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Staphylococcal cassette chromosome *mec* containing a novel *mec* gene complex, B4

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Objectives: To describe a new subclass of *mec* class B complex identified in *Staphylococcus epidermidis*.

Methods: Four *S. epidermidis* isolates obtained from bloodstream infections in patients at University Medical Center Groningen (UMCG) were analysed by phenotypic antibiotic susceptibility testing and WGS.

Results: Sequence analysis revealed a new staphylococcal cassette chromosome *mec* (SCC*mec*) structure in isolate UMCG335. In this structure, plasmid pUB110 was found to be integrated into SCC*mec* IVc, creating a new SCC*mec* subtype, IVUMCG335. SCC*mec* IVc and a copy of plasmid pUB110 were found in other isolates, UMCG364 and UMCG341, respectively, indicating a probability that SCC*mec* IVUMCG335 could have evolved at the UMCG. SCC*mec* of UMCG337 contained a new genetic organization of the *mec* complex (IS*431*-Δ*mecR1*-*mecA*-IS*431*-pUB110-IS*431*-ψIS*1272*) that we have named B4. This new subclass of *mec* class B complex originated by IS*431*-mediated inversion of the DNA segment encompassing the plasmid and most of the genes of the *mec* complex with the exception of IS*1272*. As the SCC*mec* organization in UMCG337 differed by the inversion of an ∼10 kb sequence compared with SCC*mec* IVUMCG335, we have named it SCC*mec* subtype IVUMCG337. Isolates UMCG335 and UMCG337 carrying SCC*mec* IVUMCG335 and IVUMCG337, respectively, were associated with a restriction-modification system and a CRISPR-Cas system, creating a composite island of almost 70 kb.

Conclusions: Our findings highlight the importance of IS*431* in the evolution of the SCC*mec* region. The increasing genetic diversity identified in the SCC*mec* elements imposes a great challenge for SCC*mec* typing methods and highlights possible difficulties with the SCC*mec* nomenclature.

Introduction

Methicillin-resistant staphylococci produce an altered PBP encoded by *mecA* (PBP2a) or *mecC* (PBP2c), with the genes located in a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* elements integrate into the staphylococcal chromosome at a specific site at the end of the *rlmH* (*orfX*) gene and are flanked by direct and inverted repeat sequences (DRs and IRs, respectively). Fourteen types of SCC*mec* have been distinguished to date based on the combination of the *mec* gene complex class and the type of *ccr* gene complex, which is represented by the *ccr* gene allotype.[1] The *mec* complexes differ by the presence or partial deletion of *mecA* regulatory genes (*mecR1* and *mecI*) and the presence and location of insertion sequences IS*431* and IS*1172*. SCC*mec* subtypes harbour variable J ('junkyard') regions, which contain other genetic elements, such as plasmids, transposons and antimicrobial and heavy metal resistance genes.

Specific staphylococcal species may be sources of particular SCC*mec* elements. In particular, *Staphylococcus epidermidis* appears to be a reservoir of SCC*mec* type IV that can spread to the most virulent staphylococcal species, *Staphylococcus aureus*.[2] SCC*mec* type IV harbours class B *mec* and *ccrAB* allotype 2 complexes. At least five variants of the class B *mec* complex have been reported and they have been designated B, B1, B2, B3 and B(L).[3,4] To date, 13 subtypes (IVa–IVo) have been described.[5,6] In this study, we report a new subclass of class B *mec* originating from

IS*431*-mediated integration of plasmid pUB110 into SCC*mec* IVc and subsequent inversion of the DNA segment encompassing the plasmid and most of the *mec* complex genes.

Materials and methods

Bacterial isolates

S. epidermidis isolates analysed in this study were obtained during an ongoing study on multiresistant strains recovered from bloodstream infections in patients at University Medical Center Groningen (UMCG), The Netherlands. Isolate UMCG364 was obtained from a 51-year-old patient in 2017, while isolates UMCG335, UMCG337 and UMCG341 were recovered from 40-year-old, 5-week-old and 56-year-old patients, respectively, in 2018.

Phenotypic antibiotic susceptibility testing

Resistance to β-lactam antibiotics (benzylpenicillin, oxacillin, cefoxitin and ceftaroline) was determined using Etest methodology (bioMérieux). MSSA ATCC 25923 was used as a control. Results were interpreted according to EUCAST clinical breakpoints.

Extraction of genomic DNA

The cells were lysed using lysozyme (100 mg/mL) and total DNA was purified using the MagAttract HMW DNA Kit (Qiagen). DNA was quantified using Qubit (ThermoFisher Scientific) and the quality assessed by TapeStation 2200 (Agilent Technologies). A NanoDrop 2000C spectrophotometer (ThermoFisher Scientific) was used to measure the purity of extracted DNA.

Illumina and Oxford Nanopore sequencing

Illumina genomic libraries were sequenced on a HiSeq system with a 2×150 paired-end protocol. Oxford Nanopore sequencing libraries were prepared using the Ligation Sequencing Kit (SQK-LSK109) and sequenced on a MinION device using flow cell type R9.4.1 (FLO-MIN106D).

Sequence assembly

For each isolate, nanopore data were sorted by length and the longest reads up to an expected depth of coverage of 100 *de novo* assembled with the XNG assembler (DNASTAR). Chromosomes were assembled into single contigs with a typical depth of coverage of 70, as were large plasmids. Contig consensus sequences were refined and small plasmids identified with an automated polishing workflow (DNASTAR) using the Illumina data. Polished assemblies were manually corrected using SeqMan Ultra (DNASTAR) and final sequences annotated with NCBI's Prokaryotic Genome Annotation Pipeline (version 2020–02-06.build4373).

Data analysis

The DNA sequences were aligned using BLASTn software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The MLST STs were assigned through the MLST server (www.cbs.dtu.dk/services/MLST).[7] Clustered regularly interspaced short palindromic repeats (CRISPR) were identified using the CRISPRfinder program (https://crispr.i2bc.paris-saclay.fr/Server/). Preliminary typing of SCC*mec* elements was conducted by SCCmecFinder 1.2 (https://cge.cbs.dtu.dk/services/SCCmecFinder/).[8]

Nucleotide sequence accession numbers

The chromosome sequences for UMCG335, UMCG337, UMCG341 and UMCG364 have been deposited in GenBank under the accession numbers CP064453, CP064640, CP064631 and CP064549, respectively.

Results

The four study isolates belonged to two STs: ST2 (UMCG341) and ST23 (UMCG335, UMCG337 and UMCG364). All isolates carried the *mecA* gene and were phenotypically methicillin resistant. The isolates were resistant to oxacillin, cefoxitin and benzylpenicillin, and susceptible to ceftaroline.

SCC*mec* of UMCG335 shared high nucleotide identity (99.9%) with SCC*mec* IVc of UMCG364 with an identical *mec* class B complex and *ccrAB* allotype 2, but differed by the presence of an integrated plasmid pUB110 downstream of *mecA* (Figure 1a). pUB110 carries genes for replication (*rep*), recombination (*rec*) and antimicrobial resistance, *bleO* and *aadD*, conferring resistance to bleomycin and aminoglycosides, respectively. The ST2 isolate, UMCG341, harboured SCC*mec* III with an integrated pUB110 plasmid (Figure 1a). The pUB110 sequences of isolates UMCG335 and UMCG341 were almost identical, differing by only two nucleotides, while the J regions of SCC*mec* were unrelated. This suggests that the plasmid was inserted into SCC*mec* IVc at UMCG. IS*431* elements flanked both ends of pUB110 (Figure 1a), suggesting a role in plasmid integration. This SCC*mec* of UMCG335 contains a genetic structure not previously described. Therefore, we propose that this new SCC*mec* subtype should be classified as SCC*mec* IVUMCG335. BLASTn searches of the GenBank database with SCC*mec* of UMCG335 revealed the presence of an almost identical sequence in five *S. aureus* chromosomes (accession numbers CP051982, CP051972, CP019563, CP045439 and CP045435), but none in *S. epidermidis*.

The *mec* complex in UMCG337 resembled a *mec* class B complex, but its genetic organization was divergent, due to an inversion of the DNA segment spanning pUB110 and almost the entire *mec* complex with the exception of IS*1272* (Figure 1b). It is likely that the IS*431* transposase, Tnp431, catalysed IS*431* movement to a new site and inversion of adjacent DNA as an additional IS*431* element was found downstream of pUB110. Although, a truncated *mecR1* was located upstream of *mecA*, and IS*431* downstream of *mecA*, their gene order was inverted compared with the prototypic *mec* class B. Thus, SCC*mec* of UMCG337 contained a new *mec* complex organization (IS*431*-Δ*mecR1*-*mecA*-IS*431*-pUB110-IS*431*-ψIS*1272*) that we have named B4 because of its similarity to *mec* complex B (IS*431*-*mecA*-Δ*mecR1*-ψIS*1272*). BLASTn searches did not identify any comparable sequences. As the SCC*mec* organization in UMCG337 differed by the inversion of an ∼10 kb sequence compared with SCC*mec* IVUMCG335, we have named it SCC*mec* subtype IVUMCG337.

Isolates UMCG335 and UMCG337 carried SCC*mec* IV associated with a restriction-modification system and a CRISPR-Cas system, creating a large composite island of 69 075 and 69 878 bp in size, respectively (Figure 2). The regions located between short direct repeats DR3 and DR4, and DR4 and DR2 had already been identified together with SCC*mec* type II in a composite island of *S. epidermidis* RP62a,[9] but have never been shown to be associated with SCC*mec* IV, as was determined for the isolates analysed in the current study. SCC*mec* of UMCG364 was not associated with any mobile element and was flanked by a core genome.

Discussion

The insertion, duplication and movement of IS*431* as well as homologous recombination between different copies of IS*431* play a

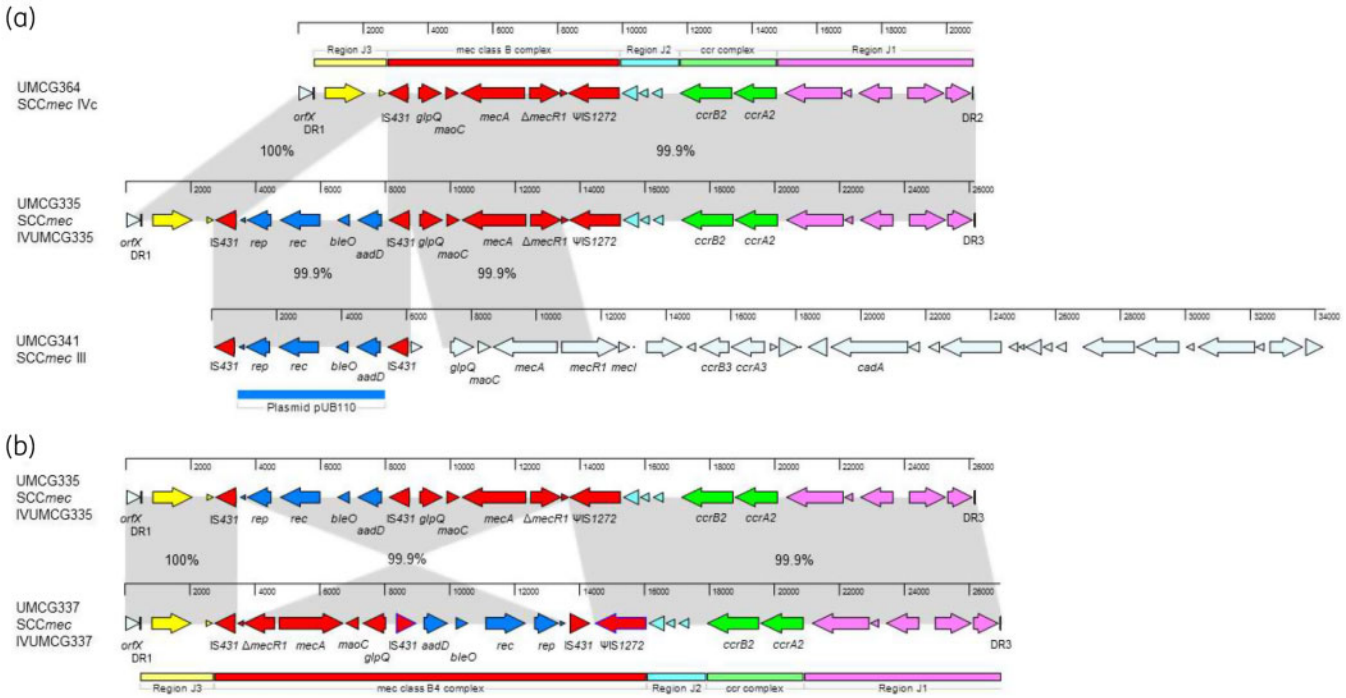

Figure 1. Evolution of SCC*mec*: (a) subtype IVUMCG335; and (b) subtype IVUMCG337. The genomic regions were compared from *S. epidermidis* isolates: UMCG335 (GenBank accession number CP064453, bases 32149.57878), UMCG337 (GenBank accession number CP064640, bases 32149.58681), UMCG341 (GenBank accession number CP064631, bases 2724906.2759262[complement]) and UMCG364 (GenBank accession number CP064549, bases 32139.52522). The arrows indicate the genes. Only the following selected genes are annotated: 23S rRNA methyltransferase RlmH (*orfX*) containing the SCC*mec* insertion site, glycerophosphoryl diester phosphodiesterase (*glpQ*), MaoC family dehydratase (*maoC*), the determinant encoding resistance to methicillin (*mecA*) and its regulatory gene (*mecR1*), the SCC*mec* cassette recombinases (*ccrA* and *ccrB*), plasmid replication-initiation protein (*rep*), plasmid recombination enzyme (*rec*), bleomycin binding protein (*bleO*), aminoglycoside *O*-nucleotidyltransferase ANT(4′)-Ia (*aadD*), cadmium-translocating P-type ATPase (*cadA*) and the transposases of IS*431* and IS*1272*. The vertical bars indicate DRs. Sequences of DRs: DR1, GAAGCATATCATAAATGA; DR2, GAAGCGTATCGTAAGTGA; and DR3, GAAGCATATCATAAATAA.

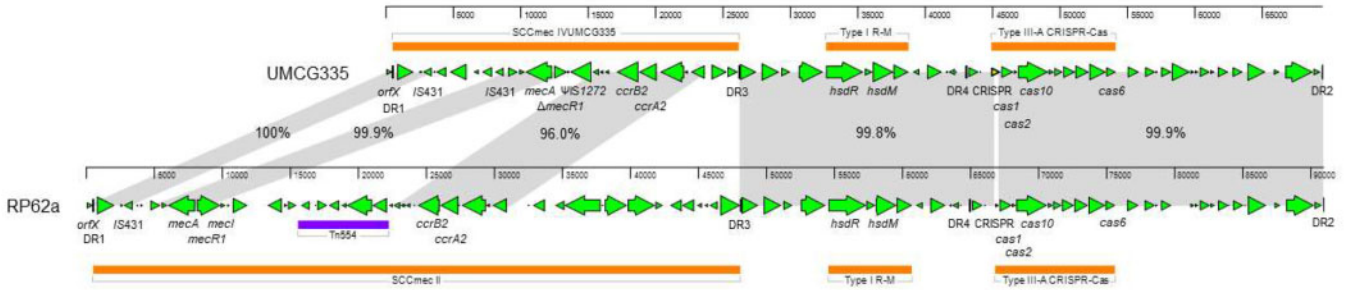

Figure 2. Structure comparison of the composite islands from *S. epidermidis* isolates: UMCG335 (GenBank accession number CP064453, bases 32149.101223) and RP62a (GenBank accession number CP000029, bases 2493635.2584193[complement]). The green arrows indicate the genes. Only the following selected genes are annotated: 23S rRNA methyltransferase RlmH (*orfX*) containing the SCC*mec* insertion site, the determinant encoding resistance to methicillin (*mecA*) and its regulatory genes (*mecR1* and *mecI*), the SCC*mec* cassette recombinases (*ccrA* and *ccrB*), type I restriction-modification system endonuclease (*hsdR*), type I restriction-modification system DNA methylase (*hsdM*), CRISPR-associated proteins (*cas1, cas2, cas6* and *cas10*) and the transposases of IS*431* and IS*1272*. The yellow arrows indicate CRISPR. The vertical bars indicate DRs. Sequences of DRs: DR1, GAAGCATATCATAAATGA; DR2, GAAGCGTATCGTAAGTGA; DR3, GAAGCATATCATAAATAA; and DR4, GAAGGGTATCGTAAGTGA.

significant role in the continuous evolution of SCC*mec*. Katayama *et al.*[10] revealed that the IS*431* translocation associated with the deletion of *mecI* and *mecR1* in the *mecA* gene complex correlated with phenotypic expression of methicillin resistance in *mecA*-carrying *Staphylococcus haemolyticus*. In another study, a potential role of IS*431* in the excision of the *mecA* gene was shown.[11] Conversely, characterization of a complex containing *mecA*, but lacking the *ccr* genes, in *S. haemolyticus* suggested that the structure containing *mecA* bracketed by two copies of IS*431* in the same orientation might have formed a composite transposon.[12] It

could explain the absence of *ccr* in some staphylococcal isolates carrying *mecA*. IS*431* was also implicated in the integration of plasmids into the chromosome.[13] In this study, we found two IS*431* copies flanking pUB110 and likely mediating its chromosomal integration.

When two IS elements of the same type are located in the same orientation and flank one or more genes they create a composite transposon (IS-unique_segment-IS). These IS elements can recombine between each other forming translocatable units (TUs) consisting of the internal DNA segment of a composite transposon and one copy of a flanking IS (IS-unique_segment or unique_segment-IS).[14] Such TUs recognize another IS of the same type as a target in the chromosome or on a plasmid. Subsequent recombination between TU and IS leads to directly oriented copies of IS at each end and the unique segment between them. This model was described for some IS elements, including IS*26*, IS*257*, IS*1216* and, recently, IS*431*.[15] The IS*431*-pUB110-IS*431* structure found in SCC*mec* IVUMCG335 could result from the recombination of an IS*431*-pUB110 TU of unknown origin with the IS*431* copy of UMCG364 SCC*mec* IVc (Figure 1a). Interestingly, pUB110 is commonly found inserted in SCC*mec* III, which is typically found in the MDR, hospital-adapted *S. epidermidis* ST2 BPH0662 lineage.[16] At UMCG, we found a member of this lineage (UMCG341), a possible source of IS*431*-pUB110 TU (Figure 1a).

SCC elements are genomic islands widely prevalent in staphylococci that carry antibiotic and heavy metal resistance genes and also virulence-associated genes.[17] Moreover, the epidemiological success of MRSA USA300 has been related to the acquisition of two mobile elements, the arginine catabolic mobile element (ACME) and the copper and mercury resistance element.[18] Genome-wide analyses revealed that different mobile elements were co-transferred as the full identity of the entire ACME-SCC*mec* composite island in two distinct MRSA lineages was determined as well as SCC*mec* and SCC-like elements could be transferred independently.[19,20] In this study, we identified composite islands in UMCG335 and UMCG337 consisting of SCC*mec* type IV together with restriction-modification and CRISPR-Cas systems responsible for protecting bacteria against foreign DNA. The presence of the CRISPR system has been rarely reported in *S. epidermidis*. Therefore, the prevalence of mobile elements containing the CRISPR-Cas system in the *S. epidermidis* ST23 lineage and its influence on mosaicisms of genomic islands should be explored.

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Transparency declarations

T.D. is an employee of and minor shareholder in DNASTAR, Inc. M.M. was an employee of DNASTAR, Inc., but left the company before the end of the project and, therefore, she has none to declare as she now works for a company that is not connected to DNASTAR, Inc. All other authors: none to declare.

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