was also repeated in plain TSB at 42°C. Plain TSB and TSB plus oxacillin confirmed the results presented above. In contrast, the mutagen mitomycin C significantly ($p = 0.0057$, unpaired *t*-test) increased the ratio of excised forms by ca. 10 times at the beginning of incubation in N315, but not in Mu50. Moreover, it returned to “normal” later on. Growth at 42°C and treatment with vancomycin (for Mu50 only) did not affect the ratio of excised forms at any time of growth.

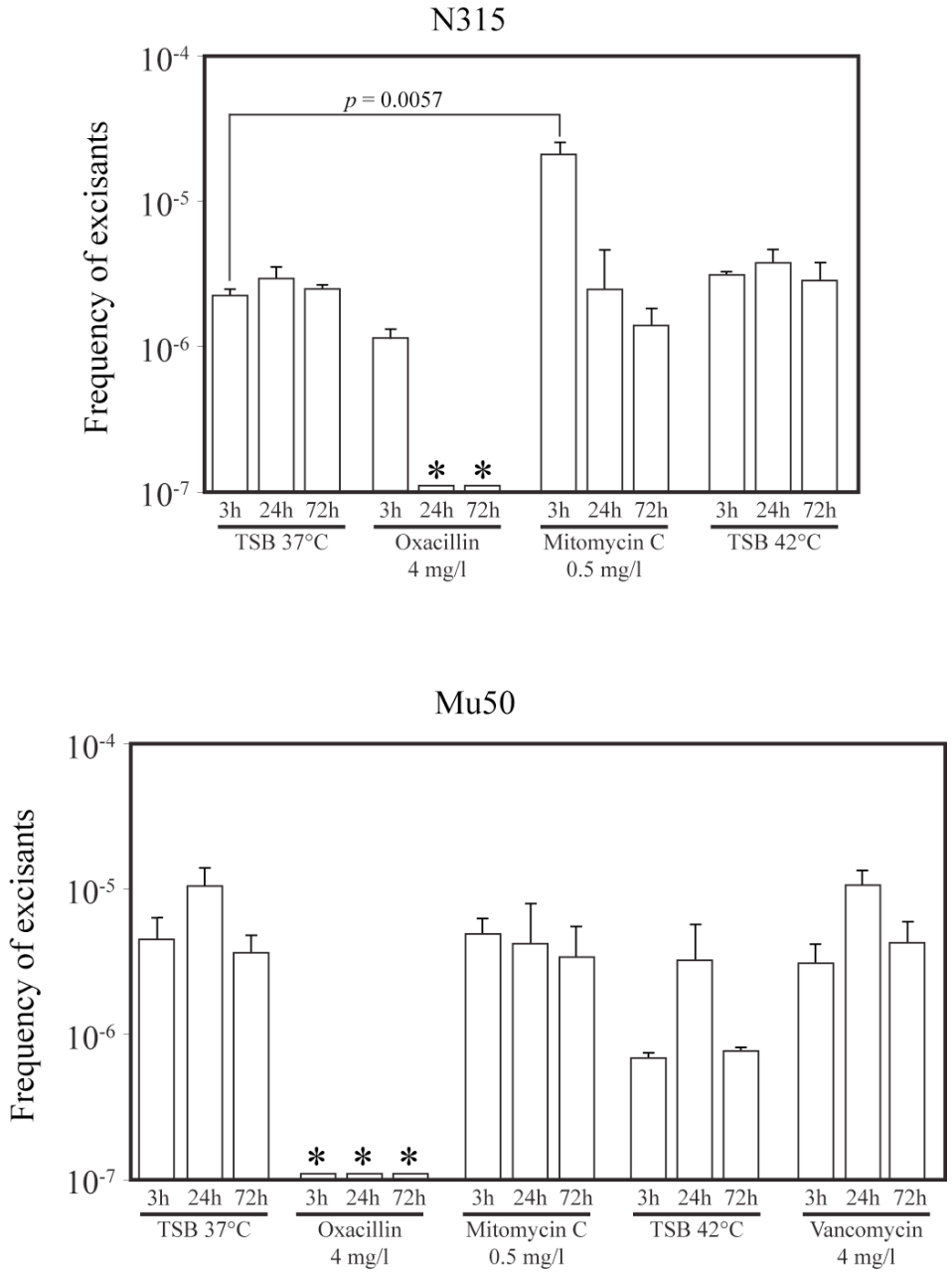

Figure 7 – Determination of rates of excisants during growth of MRSA N315 and MRSA Mu50 various stress conditions. Fresh TSB cultures were inoculated with 1/100 dilution of an overnight cultures and simultaneously exposed to either oxacillin, mitomycin C, vancomycin (only Mu50) or switched to 42°C as indicated on the Figure. Cultures were sampled at various times to determine the ratio of excisants by qPCR as described. Error bars represent the mean ± SD of three independent measurements performed in three independent cultures. Statistical differences were assessed by the unpaired *t*-test. Asterisks indicate that measurements were below the limit of detection.

5. Discussion

In the present experiments we developed a qPCR-based method to monitor the rate of excision of SCC*mec* in MRSA, taking strain N315 as model system. Moreover, we first constructed the in-house excisant N315EX, using thermosensitive plasmid pSR3-1 [25], to ensure that the parent cells and their excisants were able to grow together at similar rates in our experimental conditions.

We found that in standard conditions, i.e. growth at 37°C in plain TSB, the ratio of excised versus total chromosomes in N315 was reproducibly ca. 10^{-6}. Moreover, this proportion was already present at the first checkpoint during growth, i.e. 3 h after inoculation, and remained constant until 72 h in the late stationary growth phase.

The question then arose as to whether this constant proportion of excisants was due to trivial carry over from excisants already present in preceding cultures, and that these preexisting excisants were merely growing along without any additional excision events. However, the consecutive passage experiments as well as the ultimate dilution test clearly indicated that excision did occur *de novo* during growth in the present experimental conditions, and that this rate of excision was indeed around 10^{-6}.

The next question was whether the excisants present in the cultures resulted from a temporarily punctual event followed by propagation of the new excisant in parallel to the parent strain, or whether new excisants were constantly produced but were kept at a constant 10^{-6} proportion by some control mechanism, such as, for instance, constant parallel reinsertion of the closed circular SCC*mec* into the chromosome. The oxacillin experiments suggest that excisants were produced early during growth and became diluted off later on, due to the fact that they had lost the *mecA* gene and could not multiply due to oxacillin. Moreover, measuring the presence of closed circular SCC*mec* indicated that while this form was detectable by endpoint PCR at the very start of growth, it remained constantly below the

detection limit and did not increase during later growth. If a constant excision-reinsertion of SCC*mec* had occurred at a rate of 10^{-6}, then similar proportions of excised chromosomes and their mirror circularized SCC*mec* should have been present anytime in the cultures. Since this was not the case, it is likely that excision had occurred transiently at an early stage of growth, and that excisants further grew along in parallel to the parent cells as proposed above.

The dynamics of recombination occurring rather early on, at a given cell density, and during a limited period of time, is reminiscent of other cell-density and time-dependent recombination events, such as for instance competence for DNA transformation in certain bacteria [118, 119]. As suggested above, such a time-limited event would also explain the paucity of detectable SCC*mec* circular forms, which represent the very SCC*mec* intermediate apt to transfer. Cell density-dependent events in bacteria are classically driven by quorum sensing mechanisms, which implicate the accumulation of signaling molecules in the medium [120-122]. We did not analyze such type of regulation in the present study. However, the experiments exploring excision in different stress conditions suggest that at least the stringent response to DNA damage might also be involved in certain cases. Indeed, while oxacillin, vancomycin (in Mu50) and growth at 42°C did not affect the rates of excisants during early growth, the mutagen mitomycin C significantly increased early excision in strain N315. Although this was not the case for Mu50, other possible strain-specific differences were observed such as the fact that early excisants were totally ablated during oxacillin treatment in Mu50 as opposed to N315. Although this is based on comparing only two strains that carry similar types SCC*mec* cassettes, the possibility of inter-strain and inter staphylococcal species differences in excision events could have consequences on the transfer of the cassette in the environment, and many more strains and conditions would need to be tested to answer these questions.

Taken together, the present results indicate that excision of SCC*mec* did occur in both tested MRSA, with some intrinsic variations between them. The dynamics of the proportion of excisants during growth suggests that excision occurred rather early on, at a given cell density, and during a limited period of time. The technique described here for MRSA N315 and Mu50, which carry similar types of SCC*mec*, is amenable to study excision of other types of SCC*mec* cassettes in other MRSA backgrounds. It should be helpful to investigate additional issues, such as the precise timing of excision, whether or not it is quorum sensing dependent, and which conditions may promote or prevent it. In this regard, we would warrant against the possible false conclusion that oxacillin might prevent excision and thus spread of resistance. Indeed, while our experiments showed that oxacillin prevented the growth of the excisants, excision at a very early stage of drug exposure is not excluded. If transient excision (and transfer) does indeed occur at initiation of oxacillin or maybe other beta-lactam therapy, then it would be an ideal setting to promote the selection of new SCC*mec* staphylococcal recipients that just acquired the cassette. Only improving the detection of circular forms and evaluation of their dynamic of excision would provide definitive clues on this issue.

CHAPTER 4

Staphylococcal Cassette Chromosome *mec* insertion site in methicillin-susceptible *Staphylococcus aureus*

1. Abstract

Staphylococcus aureus isolates are classified by multilocus sequence typing (MLST), a molecular method that analyzes the polymorphism of 7 housekeeping genes. MLST defines both sequence types (STs) and clonal clusters (CCs) based on the homologies of the 7 alleles. In parallel, it was observed that methicillin-resistant *S. aureus* (MRSA), which carry the SCC*mec* island, tended to be more represented in certain CCs than in others. SCC*mec* inserts into the chromosome downstream of the conserved *orfX* gene, followed by a 15 bp *attB* attachment site. Analyses of the *orfX-attB* downstream sequences demonstrated inter-strain polymorphisms, and raised the question of whether some of these polymorphisms may be more or less prone to allow integration of SCC*mec*. Here we used direct genome sequencing to analyze the *orfX-attB* downstream sequences of 40 independent *S. aureus* isolates belonging to 10 main CCs (39 methicillin-susceptible and 1 MRSA) recovered from healthy nasal carriers, plus 6 control MRSA. The *orfX-attB* downstream sequences were highly variable, but these variations clustered precisely with the MLST-based CCs of the tested strains. Of note, MRSA clustered in a separate group, due to the insertion of SCC*mec* downstream of *attB*, but rejoined their original CC type after excision of their SCC*mec* element. We conclude that the *orfX-attB* downstream sequence, which is not taken into account by MLST-typing, closely co-evolves with its parental CC. This is compatible with the possible exclusion of SCC*mec* by certain CC types. Additional work is needed to test whether the *attB* downstream sequence is indeed responsible for this exclusion.

2. Introduction

Multilocus sequence typing (MLST) is the most frequently used sequence-based genotyping method to discriminate between different *S. aureus* isolates [123]. It relies on the single-nucleotide variations in seven housekeeping genes, defined as alleles, the combinations of which define a Sequence Type (ST). STs that share five or more alleles are clustered in larger groups named Clonal Complexes (CC) [66].

MLST analysis of 912 *S. aureus* independent isolates from 20 countries, including 553 methicillin-susceptible *S. aureus* (MSSA) and 359 methicillin-resistant *S. aureus* (MRSA), revealed a total of 162 different allelic profiles, or STs. However, the Staphylococcal Cassette Chromosome *mec* (SCC*mec*), which is responsible for methicillin resistance, was found only in 38/162 (23%) of them, with only 9 STs including more than 10 MRSA isolates [67]. This raised the question of whether some STs, which seemed to never carry SCC*mec*, were less prone to acquire the cassette, and whether specific barriers for SCC*mec* acquisition were existing.

The chance for an MSSA strain to acquired SCC*mec* depends both on the availability of donor strains in its direct environment, and on its intrinsic barriers to incorporate such exogenous element. Regarding to the opportunity of acquisition, it is noteworthy that SCC*mec* is not only restricted to *S. aureus*, but is also present in other staphylococci. The best-known example is *Staphylococcus epidermidis*, which is a very common human commensal and a potential reservoir of SCC*mec* [73, 124]. Since *S. epidermidis* and *S. aureus* often share the same anatomical niches in human (e.g. the nostrils and the skin), the opportunity of SCC*mec* transfer could be greater than expected.

Regarding to barriers of acquisition, several systems may be involved. First, *S. aureus* possesses several restriction-modification systems, which may considerably diminish acquisition of exogenous DNA [125, 126]. Second, acquisition of SCC*mec* may be

detrimental because the *mecA* gene may affect the fitness of the recipient strain [72, 127]. Third, the genetic background of the recipient strain might also play a role. On one hand the staphylococcal genome is known to influence the expression of methicillin resistance, and chromosomal factors are required for the resistant phenotype [46, 47]. On the other hand, establishment of SCC*mec* in the recipient cells requires the integration into the host chromosome, which takes place at the 3' end of the *orfX* gene. The integration process involves recombination of the SCC*mec attS* with the chromosomal *attB* site. At the *attS* site of the circular SCC*mec* form, a minimal region of 37 bp, comprising the core 15 bp sequence indispensable for insertion, is required for maximal integration frequency. In addition, even larger DNA stretches are required for optimal integration frequency at the chromosomal *attB* site, with more than 100 bp at both sides of the 15 bp core region located at the 3' of the *orfX* gene [90]. Sequence incompatibility at these sites could represent a barrier for SCC*mec* integration and consequently acquisition of methicillin resistance.

In this view, genomic sequences downstream of the *orfX* gene have been recently studied by Noto et al. [101]. The authors found that out of 42 unrelated isolates, 10 contained *attB* environments that were homologous to that the control MSSA 8325, which was able to acquire SCC*mec*. Strikingly, these 10 isolates also belonged to STs associated with MRSA. On the other hand, 27 isolates had variable sequences, some of which containing non-staphylococcal genes (suggesting insertion of other types of heterologous DNA), whereas 5 could not be sequenced [101].

These results suggest that sequence variations in the *attB* environment might indeed affect the proneness to integrate SCC*mec*. However, whether this is related to CCs or STs, or rather serendipitous within similar CCs, is less clear. Indeed, MLST typing does not take into account polymorphisms in the *orfX* environment. Moreover, as more typing data became available, SCC*mec* was observed in most STs known today [128] and new lineages of MRSA

are constantly emerging, such as MRSA of CC398, which has recently spread among pigs [129].

Here we took advantage of a large collection of MSSA isolated from healthy *S. aureus* carriers [102] to attempt assessing the relationship between the *attB* environment and the CC types in MSSA. Since PCR amplification of the sequences downstream of *orfX* might present technical limits, we performed direct sequencing of the SCC*mec* insertion site using genomic DNA.

3. Materials and methods

3.1 *Bacterial strains*

Strains of a *S. aureus* collection from healthy nasal carriers described by Sakwinska et al. were used in this study [102]. In brief, healthy carriers of *S. aureus* were detected in new hospital employees by performing nasal swabs at their first medical checkup, at a University hospital of the State of Vaud (CHUV, Lausanne, Switzerland). Carriage strains were genotyped by MLST and amplification fragment length polymorphism (AFLP). They were highly diverse, encompassing a total of 20 CCs. Of note, the large majority of them (111/113) were MSSA. Forty unique isolates representing different CCs were analyzed in this study (Table 1).

Table 1 – Genotypes of carriage strains (39 MSSA and 1 MRSA) analyzed herein

Strain	Sequence Type (ST)	Clonal Complex (CC)	Strain	Sequence Type (ST)	Clonal Complex (CC)
2	15	15	82	34	30
4	59	59	83	30	30
5	942	942	87	182	182
10	30	30	92	59	59
15	5	5	100	45	45
21A	30	30	102	8	8
23	25	25	109	5	5
27	45	45	113[a]	45	45
30	30	30	118	45	45
31	30	30	129	45	45
33	707	707	131	25	25
37	45	45	140	398	398
45	15	15	142	15	15
49	5	5	152	30	30
53	101	101	158	30	30
57	5	5	161	101	101
60	15	15	185	121	121
62	1158	30	186	508	45
63	1159	7	329	121	121
76	45	45	341	398	398

[a] Strain 113 is an MRSA

In addition, as controls we also included 5 well-defined strains of MRSA (N315 [105], MW2 [107], COL [106], MRSA252 [55], FPR3757 [129]), as well as the SCC*mec*-negative mutant N315EX, which is a MSSA derivative of MRSA N315 [105], obtained by transient overexpression of plasmid-borne *ccrAB* genes (See chapter 2).

3.2 *Genomic DNA extraction*

For *S. aureus*, genomic DNA was extracted using a protocol adapted from Bae et al. [109]. Cells were harvested by centrifugation and resuspended in 50 µl of TE supplemented with lysostaphin (final concentration 0.5 µg/ml). After 30 minutes of incubation at 37°C, 300 µl of "Nuclei lysis solution" (Promega AG, Dübendorf, Switzerland) were 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 AG) was followed by incubation for 5 minutes on ice. After centrifugation (4°C, 13'000 rpm), supernatants were collected and 300 µl of isopropanol were 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 EB (Promega AG). DNA concentrations and qualities were monitored using ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington DE, USA).

3.3 *Inverse PCR*

Genomic DNA was digested with *Sau96I* for 2 hours at 37°C, followed by 20 minutes incubation at 80°C to inactivate the endonuclease. Digested DNA was ligated O/N using T4 DNA ligase (Promega Corporation, Madison WI, USA) at 4°C and used as template for PCR amplification with specifically designed primers inv fw (CTTTGTATTCGTCATTGGCGG)

and inv rev (TGGGTCATGCGTTGGTTCA), which both anneal inside the *orfX* gene (see Figure 1).

3.4 *DNA Sequencing and data treatment*

Genomic DNA of MSSA (absence of *mecA* assessed by PCR) from the strain collection was used as template for the sequencing of the SCC*mec* insertion site. Genomic DNA was sequenced on AB3730 DNA Analyzer (Applied Biosystems) using BigDye terminator V3.1. The sequencing primer qEX fw (CGCAGTAACTACGCACTATCATTCAGC) was located at the position -46 bp with respect to the insertion site. Sequencing data were analyzed with FinchTV software (Geospiza Inc., Seattle WA, USA) and sequence alignments were performed with MegAlign software (DNASTAR Inc., Madison WI, USA) using the ClustalW algorithm.

4. Results

4.1 *Attempt to amplify the chromosomal attB insertion site using inverse PCR*

We first attempted to determine the sequence surrounding the chromosomal *attB* in MSSA using inverse PCR (Figure 1). The endonucleases *Sau96I* and *TaqI* were chosen for this purpose, due to their optimal restriction frequencies and the presence of a restriction site inside the *orfX* gene. Initial attempts with these enzymes showed that *Sau96I* was more reliable. Therefore it was chosen for further experiments. The system was initially tested in the well-defined SCC*mec*-negative mutant strain N315EX, and gave a PCR product of expected size (i.e. 343 bp) and sequence, although secondary amplifications could be observed after PCR amplification. We next attempted to test the method on chromosomal DNA from 8 MSSA of unknown sequences on two independent occasions. However, the lack of PCR amplification in some strains and the presence of multiple PCR products in others – maybe due to the length of the intervening sequence – made the method too unreliable to be pursued. Therefore it was abandoned.

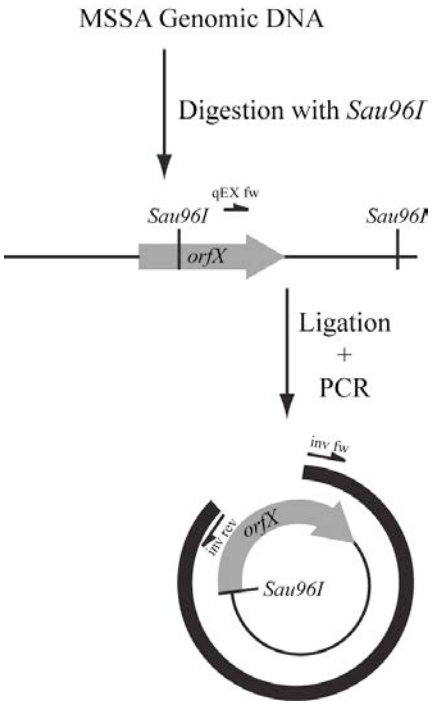

Figure 1 – Schematic representation of the inverse PCR protocol. *orfX* is located just upstream of the 15 bp *attB* site of insertion of SCC*mec* on the MSSA chromosome. *Sau96I* is expected to cut within *orfX* (which is very conserved) on one end, and outside of *orfX* on the other end. The technique of circularization and the choice on primers (located on both sides of *orfX*) should ensure that only fragments downstream of *orfX* are amplified.

4.2 *Genomic DNA sequencing*

Since *attB* sites could not be reliably determined by inverse PCR, we attempted to perform direct sequencing on genomic DNA extracted from MSSA strains, using the qEX fw primer described above (see Figure 1). First approach experiments indicated that both the concentration and purity of DNA templates were highly critical for this application. Minimum DNA concentrations of 1.5 μg/μl and absorbance ratios at 260 nm and 280 nm comprised between 1.8 and 1.9 could were indispensable for reliable sequencing.

Good sequencing results were obtained at the first run in 24 cases (Figure 1A.), while the other 16 cases needed either DNA re-extraction of manual editing of the sequencing results (Figure 1B).

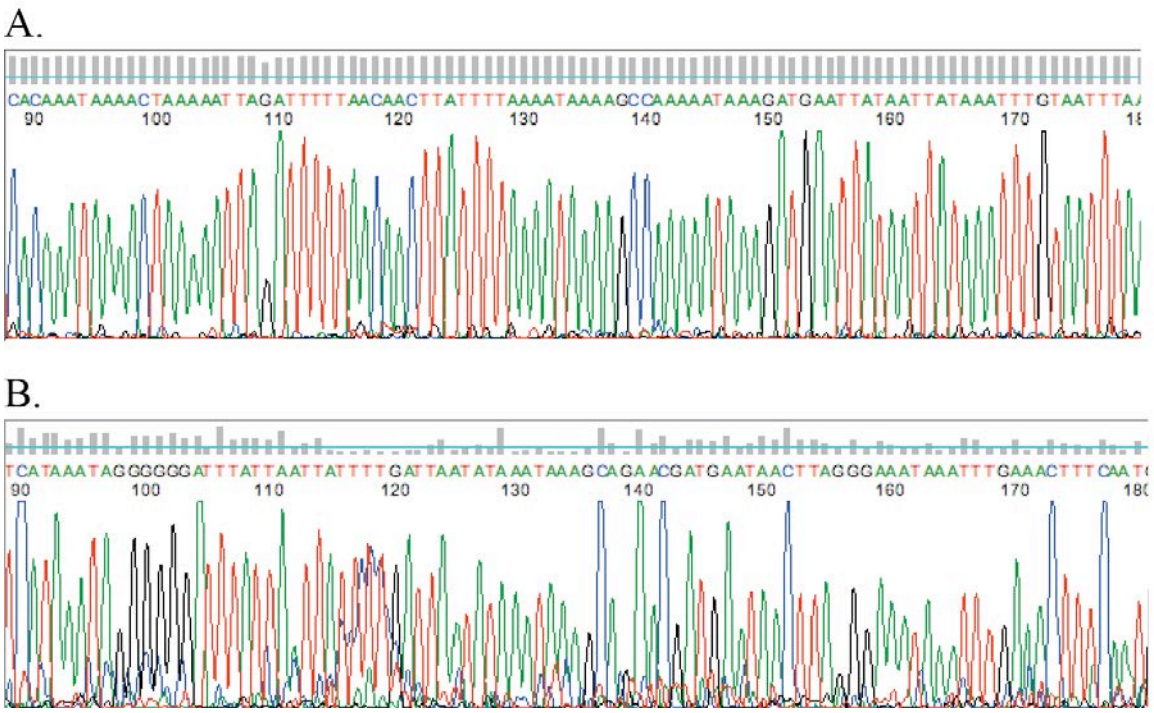

Fig. 1 – Two examples of sequencing chromatograms.
Examples of an optimal (A.) and non-optimal (B.) chromatogram result. The grey bars indicate phred quality scores with blue line at Q=20 Bars above this line indicate base calls with > 99% probability of being correct.

Table 2 shows the lengths of the reads obtained with each of the 40 MSSA tested. The median (range) length was 348 bp (174 bp to 661 bp), respectively. Because these lengths were variable, all DNA sequences were trimmed to 114 bp, in order to contain the last 57 bp of *orfX* and its adjacent downstream 57 bp. These sequences were then were aligned using ClustalW (Figure 2) and processed for phylogenic comparison (Figure 3).

Table 2 – Length of the reads obtained by sequencing of genomic DNA

Strain	Length (bp)	Strain	Length (bp)	Strain	Length (bp)
2	227	53	298	118	197
4	240	57	612	129	488
5	594	60	340	131	562
10	555	62	312	140	295
15	194	63	661	142	253
21A	338	76	466	152	372
23	400	82	449	158	517
27	458	83	246	161	430
30	400	87	393	185	512
31	228	92	419	186	356
33	419	100	183	329	180
37	179	102	174	341	427
45	244	109	183		
49	185	113[a]	182		

4.3 *Comparison of the orfX downstream sequences*

Figure 2 compares the obtained *orfX*-downstream sequences for all the tested strains. It can be seen that the 15 bp *attB* sequence (or *attR* in case of the presence of a SCC element) was highly conserved in all strains, and so was the *attB* upstream sequences belonging to *orfX*. In contrast, variations were quite frequent within the *attB*-downstream sequence and could be visually classified in three orders. First, they followed closely MLST-based CCs. Second, MRSA clustered *a priori* separately from their CC type, due to the presence of an SCC*mec* cassette, but coherently with their SCC*mec* type (e.g., USA300, MRSA252, MW2 and MRSA113 together with the type IV cassette). Third, after excision of SCC*mec*, either

physically as in strain N315EX, or *in silico* as performed in the other MRSA (Figure 2), all the MRSA strains rejoined their original MLST-based CC.

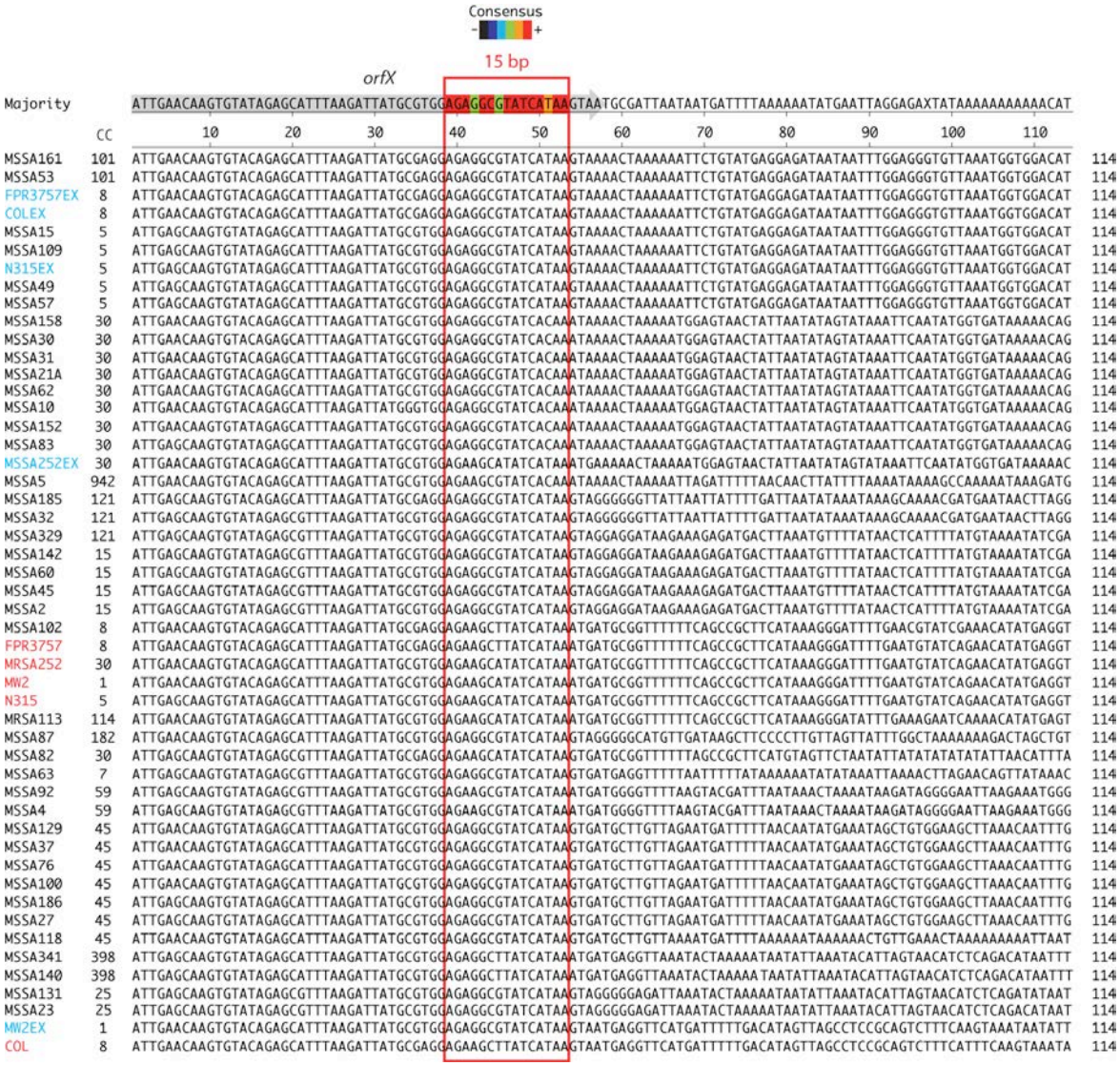

Figure 2 – ClustalW alignment of sequenced MSSA and MRSA strains.

The two first columns on the left indicate the names of the strains and their MLST-based CC affiliation. The central box (framed in red) indicates the 15 bp *attB* insertion site (or *attL* in case of the presence of a SCC), for which the color scale indicates conservation of residues. MRSA strains are highlighted in red. MRSA from which the SCC*mec* has been deleted either physically, as in N315EX, or *in silico* as in MW2, COL, MRSA252, and FPR3757, are highlighted in blue. Consensus sequence is shown for the 15 bp of *attB*.

Figure 3 depicts a more detailed phylogenetic tree based on the sequences of Figure 2. It confirms the good correlation between the 3' *attB* sequence environment if the strains and their previous characterization at the CC level [102], except for three strains.

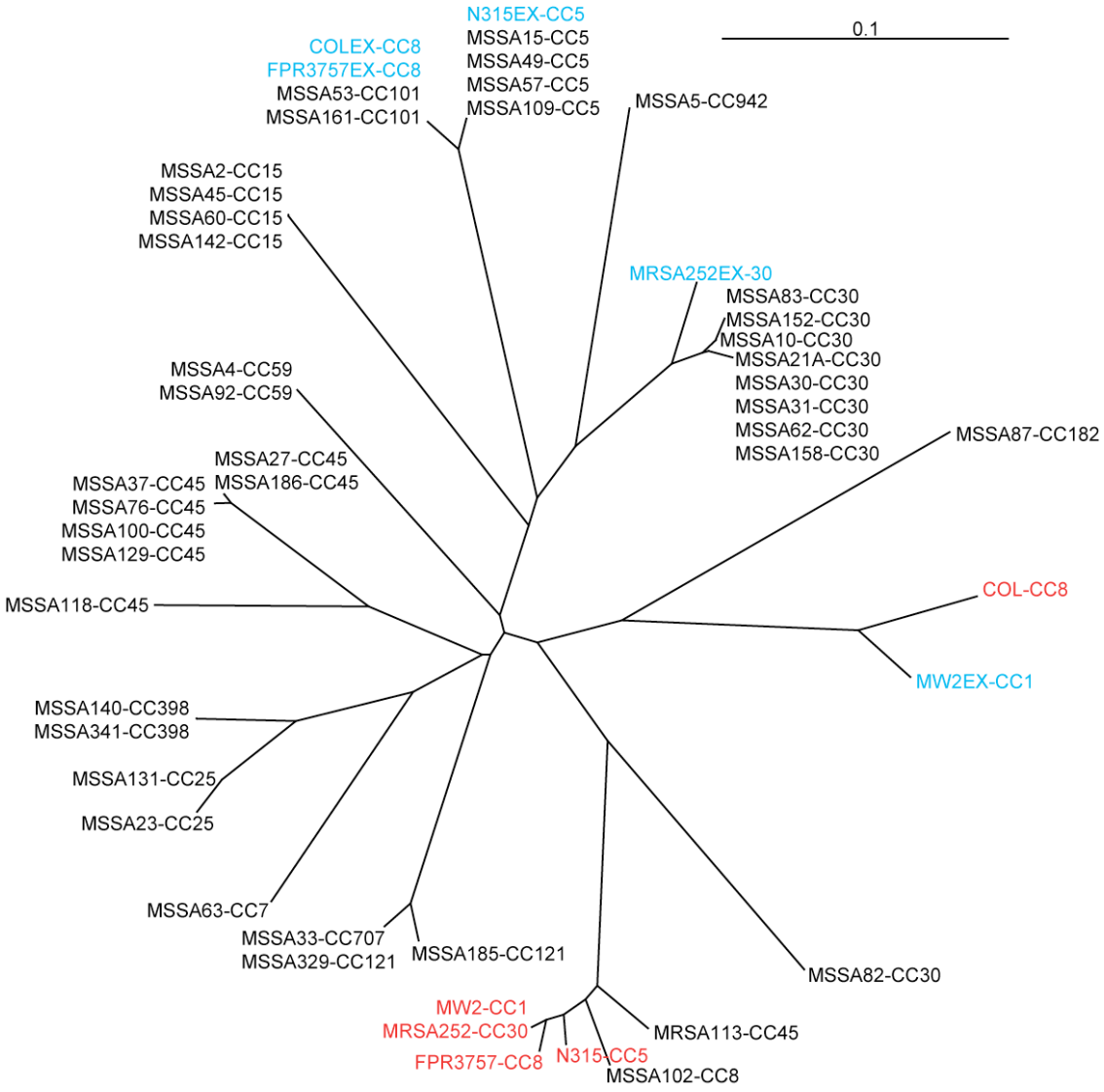

Figure 3 – Phylogenetic tree of SCC*mec* insertion sites

Phylogenetic tree was made with sequences presented in Figure 2 (see figure legend for details). CC is indicated for each strain. The scale indicates the relative distance on the phylogenetic tree. The phylogenetic tree was constructed using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

First, MSSA102 was part of CC8, but was found in the same cluster than the reference MRSA strains, indicating that it may carry a non-*mecA* SCC. Blast analysis of sequence downstream the *orfX* gene confirmed this hypothesis, since this region seems to be specific to SCC*mec*. Yet, the strain was negative for oxacillin resistance on selective plates. Second, MSSA87 presumably carried a previously characterized SCC encoding type 1 capsular polysaccharide biosynthesis gene cluster (sequence homology of 97%) [57]. Third, MSSA82 belonged to CC30, but did not cluster with other strains of the same CC included in this study. Its genomic sequence downstream *orfX* did not show any homology with the nucleotide databases (GenBank, EMBL, DDBJ and PDB sequences). All the remaining strains displayed sequence homology to known MSSA sequences previously characterized.

5. Discussion

The purpose of this study was to analyze the correlation between the variability of the 3' *attB* DNA environment, which is critical for the insertion of SCCs in general and SCC*mec* in particular, with the MLST-based CC clustering of *S. aureus*. Ultimately, the question was also whether certain *S. aureus* CCs that contain only MSSA might have a lower proneness than other CCs to acquired SCC*mec*, and thus convert to MRSA.

The experimental questions were framed on two premises. The first premise was a relatively recent study by Noto et al. [101] who analyzed the 3' *attB* environment of 40 unrelated MSSA isolates, and observed a substantial inter-strain variability of these sequences. They noticed that 10 of their isolates carried homologous sequences that resembled the sequences of staphylococcal STs known to be capable of acquiring SCC*mec*. In contrast, other strains did not have this resemblance and belonged to other STs. Therefore, they postulated that some 3' *attB* environments might be less prone to SCC*mec* integration then others, and that this could explain the quasi-absence of SCC*mec* in certain *S. aureus* CC lineages.

The second premise was based on a recent study by Sakwinska et al. in our laboratory [102], who analyzed the phylogeny of *S. aureus* isolates from 133 healthy carriers of the Lausanne geographic area. Since the frequency of MRSA in this region is notoriously low, we postulated that the chance of observing typical non-MRSA CCs could greater than in other places.

Using this strain collection, our results confirmed the inter-strain variability of the 3' *attB* DNA environment reported by Noto et al. [101]. In addition, however, they also show a unanimous correlation between the 3' *attB* environment of MSSA and their MLST-based CC types. This occurred in spite of the fact that the 3' *attB* DNA environment is not taken into account in the MLST polymorphism analyses. Therefore, this region must specifically co-

evolve along with its parent clone. In this sense, the region behaved differently than more widely conserved regions of the chromosome, such as *orfX* or the *attB* site itself, which are conserved over the whole *S. aureus* species. In the same logic, MRSA carrying similar types of SCC*mec* elements were grouped together, but outside of their MLST-based CCs, because SCC*mec* specifically insert squat downstream of *attB*. However, they rejoined their parental CC after excision of their SCC*mec* insert, either physically as in strain N315EX, of *in silico* as in strains MW2, COL, MRSA252, and FPR3757.

Besides, we also found individual MSSA exceptions. Two of them were likely to carry non-*mec* SCCs, i.e. strains 102 and 87, and a third contained DNA of unknown origin (strain 82). This was of particular interest, because it suggested that the 3' *attB* environment could act as a hotspot for the integration other MGEs than SCCs. Indeed, Noto et al. also observed such an occurrence, where one MSSA was containing DNA of lactococcal origin [101]. This is congruent with our present finding, and widens the spectrum of gene acquisition in this particular chromosomal area.

One of our original questions was whether or not some MSSA CCs might be less prone to acquire SCC*mec* due to their specific 3' *attB* DNA environment. The link between this region and specific CC lineages, which we report here, is compatible with this hypothesis, but does not confirm it. Indeed, very recent work by Wang et al. [90], challenged this possibility. Using a plasmid-based experimental system, these authors observed that sequences from supposedly insertion-refractory 3' *attB* environments did actually allow insertion of artificial SCC-like elements. If this is confirmed, putative SCC*mec*-refractory staphylococcal lineages must resist SCC*mec* acquisition through other mechanisms than by 3' *attB* restriction, as alluded to in the Introduction of this Chapter.

We showed in Chapter 2 and Chapter 3 that expression *ccrAB* recombinase genes and frequencies of SCC*mec* excision were strain-dependent. Moreover, *ccrAB* promoter activity

was driven by the genetic background of the strain rather than by the SCC*mec* element itself. Thus, there might be a symmetric strain-dependency in the proneness of SCC*mec* acquisition as well, and by extension, a CC-dependency of SCC*mec* acquisition and transfer. The results and the literature presented above are compatible with this possibility. In addition, the molecular tools presented in Chapter 2 and Chapter 3, and in the following Chapter 5, should help address these issues at both the physiological and molecular levels.

CHAPTER 5

Exploring an Extrachromosomal Reporter System to Study Excision of Staphylococcal Cassette Chromosome *mec* (SCC*mec*) in Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*

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- 71th Assembly of the Swiss Society for Microbiology, St. Gallen (Switzerland), 21-22.06.2012
  Poster presentation: "Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Excision in Methicillin-resistant Staphylococcus aureus (MRSA) assessed by an Extrachromosomal Excision Reporter".

1. Abstract

Horizontal transfer of SCC*mec* has been underestimated in the past. Recent findings suggest that new MRSA clones continuously arise due to SCC*mec* acquisition from MSSA. Excision of SCC*mec* from the donor's chromosome represent presumably a key step of its horizontal transfer. Although many epidemiological studies, few reports have focused on this issue and factors leading to SCC*mec* excision.

Here we describe a plasmid-based excision reporter system, which might clarify some key aspects of SCC*mec* excision dynamics and help understanding conditions in which this phenomenon occurs. This molecular tool, named pTRAP, consists of an SCC-like element, composed of left and right attachment sites separated by three transcriptional terminators, cloned between a constitutive promoter and the *gfp* gene. Expression of Ccr recombinases from SCC*mec* carried by MRSA strains, should result in the excision of the SCC-like element from pTRAP, which should result in a high GFP expression and thus in an easily detectable signal. This system could be exploited in different MRSA backgrounds to screen for different conditions modulating the activity of Ccr recombinases and consequently excision of SCC*mec*. This type of reporter has been successfully developed for other systems and could be also potentially exploited to study the involvement of DNA sequences in the integration/excision process of SCC*mec*.

We tested the specificity of excision of pTRAP in MRSA strain N315 and its MSSA-derivative N315EX, which resulted in a specific detection of excision only when *ccr* genes were present (N315). pTRAP was successfully introduced into different MRSA, which allowed us to test the excision process during different growth conditions.

2. Introduction

Chapters 2 and 3 of the present thesis dissertation showed that expression of *ccrAB* genes obeyed a bistable pattern, and that excision of SCC*mec* did occur spontaneously during early bacterial growth. Nevertheless, many questions remain incompletely answered regarding to factors involved in SCC*mec* excision, including what are the precise triggering circumstances, what are the regulatory factors, and what are the exact DNA-binding sites indispensable for correct site-specific excision.

As mentioned earlier, site-specific excision of SCC*mec* is driven by Ccr recombinases, which are large serine recombinases encoded on the cassette itself [25]. They are related to phage recombinases of the invertase/resolvase family, and mediate both integration and excision reactions [130]. Three distinct *ccr* genes have been described in SCC*mec*, namely *ccrA* and *ccrB*, which are transcribed from one transcription unit (*ccrAB*), and *ccrC* that is transcribed from an independent unit [112]. The two transcription units are SCC*mec*-specific and are not present simultaneously on the same cassette.

The three genes share less than 50% DNA sequence similarity. So far, four main allotypes (sequence similarity less than 87%) have been described for *ccrA* and *ccrB*. These four allotypes, combined with the *mec* complexes define eight of the eleven SCC*mec* types known today [112]. In contrast, only one allotype has been found for *ccrC* and defines the SCC*mec* types V and VII. In spite of allotypes variations, the CcrAB recombinases can mediate excision of different types of cassettes carrying the *ccrAB* complex, whereas *ccrC* are specific to type V and VII cassettes [85, 131].

All the Ccr proteins contain a characteristic catalytic domain, which includes a serine residue exchanging the DNA strands, but only CcrB and CcrC have a DNA-binding motif [81, 130]. Specific excision involves the recombination between two 15-bp direct repeats flanking SCC*mec* (*attL* and *attR*). Moreover, correct positioning of the enzymes and possibly

regulation of excision frequency seem to require accessory DNA sequences, which were in part identified as imperfect inverted repeats that are flanking the 15-bp direct repeats [81, 86, 90]. In the case of CcrAB, it was shown that CcrB was responsible for DNA binding, but required CcrA to correctly position the complex for site-specific excision [81]. Indeed, CcrB was able to catalyze excision in the absence of CcrA, but excision occurs at alternative sites [81]. Therefore, subtle protein-DNA and protein-protein interactions are involved in the process.

In this 5th chapter we sought to develop an extra-chromosomal system amenable to study factors involved in excision in different staphylococcal backgrounds. While chapters 2 and 3 described the two extremes of the process, i.e. pre-excision activation of *ccrAB* promoters and post-excision chromosomal integration sites, respectively, the experiments described below propose a new tool, referred to as pTRAP, allowing to investigate the SCC*mec* excision process in live cells. In the following we describe the construction of this new tool and the first proof of concept experiments.

3. Materials and methods

3.1 *Bacterial strains, media and culture conditions*

Staphylococci, *Escherichia coli* and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α, which was routinely used for plasmid propagation and cloning experiments, was cultivated on Luria-Bertani (LB) medium (Becton Dickinson, Sparks, MD) supplemented with 100 mg/L ampicillin (AppliChem, Darmstadt, Germany) at 37°C. Staphylococci were grown with aeration in trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) in a rotating incubator (at 180 rpm) at 37°C, or plated on trypticase soy agar (TSA). 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 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.

3.2 *DNA manipulations*

Plasmids were isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). 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 "QIAquick Gel Extraction Kit" (Qiagen Inc.) according to manufacturer's protocols.

Ligations were performed using 1 µl of T4 ligase (Promega Corp.) according to the manufacturer's specifications.

Table 1 – Bacterial strain and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Reference
Strains		
E. coli DH5α	Host for DNA cloning	Laboratory collection
S. aureus		
RN4220	Restriction-deficient derivative of RN450	[104]
N315	Hospital-acquired MRSA carrying type II SCC*mec*; Clonal complex 5	[105]
N315EX	Isogenic MSSA derivative (ΔSCC*mec*) of N315	Chapter 2
MW2	Community-acquired MRSA carrying type IV SCC*mec*; Clonal complex 1	[107]
H9008	Community-acquired MRSA carrying type IV SCC*mec*; Clonal complex 8	[71]
H19768	Community-acquired MRSA carrying type V SCC*mec*; Sequence type 152	[71]
S. epidermidis RP62A	MRSE carrying type II SCC*mec*	[106]
Plasmids		
pCN36	*E. coli-S. aureus* shuttle vector; Tc^R; ± 22 copies/cell	[108]
pCN68	*E. coli-S. aureus* shuttle vector; source of *ermC* gene	[108]
pCN50	*E. coli-S. aureus* shuttle vector; source of *blaZ* transcriptional terminator	[108]
pGFP1	Plasmid carrying the *gfpmut2* gene under the control of the constitutive promoter P1 of the staphylococcal *sar* locus [133]; P_{P1}-*gfpmut2* fusion cloned in pCN36	This study
pMrG-SCC	Synthetic plasmid carrying SCC*mec* attachment sites from N315 (Mr Gene GmbH, Regensburg, Germany)	This study

3.3 *Polymerase chain reaction (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 manufacturers' specifications. Primers used in this study are listed in Table 2.

Table 2 – Primers used in the study

Primer	Sequence	Annealing temperature (°C)
SphI-P1 fw	TTTTTTGCATGCCTGATATTTTTGACTAAACCAAATGC *SphI*	64.8
PstI-P1 rev	TTTTTTCTGCAGGATGCATCTTGCTCGATACATTTG *PstI*	66.8
GFPscreen rev	GACAAGTGTTGGCCATGGAACAGG	61.3
XbaI-T1-TT fw	TTTTTTTCTAGAATAAAACGAAAGGCCCAGTCTTTCGACTGAG *XbaI* CCTTTCGTTTTATTATTCTAAATGCATAATAAATACTGATAAC	64.8
XmaI-TT rev	TTTTTTCCCGGGTGTCACTTTGCTTGATATATGAG *XmaI*	64.2
XmaI-TT fw	TTTTTTCCCGGGTATTCTAAATGCATAATAAATACTG *XmaI*	57.0
SpeI-TT rev	TTTTTTACTAGTTGTCACTTTGCTTGATATATGAG *SpeI*	53.2
pCN fw	ACGCGGCCTTTTTACGGTTCC	57.1
M13 -46	GCCAGGGTTTTCCCAGTCACGA	61.8

3.4 *Flow cytometry*

Flow cytometry was performed on a FACS-Calibur (BD Biosciences, Erembodegem, Belgium), equipped with an aircooled argon laser (488 nm). GFP fluorescence was recorded in the FL1 channel (525 ± 15 nm). Samples were taken during exponential phase of growth and diluted in phosphate saline buffer in order to not exceed 800 events pas second and fluorescence of 20’000 events was recorded for each sample. Analysis of flow cytometry data was performed using the WinMDI software (version 2.8, Salk Institute, http://facs.scripps.edu/software.html).

When required, populations of fluorescent and non-fluorescent cells were sorted using FACS Aria (BD Biosciences, Erembodegem, Belgium). Recovered bacterial cells were resuspended in phosphate saline buffer.

4. Results

4.1 *Construction of the pTRAP excision reporter system*

The pTRAP system was aimed at capturing the important factors implicated in SCC*mec* excision as well as appraising the dynamics of excision in live cells. It was conceived as a plasmid containing two functional units, namely one excision-triggering module and one excision-reporter module. The core of the excision-triggering module consisted of an artificial SCC-like element bracketed by the two *attL* and *attR* direct repeats plus their flanking inverted repeats (IR; designed on the basis of the SCC*mec* of strain N315), and centered with three consecutive transcription terminators (see details in Figure 1).

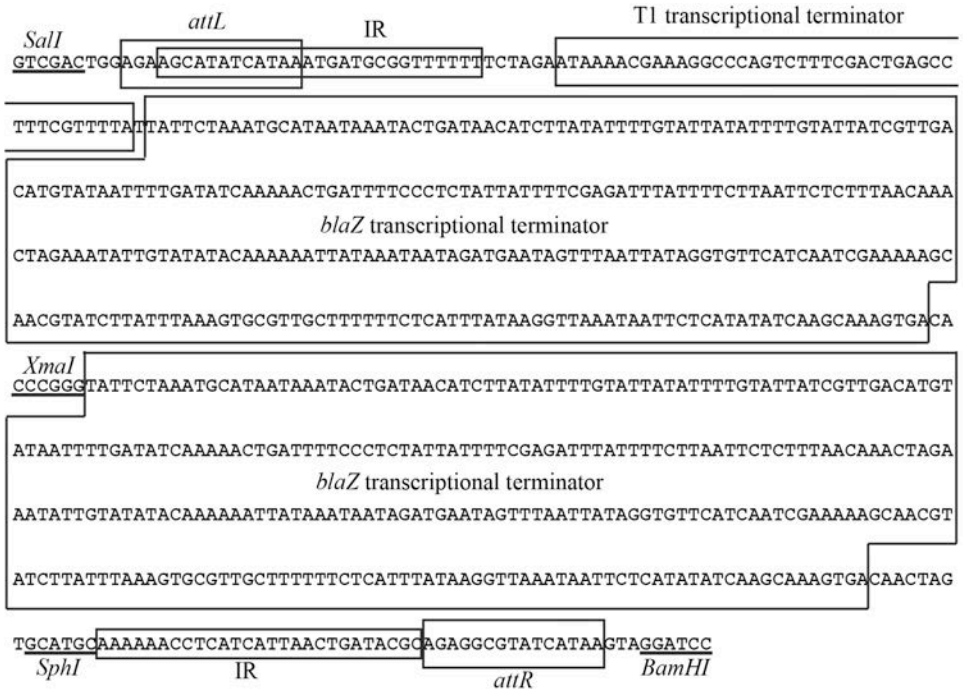

Figure 1 – Sequence of the SCC-like excision module

The sequences of the *attR* and *attL* direct repeats plus their flanking inverted repeats were amplified from the SCC*mec* of strain N315 as described in the Method section and in Figure 2A, and interspaced with three consecutive transcriptional terminators, namely the T1 terminator of the *E. coli rrnB* gene followed by two transcriptional terminators of the *blaZ* gene from plasmid pCN50 [108]. This SCC-like sequence was transformed into the GFP-expression vector pGFP1 (Table 1) to produce the pTRAP reporter system, as described in Figure 2.

The excision-reporter module consisted of the GFP-expressing vector pGFP1 (Table 1) in which the excision module described above was inserted between the promoter and the ribosomal binding site, thus disrupting GFP transcription (Figure 2).

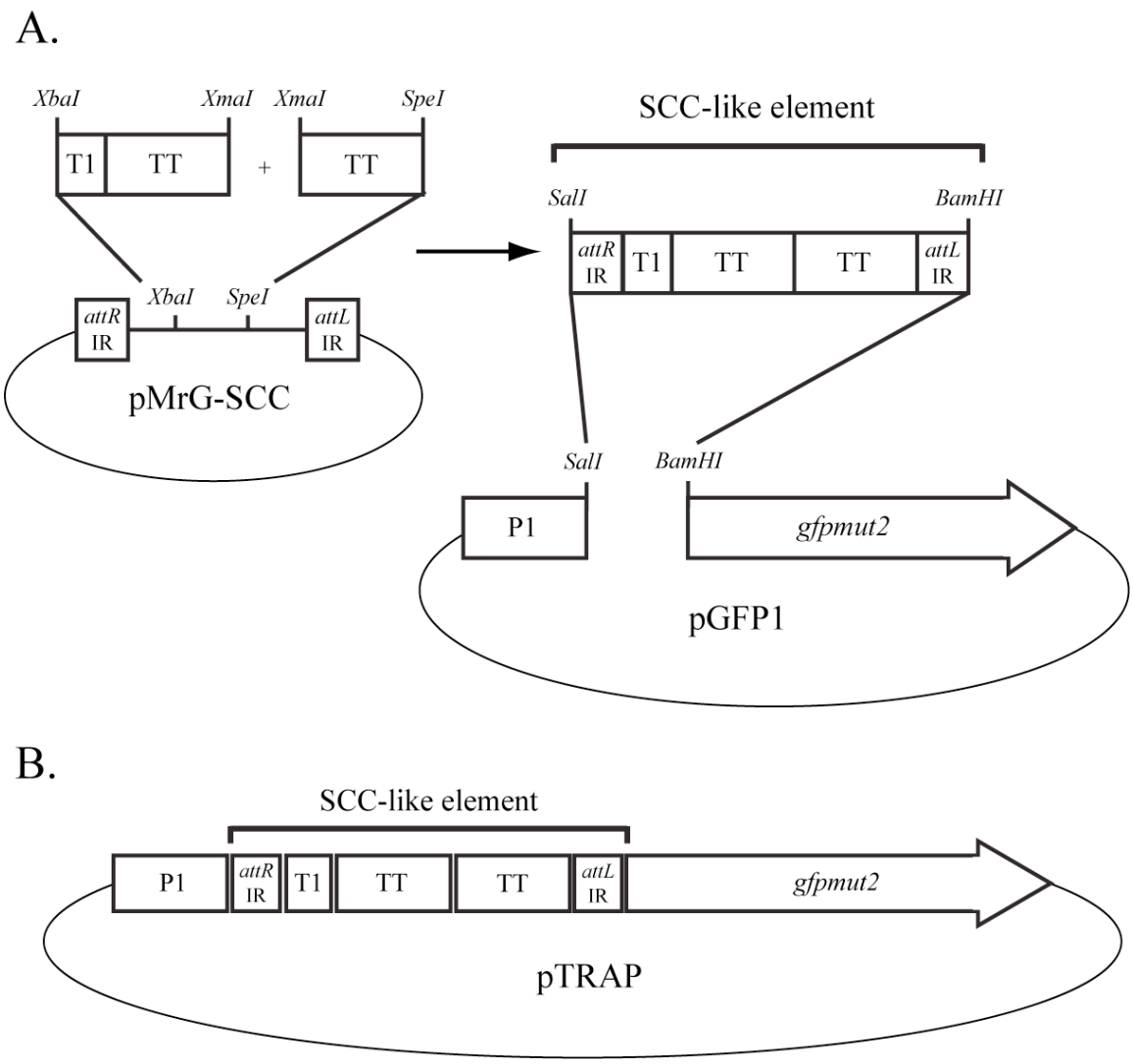

Figure 2 – Three-steps construction of pTRAP

(A) Three consecutive transcription terminators (TT) were inserted between the SCC*mec* right and left repeats in the synthetic plasmid pMrG-SCC. The two TT were amplified from the *blaZ* gene of plasmid pCN50 (Table 1) and the factor-independent T1 terminator of the ribosomal *rrnB* gene from *E. coli* was included in the forward primer. Finally, this SCC-like element, which contained the SCCmec right and left repeats separated by transcription terminators, was cloned by PCR and inserted between the promoter and the ribosomal binding site of the gfpmut2 gene in GPF-expression plasmid pGFP1 (Table 1) to yield pTRAP. (B) General scheme of the pTRAP plasmid.

This construct, named pTRAP, should not express fluorescence unless the SCC-like element is excised and thus restores the continuity between the GFP gene and its promoter. Of note, preliminary experiments in which only one transcriptional terminator (cloned from *blaZ*; Figure 2) was inserted between the SCC-like inverted repeats demonstrated GFP leakage. This was overcome by inserting three successive transcription terminators in the construct (Figure 2), which allowed GFP silencing.

4.2 *Functionality of pTRAP*

It was assumed that CcrAB recombinases expressed by methicillin-resistant staphylococci carrying SCC*mec* would catalyze the excision of the SCC-like element from pTRAP, which should result in a high GFP signal. Conversely, the SCC-like element should not be excised from pTRAP transformed into SCC*mec*-positive staphylococci, which does not express the *ccrAB* genes. A first proof of concept of these functionalities was tested by transforming pTRAP into either the parent methicillin-resistant *S. aureus* (MRSA) N315 or its SCC*mec*-negative mutant N315EX (Table 1). Transformants were selected for tetracycline-resistance, grown in TSB up to the exponential phase (3h after inoculation), and sampled for FACS analysis as described (Chapter 2). Figure 3 shows that N315 cells transformed with pTRAP contained a small subpopulation (0.4%) expressing fluorescence, indicating that excision had occurred. In comparison, none of the N315EX cells transformed with pTRAP were fluorescent, whereas all the positive control cells carrying the constitutive GFP-expression plasmid pGFP1 showed fluorescence. Thus, SCC*mec*-encoded CcrAB could indeed trigger excision of the pTRAP SCC-like element. Moreover, both CcrAB and the *attR* and *attL* plus their inverted repeats were sufficient, and presumably essential, for this process.

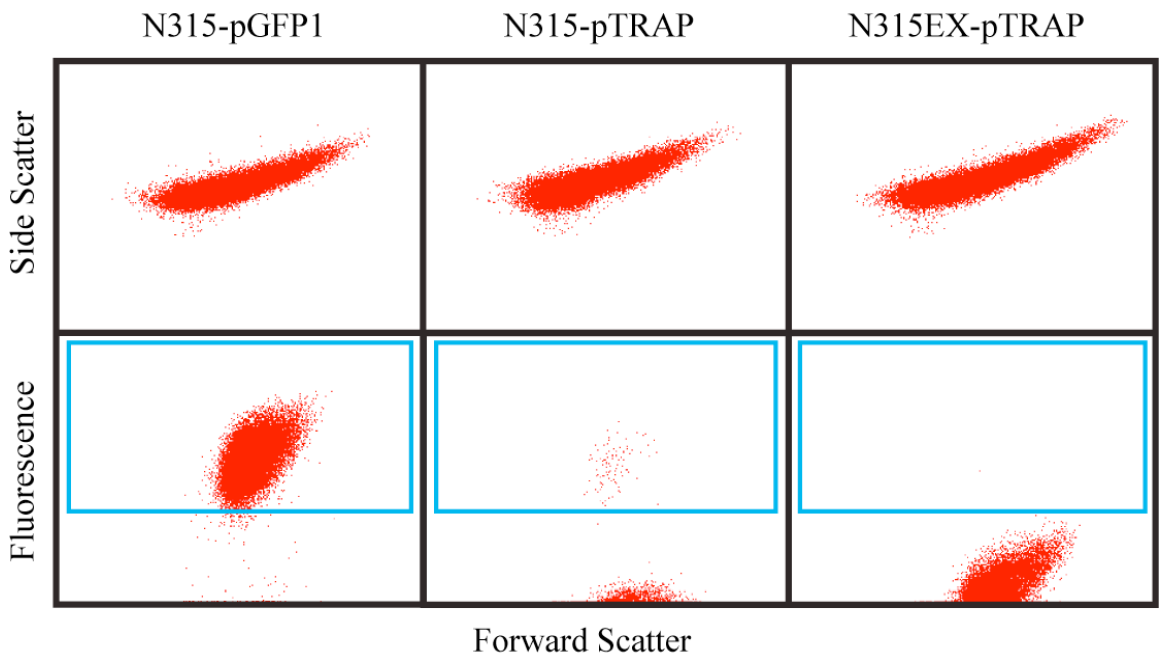

Figure 3 – Flow cytometry analysis

Fluorescence expression profiles of (A) MRSA N315 transformed with the constitutive GFP-expression plasmid pGFP1, (B) MRSA N315 transformed with the pTRAP reporter plasmid, and (C) SCC*mec*-negative mutant N315EX transformed with the pTRAP reporter plasmid. The abscissa and the forward scatter, and the ordinate indicates fluorescence and side scatter, respectively. Gated cells show the subpopulation expressing GFP. For each measurement, gates were arbitrarily determined with respect to the negative control.

4.3 *Spontaneous excision occurred in pTRAP*

To confirm the occurrence of SCC-like excision in pTRAP we sorted non-fluorescent populations (which carried an intact pTRAP) and fluorescent populations (which underwent pTRAP excision) by FACS, and spread them on tetracycline-containing TSA to ensure pTRAP stability. Plates were incubated for 24h at 37°C and visually screened for fluorescent colonies. Figure 4A and 4B show that the non-fluorescent and fluorescent populations segregated essentially in non-fluorescent and fluorescent colony progenies. However, closer examination (by a 2 time digital magnification) of the non-fluorescent colonies depicted in Figure 4A allowed visualizing intra-colony segments of fluorescent cells (depicted in Figure 4C), which indicated that *de novo* excision had occurred during overnight growth on agar

plates of these colonies. Therefore, pTRAP allowed detecting spontaneous excision both by FACS and by agar plating.

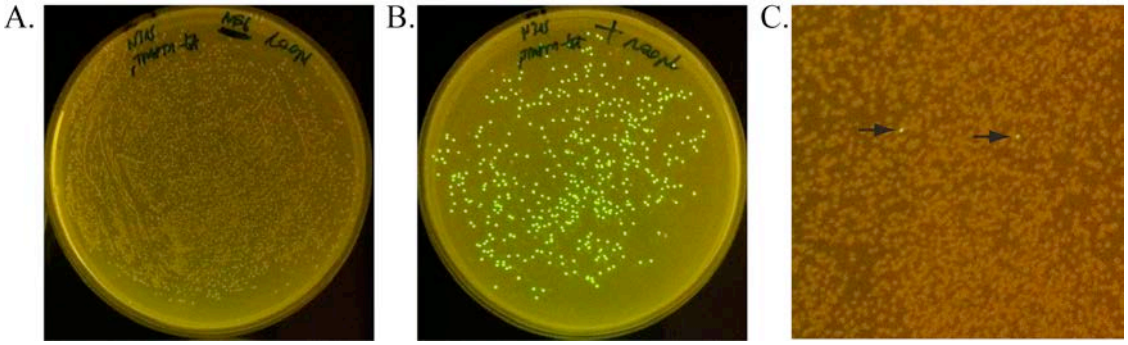

Figure 4 – Plating cells sorted by flow cytometry
Populations of fluorescent (ca. 1500 CFU) and non-fluorescent cells (ca. 400 CFU) were recovered by FACS, plated on tetracycline-containing TSA, and incubated for 24 h at 37°C. Plates were examined visually for fluorescent colonies under a transilluminator. (A) Colony progeny of a non-fluorescent population. (B) Colony progeny of a fluorescent population. (C) Colony progeny of a non-fluorescent population magnified digitally by 2 times. Arrows indicate intra-colony segments of fluorescent bacteria that underwent *de novo* excision of the SCC-like element from pTRAP during colony growth.

4.4 *pTRAP detection of excision in various staphylococcal backgrounds*

One purpose of pTRAP construction was that it could be used as an excision-reporter system in different staphylococcal backgrounds. We tested this possibility by transforming pTRAP in a series of unrelated strains of MRSA and methicillin-resistant *S. epidermidis* (MRSE) RP62A (Table 1), and exposing them to various environmental stresses. Bacteria were grown at 37°C in TSB alone or supplemented with either oxacillin (4 mg/l) or mitomycin (0.5 mg/l), and collected for flow cytometry analyses as described (Chapter 2). The results are shown in Figure 5. In TSB, a basal frequency of fluorescence activation of approximately 10^{-3} was observed in all strains. In the presence of sub-inhibitory concentrations of oxacillin, activation of fluorescence was unaffected or tended to decrease in *S. aureus* N315, MW2 and *S.*

epidermidis RP62A, which was consistent with results obtained in chapters 2 and 3. On the other hand, it substantially increased (by ≥5 times) in community-acquired MRSA (CA-MRSA) H9008 and H19768. Likewise, CA-MRSA H19768 and MRSE RP62A further increased the proportion of fluorescent cells when grown in mitomycin C. Therefore, pTRAP could detect both inter-strain and intra-strain differences in excision activity in various conditions. However, additional experiments in additional strains and conditions will be needed to assess whether a clear strain-specificity patterns exist.

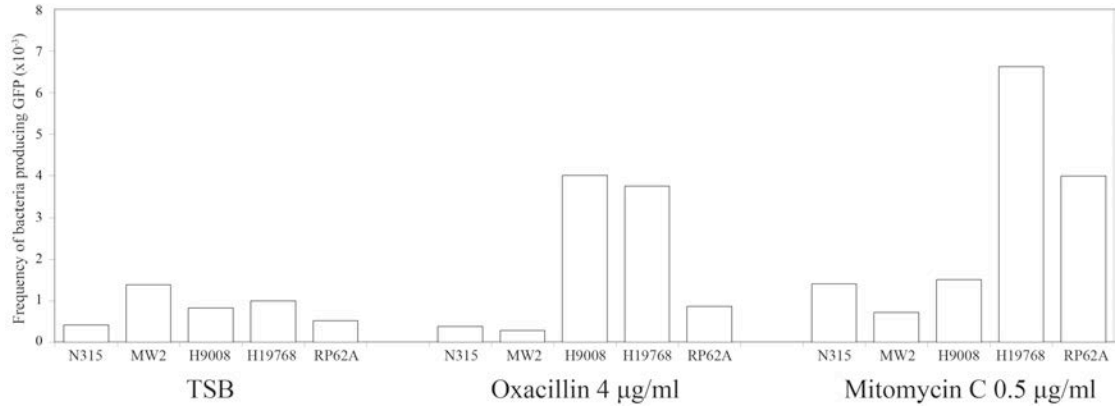

Figure 5 – Influence of external factors on pTRAP excision

The pTRAP reporter was transformed into the various methicillin-resistant staphylococci indicated at the bottom of the Figure (see also Table 1). Cultures were grown either in plain TSB at 37°C (TSB) or in TSB supplemented with 4 mg/ml of oxacillin or 0.5 mg/ml of mitomycin C. Samples were removed from the cultures 3h after inoculation (exponential growth phase), diluted in phosphate saline buffer in order not to exceed 800 FACS events per seconds, and fluorescence of 20,000 events was recorded for each sample.

5. Discussion

Excision of SCC, including SCC*mec*, is a critical step for their transfer and propagation in other strains. Therefore, understanding the circumstances and the molecular mechanisms of SCC*mec* excision is relevant to appraise the evolution of methicillin-resistance epidemiology and maybe interfere with it. Previous work by others and by us showed that excision of SCC*mec* might occur spontaneously or experimentally, that it depended on SCC*mec*-encoded recombinases as well on SCC*mec*-independent regulatory factors, and that site-specific excision involved specific DNA-flanking regions of the cassette [81, 86, 90]. Moreover, expression of the recombinases obeyed a bistable pattern, which could be influenced by bacterial density and other environmental conditions such as antimicrobial drugs (Chapter 2 and 3, [91]). However, all these results were obtained with sophisticated experimental tools (including qPCR, flow cytometry, protein-protein two hybrid systems etc.) that are not easily amenable to study SCC*mec* excision in large numbers of methicillin-resistant staphylococci, in which excision frequency may indeed vary from strain to strain and in different conditions.

Excision reporters were already used to assess the intracellular mobility of the conjugative transposon Tn916 in Gram-positive bacteria [134]. They were also used in eukaryotic systems, especially in the application of Cre-loxP excision system [135, 136]. Here we propose an excision reporter plasmid that may be appropriate for this purpose. It was constructed in a low-copy number plasmid that could replicate in a variety of staphylococcal strains and staphylococcal species [108]. Moreover, the pTRAP construction tested herein could detect excision related to several *ccrAB* allotypes, thus allowing investigating the whole SCC*mec* types sub-family, containing *ccrAB* genes. In principle, the system could easily be extended to study SCC*mec* types V and VII, by slightly modifying the construction in order to include the specific *attR* and *attL* plus their inverted repeats. Of note, however, we also tested the possibility of *ccrC* excision of a *ccrAB*-like cassette in the present experiment, i.e.

H19768. The results indicated that this was possible, which comes in contradiction with previous works [85]. Further experiments are needed to clarify the mechanisms of this issue.

In the proof of concept experiments presented above, pTRAP detected excision in SCC*mec* positive MRSA N315, but not in its SCC*mec*-negative mutant N315EX. This confirmed its dependency on SCC*mec*-related factors, i.e. primarily the *ccrAB* genes. However, the expression *ccrAB* genes themselves was dependent on additional determinants that were located outside of the SCC*mec* element in the staphylococcal genome and could vary between different strains (Chapter 2). The pTRAP reporter should be useful to study the conditions triggering these extra SCC*mec* determinants, and could help identify the circumstances and timing at which they occur. This could be critical to design time-course experiments using complementary technologies to identify the key-players of excision, including time-laps proteomics and protein-DNA and protein-proteins hybridization assays. Likewise, the pTRAP reporter could also be used to screen for conditions that might inhibit SCC*mec* mobilization, and thus help develop new approaches to block its transfer and spread.

While these are just a few examples of the potential usefulness of the pTRAP system, one can imagine many other potential applications. Indeed, beyond the construction presented herein one could test other SCC*mec* flanking regions to detect possible complementary DNA regulatory elements. For instance, in one preliminary attempt to sequence the remnant site of pTRAP after excision of its SCC-like element, it occurred that the 15 bp attachment site, which should be the hallmark of SCC*mec* excision, was missing from the remaining plasmid. In this case, one might speculate that CcrB illegitimate excision had occurred, because the inverted repeats included in the construct were too short to allow appropriate positioning of CcrB on the excision site.

Taken together, the conceptually straightforward reporter system described herein appeared useful to identify specific conditions prone to trigger SCC*mec* excision in

methicillin-resistant staphylococci. It confirmed that intrinsic rates of excision varied between different strains, and that excision in different strains varied regarding to environmental stresses. While this is pertinent to understand the spread of methicillin-resistance, which is a public health concern, the same concept might be used to study excision in other mobile genetic elements (MGEs) as well. This may be particularly pertinent to the evolution of staphylococcal pathogenesis, since the large majority of virulence genes are carried by MGEs in these organisms [11, 20]. Therefore, the system could become of much more general interest in the study of mobile DNA.

CHAPTER 6

Conclusion and perspectives

Horizontal transfer of the SCC*mec* cassette is a fascinating subject, which is of great medical concern. Like other MGEs, SCC*mec* can get inserted at a specific site of the staphylococcal chromosome, and excise itself to be transmitted to other strains under unknown conditions. Hence, several key issues in this process are still not completely understood. For instance, SCC*mec* arose from an SCC ancestor, which seems to be a more generalized cassette capable of capturing environmental genes that might be useful for the recipient bacteria. This might help the microbial host to survive in specific environmental circumstances, and thus benefits the multiplication and spread of the cassette. Moreover, SCCs are quite ubiquitous among *Staphylococcus* spp. Thus they are likely to be much more widespread than expected, and possibly participate to the trafficking of many more genes than originally imagined.

SCC*mec* was fortuitously discovered before other SCCs because it conveyed the gene (*mecA*) responsible for methicillin resistance in *S. aureus*, and thus appeared of great medical interest. Therefore, most studies have concentrated on SCC*mec* rather than on non-*mec* SCCs. However, SCCs are so generalized that they are of special interest to have further insights in gene trafficking and genome evolution in staphylococci. Before SCC themselves, it would be interesting to reconstruct their origin and their environmental niches. At the level of SCCs, it would be important to understand their reservoir, and the array of genes they convey. From this point of view, more effort should be put in understanding the role of SCCs in the *Staphylococcus* genus, especially in CoNS, which seem to carry SCC*mec* and other SCCs at high rates. Beyond SCCs, and especially regarding to SCC*mec*, it would be critical to know more about the mechanisms of their transfer, as well as the array of bacterial species, and maybe bacterial genus, in which they can evolve. Indeed, similar elements were recently described in the *Enterococcus* genus [137]. Conversely, it would also be relevant to learn more about the natural limits of their expansion, for instance through host factors such as restriction systems, incompatibilities of insertion sites, or fitness cost that would counter-

select against the element in certain ecological niches. All these questions are scientifically relevant for the understanding of biological mechanisms, epidemiological spread, and possibly for the development of means to interfere with the spread of methicillin resistance.

The results presented herein enlighten some of theses aspects. On the level of SCC*mec* excision, it is quite pertinent to learn that recombinase genes are expressed via a bistable pattern (Chapter 2), and that they are likely to be regulated by strain-dependent determinants that are not encoded on the SCC*mec* cassette itself, but elsewhere on the staphylococcal genome. This is not unique to SCC*mec*, since bistability was observed in other MGEs [82], and may help understanding strain-dependency of SCC*mec* transmission, which happens to be an ongoing question in the context of MRSA (see below). Moreover, it is intriguing from the evolutionary point of view. Indeed, it may be the "choice" of the SCC*mec* cassette to integrate into one rather than another host genome, in order to ensure either its stabilization, or its transmission, or both. In this regard, it will be very important to study more strains and additional conditions that might promote or inhibit the expression of the *ccr* recombinase genes. Such experiments are ongoing using the system described above.

The expression of recombinases should result in SCC*mec* excision. The present results indicate that *de novo* excision is likely to occur during a limited period of time, and relatively early during growth. This phenomenon is reminiscent of other cell-concentration dependent gene regulation occurrences, such as the induction of competency for DNA transformation in pneumococci and a few other organisms [118, 138], and the *agr*-related expression toxins and superantigens in *S. aureus* [139]. Since many toxins and superantigens that are carried by MGEs succeeded in putting themselves under *agr*-regulation in *S. aureus*, one may wonder whether *ccr* genes might not have done the same. Based on the present observation, the circumstances and genetic conditions influencing *ccr* gene induction and SCC*mec* excision will be further studied, for instance by using spent versus fresh growth medium, in order to

attempt purifying possible extracellular signaling molecules, of by testing various types of regulatory mutants, including *agr* mutants, mutant in two-component regulatory systems, and mutants in in DNA-binding regulators [139, 140].

There is a wide array of questions and experiments that can be imagined to attempt gaining a deeper view in these issues. One of them is the importance of the 3' *attB* polymorphism, which may be more or less prone to "accept" the integration of incoming SCC*mec* elements. Most interestingly, this polymorphism obeys the MLST-based phylogeny of *S. aureus*, in spite of the fact that the region is not part of the set of genes taken into account by MLST and was described as highly variable by other studies (Chapter 4, [101]). This reveals clone-specific co-evolution of this region. The question then arises as to whether some *S. aureus* CCs might be more prone than other CCs to integrate incoming SCC*mec* cassettes, and thus become MRSAs. This elegant hypothesis seems to be challenged by a very recent publication by Wang et al. [90], who showed that 3' *attB* polymorphisms that were thought to be refractory to SCC*mec* integration (which correlate with specific CCs in our hands) are actually not refractory when tested in a dual plasmid-based integration system testing *att*-DNA stretches of interest.

This provocative observation is quite stirring, because challenges the nascent dogma of sequence-specific restriction of SCC*mec* integration in MSSA, which could impede the transmission of methicillin resistance into certain CCs. In other words, if 3' *attB* polymorphism is not involved in the restriction of SCC*mec* spread, then other mechanisms must be responsible, such as restriction systems, fitness cost, or merely the lack of opportunity to encounter either SCC*mec* donors, or conditions promoting SCC*mec* transfer. Most importantly, the fact that we could match the 3' *attB* polymorphism with specific CCs will help us identify the refractory the CCs of interest, and study them further.

As mentioned, investigating these questions at a more global level will need appropriate molecular reporter tools that can be used at a broad scale of strain diversity. The pTRAP system described in Chapter 5 should greatly help study these features in multiple strains, at least with regard to excision. Especially, this concept of reporter system – and adaptations of it – should be amenable to study excision not only of SCC*mec*, but also of other SCC elements as well as MGEs in a larger context. Moreover, it could also be invaluable to screen for conditions of drugs that would inhibit excision, and thus interfere with transmission of methicillin resistance and maybe other virulence genes as well. Such experiments are planed in future work.

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CURRICULUM VITAE

Miloš Stojanov
Born in Novi Sad, Serbia on November 16th 1982
Nationality: Swiss and Serbian

WORK EXPERIENCE

• Dates (from – to)	03. 2007 – 05. 2007
• Name and address of employer	Institute of Occupational Health Sciences – 19, Rue du Bugnon, 1005 Lausanne, Switzerland
• Type of business or sector	Health research
• Occupation or position held	Training course
• Main activities and responsibilities	Development of a qPCR assay for the quantification of *Aspergillus fumigatus* spores

EDUCATION

• Dates (from – to)	11. 2007 – 10. 2012
• Name and type of organisation providing education and training	University of Lausanne – Faculty of biology and medicine – Doctoral school
• Title of qualification awarded	Doctorate in life sciences (PhD) *Thesis:* "Excision of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) and its crucial role in the horizontal transfer of methicillin resistance in staphylococci"
• Dates (from – to)	10. 2005 – 02. 2007
• Name and type of organisation providing education and training	University of Lausanne – Faculty of biology and medicine
• Title of qualification awarded	Master of science (MSc) in Genomics and Experimental Biology *Master thesis:* "Horizontal gene transfer and genomic islands: the *clc* element" *First Step project:* "Characterization of a novel insecticidal protein produced by *Pseudomonas fluorescens* CHA0, a beneficial root-colonizing strain"
• Dates (from – to)	10. 2002 – 07. 2005
• Name and type of organisation providing education and training	University of Lausanne – Faculty of biology and medicine
• Title of qualification awarded	Bachelor of science (BSc) in biology
• Dates (from – to)	09. 1998 – 06. 2002
• Name and type of organisation providing education and training	Cantonal High School of Locarno (Ticino)
• Title of qualification awarded	Scientific Maturity

PUBLICATIONS

Minoia M, Gaillard M, Reinhard F, **Stojanov M**, Sentchilo V, van der Meer JR. Stochasticity and bistability in horizontal transfer control of a genomic island in Pseudomonas.
Proceedings of the National Academy of Sciences of the United States of America 2008; **105**: 20792-7.

Stojanov M, Sakwinska O, Moreillon P.
Expression of SCC*mec* Cassette Chromosome Recombinases in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*.
Journal of Antimicrobial Chemotherapy (Accepted with modifications)

Stojanov M, Moreillon P, Sakwinska O.
Excision of Staphylococcal Cassette Chromosome *mec* (SCC*mec*) in methicillin-resistant *Staphylococcus aureus* (MRSA) assessed by quantitative PCR.
Submitted to *Journal of Antimicrobial Chemotherapy*

Stojanov M, Moreillon P, Sakwinska O.
Staphylococcal Cassette Chromosome mec (SCC*mec*) insertion site in methicillin susceptible *Staphylococcus aureus* (MSSA).
In preparation

CONGRESSES

International congresses

49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco (USA), 12-15.09.2009
Poster presentation: "Cassette Chromosome Recombinase expression in MRSA".

50th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston (USA), 12-15.09.2010
Poster presentation: "The dynamics of SCC*mec* excision in methicillin-resistant *Staphylococcus aureus* (MRSA)".

51st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago (USA), 17-20.09.2011
Poster presentation: "Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Excision in Methicillin-resistant Staphylococcus aureus (MRSA) assessed by an Extrachromosomal Excision Reporter".

52nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco (USA), 09-12.09.2012
Oral presentation: "Dynamics of excision of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) in Methicillin-resistant *Staphylococcus aureus* (MRSA)".

National congresses

68th Assembly of the Swiss Society for Microbiology, Lausanne (Switzerland), 04-05.06.2009
Poster presentation: "Ccr recombinase expression in methicillin resistant *Staphylococcus aureus"*

69th Assembly of the Swiss Society for Microbiology, Zurich (Switzerland), 24-25.06.2010
Poster presentation: “The dynamics of SCC*mec* excision in methicillin-resistant *Staphylococcus aureus* (MRSA)”

71th Assembly of the Swiss Society for Microbiology, St. Gallen (Switzerland), 21-22.06.2012
Poster presentation: “Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Excision in Methicillin-resistant Staphylococcus aureus (MRSA) assessed by an Extrachromosomal Excision Reporter”.

5th Swiss Molecular Microbiology Workshop, Thun (Switzerland), 24-26.03.2010
Poster presentation: “Excision of the genomic island SCC*mec*”