Novel Type XII Staphylococcal Cassette Chromosome *mec* Harboring a New Cassette Chromosome Recombinase, CcrC2

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Excision and integration of staphylococcal cassette chromosome *mec* (SCC*mec*) are mediated by cassette chromosome recombinases (Ccr), which play a crucial role in the worldwide spread of methicillin resistance in staphylococci. We report a novel *ccr* gene, *ccrC2*, in the SCC*mec* of a *Staphylococcus aureus* isolate, BA01611, which showed 62.6% to 69.4% sequence identities to all published *ccrC1* sequences. A further survey found that the *ccrC2* gene was mainly located among coagulase-negative staphylococci (CoNS) and could be found in staphylococcal isolates from China, the United States, France, and Germany. The *ccr* gene complex harboring the *ccrC2* gene was designated a type 9 complex, and the SCC*mec* of BA01611 was considered a novel type and was designated type XII (9C2). This novel SCC*mec* element in BA01611 was flanked by a pseudo-SCC element (Ψ SCC_{BA01611}) carrying a truncated *ccrA1* gene. Both individual SCC elements and a composite SCC were excised from the chromosome based on detection of extrachromosomal circular intermediates. We advocate inclusion of the *ccrC2* gene and type 9 *ccr* gene complex during revision of the SCC*mec* typing method.

The worldwide emergence of health care-associated, community-associated, and livestock-associated (LA) methicillin-resistant *Staphylococcus aureus* (MRSA) strains emphasizes the importance of understanding the genetic basis for acquisition of a methicillin resistance determinant (1). MRSA is generated when a methicillin-susceptible *Staphylococcus aureus* (MSSA) strain exogenously acquires a mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*), which carries the *mecA* gene (2, 3). Excision and integration of SCC*mec* elements are mediated by cassette chromosome recombinases (Ccr), which are large serine recombinases of the resolvase/invertase family (4). Ccr recombinases are encoded by three phylogenetically distinct *ccr* genes, *ccrA*, *ccrB*, and *ccrC*, with nucleotide similarities among them below 50%. In general, *ccr* genes with nucleotide identities of more than 85% are assigned to a same allotype. Until now, five *ccrA* allotypes and seven *ccrB* allotypes had been described. In contrast, there has been only one known *ccrC* allotype since its first description in 2004 (5), because all identified *ccrC* variants to date have shared identities of more than 87% and are described as different alleles. On the basis of different *ccr* allotypes and their combinations, eight types of the *ccr* gene complex have been identified in staphylococcal species (3, 6).

SCC*mec* elements are highly diverse in their structural organization and genetic content and have been classified into 11 types (6), based on different combinations of *ccr* gene complexes and *mec* gene complexes. Among them, nine SCC*mec* elements carry the *ccrA* and *ccrB* genes (*ccrAB*), whereas only type V and type VII SCC*mec* elements carry the *ccrC* gene. It is now a common practice to define MRSA clones by the combination of the SCC*mec* type and the chromosomal background (3). However, we failed to determine the SCC*mec* type of a MRSA isolate, BA01611, using the latest SCC*mec* typing strategy (6), because we could not amplify any known *ccr* genes from the bacterium. To gain insights into genetic characteristics of this MRSA isolate, we sequenced and characterized its SCC*mec* element in this study.

MATERIALS AND METHODS

Antibiotic resistance, genotyping, and genome sequencing. MRSA strain BA01611 was isolated from a milk sample of a Holstein cow with clinical mastitis in northwest China and was identified as *S. aureus* according to the results of 16S rRNA gene sequencing and the coagulase test. MICs for various antimicrobial agents were determined for BA01611, using an agar dilution method recommended by the Clinical and Laboratory Standards Institute (7). Multilocus sequence type (MLST) analysis and a *spa* typing assay were carried out according to previously described methods (8, 9).

The whole-genome sequencing (WGS) of BA01611 was carried out employing Solexa paired-end-sequencing technology. A genomic DNA library with an insertion size of 500 bp was prepared. WGS sequence data were *de novo* assembled into contigs using SOAPdenovo software (version 2.21). Furthermore, the contigs were joined into scaffolds using paired-end information. The order of the scaffolds was determined by alignment to a reference genome of *S. aureus* strain VC40 (GenBank accession no. NC_016912) using SOAPaligner software (version 2.21).

Assembly and identification of SCC elements in BA01611. To map SCC elements, every scaffold of BA01611 was searched against marker genes, including *orfX*, *mec* gene complexes, and *ccr* gene complexes, using BLASTN (10). Positive scaffolds were aligned to SCC elements of *S. aureus* strain JCS6690 (GenBank accession no. AB705453) to reveal their genetic organization. Gaps were closed by PCR amplification, followed by cloning into a pEASY-Blunt Zero vector (Transgen, China) for Sanger sequencing. Gene prediction and annotation for BA01611 were per-

Received 25 July 2015 Returned for modification 21 August 2015

Accepted 20 September 2015

Accepted manuscript posted online 28 September 2015

Citation Wu Z, Li F, Liu D, Xue H, Zhao X. 2015. Novel type XII staphylococcal cassette chromosome *mec* harboring a new cassette chromosome recombinase, CcrC2. Antimicrob Agents Chemother 59:7597–7601. doi:10.1128/AAC.01692-15.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.01692-15>.

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FIG 1 (a) Schematic representation of the composite SCC elements ($\text{CI}_{\text{BA01611}}$) in *S. aureus* BA01611. ORFs are shown as arrows indicating the transcription direction, and the colors of the arrows represent different fragments. Red arrowheads (DR1, DR2, and DR3) indicate the locations of integration site sequences (ISSs). Blue thin arrows indicate primers used to detect excisions. Black thick arrows indicate primers used to close gaps. (b) Comparative structure analyses of $\text{CI}_{\text{BA01611}}$ against SCC elements of *S. aureus* strains JCSC6690, A69, A71, and 1110803248. Homologous gene clusters in different strains are shaded in gray. The characteristics of aligned SCC elements are showed in the inserted table.

formed using the Rapid Annotations Subsystems Technology (RAST) server (11). The nucleotide sequences of predicted genes and their deduced amino acid sequences were subsequently compared to the nucleotide collection (nr/nt) database and the nonredundant protein database provided by NCBI, respectively.

PCR for excision of SCC elements and for determination of the presence of *ccrC2* in staphylococcal isolates. A set of PCRs were performed for investigating excision of SCC elements according to the method described by Ito et al. (2). Extracted genomic DNA of BA01611 from logarithmic-phase cultures was used as the template. The products were then cloned into a pEASY-Blunt Zero vector (Transgen, China) for Sanger sequencing. Primer sequences are listed in Table S1 in the supplemental material.

A local collection of 97 staphylococcal isolates in our laboratory was used for determining the presence of *ccrC2*, using the specific primers listed in Table S1 in the supplemental material. The primer set can discriminate *ccrC2* from *ccrC1* with an amplicon size of 761 bp. These staphylococci were isolated from bovine mastitis milk in northwest China and have been subjected to 16S rRNA gene sequencing, the coagulase test, and the oxacillin susceptibility test.

Bioinformatics analyses. In order to determine phylogenetic relations between this novel SCC_{mec} and other reported SCC_{mec} , the NCBI nucleotide blast software was used for genome alignments in the nr/nt and wgs databases with default parameters (accessed on 30 April 2015). The query sequence was the identified nucleotide sequence of Composite Island ($\text{CI}_{\text{BA01611}}$), with a length of 49.3 kb. The top matched sequences with both a maximum score of greater than 10,000 and a total score of greater than 30,000 were chosen for detailed analyses.

The comparison of different SCC_{mec} elements was conducted using BLASTN with Mauve software (12) and Easyfig software (13). For the phylogenetic analysis, *ccrC* genes were aligned by the ClustalX 2.1 program. A phylogenetic tree was constructed employing the MEGA pro-

gram (version 6.0) with the neighbor-joining method. Bootstrap values were obtained from 1,000 repetitions and are listed as percentages at the nodes.

The existence of *ccrC2* in staphylococcal species in GenBank was determined by searching the nr/nt and wgs databases from NCBI, utilizing the nucleotide sequence of the *ccrC2* gene as the query.

Nucleotide sequence accession number. The nucleotide sequence of this composite SCC, designated Composite Island $\text{CI}_{\text{BA01611}}$ (described below), has been deposited in the GenBank database under accession no. [KR187111](https://www.ncbi.nlm.nih.gov/nuccore/KR187111).

RESULTS AND DISCUSSION

WGS was carried out in order to understand the genetic background of MRSA isolate BA01611. A total of 360 Mb of sequencing data from BA01611 were obtained and assembled into 50 scaffolds (GenBank accession no. [LIRC00000000](https://www.ncbi.nlm.nih.gov/nuccore/LIRC00000000)). Among those scaffolds, five were identified as containing SCC elements. Four PCRs were performed to close these gaps. Finally, a composite SCC, designated Composite Island ($\text{CI}_{\text{BA01611}}$), with a total length of 49.3 kb was revealed. It consisted of a pseudo-SCC element named $\Psi\text{SCC}_{\text{BA01611}}$ and an intact SCC_{mec} designated $\text{SCC}_{\text{mecBA01611}}$ (Fig. 1a). In addition, three integration site sequences (ISS) comprising directly repeated (DR) sequences were identified in $\text{CI}_{\text{BA01611}}$.

BA01611 showed multidrug resistance to oxacillin (MIC = 32 $\mu\text{g/ml}$), kanamycin (MIC > 256 $\mu\text{g/ml}$), gentamicin (MIC = 256 $\mu\text{g/ml}$), penicillin (MIC = 32 $\mu\text{g/ml}$), erythromycin (MIC > 256 $\mu\text{g/ml}$), cefoxitin (MIC = 128 $\mu\text{g/ml}$), neomycin (MIC > 256 $\mu\text{g/ml}$), chloramphenicol (MIC = 32 $\mu\text{g/ml}$), and sulfamethoxazole (MIC > 256 $\mu\text{g/ml}$). However, it was susceptible to vanco-

mycin. In addition, the composite SCC (CI_{BA01611}) in BA01611 did not contain any antibiotic resistance gene other than *mecA*. This phenomenon seems contradictory to previous reports that composite SCC elements usually contain more resistance genes (14–16). Nevertheless, antibiotic resistance genes such as *blaZ*, *tetL*, *aacA-aphD*, *aadD*, *aadE*, *lnuB*, *isaE*, and *tcaA* were found elsewhere to be carried by many insertion sequences (IS256 and IS431) or recombinant plasmids located in the chromosome of BA01611 (see Table S2 in the supplemental material).

In order to study the structural organization and genetic contents of the SCCmec in BA01611, predicted open reading frames (ORFs) were analyzed. SCCmec_{BA01611} was estimated to be 25 kb in size with 31 ORFs and to be demarcated by DR2 and DR3 (Fig. 1a). A novel *ccrC* allotype (*orf37*) was identified in SCCmec_{BA01611}, which shared nucleotide identities of 62.6% to 69.4% with the *ccrC1* alleles (see Table S3 in the supplemental material). Results from the phylogenetic tree also confirmed that this novel *ccrC* allotype was phylogenetically distinct from all *ccrC1* genes (see Fig. S1 in the supplemental material). According to the proposed criteria from International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (3), we gave the novel *ccr* gene the designation *ccrC2*. The *ccr* gene complex (*orf32* to *orf38*) was consequently designated a type 9 complex. This complex had nucleotide identity of 66.2% with the type 5 *ccrC* gene complex of *S. aureus* strain JCSC6082 (GenBank accession no. AB373032). This novel *ccrC* gene complex was further classified as a group 1 *ccrC* gene complex, according to an additional *ccrC* classification scheme (17). A class C2-like *mec* gene complex (*orf44* to *orf50*), composed of Δ IS431-1, Δ *mvaS*, *ugpQ*, *maoC*, *mecA*, Δ *mecR1*, and IS431-2 genes, was located downstream of the *ccrC* gene complex. Unlike the class C2 *mec* gene complex, the IS431-1 gene was truncated in BA01611. On the basis of the results presented above, SCCmec_{BA01611} was identified as a novel type XII SCCmec (9C2). The discovery of *ccrC2* added a new member to the *ccr* gene family. Since this novel allotype cannot be detected by the latest SCCmec typing strategy (6), we advocate revision of the SCCmec typing method to include *ccrC2* and the type 9 *ccr* gene complex.

Two type III restriction-modification (RM) system methylation subunits (Mod) (*orf51* and *orf55*) were identified in the SCCmec of BA01611. Defensive systems such as restriction-modification systems are frequently reported in SCCmec of staphylococci and play a crucial role in the stability of certain regions of the *S. aureus* chromosome (5). While type I and type II RM systems other than the composite SCC (CI_{BA01611}) existed in regions of the chromosome of BA01611, there was no type IV RM system in its chromosome. Type III RM systems appear to be extremely rare in *S. aureus* and to date have been identified in only two *S. aureus* isolates, KLT6 and 118 (18).

The Ψ SCC_{BA01611} pseudo-SCC element, carrying 30 ORFs, was immediately downstream of *orfX* with a length of 24.3 kb and was delineated by DR1 and DR2 (Fig. 1a). Ψ SCC_{BA01611} shared 99.9% nucleotide sequence identity with Ψ SCC_{JCSC6690} in *S. aureus* strain JCSC6690 (Fig. 1b). An incomplete type 1 *ccr* gene complex which contained a truncated *ccrA1* was identified in Ψ SCC_{BA01611} (Fig. 1a).

CcrAB or CcrC1 can carry out both excision and integrative recombination. Moreover, a CcrA protein has little or no recombination activity on its own and requires a CcrB protein for its functions (19). Interestingly, CcrAB recombinases can excise dif-

TABLE 1 Staphylococcal isolates which carried the *ccrC2* gene from a local collection

Isolate	Species	<i>ccrC2</i>	<i>mecA</i>	MIC of oxacillin (μg/ml)
BA01611 ^a	<i>S. aureus</i>	+	+	32
BC03632	<i>S. aureus</i>	+	—	<0.5
BA00841	<i>S. epidermidis</i>	+	—	<0.5
BA00821	<i>S. epidermidis</i>	+	—	<0.5
BA01221	<i>S. epidermidis</i>	+	—	<0.5
BA01211	<i>S. epidermidis</i>	+	—	<0.5
BB02612	<i>S. epidermidis</i>	+	+	16
BB02622	<i>S. epidermidis</i>	+	+	16
BB02632	<i>S. epidermidis</i>	+	+	16
BB02642	<i>S. epidermidis</i>	+	+	16
BB02441	<i>S. epidermidis</i>	+	—	<0.5
BC02711	<i>S. epidermidis</i>	+	—	<0.5
BC03831	<i>S. epidermidis</i>	+	—	<0.5
BC03841	<i>S. epidermidis</i>	+	+	16
BC05311	<i>S. epidermidis</i>	+	—	<0.5
BA00142	<i>S. haemolyticus</i>	+	—	<0.5
BC03531	<i>S. haemolyticus</i>	+	+	16
BC04121	<i>S. haemolyticus</i>	+	—	<0.5
BC04131	<i>S. haemolyticus</i>	+	—	<0.5
BC04141	<i>S. haemolyticus</i>	+	—	<0.5
BC04821	<i>S. haemolyticus</i>	+	—	<0.5
BC04531	<i>S. haemolyticus</i>	+	—	<0.5
BC04621	<i>S. haemolyticus</i>	+	—	<0.5
BC04631	<i>S. haemolyticus</i>	+	—	<0.5
BA01931	<i>S. agnetis</i>	+	—	<0.5
BC04632	<i>S. warneri</i>	+	—	<0.5

^a BA01611 was coagulase positive, while all of the other isolates were coagulase negative.

ferent types of SCCmec elements, while a CcrC1 recombinase is specific to the type of SCCmec element that contains it (5). Previous research found recombination between two phylogenetically close *ccr* genes, *ccrC1* allele 8 and *ccrC1* allele 10, causing loss of the *mec* gene complex (20). In order to investigate whether excision of SCC elements occurred in BA01611, extrachromosomal circular intermediates of each element situated between DRs were identified by PCR using primer sets of 1R and 1F, 2R and 2F, and 1R and 2F (Fig. 1a). DNA fragments giving positive results were successfully amplified by all primer sets, indicating formation of the circular intermediates from both single SCC elements (SCC-mec_{BA01611} and Ψ SCC_{BA01611}) and the composite SCC (CI_{BA01611}). Nucleotide sequencing confirmed that excision of these elements occurred precisely at the DR sites. These results suggest that CcrC2 is functional and responsible for the potential spread of SCC elements in BA01611. Recent studies indicated that horizontal spread of SCCmec is more frequent than was believed before (21, 22). It has been suggested that an increase in expression of the *ccrAB* genes might be associated with a high frequency of SCCmec excision (14). Higgins et al. (23) found upregulated expression of *ccrA* in *S. aureus* under conditions of exposure to β -lactam antibiotics. An *in vivo* transfer of the SCCmec into a MSSA isolate after β -lactams were used was also observed (24). Therefore, treatment with β -lactams has the potential to promote the transfer of SCCmec. The involvement and regulation of *ccrC2* in horizontal transmission of SCCmec need to be further investigated.

A local collection of 97 staphylococcal isolates in our laboratory was used for examining the presence of *ccrC2*. As shown in Table 1, 26 isolates were positive for *ccrC2*. The *ccrC2* gene was

TABLE 2 The presence of *ccrC2* in staphylococcal strains represented in GenBank

Strain	Species	% sequence identity	Country	GenBank accession no
A69	<i>S. aureus</i>	100	China	JJOP00000000.1
A71	<i>S. aureus</i>	100	China	JJO000000000.1
1110803248	<i>S. aureus</i>	100	United States	JJDZ00000000.1
VCU013	<i>S. epidermidis</i>	96	United States	JHTZ00000000.1
VCU014	<i>S. epidermidis</i>	96	United States	JHQB00000000.1
NIH05001	<i>S. epidermidis</i>	96	United States	AKHE00000000.1
1057	<i>S. epidermidis</i>	96	Germany	JHTZ00000000.1
12142587	<i>S. epidermidis</i>	90	France	AMSJ00000000.1

mainly carried by coagulase-negative staphylococci (CoNS), especially *S. epidermidis* and *S. haemolyticus*, and was present in both oxacillin-resistant and oxacillin-susceptible isolates. The existence of *ccrC2* in staphylococcal species whose data are available in GenBank was also determined by searching the NCBI nr/nt and wgs databases, utilizing the nucleotide sequence of *ccrC2* gene as the query. Results showed that the *ccrC2* gene was present in eight staphylococcal isolates (3 *S. aureus* and 5 *S. epidermidis*), with sequence identities of 90% to 100% to the *ccrC2* gene in BA01611 (Table 2). These bacteria were collected from China (2 isolates), the United States (4 isolates), France (1 isolate), and Germany (1 isolate). Among them, *S. aureus* isolates A69 (China), A71 (China), and 1110803248 (United States) shared 99.9% nucleotide sequence identity with part of *CI*_{BA01611} (Fig. 1b), suggesting that the SCC elements in these three isolates as well as in BA01611 shared a closely related ancestor.

BA01611 belonged to *spa* type t899 and ST9. ST9-t899 MRSA isolates predominated in swine farms from four Chinese provinces and could be isolated from two swine workers, indicating its interspecies transmission (25). ST9-t899 MRSA is the major livestock-associated MRSA (LA-MRSA) strain in pigs in China (26). On the other hand, ST398 strains are the most frequently found LA-MRSA strains in human and animal hosts in European countries and North America (27, 28) and have been reported less frequently in China (29). Considering the fact that LA-MRSA ST9 appears to be capable of spreading the resistance phenotype to humans, proliferation of ST9 MRSA isolates carrying type XII SCCmec in staphylococci from cows could pose a threat to both animal and human health.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Science Foundation of China (grant number 31372282), by the China Thousand Talents program, and by two University Scientific Research Fund projects (Fund number Z111021305 and Fund number Z109021431). This work was also partially supported by a discovery grant from the Natural Science and Engineering Research Council of Canada.

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