**Grant Year** * 2010

**Project Title** * Search for the missing links in the evolution of the resistance determinant SCCmec

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**Country** * Portugal

**Short summary of project outcome (max. 100 words)**

We aimed to contribute to the clarification of the roles of Staphylococcus sciuri and other coagulase-negative staphylococcal species in the evolution and assembly of the mobile element (SCCmec), carrying the determinant of resistance to beta-lactams. We found that different Staphylococcus species are reservoirs of different types of cassette chromosome recombinase (ccr), one of the central structural elements of SCCmec that promote the excision and integration of SCCmec from the chromosome. Moreover, we found that hospital environment promotes diversification of the SCCmec.

**Published articles originating from your Research Grant project**


**Communications originating from your Research Grant project (e.g. oral, poster, extended abstract)**

**INVITED LECTURES IN INTERNATIONAL AND NATIONAL MEETINGS**


**POSTER COMMUNICATIONS IN INTERNATIONAL MEETINGS**

4. Miragaia, M., J. Rolo, O. Bouchami, N. Faria, H. Westh, and H. de Lencastre. Coagulase-negative staphylococci species are donors of specific cassette chromosome recombinases (ccr) for staphylococcal chromosomal cassette
Search for the missing links in the evolution of the resistance determinant SCCmec

Maria Miragaia Ryder
SUMMARY

The methicillin resistance determinant (mecA) in staphylococci is carried by SCCmec that is characterized by mec complex (mecA, mecl/mecR1) and ccr complex - responsible for SCCmec mobility. Ten SCCmec types have been described in S. aureus that are combinations of different mec complex classes and ccr allotypes. But, the stages in SCCmec evolution are unknown. In this study we aimed to understand the contribution of each CoNS species for the assembly of SCCmec.

A total of 433 S. epidermidis (236 MRSE, 197 MSSE), 85 S. hominis (48 MRSHo, 37 MSSHo) and 69 S. haemolyticus (50 MRSH, 19 MSSH) were analyzed. The mecA and ccr genes were detected by Southern hybridization and the mec complex class and ccr allotypes were determined by PCR. Internal fragments of ccrB were amplified by PCR and their nucleotide sequence was determined.

The ccrAB2 was the most frequent allotype among MSSE (70%) and MRSE (50%). The ccrAB1 and ccrAB4 were almost restricted to MSSHo (19%, each) and were the most frequent among MRSHo (49%, 36%, respectively); ccrC was the most frequent among MSSH (50%) and MRSH (64%). Interestingly, we found >95% nucleotide homology between: ccrB2 from MSSE and SCCmec IV; ccrB1 from MSSHo and SCCmec I; ccrB4 from MSSHo and SCCmec VI.

The results suggest that each CoNS could have had a particular role in SCCmec evolution: S. epidermidis as the donor of ccrAB2 for SCCmec IV; S. haemolyticus as the origin of ccrC for SCCmec V; and S. hominis as the donor of ccrAB1 and ccrAB4 for SCCmec I and VI.
INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) are one of the major nosocomial human pathogens causing disease worldwide. The genetic determinant of broad-spectrum β-lactams resistance in *S. aureus* is the *mecA*, which encodes the low-affinity penicillin-binding protein 2A (PBP2A). The *mecA* gene is known to be carried within a mobile genetic element called the *Staphylococcal Chromosome Cassette mec* (SCCmec) that can be transferred among different staphylococcal strains and species (10, 14). The two central elements of SCCmec are the *mec* complex, composed of *mecA* and its regulators (*mecI* and *mecR1*) and the *ccr* complex that comprises recombinases that are responsible for SCCmec mobility. Moreover, this element is known to be flanked by direct and inverted repeats and to integrate at a unique and specific site in the chromosome, an open reading frame with unknown function called *orfX*. Up until now seven different types of SCCmec have been described (I-VII) in *S. aureus* that vary in the composition of *mec* complex and *ccr* complex and in the remaining J regions (from junkyard) (10, 13, 22). In spite of the clinical importance of SCCmec, not much is known about its evolution and mechanism of transfer.

Several observations support the proposition that the *mecA* present in MRSA strains have evolved from a homologous gene present in *Staphylococcus sciuri*, a staphylococcal species distantly related to *S. aureus*, and most frequently recovered from rodents and primitive mammals (5). Although much effort has been made to prove the origin and evolution of the *mecA*, little information is available regarding the steps leading to the assembly of the *mecA* precursor into the mobile genetic element SCCmec widely disseminated among MRSA strains and methicillin resistant *S. epidermidis* (MRSE).

The existence of similar regions among different SCCmec types, like the J1 region between SCCmec type II and IV, or the *mec* complex B between SCCmec I and IV (4) as well as the identification of similarities between SCC non-*mec* and SCCmec regions (18), suggests that SCCmec have undergone several sequential recombinational events, giving rise to mosaic-like structures. Some studies in which the *mec* complex region was characterized in clinical MRSA and MR-CoNS strains have revealed that the *mecA* regulators and the IS431-R together with *mecA*, were once the original components of *mec* region DNA and that deletion of the regulator genes by IS occurred at a later time in evolution, probably in a CoNS species, where IS431 and IS1272 are frequent (16, 27). Recent studies have demonstrated that SCCmec type IV was the most frequent among
S. epidermidis, suggesting that this species might have been the source of SCCmec for S. aureus (21, 29). Besides these data, no clues on the order of the occurrence of events leading to the assembly of the seven well characterized SCCmec types or on the role of the different staphylococcal species in SCCmec evolution exist.

In the first year of the project we focused our studies on the clarification of the role of the different CoNS in the assembly of the SCCmec (Task 2 of the project).
MATERIALS AND METHODS

Bacterial isolates. A collection of 433 *S. epidermidis* (236 MRSE and 197 MSSE) from hospital and community origins, and 69 *S. haemolyticus* (50 MRSH and 19 MSSH) and 85 *S. hominis* (48 MRSHo, 37 MSSHo) nosocomial isolates were analyzed.

Species identification. All isolates were tested for mannitol fermentation and coagulase production (BBL Coagulase Plasma Rabbit test, Becton Dickinson Microbiology systems, Cockeysville, USA). Isolates were characterized at the species level by ITS-PCR, as described [31].

DNA preparation. DNA for PFGE was prepared as previously described (1). Genomic DNA for PCR was extracted by the isothiocyanate method as described before (3). DNA probes for *mecA*, *ccrAB1*, *ccrAB2*, *ccrAB4* and *ccrC* were prepared using previously described primers (2-4) followed by purification by the Wizard PCR preps DNA Purification System (Promega, Madison, WI).

Pulsed-field gel electrophoresis (PFGE). *S. epidermidis* and *S. haemolyticus* isolates were characterized by PFGE as previously described (1). PFGE for *S. hominis* was performed as described (1) with some modifications. XhoI (20 units/disk) was chosen as the restriction enzyme for PFGE. The running conditions were the following: block1- pulse times 2 to 20s, running time 11h and block2- pulse times 2 to 7s, running time 15h; voltage 6V; angle 120° [37]. Low Range Lambda ladder DNA (New England BioLabs, Beverly, USA) was used as molecular weight PFGE marker. *S. epidermidis* RP62A was used to access inter-gel reproducibility. XhoI PFGE restriction band patterns were analyzed by visual inspection by counting the number of band differences; and automatically using BioNumerics Software (version 4. 5) from Applied Maths (Sint-Martens-Latem, Belgium). Clusters (PFGE types) were defined using the Dice similarity coefficient and the unweighted pair group method with arithmetic means (UPGMA), with 1% of tolerance and 0.8% optimization, using a cutoff similarity value of 90%. PFGE types were identified by letters; and subtypes were identified by letters followed by a numeric subscript.

Southern blotting and DNA hybridization. Smal and XhoI DNA fragments in PFGE gels, were transferred by vacuum blotting according to manufacturer’s instructions and hybridized with DNA probes for *mecA*, *ccrAB2* and *ccrC* using ECL direct Prime Labeling and detection systems (Amersham Biosciences, Buckinghamshire, United Kingdom), according to manufacturer's instructions.
**ccr typing.** The nucleotide sequence of an internal region of *ccrB* was determined by the *ccrB* typing method (5). The *ccrB* nucleotide sequences were compared by the construction of an unrooted phylogenetic tree using the average distance clustering method and the default parameters set in the *ccrB* typing tool (http://www.ccrbtyping.net). The measurement of statistical confidence of the clustering was performed by bootstrap resampling (1,000).

**SCCmec typing.** SCC*mec* type was determined by the amplification by PCR of the *mec and ccr* complexes as described previously (4).
RESULTS

SCCmec distribution in MR-CoNS

The molecular characterization of SCCmec carried by MRSE showed that SCCmec type IV was the most common (39.8%, 94/236), followed by SCCmec III (16%, 38/236), SCCmec II (7.6%, 18/236) and SCCmec V (5.5%, 13/236). SCCmec types I, VIII were poorly represented (2.1% and 1.7%, respectively), but a relatively high proportion of isolates carried non-typeable (12.3%, 29/236) or new (11.0%, 26/236) SCCmec structures. The new SCCmec types had the following structures: 5A (17/236), 3B (2/236), 5B (1/236), 2C (5/236) and 5D (1/236).

Noteworthy, half of MRSE (50.0%) carried ccrAB2 genes and in this species as many as eleven different SCCmec structures were found.

The molecular characterization of the SCCmec carried by MRSHo showed that SCCmec VI (4B) was carried by 22.9% (11/48) of the isolates, and SCCmec type VIII (4A) by 10.4% (5/48). Interestingly, a high proportion of MRSHo strains (42%, 20 out of 48) carried a unique new association between the mec complex class and the ccr complex, corresponding to ccrAB1 associated to mec complex type A (1A). In addition, a high number of non-typeable SCCmec structures (25%, 12/48) were also found, that resulted from the association of a single mec complex to two different ccr complex allotypes. They included mec complex class A associated to two different ccr allotypes: ccrAB1 and ccrC (NT2) (three isolates); ccrAB1 and ccrAB4 (NT1) (two isolates); ccrAB4 and ccrC (NT3) (one isolate). Moreover, three isolates carried mec complex type A but none of the ccr genes described so far and in two isolates neither ccr nor mec complexes were typeable, although mecA was present.

Overall a high frequency of mec complex class A (68.8%) and ccrAB1 (49.1%) and ccrAB4 (35.5%) was observed among MRSHo.

The molecular characterization of SCCmec in MRSH showed that SCCmec type V (5C) was the most common, being identified in 42% (21/50) of the collection analyzed. Interestingly, however, mec complex C was found to be also associated to other SCCmec types besides SCCmec V in this species. In five isolates new associations between the mec complex C and the ccr complex were found, namely: mec complex C1/ccrAB4 (4C1); mec complex C2/ccrAB4 (4C2); and mec complex C1/ccrAB1 (1C1). In addition, one isolate carried mec complex C associated to no ccr complex or to an
unknown ccr allotype. The remaining 23 isolates harbored SCCmec with mec complex class A associated to ccrAB4 or ccrC (one isolate each) or non-typeable SCCmec (21 isolates), either because they carried non-typeable mec complex, non-existent/non-typeable ccr, or combination of both.

Overall a high prevalence of ccrC (26.3%) and mec complex C (56%) was found in S. haemolyticus, not necessarily associated to SCCmec V. Moreover, as much as 40% of the isolates carried non-typeable mec complex classes.

![Figure 1](image)

**Figure 1** – Distribution of SCCmec types among MRSE, MRSH and MRSHo.

**Distribution of ccr allotypes among MS-CoNS**

The presence of ccr genes among MS-CoNS isolates was tested by Southern hybridization followed by ccr typing.

The molecular characterization of ccr genes carried by MSSE showed that as much as 27.4% of the isolates (54 isolates) carried ccr genes. Interestingly, we found that 70.4% (38/54) of the isolates carried ccrAB2 allotype either alone (30/54) or associated to ccrC (7/54) or ccrAB4 (1/54). The remaining isolates carried ccrC alone (33.3%, 11/54), ccrAB4 (5.5%, 2/54) or non-typeable ccr allotype (three isolates).

Among the 37 mecA-negative S. hominis isolates analyzed, 43% (16/37) carried ccr genes: six harbored ccrC, two carried ccrAB1, two ccrAB4, one isolate carried ccrAB2, and four isolates carried non-typeable ccr allotypes.
Regarding MSSH, 32% (6/19) of the isolates carried ccr genes, the great majority (83%, 5/6) belonged to ccrC allotype, and one isolate to ccrAB2.

Nucleotide homology between ccrB from CoNS and S. aureus

a) Homology between ccrAB1 and ccrAB4 from S. hominis and S. aureus

In order to verify if ccrAB4 and ccrAB1 found in S. hominis were similar to those previously described, ccrB typing was performed for representative 28 MRSHo and 5 MSSHo carrying a single ccrAB allotype. A high homology (97-99%) was found between ccrB1 from S. hominis isolates from our study and those of ccrB1 of other S. hominis isolates in the database (SH13-27 and SH8-39). More interesting, a similar homology was found between ccrB1 from our collection and the ccrB1 from S. epidermidis (SE6-42). Likewise, S. hominis ccrB4 showed a high nucleotide sequence homology (92 to 96.5%) with ccrB4 from S. aureus (HDE288) (Table 1).

<table>
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<th>Strain</th>
<th>mecA</th>
<th>Closest Prototype match</th>
<th>Similarity (%)</th>
<th>ccrB allele</th>
<th>Predicted ccrAB Allotype</th>
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<td>2762b</td>
<td>+</td>
<td>NEW-Closest:HDE288</td>
<td>92.7%</td>
<td>604</td>
<td>4</td>
</tr>
<tr>
<td>5486</td>
<td>+</td>
<td>NEW-Closest:HDE288</td>
<td>96.5%</td>
<td>605</td>
<td>4</td>
</tr>
<tr>
<td>4793a</td>
<td>+ +</td>
<td>NEW-Closest:HDE288</td>
<td>93.4%</td>
<td>606</td>
<td>4</td>
</tr>
<tr>
<td>3140aë</td>
<td>+</td>
<td>NEW-Closest:HDE288</td>
<td>96.5%</td>
<td>605</td>
<td>4</td>
</tr>
<tr>
<td>5162a</td>
<td>+</td>
<td>NEW-Closest:HDE288</td>
<td>96.5%</td>
<td>605</td>
<td>4</td>
</tr>
<tr>
<td>1561</td>
<td>+</td>
<td>NEW-Closest:HDE288</td>
<td>96.5%</td>
<td>605</td>
<td>4</td>
</tr>
<tr>
<td>4364a</td>
<td>+</td>
<td>NEW-Closest:HDE288</td>
<td>92.1%(T); 91.9%(A)</td>
<td>NEW-Closest:600</td>
<td>4</td>
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<td>3070a</td>
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<td>91.9%(T); 92.1%(A)</td>
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<td>2573b</td>
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<td>2910</td>
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<td>312a</td>
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<td>99.8%(T); 100%(C)</td>
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Table 1. Summary of nucleotide sequence showing the percentage identity values (%) for ccrB1 and ccrB4 sequences between S. hominis from this study and reference strains.

*HDE288 is a S. aureus prototype strain for SCCmec type VI; SH corresponds to S. haemolyticus; SE corresponds to S. epidermidis.

*Strains with two similarity percentages correspond to strains in which the first position of the ccrB sequence analyzed has two superimposed peaks for the nucleotides in brackets (these results were repeated at least two times). In order to get the similarity percentages for those strains, we run the ccrB typing tool using each one of the two possible nucleotides for the first position of the ccrB sequence.

Thirteen out of the 33 isolates were non-typeable by ccrB typing methodology, because of the presence of several superimposed peaks in sequence’s traces. As ccrB typing makes use of degenerated primers, the existence of superimposed peaks may indicate...
the presence of multiple ccr alleles of the same allotype, which PCR multiplex methodology is enable to detect.

We also found four isolates for which PCR-based typing indicated the presence of ccrAB1, but ccrB sequencing indicated the presence of ccrAB4 (5162a, 3140aé, 5486, and 1561). The results suggest that these isolates carry the two ccrB allotypes (ccrB1 and ccrB4). However they could not be both detected by the same methodology most probably due to nucleotide differences in the primer regions used.

Altogether, these apparently discrepant results, suggests a large pool of ccrAB1 and ccrAB4 alleles in S. hominis.

A total of four different ccrB1 and 10 different ccrB4 alleles were found in the 14 isolates from our collection, for which a ccrAB allotype could be determined. The phylogenetic relationships among ccrB1 and ccrB4 alleles found in this study with those of control strains available in the database [43] are displayed in Figure 1. The ccrB4 alleles were clustered into three different branches: one containing the ccrB4 from the SCCpbp4 of S. epidermidis strain ATCC12228; the other containing SCCmec VI prototype S. aureus strain (HDE288), SCCmec VIII prototype S. aureus strain (10682) and four isolates from our study (5162a, 3140aé, 5486, and 1561); and the last one containing only four MRSHo (4793a, 2762b, 3070a, 4364a) and two MSSHo isolates (2573b, 2910) from our collection.

The ccrB1 was present in several species in ccrB database, being distributed in one major cluster that contained three prototype S. aureus strains for ccrAB1 (COL, PER184, PL72), one S. epidermidis isolate (SE 6-42), one S. hominis (SH 13-27) isolate from the database and three S. hominis isolates from our collection (two MRSHo and one MSSHo). The remaining five isolates were distributed by five different branches (S. fleuretti H65; S. aureus MSSA4476, S. hominis 2189 from our study, S. hominis SH8-39, and S. haemolyticus D64).

The phylogenetic analysis showed that ccrAB1 and ccrAB4 from S. hominis and those found in S. aureus SCCmec are highly similar and must have had a common origin.
b) Homology between ccrAB2 from S. epidermidis and S. aureus

In order to verify if ccrAB2 found in S. epidermidis were similar to those previously described in S. aureus, ccrB typing was performed for representative MSSE carrying ccrAB2 allotype (9/38 MSSE). A total of eight different alleles were identified (401, 405, 407-412), suggesting a high genetic diversity. However all the alleles found had between 96.3-100% homology in nucleotide sequence with the ccrB2 alleles associated to S. aureus prototype strains carrying SCCmec IV (see Table 2).

Table 2. Summary of nucleotide sequence showing the percentage identity values (%) for ccrB1 and ccrB4 sequences between S. hominis from this study and reference strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>mecA</th>
<th>Closest Prototype match*</th>
<th>Similarity (%)*</th>
<th>ccrB allele</th>
<th>Predicted ccrAB allotype</th>
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<td>100</td>
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<td>2</td>
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<td>DFT93N</td>
<td>-</td>
<td>8/6-3P (IVb)</td>
<td>100</td>
<td>401</td>
<td>2</td>
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<tr>
<td>DFT626N</td>
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<td>8/6-3P (IVb)</td>
<td>98.9</td>
<td>405</td>
<td>2</td>
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<tr>
<td>DFT148N</td>
<td>-</td>
<td>MW2 (Iva)</td>
<td>96.3</td>
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<td>DFT250N</td>
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<td>MW2 (Iva)</td>
<td>99.1</td>
<td>408</td>
<td>2</td>
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<td>DFT800N2</td>
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<td>MW2 (Iva)</td>
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<td>409</td>
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<td>Q2314 (IVc)</td>
<td>99.1</td>
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In order to verify the similarity and relatedness of the ccrB nucleotide sequences obtained a phylogenetic tree was constructed (see Figure 2). The global analysis of the tree revealed the definition of three different clusters, each containing a prototype strain.
for a different SCCmec IV subtype (IVA, IVB and IVC). The sequences obtained for two isolates (56N and 93N) were 100% identical to the prototype strain of SCCmec IVB (8/6-3P). The sequences obtained for DFT800N2 e DFT148N isolates were highly homologous (89%) to the S. aureus prototype strain for SCCmec IVA (MW2) and sequences for isolates DFT676NR1 and DFT783N2 were related to IVC. Globally the results suggest that the ccrB2 found in SCCmec IVA, IVB and IVC could have been originated in S. epidermidis.

Conclusions

The dissimilar distribution of ccr allotypes among the different CoNS species both in MR and MS isolates suggests that each CoNS species appears to have been the donor of specific ccrAB allotypes for the assembly of SCCmec types frequently found in S. aureus. S. epidermidis as the donor of ccrAB2 for SCCmec IV and S. hominis as the donor of ccrAB1 and ccrAB4 for SCCmec VI and VIII. Although ccrC was the most predominant ccr allotype among both MRS and MSSH, suggesting that S. haemolyticus could have been the donor of ccrC for SCCmec V, ccrC allotype was also well represented in the other two species analyzed and more detailed studies are needed to prove its origin.
REFERENCES


Communications that resulted from this work

Communications in poster


Oral Communications

Financial Report

Table 3. Expenses description for the project.

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