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The evolutionary pathway of the staphylococcal cassette chromosome element

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Abstract: The staphylococcal cassette chromosome (SCC) element can carry resistance genes to antibiotics, disinfectants, and heavy metals, contributing to the survival of strains in the environment and causing difficulties in the treatment of staphylococcal infections. Methicillin resistance in staphylococci, which is of particular clinical significance, is encoded by staphylococcal cassette chromosome *mec* (SCC*mec*). Despite the importance of the SCC element and description of multiple nucleotide sequences, the information about its origin and evolution is still scarce. Here, we present a phylogenetic analysis of SCC elements that is unique in the use of whole SCC sequences. A phylogenetic tree for a noteworthy number of 81 SCC elements based on global sequence alignment was constructed. The SCC clustering did not reflect the genetic relationships of bacteria containing the SCC elements, but was done according to type, determined by the combination of *mec* gene complex class and *ccr* gene complex type. The results emphasise the horizontal gene transfer as a means of spread of SCC elements in bacterial strains. Overall, this study contributes to the understanding of SCC emergence, evolution, and dissemination.

Key words: *Staphylococcus*; methicillin-resistant *Staphylococcus aureus* (MRSA); staphylococcal cassette chromosome; evolution; mobile genetic elements.

Abbreviations: ACME, arginine catabolic mobile element; CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*; CC, clonal complex; *ccr*, chromosome cassette recombinase; CoNS, coagulase-negative staphylococci; HA-MRSA, hospital-acquired methicillin-resistant *Staphylococcus aureus*; HGT, horizontal gene transfer; LCB, locally collinear block; MGE, mobile genetic element; MLST, multilocus sequence typing; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; SCC, staphylococcal cassette chromosome; SCC*mec*, staphylococcal cassette chromosome *mec*; ST, sequence type.

Introduction

Staphylococci are major human and veterinary pathogens causing local and systemic infections. The pathogenicity of these bacteria depends on the production of virulence factors and content of antimicrobial resistance determinants. Genes for the virulence and resistance factors are carried on different types of mobile genetic elements (MGEs), such as genomic islands, pathogenicity islands, prophages, plasmids, and staphylococcal cassette chromosome (SCC), which can be horizontally transferred. The newly acquired genes responsible for virulence and antibiotic resistance are thus rapidly disseminating in the staphylococcal population, resulting in the formation of new successful clones. Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major medical problem in the recent years.

Methicillin resistance is associated with the acquisition of the *mecA* gene through horizontal gene transfer (HGT). The methicillin resistance gene is carried on a mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*), which inserts itself in the *orfX* gene encoding a ribosomal methyltransferase (Boundy et al. 2013). The cassette has a mosaic structure and can carry multiple genes for virulence and resistance factors. To date, 12 types of SCC*mec* elements (I-XII) have been described (IWG-SCC 2009; Wu et al. 2015). The SCC*mec* classification is based on the combination of two gene complexes: the *mec* gene complex, containing the *mecA* gene, and the chromosome cassette recombinase (*ccr*) gene complex, containing one *ccrC* or two *ccrAB* serine recombinases (Shore & Coleman 2013).

In the beginning, MRSA was predominantly a nosocomial pathogen; the first hospital-acquired MRSA (HA-MRSA) strain was isolated shortly after the introduction of methicillin in clinical practice, in 1961 (Jevons et al. 1961). This HA-MRSA clone belonged to sequence type (ST) 250 from clonal complex (CC) 8 and carried SCC*mec* I (Enright et al. 2002). Several pandemic waves of HA-MRSA with SCC*mec* I, II, or

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III have been observed since then (DeLeo & Chambers 2009). Afterwards, the first case of community-acquired MRSA (CA-MRSA) infection was documented in 1993 in Western Australia (Udo et al. 1993). CA-MRSA typically carries SCC*mec* IV (Okuma et al. 2002). The number of CA-MRSA infections was increasing in the 1990s and some of the CA-MRSA clones (e.g. MRSA-ST8-IV) have been replacing typical HA-MRSA strains in hospitals. Nowadays, five main pandemic clones ST5 (CC5), ST8 (CC8), ST22 (CC22), ST36 (CC30), and ST45 (CC45) defined by multilocus sequence typing (MLST) have been established as a cause of nosocomial infections (DeLeo et al. 2010). Moreover, in the past two decades there has been an increase in MRSA infections and carriage in individuals with no connection to health care, but with exposure to livestock. These livestock-associated MRSA belong to CC398 and were first recognized among pig farmers in France and the Netherlands in the early 2000s (Armand-Lefevre et al. 2005; Voss et al. 2005). Since then, *S. aureus* isolates from CC398 have been reported in diverse livestock hosts in many countries around the world (Verkade & Kluytmans 2014).

SCC elements are not found only in *S. aureus*, but across the whole genus *Staphylococcus*. The origin of SCC*mec* is still discussed, but it was suggested that *S. aureus* acquired the element from coagulase-negative staphylococci (CoNS). This mobile element could originate in *Staphylococcus sciuri* group and it seems that *Staphylococcus fleurettii* was the source of the *mecA* region (Tsubakishita et al. 2010b). Since many SCC*mec* and related elements were found in CoNS, such as *Staphylococcus epidermidis* (Wisplinghoff et al. 2003), *Staphylococcus hominis* (Bouchami et al. 2011), *Staphylococcus haemolyticus* (Urushibara et al. 2011), and other species (Zong et al. 2011; Vanderhaeghen et al. 2012; Perreten et al. 2013), CoNS may represent a potential reservoir for this element (Otto 2013). However, the possibility that the main source of SCC*mec* could be MRSA itself cannot be ruled out (Aires de Sousa & de Lencastre 2004).

The acquisition of the SCC*mec* element by methicillin-susceptible *S. aureus* (MSSA) is crucial for the bacterium to become a successful MRSA pathogen. Yet, little is known about the origin and course of evolution of this element. To address this issue, we studied the phylogenetic relationships between the SCC elements using a computational analysis. Unlike previous studies, we used whole sequences of the SCC-elements. The results of the work clarify some of the evolutionary pathways of the SCC elements and broaden the general knowledge about their origin and spread in MRSA strains.

Material and methods

Sequence data

Sequences of SCC elements were downloaded from the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore). The borders of SCC elements were determined either from the annotation of the sequence or were established by the identification of the direct and inverted repeats in the attachment site of the *orfX* gene. Composite islands in *Staphylococcus saprophyticus* strain ATCC 15305 (accession number AP008934) and *S. aureus* strain WAMRSA40 (accession number JQ746621) were divided into separate SCC elements. SCC*mec* elements in *S. aureus* strain M1, M08/0126, and Sa0059 were included in the analysis with the insertion of arginine catabolic mobile element (ACME). The inclusion of the ACME traces the evolution of the examined chromosomal region.

MLST types of strains analysed in this study were obtained from the web-sites http://pubmlst.org (Jolley & Maiden 2010) and http://saureus.mlst.net (Enright et al. 2000).

Phylogenetic analysis

SCC sequences were aligned using Mauve v.2.3.1. with the progressive Mauve algorithm (Darling et al. 2010; http://darlinglab.org/mauve/). Mauve divides the whole sequences into short segments – the locally collinear blocks (LCBs). The LCBs are separately aligned in a specific format, so custom-designed script was used to concatenate the LCBs of each sequence into a fasta file. Afterwards, the concatenated LCBs covering the whole sequences were realigned using Mafft v.7.158 with the auto-method (Katoh & Standley 2013; http://mafft.cbrc.jp/alignment/software/). For evolutionary model prediction, the JmodelTest2 (Guindon & Gascuel 2003; Darriba et al. 2012; https://github.com/ddarriba/jmodeltest2/) software was used with the Akaike information criterion test setting. As the best model, the program predicted GTR + gamma + inv; hence the GTR + gamma model was employed when constructing the maximum-likelihood tree using RAxML v.7.2.8. (Stamatakis et al. 2007; http://sco.h-its.org/exelixis/web/software/raxml/). Node support was evaluated using 5,000 bootstraps cycles.

Results and discussion

Computational analysis

In order to understand the evolution of the SCC elements, we constructed a phylogenetic tree for 81 SCCs downloaded from the NCBI GenBank database. Earlier, Lina et al. (2006) have inferred the evolution of SCC*mec* from *ccr* complex genes and similarly, Urushibara et al. (2011), Rolo et al. (2014), and Park et al. (2013) have focused on *ccr*A and *ccr*B genes; however, we took SCC elements as a whole for the *in silico* analysis. The crucial step of the analysis was the alignment of the SCC element sequences. SCC elements have unique mosaic structures and their evolution is dependent on large scale element rearrangements, including loss and gain of other MGEs (like plasmids and insertion sequences). Most of the computational tools were unable to create an alignment, but Mauve, since its algorithm uses the anchored alignment technique allowing the order of alignment anchors to be rearranged that permits identification of genome rearrangements (Fig. 1). The whole sequences were divided by Mauve into LCBs, which determined the related regions of the SCC elements. The LCBs of each SCC sequence were concatenated and the concatenate was used for alignments. Hence all parts of

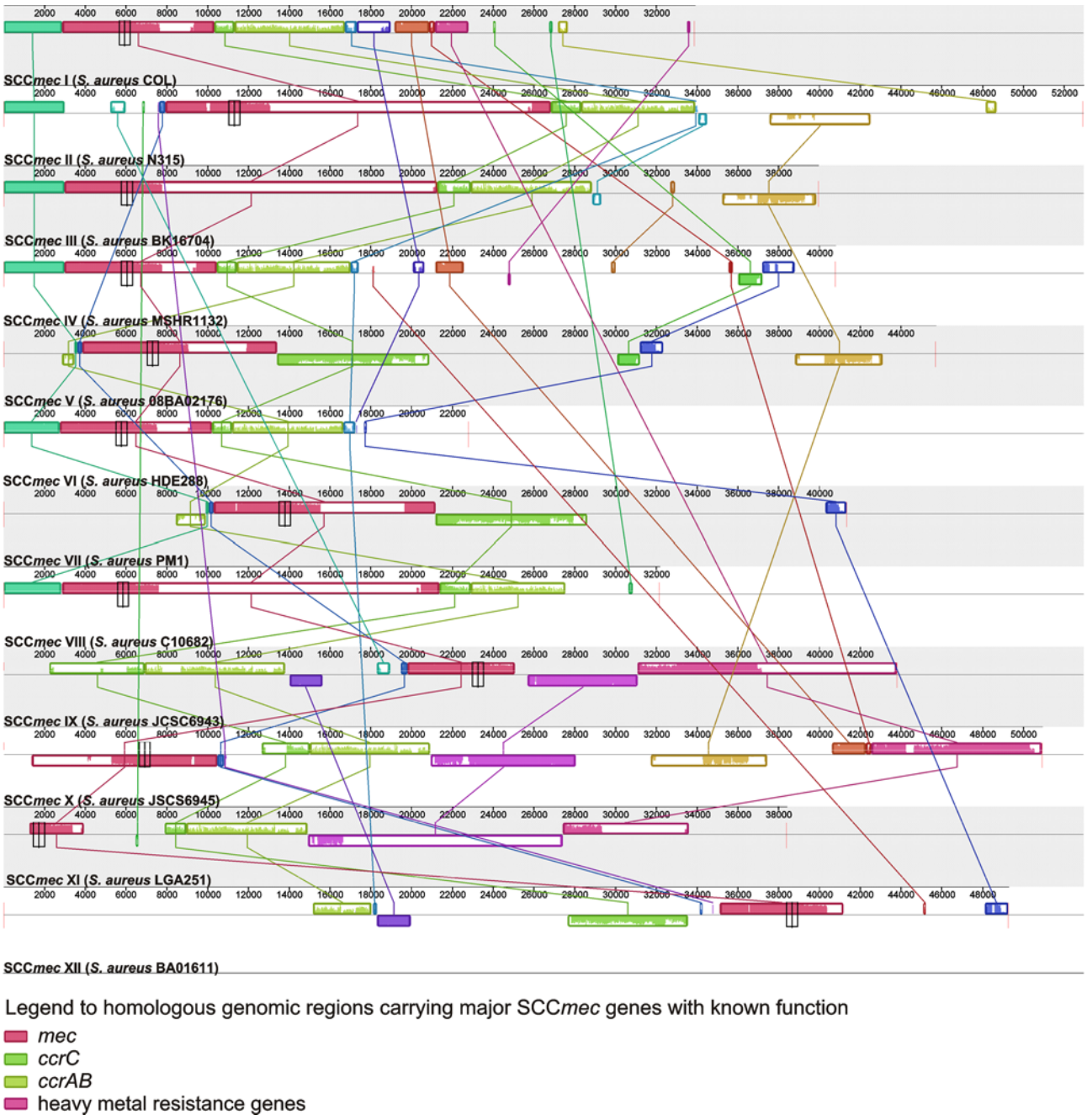

Fig. 1. Mauve-based alignment of 12 SCC*mec* type representatives (I – XII) from *S. aureus* strains. The coloured blocks represent regions of homology between strains as determined by progressive Mauve alignment on default settings. The height of the coloured blocks indicates the alignment score. Blocks below the central line represent sequences that are inverted in comparison to the strain COL (SCC*mec* I) arrangement.

the SCC elements were taken in the phylogenetic analysis. Due to the quantity of the data, RAxML (Stamatakis et al. 2007) was evaluated as the best program for phylogenetic tree construction. The computation with other used programs, such as MrBayes (Ronquist & Huelsenbeck 2003) and PhyML (Guindon et al. 2010) was extremely slow, despite the process parallelization and usage of 12 CPUs/60 GB.

Association of SCCs with clonal complexes

The SCC*mec* of the same type grouped together in the maximum likelihood phylogenetic tree (Fig. 2). This shows that SCCs share an essential part of genetic information. The shared genes are the *mec* and *ccr* gene complexes; hence the classification of SCC*mec* types (IWG-SCC 2009) reflects well their phylogenetic relationships. The topology of the phylogenetic tree shows that SCCs from different STs (CCs) are dispersed throughout several clusters. This may be significant evidence for the introduction of SCC*mec* into some ST (CC) on several occasions as suggested by the evolutionary models of MRSA emergence by Robinson & Enright (2003). It is also possible that strains from one ST (CC) can lose a SCC*mec* and acquire another one (Diep et al. 2006).

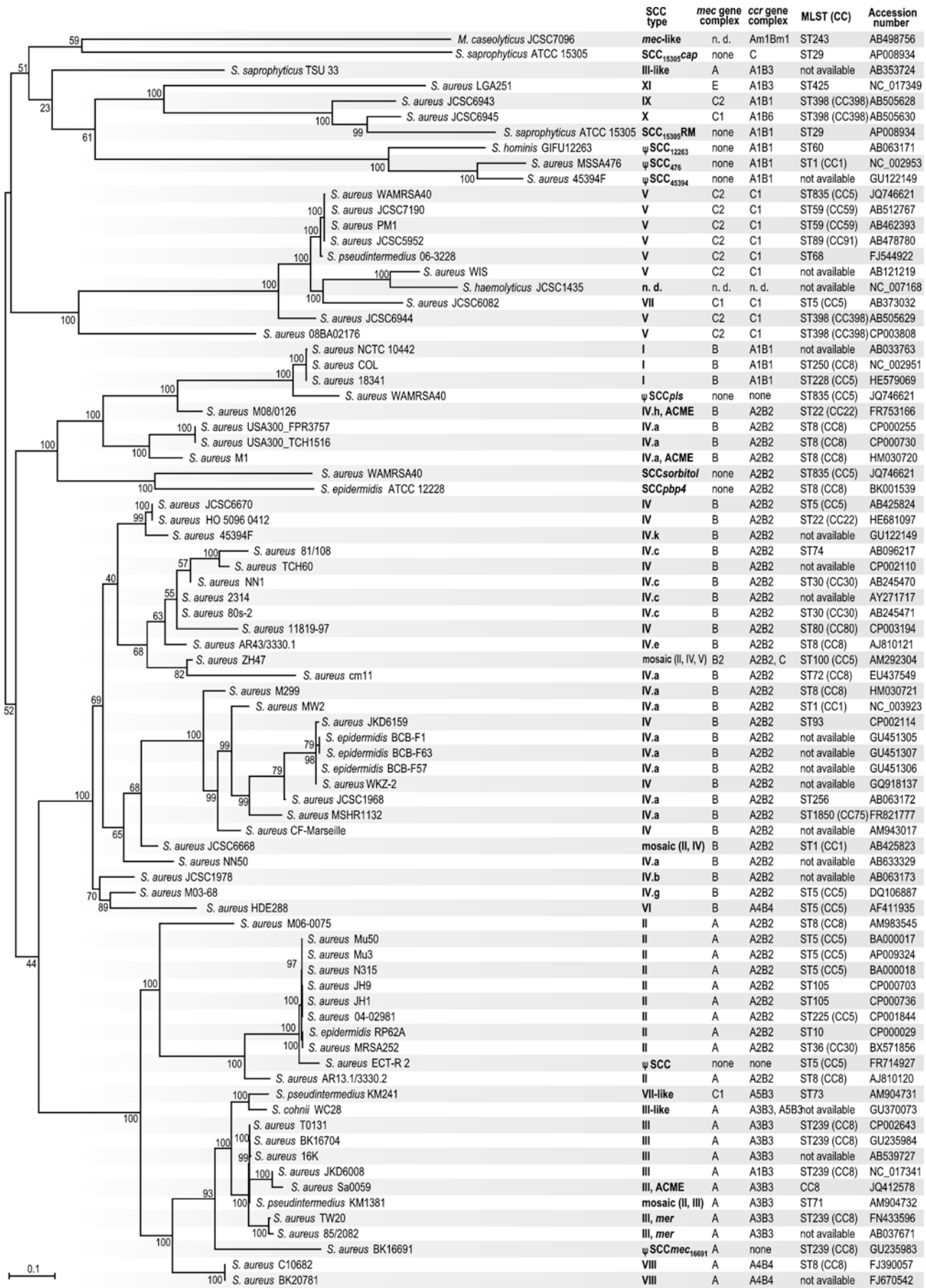

Fig. 2. Unrooted maximum likelihood tree showing phylogenetic relationships among SCC elements in the genus *Staphylococcus*. The phylogenetic tree was inferred using the GTR + gamma nucleotide substitution model. Support after 5000 bootstrap cycles is shown for nodes with confidence value higher than 50. The scale bar indicates an evolutionary distance of 0.1 nucleotides per position in the sequence. n.d., not determined.

Characterization of SCCmec types

SCCmec I, II, and III. SCC*mec* types I, II, and III are considered to be the evolutionary oldest SCC*mec* elements in *S. aureus*. In the analysed data set, SCC*mec* I elements showed a quite close phylogenetic relationship, although their sequences were from two clonal complexes CC5 and CC8. The presence of similar SCC*mec* I in divergent genotypes is perhaps a consequence of HGT (Enright et al. 2002). Similarly, clusters of SCC*mec* II and SCC*mec* III look quite homogeneous with high bootstrap support and the evolution of these SCC*mec* types seems to be a rather slow process. These elements are up to 80 kb long so their intact transfer is difficult. Therefore they may have been introduced into MSSA on only few occasions. The antibiotic pressure in health care facilities may have promoted the maintenance of the MRSA strains with these long elements (Robinson & Enright 2003). The selection pressure also enhances the evolution of SCC*mec* I, II, and III, mainly the capture of other resistance and virulence determinants. Many mobile genetic elements were identified in SCC*mec* I, II, and III, such as pUB110 plasmid encoding resistance to aminoglycosides in SCC*mec* IA (Oliveira & de Lencastre 2002), pT181 plasmid with the mercury resistance operon *mer* and tetracycline resistance genes in SCC*mec* II (Ito et al. 2001), or Tn*554* carrying resistance to macrolides, lincosamides, and cadmium in SCC*mec* III (Ito et al. 2001). The gene encoding phenol-soluble modulin was found in the J1 region of SCC*mec* II and III (Queck et al. 2009). As these SCC*mec* types can be a fitness burden due to their length (Ender et al. 2004; Lee et al. 2007; Knight et al. 2013), clones with smaller SCC*mec* are becoming predominant nosocomial pathogens.

SCCmec IV. SCC*mec* IV elements were the most abundant in the data collection, disseminated in nearly all clonal complexes. Despite their diverse origin, SCC*mec* IV elements formed a compact cluster in the phylogenetic tree with high confidence, which is a good evidence of the tree stability. Our results promote a currently described hypothesis that SCC*mec* IV was repeatedly introduced into different STs (CCs), as was evidenced for CC8. SCC*mec* IV elements from CC8 in the analysis were more distantly related to each other than to SCC*mec* IV from other CCs.

The acquisition of SCC*mec* IV is facilitated mainly by its small size (20-35 kb) (Robinson & Enright 2003) and due to its low cost in fitness, SCC*mec* IV is widely disseminated in the staphylococcal population and is probably the most prevalent type of SCC*mec* in MRSA (Daum et al. 2002). Consequently, MRSA strains with SCC*mec* IV are becoming predominant nosocomial pathogens, as is the case of MRSA strains from ST22 (CC22) (Albrecht et al. 2011; Knight et al. 2012; Kinnevey et al. 2014). It seems that one of the SCC*mec* IV reservoirs for the introduction into *S. aureus* is *S. epidermidis* (Barbier et al. 2010), because SCC*mec* IV.a elements from three isolates of *S. epidermidis* strain BCB included in the analysis were closely related to SCC*mec* IV.a from *S. aureus* strain WKZ-2 (accession number GQ918137). We assume on the basis of our analysis that SCC*mec* IV has evolved on several independent occasions. Lina et al. (2006) have suggested that SCC*mec* IV evolved from SCC*mec* I, and our interpretation of the analysis supports their hypothesis for SCC*mec* IV from *S. aureus* USA300 related strains (USA300_FPR3757, USA300_TCH1516, M08/0126, and M1). The elements from these strains were in a distinct clade together with SCC*mec* I elements, whereas all other SCC*mec* IV elements grouped together in a separate clade. In the aforementioned clades, SCC*mec* IV probably evolved from different ancestral SCC*mec* elements and diversified throughout the transmission in the staphylococcal population.

SCCmec V and VII. The phylogenetic analysis ranged SCC*mec* V in a separate branch apart from other SCC elements, which indicates its independent evolution from other types. The phylogenetic tree topology also reveals that SCC*mec* V and SCC*mec* VII are closely related. This result implies that SCC*mec* V and SCC*mec* VII may have been evolving together, possibly in several recombination events. The independent formation of SCC*mec* V and SCC*mec* VII may have been due to the specificity of CcrC recombinase (Ito et al. 2004). Several *ccrC* genes were identified in chromosomes of CoNS species; therefore, SCC*mec* elements might have emerged in one of the CoNS species. Candidate species for the creation of SCC*mec* V could be *S. haemolyticus* (Bouchami et al. 2012), *S. saprophyticus* (Kuroda et al. 2005), or *S. pseudintermedius* (Descloux et al. 2008; Black et al. 2009). The SCC*mec* V transfer from a CoNS species into *S. aureus* could be frequently happening among staphylococci inhabiting animals, since many SCC*mec* V elements were found in *S. aureus* strains CC398, predominantly associated with animals (Argudin et al. 2010; Price et al. 2012). The exchange of SCC*mec* V element might be enhanced by antibiotic use in the animal farming.

Sporadically detected SCCmec types and non-mec SCC. Sequences of SCC*mec* types VI, VII, VIII, IX, X, and XI, though of limited count, were included in the analysis to obtain more precise results and to ascertain their evolution. Clustering of these types reflected the SCC*mec* classification well; they grouped with other SCC*mec* types with similar *mec* and *ccr* gene complexes. The recently described SCC*mec* type XI carries a variant of the *mecA* element designated as the *mecC* allotype. The *mecC* gene shares 70% nucleotide identity with *mecA* and was identified in *S. aureus* from numerous sources including humans and a range of animals (Cuny et al. 2011; Garcia-Alvarez et al. 2011), as well as in *Staphylococcus xylosus* (Harrison et al. 2013) and *S. sciuri* (Harrison et al. 2014). The *mecC* gene is part of *mec* gene complex class E and this complex is structurally similar to *mec* gene complex from *Macrococcus caseolyticus* with *mecB* gene (Tsubakishita et al. 2010a). However, SCC*mec* XI and SCC*mec*-like from *M. caseolyticus* are probably not related, because they are localized quite distantly in the phylogenetic tree. The SCC*mec*-like elements in *M. caseolyticus* are found

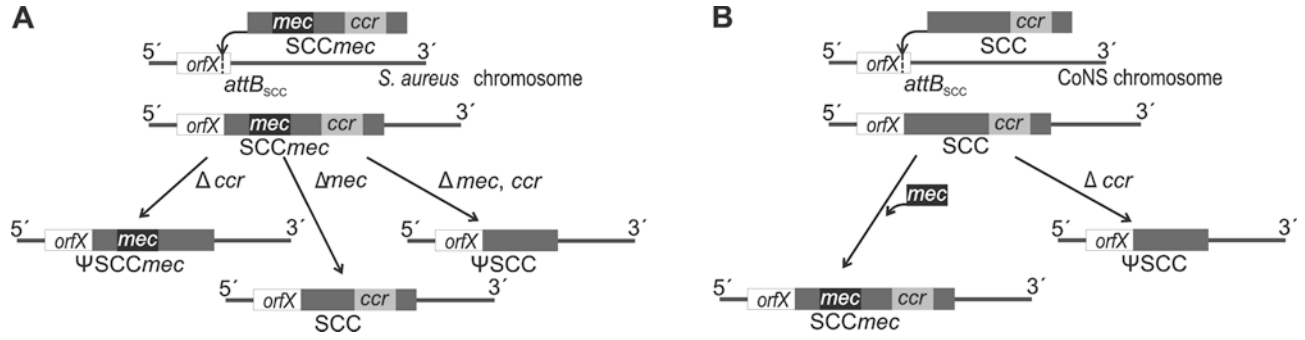

Fig. 3. Proposed hypotheses of the formation and spread of the SCC elements. A. SCC*mec* first, B. SCC first.

in variable parts of the genome including plasmids (Baba et al. 2009). Such extrachromosomal elements could be easier spreading in the bacterial population via HGT.

The non-*mec* SCC elements, such as ψSCC$_{12263}$, ψSCC$_{476}$, and ψSCC$_{45394}$ and SCC carrying various genes SCC*sorbitol*, SCC$_{cap}$ (capsule), SCC$_{15305}$RM (restriction-modification), SCC*pls* (plasmin), and SCC *pbp4* (penicillin-binding protein 4) were in separate clusters and quite distantly related to SCC*mec*.

Dissemination of SCC elements

The results unambiguously support the hypothesis that the SCC element is horizontally transferred probably across the species barrier, since the SCC elements from different species cluster together. A plausible transfer mechanism seems to be the phage mediated transduction (Scharn et al. 2013; Chlebowicz et al. 2014). SCC*mec* or a part of SCC are packaged into phage capsids (Mašlaňová et al. 2013) and if such DNA is injected into the recipient bacterium already carrying an SCC element, new SCC types might be created via recombination (Rolo et al. 2012). Also mutations (Lina et al. 2006) and insertion/excision of the *mec* or *ccr* gene complexes in diverse SCC types (Hanssen et al. 2003) are playing a role in the evolution of SCC. The SCC evolution differs among species of the genus *Staphylococcus*. *S. aureus* harbours the SCC elements irregularly, whereas in other species, e.g. *S. sciuri*, these elements are found in the majority of isolates. We present two hypotheses describing the formation and spread of the SCC and SCC*mec* elements in staphylococci.

The first hypothesis, the "SCC*mec* first", assumes that SCC*mec* harbouring both *mec* and *ccr* complexes was introduced into the staphylococcal chromosome first, and the *mec* or even *ccr* gene complex may have been deleted later (Fig. 3). This mechanism occurs predominantly in *S. aureus*, because *in vivo* recombination and loss of methicillin resistance were observed in this pathogen (Chlebowicz et al. 2010) and remnants of SCC*mec* were detected in clinical MSSA isolates (Donnio et al. 2007).

The second hypothesis, the "SCC first", postulates that SCC elements were inserted in the staphylococcal genome first, and the *mec* gene complex was gained later. This evolutionary step might have happened in a similar way to the possible formation of a SCC*mec*-like element in *M. caseolyticus*. The *mecB* gene in *M. caseolyticus* was found on transposon Tn*6045*. In strain JCSC7096 transposon Tn*6045* was located on the chromosome close to *orfX* next to the SCC element carrying *ccrAB* genes (Tsubakishita et al. 2010a). An inactivation of transposase or deletion of the direct repeats separating the two elements would be sufficient for the creation of a new SCC*mec*.

The CcrAB homologues were found not only in staphylococci and macrococci, but also in enterococci (Bjorkeng et al. 2010), which is of particular interest, because enterococci are mostly methicillin resistant (de Fatima Silva Lopes et al. 2005). Also, enterococcal genes *pbp5*, *pbp3r*, and *pbp4* showed a close relationship to the *mecA* gene from staphylococci and the *mecB* gene from *M. caseolyticus* (Tsubakishita et al. 2010a). It is tempting to speculate that enterococcal *pbp*s, *mecB*, and *mecA* are either paralogues or their evolutionary precursor was horizontally transferred among these species. As hypothesized by Hiramatsu et al. (2013), antecedents of staphylococci could have originally carried the *mecA* gene on their chromosome and later could have lost the methicillin resistance, because staphylococci associated with distinctive mammals had become protected from the β-lactam-producing organisms by the immune system of the host. The *mecA* gene could have been maintained in *S. sciuri* and other CoNS not inhabiting such mammals. When the conditions favour the methicillin-resistant bacteria, the *mecA* gene might be readily assembled with *ccr* genes into SCC*mec* in one of the CoNS (Kuroda et al. 2005; Descloux et al. 2008; Black et al. 2009; Tsubakishita et al. 2010b; Bouchami et al. 2011; Fluit et al. 2013; Rolo et al. 2014).

SCC and SCC*mec* have a dynamic constitution prone to the accumulation of MGEs, which leads to further development of these elements. Antibiotic pressure in the environment could induce the insertion of other resistance genes into the SCC elements. Such resistance determinants are usually located on transposons or plasmids. The SCC elements carrying several MGEs have a mosaic structure predisposed to recombination and loss of the genes of no use when the selection pressure changes. So this flexible system promotes the emergence of new resistant clones and complicates the treatment of staphylococcal infections.

Conclusion

The high number of complete SCC sequences allowed an analysis of the relationships between SCC elements. This study demonstrated that SCC elements, despite their mosaic structure, form distinctive clusters according to types, and suggested that the *mec* gene complex and *ccr* gene complex represent significant conserved segments relevant to the current classification of SCCs. The analysis of the phylogenetic tree topology together with the data on strain sequence types support the hypothesis that SCC*mec* emerged on several occasions and are frequently horizontally transferred among staphylococci. This addition to the body of knowledge on SCCs contributes to a better understanding of the evolution of these elements which substantially affect the antimicrobial resistance among staphylococci.

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