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Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection:

Staphylococcal commensal species such as *Staphylococcus epidermidis* are being recognized as important sources of genes promoting MRSA colonization and virulence

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Abstract

Recent research has suggested that *Staphylococcus epidermidis* is a reservoir of genes that, after horizontal transfer, facilitate the potential of *Staphylococcus aureus* to colonize, survive during infection, or resist antibiotic treatment, traits that are notably manifest in methicillin-resistant *S. aureus* (MRSA). *S. aureus* is a dangerous human pathogen and notorious for acquiring antibiotic resistance. MRSA in particular is one of the most frequent causes of morbidity and death in hospitalized patients. *S. aureus* is an extremely versatile pathogen with a multitude of mechanisms to cause disease and circumvent immune defenses. In contrast, most other staphylococci, such as *S. epidermidis*, are commonly benign commensals and only occasionally cause disease. Recent findings highlight the key importance of efforts to better understand how genes of staphylococci other than *S. aureus* contribute to survival in the human host, how they are transferred to *S. aureus*, and why this exchange appears to be uni-directional.

Keywords

Staphylococcus aureus; MRSA; *Staphylococcus epidermidis*; horizontal gene transfer; CRISPR

Introduction

Staphylococcus aureus is a frequent and dangerous human pathogen. It is most notorious for causing hospital- and community-acquired skin and lung infections. *S. aureus* is also a premier cause of endocarditis, osteomyelitis, and septicemia. Importantly, *S. aureus* infection is a major cause of death in hospital-associated infections, particularly when patients have underlying conditions such as immune deficiencies or primary infections by other pathogens, for example influenza [1].

S. aureus strains may produce a large variety of toxins. *S. aureus* cytolytic toxins include leukocidins, alpha-toxin, and the phenol-soluble modulins (PSMs) [2,3]. Further important toxins are the *S. aureus* superantigens, which include toxic shock syndrome toxin and lead to an exacerbated immune response by polyclonal T cell activation and massive cytokine release [4]. *S. aureus* also produces a large series of toxins that interfere with almost every mechanism of human innate host defense [5].

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Notably, toxins are the prime determinants of a specific *S. aureus* strain's virulence or aggressiveness. Some *S. aureus* strains may cause particular toxin-related diseases such as toxic shock or scalded skin syndrome [6,7]. These diseases are directly linked to the presence of specific toxin genes in those strains (in these examples, genes encoding toxic shock syndrome toxin and exfoliative toxins, respectively). Similarly, in more common forms of *S. aureus* diseases, the severity of infection may be related to the toxin repertoire of the infecting strain, which may differ considerably from isolate to isolate. Even for toxin genes that are found in all or almost all *S. aureus* isolates, differential gene expression may lead to substantially varying aggressiveness [8].

Despite the undisputed importance of aggressive toxins, there are a series of other factors that determine the survival of *S. aureus* during infection and thus its capacity to cause disease. These include molecular determinants that “shield” *S. aureus* from the immune system without directly attacking it [9]. Capsular polysaccharides, any substance participating in the formation of extracellular matrix, or the surface protein, protein A, belong to that group of molecules. Importantly, most infections with *S. aureus* originate from colonizing strains, because at least a considerable subset of the human population is permanently or transiently colonized with *S. aureus*, mostly in the nose [10,11]. Therefore, any factor promoting asymptomatic colonization may be regarded a risk factor for the subsequent development of infection.

Frequent and often multiple antibiotic resistances considerably narrow therapeutic options to treat *S. aureus* infections. *S. aureus* strains may be resistant to virtually all but very recently developed antibiotics [12]. Resistance to some antibiotics, such as penicillin, is so widespread that these antibiotics cannot be considered valuable therapeutics for *S. aureus* anymore. Because methicillin is a first-choice, efficient drug for *S. aureus* infections, methicillin-resistant *S. aureus* (MRSA) is a particular concern for public health. Methicillin resistance rates among infectious *S. aureus* isolates are high. In many countries, they reach or exceed 50% [13].

Coagulase-negative staphylococci (CoNS) are systematically distinguished from *S. aureus* by the lack of coagulase. This enzyme promotes blood clotting, and the resulting fibrin coat on the bacterial surface may facilitate immune evasion. CoNS comprise a multitude of species, many of which are opportunistic pathogens [14]. *Staphylococcus epidermidis* is the most frequently encountered CoNS species on human skin and by far the most frequent source of CoNS infections [15]. *S. epidermidis* and other CoNS differ from *S. aureus* in that they are less virulent and typically cause chronic rather than acute infections. In fact, they may have an important role in contributing to immunity of the skin and mucous surfaces toward more harmful pathogens [16]. However, serious complications can arise from chronic CoNS infections, particularly in immune-compromised, hospitalized, and very young or old patients. Notably, *S. epidermidis* is the most important cause of infections on indwelling medical devices [17].

In accordance with their considerably lower virulence compared to *S. aureus*, CoNS typically do not produce aggressive toxins [15]. There are rare reports about enterotoxins in CoNS, but it is not clear whether in these cases the toxin genes cause increased virulence. Furthermore, most CoNS have *psm* genes [18]. The virtual omnipresence of PSM peptides in CoNS may be due to the general functions of those surfactant-like peptides in biofilm formation and surface colonization, while it appears that CoNS – in contrast to *S. aureus* – do not have genes for strongly cytolytic PSMs or do not express them at considerable amounts [19]. In general, pathogenicity of CoNS appears to stem from molecular determinants that evolved for commensal life on the skin, but may rise to additional use during infection [15,20].

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Thus, among extant staphylococcal species most toxins appear to be limited to *S. aureus*. However, there are several recent findings indicating that *S. aureus* acquired other factors that facilitate survival during infection - such as antibiotic resistance determinants or molecules promoting immune evasion - from *S. epidermidis* or other CoNS. Direct evidence for such a process is understandably hard to achieve, but the facts that these factors are more frequently found in CoNS and located on mobile genetic elements indicate a CoNS origin. Here, important examples of such determinants will be discussed to substantiate the overarching hypothesis of a key role of *S. epidermidis* and other CoNS as a reservoir for genes facilitating the evolution of *S. aureus* as a successful pathogen. Because MRSA represent the most important *S. aureus* strains from a clinical point of view and methicillin resistance itself is an important example of a phenotype likely acquired form CoNS, I will focus on MRSA. On the CoNS side, the focus will be on *S. epidermidis*, as most information is available about this CoNS species, both in general and as a donor of genes to MRSA.

Genes are transferred among and between staphylococcal species by horizontal gene transfer

Many antibiotic resistance and virulence determinants in *S. aureus* are located on mobile genetic elements (MGEs), such as pathogenicity islands, chromosomal cassettes, transposons, or plasmids [21]. The diversity of *S. aureus* as a species stems mainly from the different presence of such MGEs, which may be readily lost and acquired, while the remaining genes represent a skeletal “core genome” [22].

As different staphylococcal species live in the same or similar niches on human epithelial surfaces, they are in close enough contact for genetic exchange to occur. In general, genetic material may be exchanged by the use of conjugation, phage transduction, or the uptake of “naked” DNA by natural competence. Competence has not been described in staphylococci [23]. However, it is often assumed that natural competence exists in many bacteria. Direct evidence for competence-mediated horizontal gene transfer and knowledge about competence systems are lacking only because the conditions under which bacteria become competent are hard to established in the laboratory [24]. In contrast, the transfer of virulence and antibiotic resistance genes - carried on conjugative plasmids or transduced by phages - has frequently been described in *S. aureus* [25]. There is also evidence for transfer between species. For example, a self-transmissible plasmid can transfer between CoNS and *S. aureus* [26]. However, the mechanism of transfer of MGEs containing methicillin resistance genes, which will be discussed below, is still unclear.

Methicillin resistance is encoded on SCC*mec* elements, which originated from CoNS

Methicillin resistance in staphylococci is encoded on MGEs called staphylococcal cassette chromosome *mec* (SCC*mec*) [27]. SCC*mec* elements are classified by differences in their key components, the methicillin resistance and immunity systems (*mec* gene complex) and the recombinase (*ccr*) genes, and overall genetic composition [28] (Fig. 1). New types of SCC*mec* elements are frequently reported; the current number of known SCC*mec* elements is 11.

Resistance to methicillin is due to the *mecA* gene, which encodes an alternative penicillin binding protein with decreased binding affinity to methicillin [29]. Most *S. aureus* and other staphylococcal strains contain this type of *mecA*, but homologues of *mecA* with high similarity (80-90% on the nucleotide level) are found in some *S. sciuri* and *S. vitulinus* strains [30,31], now named *mecA1* and *mecA2* [32]. In addition, homologues of less pronounced similarity to *mecA* (<70%) were found in *S. aureus* strain LGA251 and *Macrococcus caseolyticus* [33,34], which are now named *mecB* and *mecC*, respectively [32]. Other parts of the *mec* gene complex are *mecR1* and *mecI*, which encode a

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transcriptional repressor and a signal transduction protein involved in regulation of *mecA*. They are not present on all SCC*mec* types. The *ccr* gene complex contains homologues of the *ccrA*, *ccrB*, or *ccrC* recombinase genes. Other genes found in the SCC*mec* elements vary. Among them are antibiotic resistance genes, insertion elements such as IS*431*, plasmids such as pT181, or transposons such as Tn*554*.

Recently, the *psm-mec* gene was identified in SCC*mec* elements of types II, II, and VIII as conserved part of the class A *mec* gene complex [35,36] (Fig. 1). PSM-mec is also found frequently among methicillin-resistant *S. epidermidis* (MRSE) and represents an exception to the rule that *S. epidermidis* does not transfer toxin genes to *S. aureus*. This exception may be explained by the fact that the PSM-mec cytolysin, like other PSMs, has additional functions in basic staphylococcal physiology. Furthermore, it only "hitchhikes" on an MGE with a primary function in antibiotic resistance.

Notably, SCC*mec* elements have only been reported in staphylococci. The abovementioned *M. caseolyticus*, which is closely related to staphylococci, represents a noticeable exception. While the origin of SCC*mec* elements is unknown, several lines of evidence indicate that they came from CoNS. The proposed evolutionary scenario is the following: The *mecA* gene appears to have a common staphylococcal ancestor. This ancestor was originally assumed to be *S. sciuri* [37], whereas more recent findings point to *S. fleuretti* [38]. The *mec* and *ccr* gene complexes were likely assembled in CoNS, but the source is not known. In CoNS, some alterations occurred, such as *mec* gene complex deletions, upon which the SCC*mec* elements were transferred to *S. aureus*.

The evidence for a transfer of SCC*mec* elements specifically from *S. epidermidis* is quite strong. First, the homology of DNA sequences between SCC*mec* elements of *S. aureus* and *S. epidermidis* is very high [39]. For example, *S. epidermidis* SCC*mec* type IV shows 98-99% homology to SCC*mec* IVa of *S. aureus* (Fig. 1), while the overall similarity of genomic sequences between the two species is much lower [40]. SCC*mec* type IV was also observed in *S. epidermidis* much earlier, in the 1970s, than in *S. aureus* [40]. Second, the insertion element IS*1272* is found much more often in *S. epidermidis* (and *S. haemolyticus*) than in *S. aureus* [41]. SCC*mec* types I and IV in *S. aureus* and *S. epidermidis* have identical IS*1272*-Δ*mecR1* junctions [40]. Finally, overall, methicillin resistance is much more frequent among *S. epidermidis* than *S. aureus* [42,43]. Altogether, these observations indicate that SCC*mec* elements were transferred from *S. epidermidis* to *S. aureus*, although some parts may have originated from other CoNS before their transfer to *S. epidermidis*.

As stressed above, the mechanism of SCC*mec* transfer is unknown. In laboratory experiments, the *mec* locus could not be transferred by conjugation [44]. Furthermore, there appear to be difficulties introducing SCC*mec* into staphylococci by common laboratory transformation procedures. Phage-mediated generalized transduction was successful between two *S. aureus* strains [45], but phage-mediated transfer of genes between different staphylococcal species is rare [27]. It has only been described for the transfer of SaPIbov2, a pathogenicity island encoding the biofilm-associated *bap* gene, from *S. aureus* to several CoNS species [46]. In vivo, there is evidence that *mecA* was transferred from *S. epidermidis* to *S. aureus* [47]. Undoubtedly, the SCC*mec* transfer mechanism represents one of the major unsolved questions in staphylococcal molecular biology. Furthermore, given that there are reports of SCC elements that lack *mec*, the transfer of such elements may have implications for genetic shuffling between staphylococcal species that is not limited only to antibiotic resistance.

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The mobile genetic element ACME represents an excellent example of DNA obtained from *S. epidermidis* that facilitates MRSA colonization and fitness

The arginine catabolic mobile element (ACME) is a ~ 31 kb MGE that was found first in the CA-MRSA strain USA300 located next to the SCC*mec* element of type IV [48] (Fig. 2). ACME encodes an arginine deiminase (*arc*) gene cluster and an oligopeptide permease (*opp3*) system. Other *arc* and *opp* clusters are found in the *S. aureus* core genome, which are believed to have basic functions in metabolism, but there is evidence indicating that ACME has a different role. Both ACME and SCC*mec* of USA300 could be excised using *ccrAB* recombinases, together and separately [49]. Analysis of these deletion mutants revealed that ACME affected survival of strain USA300 in a rabbit model of bacteremia, in contrast to SCC*mec*. However, ACME was shown not to impact the capacity of USA300 to cause rat skin abscess and necrotizing pneumonia [50], the two disease manifestations predominantly associated with the USA300 CA-MRSA epidemic [51]. It was concluded that ACME may somehow contribute to competitive fitness of USA300 with the *arc* or *opp* gene clusters facilitating colonization or transmissibility capacities of USA300. In fact, it has frequently been reported that *opp* gene clusters have roles in pathogenesis, such as by facilitating transport of quorum-sensing pheromones or antimicrobial peptides, while the *arc* gene cluster was speculated possibly to neutralize the acid environment on the skin by producing ammonia [52].

The first mechanistic insight into how ACME may play a role in enhancing survival of USA300 came from a recent study by Joshi *et al.*, who could attribute that role to another part of ACME, a gene termed *speG* [53]. SpeG is a polyamine N-acetyltransferase that detoxifies polyamines, such as spermin or spermidin, by acetylation. Notably, in contrast to most organisms, *S. aureus* does not produce polyamines and is hypersensitive to their toxic effects. Enhanced survival characteristics of USA300 on human skin compared to other *S. aureus*, as proposed previously, may thus at least in part be due to *speG*. However, *in-vivo* evidence for such a role of *speG* still needs to be produced. Furthermore, it remains to be investigated what roles, if any, the other parts of ACME may have for USA300 pathogenesis or transmissibility.

More recently, ACME elements that differ from that originally found in USA300 were identified in *S. aureus*. ACME type I, the original type, has *opp3* and *arc*, type II has only *arc*, and type III only *opp3*. While the USA300 ACME element (type I) was exclusively detected among clones of sequence type (ST) 8, the ST to which USA300 belongs, ACME type II, which lacks *opp3*, was found in ST5 and ST59 [54,55]. In some *S. aureus* strains of ST22, the *arc* gene cluster is up- rather than downstream of SCC*mec* type IV and *opp3* is missing [56]. These findings indicated separate ancestors of *S. aureus* ACME types.

ACME occurs in *S. epidermidis* at a high frequency [57]. In a detailed investigation of a large number of *S. epidermidis* strains, Miragaia *et al.* found that 52% of global *S. epidermidis* clones - representing a broad spectrum of the *S. epidermidis* genetic and geographic diversity - harbored an ACME allotype [58]. Importantly, a close relative of the USA300-type ACME element (now termed ACME I.01), termed ACME I.02, was by far the most predominant among *S. epidermidis* isolates (27% of all isolates) [58] (Fig. 2). Furthermore, ACME I.02 was particularly frequent in *S. epidermidis* isolates of clonal complex (CC) 2, a large and widely distributed *S. epidermidis* strain group associated with hospital infection. Moreover, a large part of the ST22 ACME type has high homology to the ACME composite island of the genome-sequenced *S. epidermidis* strain ATCC12228 [58].

In conclusion, these findings support the notion that most parts of the ACME elements found in *S. aureus* originated from *S. epidermidis*. Only minor parts may have come from other CoNS, such as the *opp3* genes that likely originated from *S. haemolyticus* [59].

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Notably, ACME is present in the most successful *S. epidermidis* lineage and the most widespread clone of *S. aureus* in the U.S., USA300, supporting the idea that it has an important role in facilitating colonization and/or transmissibility. Mechanistically, this role may be in part due to the recently described *speG* gene [53], which is present in the widely distributed ACME types I.01 and I.02, but absent from other, less frequent types. SpeG is a polyamine acetyltransferase, which abrogates the unique hypersensitivity of *S. aureus* to these host-produced molecules.

SasX: Epidemiological evidence for the importance of a colonization and virulence factor obtained from *S. epidermidis*

The molecular factors underlying the rise of novel HA-MRSA clones are still poorly understood. As with CA-MRSA, the spread of successful clones may not only be linked to virulence *sensu stricto*, but also non-symptomatic colonization from which infection may arise [10]. While several molecules contributing to *S. aureus* nasal colonization have been described, no factor has yet been linked to the spread of a particular HA-MRSA clone.

Recently, Li *et al.* described a novel surface protein, termed SasX, that is located on a prophage and can thus be easily transferred between MRSA strains [60]. SasX was shown in a mouse model to facilitate nasal colonization. Interestingly, SasX also promoted survival of MRSA during experimental skin and lung infection, which likely is a consequence of its impact on bacterial aggregation, which prevents efficient phagocytosis by white blood cells. Notably, the frequency of *sasX*-positive clones increased significantly among invasive MRSA isolates in eastern China. It was predominantly found in clones of ST239, the most prominent MRSA type in most parts of Asia, and may be in part responsible for the success of that ST in this geographical region. More recently, *sasX* is spreading to other STs, increasing the recipient clones' virulence and colonization capacity. Importantly, these findings give first insight into the molecular mechanisms underlying epidemic outbreaks of MRSA [61].

The 127-kb ΦSPβ-like prophage that harbors the *sasX* gene at its end is very similar to a prophage found in the genome of *S. epidermidis* strain RP62A [62]. The striking similarity suggests that the prophage was acquired from *S. epidermidis* by horizontal gene transfer, which is supported by earlier findings reporting high frequency of the S. epidermidis *sasX* homologue, *sesI*, among *S. epidermidis* strains [63]. However, a more exhaustive analysis of the frequency of the phage in *S. epidermidis* strains will be needed to substantiate that hypothesis.

CRISPR: A mechanism directing the gene flow from *S. epidermidis* to *S. aureus*?

While these findings indicate that *S. aureus* frequently takes up genetic material from CoNS, especially *S. epidermidis*, and stably includes it in its own genome, *S. epidermidis* does not appear to acquire genes from *S. aureus*. If *S. epidermidis* were to do so, one would expect that many of the multiple toxins and other virulence determinants of *S. aureus* be transferred to *S. epidermidis* over time. However, *S. epidermidis* clearly lacks the vast repertoire of aggressive virulence factors known from *S. aureus* [14,15], a situation that has been hypothesized to fit perfectly to the distinctly different lifestyles of these two closely related bacteria [64].

What is the mechanism underlying this virtually unidirectional gene transfer? The answer may lie in the recently discovered CRISPR (clustered, regularly interspaced, short palindromic repeat loci) [65], a bacterial system of self versus non-self discrimination [66] (Fig. 3). CRISPR loci contain sequences that, when transcribed to RNA, bind non-host DNA specifically, and target it for degradation. That process protects bacteria against phages, for

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example, but it could also be the mechanism preventing foreign DNA from a related bacterial species being incorporated into the *S. epidermidis* genome. Interestingly, much of the work on CRISPR has been performed using *S. epidermidis*. *S. epidermidis* strain RP62A, several other *S. epidermidis* strains [67], as well as ~40% to ~90% of sequenced bacterial genomes contain CRISPR sequences [68]. However, CRISPR sequences are much less frequent in *S. aureus*. Therefore, CRISPR sequences may be involved in preventing uptake of foreign DNA in *S. epidermidis*. In contrast, *S. aureus* as a species appears to take the risk of incorporating harmful DNA, such as viruses, to benefit from the possibility to accumulate virulence factors from other bacteria.

Certainly, the role of CRISPR in directing the gene flow from *S. epidermidis* to S. *aureus* is still hypothetical. First, CRISPR is only found in some *S. epidermidis* genomes. Second, some *S. aureus* strains contain CRISPR sequences but also MGEs. However, in those cases, MGEs may have been present before the acquisition of CRISPR. Third, one would expect characteristic nucleotide sequences of MGEs found in *S. aureus* to be present in *S. epidermidis* CRISPR spacer regions. Substantiating the role of CRISPR in limiting gene transfer from *S. aureus* to *S. epidermidis* will require exhaustive further investigation. For example, genome sequencing of more *S. epidermidis* strains should be performed to show whether CRISPR loci are more frequently present in *S. epidermidis* than *S. aureus*. Draft sequences obtained by pan-genome analysis do not necessarily allow a rigorous analysis of CRISPR presence, in part because CRISPR heterogeneity complicates identification. Furthermore, analysis of CRISPR spacer sequences may reveal sequences homologous to bacteriophages involved in gene transfer between staphylococci. So far, spacer analysis of *S. epidermidis* CRISPR sequences has not revealed such sequences; however, this may be related to our general lack of knowledge on interspecies phage-dependent gene transfer between staphylococci, which also requires more emphasis. Finally, retrospective analysis of historical strains may allow insight into whether CRISPR sequences were acquired by CRISPR-positive *S. aureus* strains before or after acquisition of MGEs.

Conclusions

Recent studies have provided additional examples supporting the hypothesis that *S. epidermidis* and other CoNS have an important function as providers of genes that contribute to the survival of *S. aureus* during infection and as a commensal. This hypothesis is based on the higher prevalence and, where information is available, earlier appearance of transferred determinants in *S. epidermidis* compared to *S. aureus*. The opposite scenario with *S. aureus* as the source appears much less likely because of (i) the general paucity of characteristic *S. aureus* genes that are found in *S. epidermidis*, and (ii) the fact that the transferred determinants are not restricted to a specific *S. epidermidis* clone, which would suggest more recent acquisition from *S. aureus*. However, more genetic data will be needed to confirm this scenario.

Possibly, CRISPR-mediated immunity of CoNS strains to DNA uptake may play a role in directing the gene flow between *S. aureus* and *S. epidermidis*. However, other yet unidentified mechanisms probably exist that resulted in the divergent evolution of *S. aureus* as a virulent pathogen on the one hand and CoNS as usually harmless commensals and only occasionally invasive pathogens on the other. Notably, these findings highlight the importance of research providing molecular insight into the function of genes facilitating survival of CoNS species in the human host, because they may ultimately contribute to the virulence potential of a dangerous pathogen.

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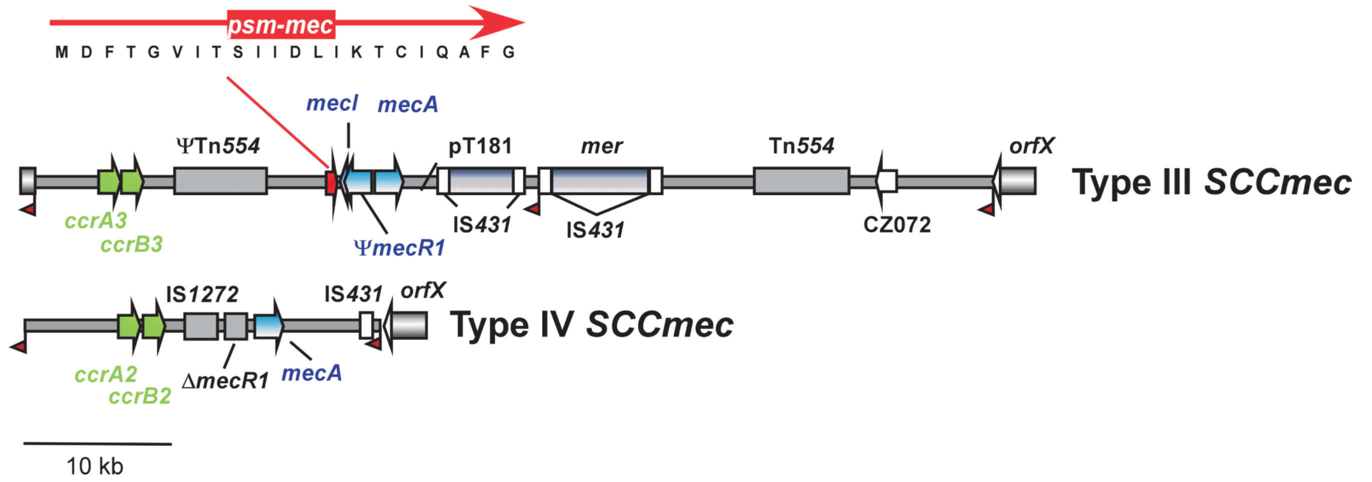

Fig. 1.

Examples of SCC*mec* element composition. Shown are SCC*mec* elements of type III and type IV. Type III elements are common in HA- and type IV elements are characteristic of CA-MRSA. The latter are smaller in total size, which may contribute to increased fitness compared to MRSA strains harboring larger SCC*mec* elements. Genes of the *mec* gene complex are shown in blue, recombinases (*ccr* genes) in green. Some other important, varying components are shown in grey. Red flags depict *att* recombination sites. The location of the *psm-mec* cytolysin gene and the amino acid sequence of the PSM-mec protein are shown.

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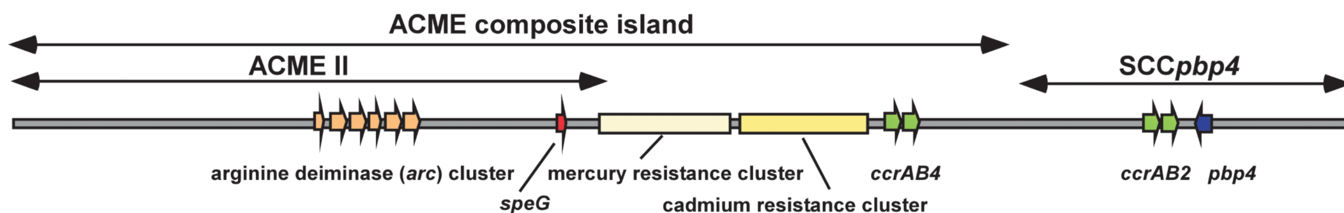

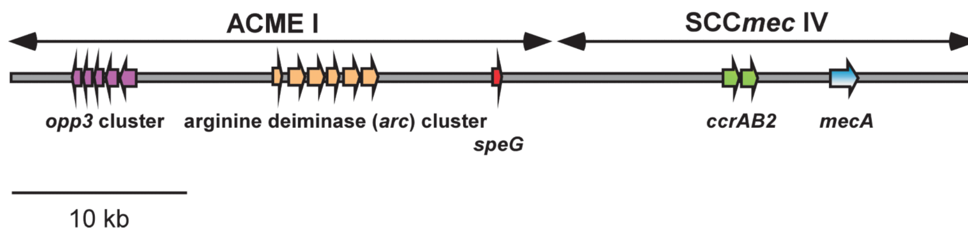

Fig. 2.

ACME in *S. epidermidis* and *S. aureus*. Important genes on the arginine catabolic mobile element (ACME), and in its vicinity, in the genome-sequenced strains *S. epidermidis* ATCC12228 and *S. aureus* USA300 (FPR3757) are shown. Genes of the *mec* gene complex are shown in blue, recombinases (*ccr* genes) in green. *opp*, oligopeptide permease; *pbp*, penicillin-binding protein; *speG*, spermidin N-acetyltransferase.

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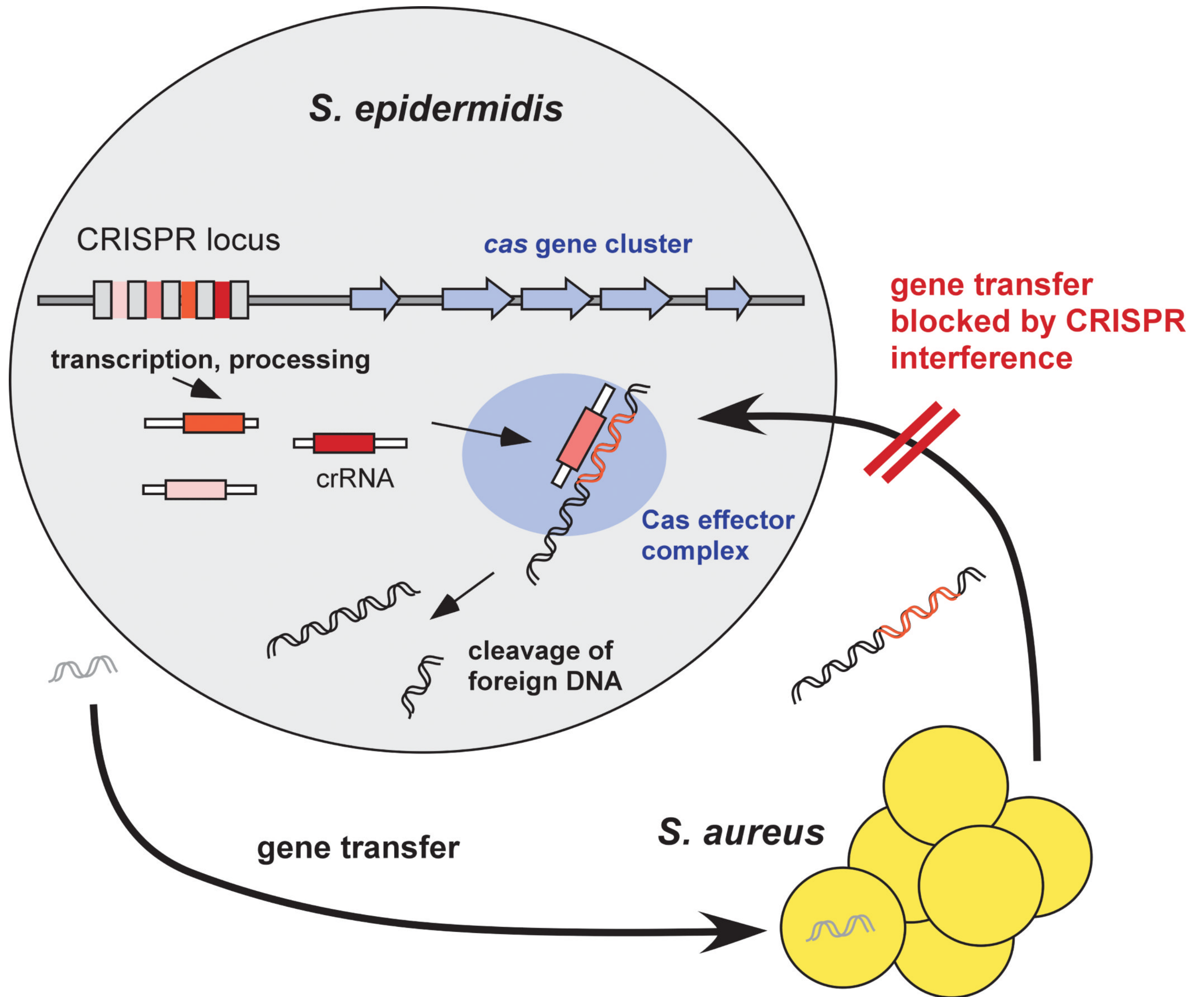

Fig. 3.

Directionality of gene transfer between *S. epidermidis* and *S. aureus* explained by CRISPR interference. *S. aureus* is known to collect a large variety of toxin and other virulence determinant genes from mobile genetic elements – in clear contrast to *S. epidermidis*, which virtually lacks such genes. This may be explained at least in part by the presence of CRISPR loci in *S. epidermidis* and their absence in *S. aureus*. CRISPR loci (clustered, regularly interspaced, short palindromic repeat loci) consist of repeats, between which DNA sequences are found that hybridize with components of foreign DNA. CRISPR RNA, which arises from CRISPR loci after transcription and processing, hybridizes to such foreign sequences and the foreign DNA is cleaved by products of the *cas* (CRISPR-associated genes) complex.