Characterization of a New SCCmec Element in Staphylococcus cohnii

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Abstract

**Background:** Many SCCmec elements of coagulase-negative staphylococci (CoNS) could not be typed using multiplex PCR. Such a ‘non-typable’ SCCmec was encountered in a *Staphylococcus cohnii* isolate.

**Methodology/Principal Findings:** The SCCmec type of methicillin-resistant *S. cohnii* clinical isolate WC28 could not be assigned using multiplex PCR. Newly-designed primers were used to amplify ccrA and ccrB genes. The whole SCCmec was obtained by three overlapping long-range PCR, targeting regions from left-hand inverted repeat (IRL) to ccrA/B, from ccrA/B to mecA and from mecA to orfX. The region abutting IRL was identified using inverse PCR with self-ligated enzyme-restricted WC28 fragments as the template. WC28 SCCmec had a class A mec gene complex (mecI-mecR1-mecA). The ccrA and ccrB genes were closest (89.7% identity) to ccrA/B of *Staphylococcus haemolyticus* strain H9 and to ccrB3 (90% identity) of *Staphylococcus pseudointermedius* strain KM241, respectively. Two new genes potentially encoding AAA-type ATPase were found in J1 region and a Tn554 transposon was present in J2 region, while J3 region was the same as many SCCmec of *Staphylococcus aureus*. WC28 SCCmec abutted an incomplete SCC element with a novel allotype of ccrC, which was closest (82% identity) to ccrC1 allele 9 in *Staphylococcus saprophyticus* strain ATCC 15305. Only two direct target repeat sequences, one close to the 3’-end of orfX and the other abutting the left end of WC28 SCCmec, could be detected.

**Conclusions/Significance:** A new 35-kb SCCmec was characterized in a *S. cohnii* isolate, carrying a class A mec gene complex, new variants of ccrA5 and ccrB3 and two novel genes in the J1 region. This element is flanked by 8-bp perfect inverted repeats and is similar to type III SCCmec in *S. aureus* and a SCCmec in *S. pseudointermedius* but with different J1 and J3 regions. WC28 SCCmec was arranged in tandem with an additional SCC element with ccrC, SCCWC28, but the two elements might have integrated independently rather than constituted a composite. This study adds new evidence of the diversity of SCCmec in CoNS and highlights the need for characterizing the ‘non-typable’ SCCmec to reveal the gene pool associated with mecA.

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Introduction

Coagulase-negative staphylococci (CoNS) are opportunistic pathogens [1] and are usually resistant to methicillin [2]. In staphylococci, methicillin resistance is mainly dependent on the expression of the mecA gene, which encodes PBP2a, a transpeptidase with a low affinity for β-lactams [3–4]. mecA together with its regulatory genes and associated insertion sequences forms the mec gene complex, which is carried by a mobile genetic element (MGE) termed the staphylococcal cassette chromosome (SCCmec) [5]. SCCmec is bounded by terminal inverted repeats (IRs) and integrates site specifically in the staphylococcal chromosome close to the 3’ end of orfX [6], a gene of unknown function located close to the origin of the chromosomal replication. The integrase site sequence (ISS) usually contains the consensus sequence GA/A/G/GC/A/G/TATCA/G/C/T/A/A/A/G/T/A/G/A/G [7–8]. A 15 bp sequence is duplicated as direct target repeats (DR) on insertion of SCCmec [6–7]. Integration and excision of SCCmec are due to recombinases encoded by a set of cassette chromosome recombinase (cer) genes (cerC or the pair of cerA and cerB) [6,9]. The cer gene(s) and surrounding genes constitute the cer gene complex [6,9]. In addition to cer and mec gene complexes, SCCmec contains a few other genes, many of which have unknown functions, and various other MGE, e.g. insertion sequences, transposons and plasmids. These genes and MGE are located in three joining regions, i.e. J1 between the left-hand IR (IRL) and the cer gene complex, J2 between the cer and mec gene complexes, and J3 between the mec gene complex and the right-hand IR (IRR) [9].

Eight types (I to VIII) of SCCmec have been assigned for *Staphylococcus aureus* based on the classes of the mec gene complex and the types of the cer gene complex [9]. As methicillin resistance is more prevalent in CoNS than in *S. aureus*, CoNS may serve as a larger reservoir of SCCmec available for *S. aureus* to form methicillin-resistant *S. aureus* (MRSA) [6]. However, compared to MRSA, much less is known about the genetics of mecA in CoNS [10]. According to the available data [10–21], SCCmec elements are more diverse in CoNS, with new variants of cer genes.
continuing to be identified [13,20–22]. Although type III and IV SCCmec are prevalent in CoNS, many SCCmec elements of CoNS could not be typed using currently-available schemes based on multiplex PCR [6,21]. In a study of SCCmec in local CoNS clinical isolates, a *Staphylococcus cohnii* isolate containing a “non-typeable” SCCmec was encountered. This “non-typeable” SCCmec was characterized in detail and is reported here.

### Methods

#### Strain and SCCmec typing

CoNS isolate WC28 was recovered from a clinical specimen (wound secretion) collected in West China Hospital, Chengdu, western China. This isolate was identified as *S. cohnii* by partially sequencing the 16s rRNA gene amplified with the universal primers 27F and 1492-R (Table 1) [23]. WC28 could grow on plates containing 4 µg/ml cefoxitin (Sigma, St Louis, MO). The mecA gene and its regulatory genes mecI and mecR1 were detected by PCR as described previously [24]. The SCCmec typing was carried out using multiplex PCR as described previously [24].

#### Identification of ccr genes

Since primers targeting *ccrAB1*, *ccrAB2*, *ccrAB3* and *ccrC* [24] failed to detect the *ccr* genes in WC28, *ccrA* and *ccrB* of WC28 were obtained using new primers (Table 1) designed from an alignment of known *ccrA* and *ccrB* sequences retrieved from GenBank.

#### PCR mapping

Three overlapping long-range PCR (Fermentas, Burlington, ON, Canada; Figure 1) were used to obtain the whole SCCmec and to confirm the links between different genetic components. These three PCR linked IRL to *ccrA*, the *ccrAB* genes to *mecA*, and *mecA* to orfX (Figure 1).

### Table 1. Primers used for PCR.

| Primer | Sequence (5’-3’) | Target/location | Reference |
|--------|-----------------|-----------------|-----------|
| 27F    | GGTTACCTTGTTACGACTT | 16s rRNA gene | [23] |
| 1492R  | AGAGTTTGATCCTGGCTCAG | | [23] |
| MecA147-F | GTGAAGATATACCAAGTGATT | mecA | [24] |
| MecA147-R | ATGCGCTATAGATTGAAAGGA | mecI | [24] |
| mecl-F  | CCCTTTTCTTACAATCTCGTT | meci | [24] |
| mecl-R  | ATATCATCTGCAAGATGG | | [24] |
| ccrA-UF1 | AATGTGAHGTATTATGTTGYTA | ccrA | This study |
| ccrA-UR1 | GGTTCATTTTTDAARTAGAT | | This study |
| ccrB-UF1 | CGTGTATCAACGDAATVCAA | ccrB | This study |
| ccrB-UR1 | CTTTATCACCTTGGAYWATTTC | | This study |
| orfX-F1 | GAAACGGACCCWGAAMATAGG | orfX | This study |
| IR-L SCC | TATCRGWTRATGATGMGITT | IRL of SCCmec | This study |
| ccrA_28-R1 | TGGTTGATAGACAGACAGACACA | ccrA | This study |
| 28-7 | TTCCTCTTCATTCTCTGG | orf2 | This study |
| Trn554-UR1 | TTCTATGCGAGAGATGTGG | Trn554 | This study |
| 28-10 | AATTGGATTGTCACAGTACAGG | 5’ end of orf15 | This study |
| HMG-up | ATTTGTCGTAAGCTAGCTTG | 3’ end of orf19 | This study |
| 28-11 | CCCATTATGGAGCCCTTGT | orfA | This study |
| orf28-F1 | TGTCCCAATTAAAAAGGTTGTTT | orfL | This study |
| orf28-R1 | GCCAACCCCCGTAACCTACT | orfL | This study |
| orf28-R2 | ATTTTACACAGCTGCCATT | orfL | This study |
| 28-14 | GCAGGTGTTATGGCAAGCAGA | orfB | This study |
| 28-17 | TTTGTTTCTTACTACATTGG | orfC | This study |
| 28-18 | TGTAGTCGTTCCCTGAGAAAG | orfC | This study |
| 28-21 | CGTACAAATAAAAGCCACAGA | orfF | This study |
| 28-22 | CCATGCAAGATGAAAAAGGTA | orfF | This study |
| 28-23 | CCGAATCTGTAGTGCGCTCA | ccrC-orfF spacer | This study |
| 28-24 | GGAGAGATCAGGATGGGA | ccrC | This study |
| 28-13 | TTAGGACATCCGGTTTCTTC | orf3 | This study |
| 28-32 | ACCAACCAATACCTCAAGCAGA | orfI | This study |
| 28-26 | AGCTTTCAAGAGCCTATTTTT | ccrC | This study |
| 28-39 | CCAACGGTATACAGACAGACAC | upstream of orfN | This study |

* D: A, G or T; H: A, C or T; M: A or C; R: A or G; W: A or T; Y: C or T; V: A, C or G.

*Description of orfs are available in Table S1 and 3.

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Invers PCR

A few inverse PCR reactions were employed to identify the region abutting IRL with pairs of outwards-facing primers (Table 1 and Figure 1). Genomic DNA of WC28 prepared using a commercial kit (Tiangen, Beijing, China) was restricted with a restriction enzyme (Figure 1), self-ligated with T4 DNA ligase and then used as a template for inverse PCR. The links between genetic elements might therefore be slightly different mecA (listed in Table S1) and SCCWC28 (listed in Table 3), respectively. WtN554 contains trnB, trnP, ccdC and ccdB. The 15 bp sequences abutting the IR are shown with nucleotides that differ in lower case. The region similar to type III SCCmec of S. pseudintermedius KM241 is highlighted with a grey background. PCR primers and amplicon sizes are indicated. Several self-ligated restricted fragments were used as templates for inverse PCR with the names and restriction locations of the enzymes being shown.

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Sequencing

Amplicons were sequenced by primer walking using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) at the Beijing Genomics Institute (Beijing, China). Sequences were assembled using the SeqMan II program in the Lasergene package (DNASTAR Inc, Madison, WI) and similarity searches were carried out using BLAST programs [http://www.ncbi.nlm.nih.gov/BLAST/].

Nucleotide sequences accession number. The complete sequence of the WC28 SCCmec is deposited in GenBank as GU370073.

Results and Discussion

WC28 contained mecA gene but its SCCmec type could not be assigned using multiple PCR, suggesting that WC28 might harbor a new SCCmec element.

WC28 SCCmec had perfect IRs but imperfectly-matched abutting sequences

IRs vary in size and can be imperfect in different SCCmec [6–7]. Nonetheless, the IRs of SCCmec type I (strain NCTC10442), II (N315), III (85/2082) and IVa (CA05) in S. aureus contain a consensus 8-bp sequence GC(A/G/T)TATCA at the end [7,25]. In WC28 GC(T)ATCA bounded the SCCmec and constituted the 8-bp perfect IR. The 15 bp sequences abutting both ends of the WC28 SCCmec were not perfectly matched, with three nucleotide differences (Figure 1), suggesting that the WC28 SCCmec might have been formed by recombination. However, based on SCCmec excision experiments [7], it appears that nucleotide mutations are likely to be introduced during the insertion of SCCmec, generating target repeats that are not perfectly matched. The 15-bp sequences abutting the WC28 SCCmec may therefore be slightly different simply as a result of direct insertion of this element in orfX.

WC28 SCCmec carried a class A mec gene complex

The SCCmec of WC28 had a class A mec gene complex composed of mecA, mecI mecR1, several other genes and a single copy of insertion sequence IS431 downstream of mecA (Figure 1 and Table S1 in Online Supporting Information). The class A mec gene complex is also present in SCCmec types II, III and VIII and SCCmec of unassigned types in Staphylococcus pseudintermedius strain KM241 [21] and Staphylococcus saprophyticus strain TSU33 [20]. The class A mec gene complex in WC28 was most similar to that in S. saprophyticus TSU33 with only two nucleotide differences.

New variants of ccrA and ccrB representing challenges for the present classification scheme

The WC28 SCCmec contained a ccr gene complex with new ccrA and ccrB variants. The WC28 ccrB gene (ccrBC28) was 1503 bp in length, shorter than most other ccrB genes (1629 bp) reported previously [9]. ccrBWC28 was most similar (90% identity) to ccrB3 (S. pseudintermedius KM241) [21] and was 88.9% identical to ccrB5SH (Staphylococcus haemolyticus H9) [13] and 88.7% to ccrB3 (S. aureus 85/2082) [7] (Table 2). According to the guidelines for reporting novel SCCmec elements [9], ccr genes with greater than 85% nucleotide identity should be classified into the same allotype. ccrBWC28 is therefore a new variant of ccrB3.

The WC28 ccrA gene (ccrAWC28; 1350 bp) had the highest identity (89.7%) with ccrA5SH (S. haemolyticus H9) and was 85.7% identical to ccrA5 (85/2082) and 85.0% to ccrA5 (S. pseudintermedius KM241) (Table 2). It appears that ccrA5SH could be a member of the ccrA3 or ccrA5 allotype, illustrating a problem with the current classification system [9]. Nonetheless, ccrA5SH, the closest match to ccrA5SH, is closer to ccrA5 (KM241) than to ccrA5 (85/2082; 86.6 vs 85.0% identity), and therefore should be clustered with ccrA5 based on the 85% cutoff value. Accordingly, it seems more appropriate that ccrA5SH should be designated as the ccrA5, rather than the ccrA3, allotype. Like S. haemolyticus H9 and S. pseudintermedius KM241, WC28 had a ccrA5B3 type ccr gene complex, different from all ccr complex types identified in S. aureus so far.

Compared with those in S. aureus, the ccrAB sequences in CoNS appear to be more diverse with several new variants reported recently [6,13,20–21]. ccrAB sequences in CoNS could have more than 85% identity with more than one designated allotype, exemplified by ccrABWC28 here and ccrBS of S. pseudintermedius KM241, which is 91.4% identical to ccrB3 (85/2082) and 85.5% to ccrB1 (S. aureus MSSA476). This dilemma may need to be considered when developing the classification guidelines for...
SCC\textit{mec} in CoNS. It seems reasonable to assign a \textit{ccr} variant to its closest allotype when it had more than 85\% identity with two or more designated allotypes.

The joining regions in WC28 SCC\textit{mec} contained several new features

Five genes were identified between IRL of SCC\textit{mec} and \textit{ccr}. The three genes adjacent to \textit{ccr} were similar to the counterparts in \textit{S. pseudintermedius} KM241 and appear to be part of the \textit{ccr} gene complex. The remaining two genes (orf1 and -2) closest to IRL had no significant matches with any staphylococcal sequences currently deposited in GenBank but had the highest identities to a gene \textit{bwe9773} (62\% identical to orf1) in \textit{Listeria welshimeri} SLCC5534 (NC_005555) and a gene \textit{MSC_1061} (64\% identical to orf2) in \textit{Mycoplasma mycoides} PG1 (NC_005364). These two genes are likely to encode proteins of the AAA-type ATPase superfamily. AAA refers to ATPases associated diverse cellular activities such as protein degradation and intercellular transport [26]. The presence of these two novel genes suggests that the J1 region in the WC28 SCC\textit{mec} is different from those reported previously.

Like SCC\textit{mec} type III of \textit{S. aureus} 85/2082 and the SCC\textit{mec} of \textit{S. pseudintermedius} KM241, the \textit{ccr} and the \textit{mec} gene complexes in the WC28 SCC\textit{mec} were separated by a few genes, most of which have unknown functions, and \textit{\psi Tn554} carrying cadmium resistance determinants (Table S1 and Figure 1). Of note, there is a single nucleotide deletion in the transposase \textit{B} gene, \textit{topB}, of \textit{\psi Tn554} in WC28 compared with those reported before. This deletion is not due to an error as it was confirmed by sequencing at both directions. Due to the deletion, two smaller open reading frames instead of a complete \textit{topB} gene were present in WC28 but the impact of this deletion on the function of \textit{\psi Tn554} remains unexplored. In general, this J2 region in the WC28 SCC\textit{mec} is almost identical to those in the KM241 SCC\textit{mec} and SCC\textit{mec} type III (85/2002), except a few nucleotide differences, most of which were in \textit{\psi Tn554}.

Downstream of the \textit{mec} gene complex, the J3 region of WC28 contained one gene of unknown function (Table S1). The same J3 region has also been seen in many SCC\textit{mec} elements of different types or subtypes, e.g. type I, IIb, IVa and VI in \textit{S. aureus} [9] and an unassigned type in \textit{S. saprophyticus} TSU33 [20]. This structure was termed the downstream constant segment (dcs) [9,27]. Of note, the dcs is not present in \textit{S. pseudintermedius} KM241, suggesting that the WC28 and KM241 SCC\textit{mec} had different J3 regions.

WC28 SCC\textit{mec} abuts another SCC carrying a novel allotype of \textit{ccrC}

A 16 kb region was identified abutting the IRL of WC28 SCC\textit{mec} on one side and abutting a gene, designated orfN here,
which putatively specified an FMN-binding flavin reductase on the other side. Variants of this flavin reductase-encoding gene were present in all S. aureus and Staphylococcus epidermidis genomes available in GenBank, suggesting that this gene was part of the staphylococcal core genome.

A ccrC gene was identified in this 16 kb region. All ccrC genes identified previously shared more than 87% identity and therefore were variants of a common ccrC allotype based on the 85% cutoff value [9]. These variants included ccrC1 allele 1 (in SCCmec V) (Accession no. AB121219), 2 (AY894416), 3 (AB037671) (in SCCmec III), 4 (U10927), 5 (AP006716), 6 (EF190467), 7 (EF190468), 8 (AB462393), 9 (NC_007350) and 10 (GQ902038) from S. aureus and several unassigned ccrC alleles in coagulase-negative staphylococci. The 1677-bp ccrC in WC28 was a novel ccrC allotype, closest (82% identity) to ccrC1 allele 9 in S. saprophyticus ATCC15305 and 81% identical to ccrC1 allele 1 in S. aureus (Table 2). Based on the 85% cutoff value [9], ccrC in WC28 could be therefore designated ccrC1 allele 1.

The presence of ccrC suggested that this 16 kb region was likely to be a SCC element, therefore designated SCCWC28 here, which was arranged in tandem with WC28 SCCmec. The presence of two SCC elements in tandem could result from separate integration of the two elements, but the two SCC elements could also constitute a composite generated by fusion of the two elements following deletion of the original junction region containing the DR [9]. Nonetheless, only two DR sequences, one close to the 3’-end of oriX and the other abutting the IRL of WC28 SCCmec, could be detected. This suggested that WC28 SCCmec and SCCWC28 might have integrated independently rather than constituted a composite.

In addition to ccrC, SCCWC28 contained a few other genes (Table 3), most of which have counterparts seen in SCCmec or in SCCmec type V, but function of most of these genes remained

### Table 3. Genes in SCCWC28

| Gene | Position | Product | Closest match |
|------|----------|---------|---------------|
| orfA | 16947-15829 | Hypothetical protein | 67% identical to a gene (BCQ_477, function unknown) in Bacillus cereus Q1 (CP000227) |
| orfb | 15359-15057 | Hypothetical protein | No significant matches |
| orfC | 15046-13550 | Hypothetical protein | 88% identical to a gene (SSP0042, function unknown) of SCCmec in S. saprophyticus ATCC15305 (NC_007350) |
| orfD | 13324-12224 | Putative DNA/RNA polymerase | 79% identical to a gene (function unknown) of SCCmec type V, e.g. in S. aureus PM1 (ORF no. 25, AB462393) |
| orfE | 12231-11860 | Hypothetical protein | 94% identical to a gene (function unknown) of SCCmec type V, e.g. in S. aureus PM1 (ORF no. 26) |
| orfF | 11860-10565 | Putative phage/plasmid primase | 85% identical to a gene (function unknown) of type V SCCmec, e.g. in S. aureus PM1 (ORF no. 27) |
| ccrC | 9998-8322 | CcrC Recombinase | 82% identical to ccrC1 allele 9 in S. saprophyticus ATCC15305 (NC_007350) |
| orfG | 8217-7476 | Hypothetical protein, DUF 950 superfamily | 81% identical to a gene (SSP0034, function unknown) of SCCmec in S. saprophyticus ATCC15305 |
| orfH | 7865-7469 | Hypothetical protein, DUF 960 superfamily | 84% identical to a gene (function unknown) in SCCmec, e.g. in TW20 (SATW20_00450, FN433596), and also in SCCmec in S. pseudintermedius KM241 (AM904731) and KM1381 (AM904732) |
| orfI | 7453-6947 | Hypothetical protein, DUF 960 superfamily | 84% identical to a gene (function unknown) of SCCmec type V, e.g. in S. aureus PM1 (ORF no. 11) |
| orfJ | 6965-6477 | Putative DNA repair protein, RadC | 82% identical to a gene encoding a putative RadC of SCCmec in S. saprophyticus T5U33 (AB353724) |
| orfK | 6070-5432 | Hypothetical protein | 79% identical to a gene (SATW20_00450, function unknown) of SCCm_2 in S. aureus TW20 |
| orfL | 5394-4927 | Hypothetical protein | No significant matches |
| orfM | 3678-3208 | Hypothetical protein | 84% identical to a gene (SATW20_00490, function unknown) of SCCmec in S. aureus TW20 |

*Positions are according to GenBank accession no. GU370073.

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Figure 2. A proposed model for double crossover-mediated exchange between two SCCmec. When two different SCCmec (not to scale) contain two sequences of homology, exemplified by ccrB3 and IS431 here, two homologous recombination events (the upper panel) occurring between the two sequences can result in exchange of the intervening components (lines of different thicknesses) between the two SCCmec (the lower panel).

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undetermined. No MGE such as IS431 and Tn4001 were present in SCCWC28. Of note, no DR sequences could be detected flanking SCCWC28, suggesting that SCCWC28 was probably incomplete and also carries two novel genes in the J1 region. WC28 generated 15-bp DR with nucleotide mutations on insertion. This element in WC28 is a new SCCmec since it contains a new crf gene complex and also carries two novel genes in the J1 region. WC28 SCCmec was arranged in tandem with an additional SCC element, SCCWC28, with a novel crf allele, crfC2. However, the two elements might have integrated independently rather than constituted a composite.

As a whole, the WC28 SCCmec is very similar to that of S. pseudintermedius KM241 except at both ends (Figure). Based on characteristics of the mec and ccr gene complexes, the WC28 and KM241 SCCmec should be considered together as a new type, while the different J1 and J3 regions suggest that these two SCCmec are of two distinct subtypes. The WC28, KM241 and type III (S. aureus 85/2082) SCCmec share a similar “core” including the ccr and mec gene complexes and the J2 region suggesting a possible common origin. The divergent J1 and J3 regions in these three SCCmec might have resulted from two recombination events occurring in two regions of homology, one of which appears to be IS431 downstream of mecA and another might be ccrB3 or adjacent sequences (a proposed scheme is shown in Figure 2). The similarity and divergence between SCCmec in CoNS and those in S. aureus highlights the need to characterize SCCmec elements in CoNS, particularly those not identified by PCR-based typing schemes. The information generated is essential for revealing the potential reservoir of components that could allow formation of diverse elements carrying mecA and for appreciating the origin and the evolution of SCCmec.

Supporting Information

Table S1 Genes in the WC28 SCCmec.

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Author Contributions

Conceived and designed the experiments: ZZ XL. Performed the experiments: ZZ. Analyzed the data: ZZ. Contributed reagents/materials/analysis tools: ZZ. Wrote the paper: ZZ.

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