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Novel Types of Staphylococcal Cassette Chromosome *mec* Elements Identified in Clonal Complex 398 Methicillin-Resistant *Staphylococcus aureus* Strains▽‡

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The structures of staphylococcal cassette chromosome *mec* (SCC*mec*) elements carried by 31 clonal complex 398 (CC398) methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from the participants at a conference were analyzed. The SCC*mec*s were classified into novel types, namely, IX, X, V(5C2&5) subtype c, and IVa. Type V(5C2&5) subtype c, IX, and X SCC*mec*s carried genes conferring resistance to metals. The structures of SCC*mec*s from CC398 strains were distinct from those normally found in humans, adding to the evidence that humans are not the original host for CC398.

Recent reports of methicillin-resistant *Staphylococcus aureus* (MRSA) in livestock, particularly pigs, and in individuals who have contact with livestock provided evidence of the existence of a true livestock-associated MRSA reservoir (1, 13, 16, 18–19). Livestock-associated MRSA strains are nontypeable by pulsed-field gel electrophoresis using SmaI (2) and belong to specific *spa* types that group into multilocus sequence types (MLST) of clonal complex 398 (CC398), and the vast majority of strains are resistant to tetracycline (6, 12).

Although CC398 MRSA strains have been studied extensively (e.g., the whole genome sequence has been determined [15]), structures of staphylococcal cassette chromosome *mec* (SCC*mec*) elements carried by these strains are not yet fully understood. This study was undertaken to clarify the structures of SCC*mec* elements carried by CC398 MRSA strains in order to gain more understanding of their genetic background.

A total of 31 MRSA strains (Table 1) were collected from participants at the 19th International Pig Veterinary Conference in Denmark, 2006, including participants from Europe ($n = 29$), Thailand ($n = 1$), and Canada ($n = 1$); these strains were originally found to belong to *spa* types associated with CC398 in a previous study (20), which was confirmed by multilocus sequence typing (MLST) of 15 representative isolates (Table 1) (7).

The SCC*mec* elements were typed using a multiplex-PCR (M-PCR) assay described by Kondo et al. (14). Among 29 strains recovered from European participants, 25 (86.2%) carried type V SCC*mec* and 4 (13.8%) carried type IVa SCC*mec* (Table 1). Type IVa SCC*mec* of strain JCSC7158 contained a larger-than-normal class B *mec* gene complex [termed B(L) in Table 1]. Sequence analysis showed that a free copy of IS*256* was inserted 45 bp downstream of the *mecRI* start codon. In contrast, strains JCSC6943 and JCSC6945 recovered from a Thai and a Canadian participant, respectively, carried SCC*mec*s that could not be classified into extant types. JCSC6943 was shown to carry a novel combination of the *ccr* gene complex (type 1) and *mec* gene complex (class C2), whereas neither *ccr* nor *mec* gene complexes in strain JCSC6945 could be amplified. The nucleotide sequences of the untypeable SCC*mec* elements carried by strains JCSC6943 and JCSC6945, as well as by a representative of the predominant type V SCC*mec* carried by strain JCSC6944, were determined using amplified DNA fragments with long-range PCR and fosmid clones that were prepared as described previously (3) and chosen by PCRs with pairs of primers identifying genes in SCC*mec*s (*mecA*, *copB*) and the chromosomal regions flanking SCC*mec*s. Open reading frames (ORFs) (≥100 bp) were identified with the In-silico Molecular Cloning genomics edition program (*in silico* biology, inc., Yokohama, Japan) and were compared to nucleotide sequences in the databases at the National Center for Biotechnology Information (Bethesda, MD) using BLAST for identifying homologues and predicting functions.

The structures of the SCC*mec* elements are illustrated in Fig. 1. The nucleotide sequences at the left and right chromosomal junctions of all three SCC*mec*s were similar to those of extant SCC*mec*s; they were demarcated by direct repeats containing the integration site sequence of the SCC (ISS), carried characteristic inverted repeats at both ends, and were integrated downstream of *orfX*.

JCSC6943's SCC*mec* (43,675 bp) carried a type 1 *ccr* gene

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TABLE 1. Characteristics of 31 MRSA strains isolated from the participants of an international conference

Country of participants (no. of strains)	*spa* type (no. of strains)	No. of strains of MLST type ST398/ no. of tested strains	SCC*mec* type (no. of strains)	*ccr*[a]	*mec*[b]
Belgium (1)	t011 (1)	1/1	IVa (1)	2	B
Canada (1)	t034 (1)	1/1	X (new) (1)	NT	C1
Germany (13)	t011 (8)	1/8	V (13)	5	C2
	t034 (4)	1/4			
	t108 (1)	1/1			
Italy (7)	t899 (1)	1/1	IV (1)	2	B
	t108 (1)	1/1	V (6)	5	C2
	t899 (5)	1/5			
Netherlands (6)	t011 (2)	1/2	IVa (2)	2	B(L), B
	t011 (1)	1/1	V (4)	5	C2
	t571 (1)	1/1			
	t567 (1)	1/1			
	t108 (1)	1/1			
Spain (2)	t011 (2)	1/2	V (2)	5	C2
Thailand (1)	t034 (1)	1/1	IX (new) (1)	1	C2

[a] NT, nontypeable. A weak band was observed in some cases.
[b] B(L) signifies that the size of the DNA fragment amplified by a primer pair identifying the *mecA* gene lineage as IS*1272* was longer than that of standard strains carrying the class B *mec* gene complex. A long-range PCR using primers mA7 and IS7 for the class B *mec* gene complex was performed, which produced a 4,159-bp amplicon, compared to a 2,827-bp amplicon produced by the standard strain, CA05. A 4,159-bp amplicon (GenBank accession no. HQ157182) containing a free copy of IS*256* was inserted 45 bp downstream of the *mecRI* start codon on the opposite strand, which generated an 8-bp target site duplication (CATT GCTC) upon transposition.

complex and class type C2 *mec* gene complex, although the nucleotide identities of JCSC6943's *ccrA1* and *ccrB1* genes to those of NCTC10442's SCC*mec* (type I) are 94.1% and 92.2%, respectively, and six ORFs surrounding the *ccr* genes showed 58.3 to 65.9% nucleotide identities to those of NCTC10442's SCC*mec*. The *ccr* gene complex was located between *orfX* and the class C2 *mec* gene complex, which is similar to the structure of JCSC6082's SCC*mec* (3). Based upon the novel combination of *ccr* and *mec* gene complexes (1C2), JCSC6943's SCC*mec* was classified as novel type IX.

JCSC6945's SCC*mec* (50,803 bp) carried a *ccrA1* gene with 94.1% nucleotide identity to that of NCTC10442's SCC*mec* and a *ccrB* gene; *ccrB* was phylogenetically closer to *ccrB6* of *Staphylococcus saprophyticus* ATCC 15305 than to *ccrB1* of NCTC10442, with nucleotide identities of 89.1% and 87.0%, respectively (Fig. 2). Nucleotide identities of ORFs surrounding the *ccr* genes to those of SCC*mec* in NCTC10442 were 59.9 to 85.5%. Based on the novel combination of *ccrA1* and *ccrB6*, the *ccr* gene complex was classified as a novel type, type 7. SCC*mec* in JCSC6945 carried a novel class C1-like *mec* gene complex (6,422 bp), which was distinct from the class C1 *mec* gene complex (7,212 bp) carried by type VII SCC*mec* in a Swedish community-associated MRSA strain, JCSC6082, in two respects: (i) SCC*mec* in JCSC6945 and SCC*mec* in JCSC6082 carry different direct repeat unit (*dru*) types (14f

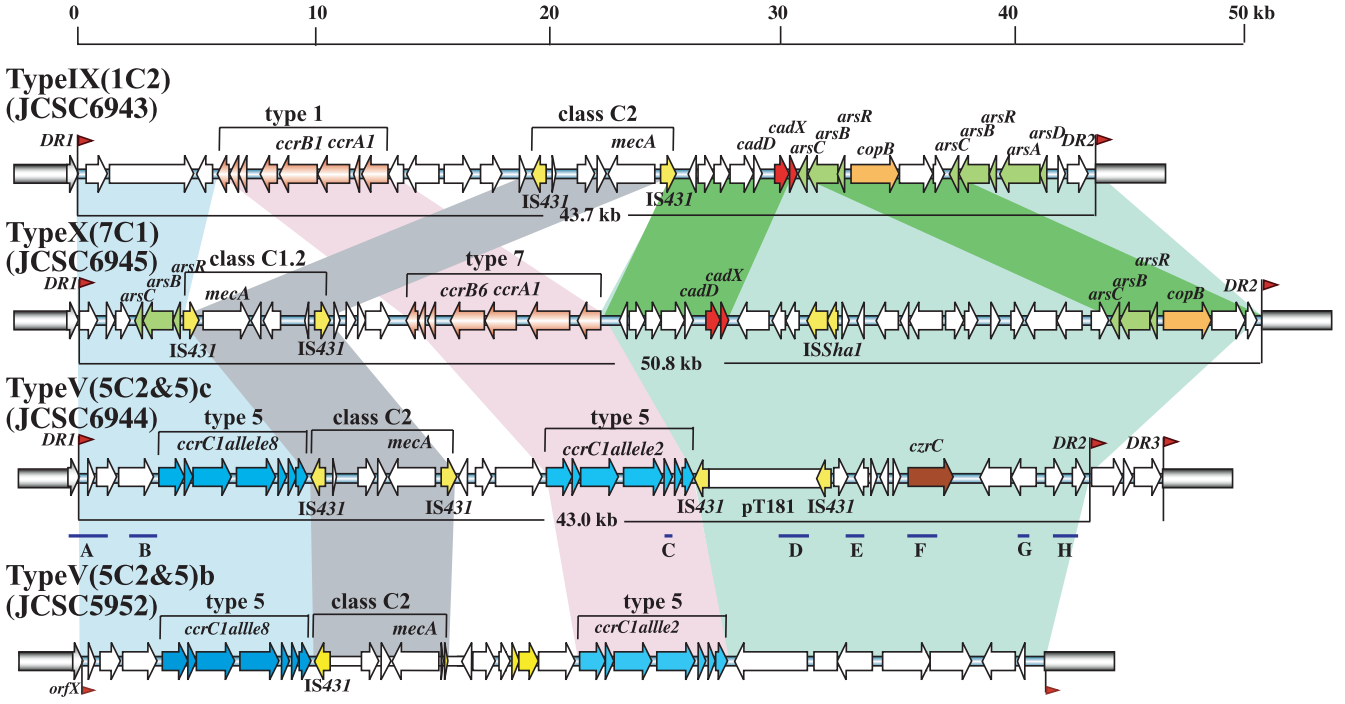

FIG. 1. Structures of three SCC*mec* elements identified in CC398 MRSA strains. The structures of type V (5C2&5)c, IX (1C2), and X (7C1) SCC*mec* elements from MRSA CC398 strains JCSC6944, JCSC6943, and JCSC6945, respectively, and type V(5C2&5)b SCC*mec* from strain JCSC5952 are illustrated based on the nucleotide sequences deposited in the DDBJ/EMBL/GenBank databases under accession no. AB505628 to AB505630 and AB478780. Blue bars indicate structures (A to H) distributed over the entire JCSC6944 SCC*mec* element, which were detected by a PCR-based approach (see the text). Red arrowheads indicate the locations of integration site sequences (ISS) for the SCC. Insertion sequences are indicated in yellow. Genes conferring resistance to metals are colored as follows: *cadDX* (red), *arsRBC* and *arsDARBC* (green), *copB* (orange), and *czrC* (brown). The *ccr* gene complexes, *mec* gene complexes, J3 regions, and J1 regions are shaded in pink, gray, blue, and light green, respectively. Structures of the J1 region that are conserved between JCSC6943's SCC*mec* and JCSC6945's SCC*mec* are shaded in deep green. The primers used for the amplification of DNA fragments, screening of fosmid clones, and structures A to H in JCSC6944's SCC*mec* are listed in Table S1 in the supplemental material. DR, direct repeat.

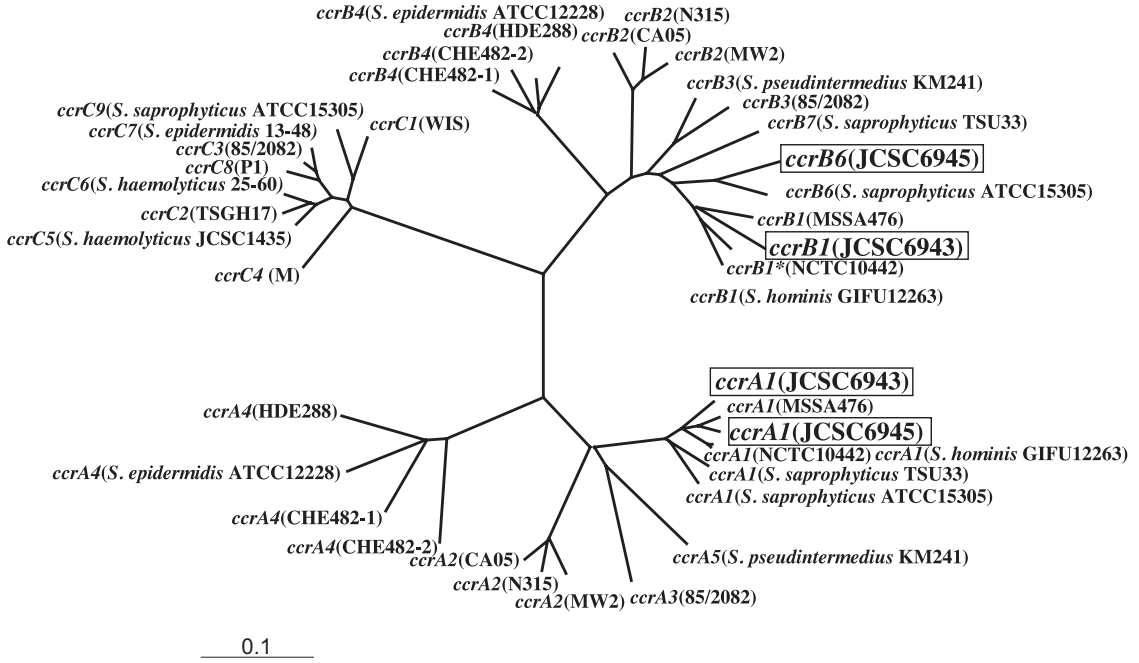

FIG. 2. Phylogenetic relations of *ccr* genes. The *ccrA* and *ccrB* genes from the following strains were used. DDBJ/EMBL/GenBank database accession numbers are indicated in parentheses, and species are not indicated in the cases of *S. aureus* strains. *ccrA1* and *ccrB1*, NCTC10442 (AB033763), MSSA476 (BX571857), and GIFU12263 (AB063171); *ccrA2* and *ccrB2*, N315 (D86934), CA05 (AB063172), and MW2 (NC003923); *ccrA3* and *ccrB3*, 85/2082 (AB037671); *ccrA4* and *ccrB4*, HDE288 (AF411935), ATCC 12228 (AE015929), CHE482-1 (EF126185), and CHE482-2 (EF126186); *ccrA1* and *ccrB6*, ATCC 15305 (NC007350); *ccrA1* and *ccrB7*, TSU33 (AB353724); *ccrA5* and *ccrB3*, KM241 (AM904731); and *ccrC1*, WIS (AB121219), TSGH17 (AY894416), 85/2082 (AB037671), M (U10927), *S. haemolyticus* JCSC1435 (AP006716), *S. haemolyticus* 25-60 (EF190467), *S. epidermidis* 13-48 (EF190468), P1 (AB656125), and *S. saprophyticus* ATCC 15305. The nucleotide sequences of 14 *ccrA* genes, 14 *ccrB* genes, and 9 *ccrC* genes were aligned by using the ClustalX program. The phylogenetic tree was generated by the neighbor-joining method by creating 2,000 bootstrap replicates. The tree was visualized with TreeView software, which was obtained from the website http://taxonomy.zoology.gla.ac.uk/ROD/treeview.html. The branch length indicates the distance, which is expressed as the number of substitutions per 100 bases.

and 10a, respectively); IS*431* is inserted into the *mecR1* gene at different positions (17 bp and 968 bp downstream of the start codon, respectively). Furthermore, the orientation of the class C1 *mec* gene complex of JCSC6945 is opposite to that of *mec* gene complexes carried by type I to VII SCC*mec* elements. These data indicated that the class C1 *mec* gene complexes of SCC*mec* in JCSC6082 and of SCC*mec* in JCSC6945 have evolved independently. SCC*mec* in JCSC6945 was classified as a novel type, type X, which carries the novel combination of a type 7 *ccr* gene complex and a class C1 *mec* gene complex.

SCC*mec* in JCSC6944 (43,381 bp) was a composite of an SCC carrying *ccrC1* allele 2 (10 kb) and a type V SCC*mec* carrying *ccrC1* allele 8 and a class C2 *mec* gene complex (17 kb). It was nearly identical to type V(5C2&5) SCC*mec* of the genome-sequenced ST398 MRSA strain S0385 (15). However, SCC*mec* in JCSC6944 carried an integrated plasmid, pT181, and the *mec* gene complexes of JCSC6944's SCC*mec* and S0385's SCC*mec* differed in their *dru* types (11a and 10a, respectively).

The joining (J) regions (J1, J2, and J3) constitute SCC*mec* components other than *mec* and *ccr* gene complexes in SCC*mec*s, and structural differences between the J regions within the same SCC*mec* type are used for defining subtypes (10). Whereas the first 27 ORFs of SCC*mec* in JCSC6944 showed very high similarity to the type V(5C2&5) SCC*mec*s carried by strains TSGH17 and PM1 (Taiwanese community-associated MRSA [CA-MRSA] isolates) and JCSC5952 (a Japanese CA-MRSA isolate), the last 18 ORFs, corresponding to the entire J1 region, were unique to SCC*mec* in JCSC6944 (Fig. 1). To distinguish them, we tentatively express the differences in J1 regions by using small letters while we await the decision of the International Working Group on the Classification of SCC*mec* Elements (IWG-SCC) (10): type V(5C2)a for the type V SCC*mec* identified in Australian CA-MRSA WIS; type V(5C2&5)b for the SCC*mec* elements identified in strains TSGH17, PM1, and JCSC5952; and type V(5C2&5)c for the SCC*mec* identified in JCSC6944.

Interestingly, the J regions of type V(5C2&5)c, IX, and X SCC*mec* elements carried genes related to the detoxification of heavy metals, such as cadmium, copper, zinc, and arsenate (Fig. 1). The J1 region (18 kb) of type IX SCC*mec* contained a *cadDX* operon, a *copB* gene, and two arsenate resistance operons, *arsRBC* and *arsDARBC*. All 20 ORFs in the J1 region were highly homologous to the ΨSCC*mec* elements in *Staphylococcus haemolyticus* JCSC1435, with nucleotide identities of 83.8 to 100%. The type X SCC*mec* elements contained a *cadDX* operon, a *copB* gene, and an *arsRBC* operon in the J1 region (28 kb) and an *arsRBC* operon in the J3 region.

In type X SCC*mec*, a 6-kb region containing the *cadDX* operon and a 7-kb region containing the *copB* gene and one of the *arsRBC* operons were highly homologous to the J1 region of type IX SCC*mec*, with nucleotide identities of 98.9% and

94.5%, respectively (indicated in dark green in Fig. 1). In addition, ISS*ha1*, an insertion sequence typically identified in *S. haemolyticus* (17), was present in the J1 region of type X SCC*mec*.

The J1 region of type V(5C2&5)c SCC*mec* (17 kb) carried a *czrC* (cadmium zinc resistance C) gene, which is responsible for cadmium and zinc resistance (4), as well as an integrated plasmid, pT181, bearing *tet*(K). Notably, this plasmid was present as a free plasmid in the genome-sequenced MRSA ST398 strain S0385 but absent from SCC*mec* of S0385 (15).

To investigate whether the remaining type V SCC*mec* element identified in this study was identical or closely similar to JCSC6944's SCC*mec*, we designed a PCR assay to detect structures (A to H) distributed over JCSC6944's entire SCC*mec* (Fig. 1). A total of 18 strains (72.2%; 18/25), including strain JCSC6944, contained all eight structures (A to H), whereas three strains were negative for pT181/*tetK* (structure D), two strains were negative for *czrC* (structure F), and two strains were negative for ORF42 (structure H). These data support the idea that the majority of type V SCC*mec*s are highly similar and that the observed structural differences in a subset of strains are confined to the J1 region.

The J regions of type II and III SCC*mec* elements carried by hospital-associated MRSA strains also encode additional resistance determinants (11), which may cause a fitness advantage in the presence of antibiotic selection pressures. Given that antibiotics and metals are used worldwide for growth promotion in animal agriculture (8), it is likely that CC398 MRSA strains are under similar selection pressures and that the spread and persistence of CC398 MRSA strains are results of coselection by metal resistance-encoding genes in the J regions of type V(5C2&5)c, IX, and X SCC*mec* elements.

The majority of type V(5C2&5)c SCC*mec* elements in the collection of MRSA CC398 strains also contained the tetracycline resistance gene *tet*(K). However, the role of the *tet*(K) gene in the coselection of methicillin resistance remains unclear, given that another tetracycline resistance gene, *tet*(M), is widely distributed among CC398 strains (6, 12). The *czrC* gene carried by type V(5C2&5)c SCC*mec*s is closely related to those identified in the type VIII SCC*mec*s of a Canadian epidemic MRSA strain, C10682, and in SCC*pbp4* of *Staphylococcus epidermidis* ATCC 12228. In addition, the *copB* genes carried by type IX SCC*mec*s and type X SCC*mec*s are closely related to each other and phylogenetically related to those identified in SCC elements of *S. epidermidis* ATCC 12228 and *S. haemolyticus* JCSC1435 as well as on the chromosome of *S. aureus* strains, e.g., FPR3757. These data indicate that genes conferring resistance to metals are disseminated widely among staphylococci and suggest that the genes might have been acquired from other species, e.g., *S. haemolyticus*, by horizontal gene transfer. This hypothesis is further supported by the presence of the *S. haemolyticus* insertion sequence ISS*ha1* in the J1 region of type X SCC*mec*.

Three SCC*mec* elements sequenced in this study are clearly distinct from SCC*mec*s previously identified in human isolates. The oppositely oriented class C1-like *mec* gene complex (although it should be classified as a subclass of C1), was identified in type X SCC*mec*. It suggests that other novel *mec* gene complexes will be generated in the future and adds to the evidence that SCC*mec* is formed by the acquisition of the *mec* gene complex by SCC. In contrast to the well-conserved *mecA* gene and its flanking region, the *ccr* gene complex and J regions are very diverse (Fig. 2). Although JCSC6943's SCC*mec* was judged to carry a type 1 *ccr* gene complex by PCR and nucleotide sequence comparisons, *ccrA1* and *ccrB1* in JCSC6943's SCC*mec* differed from prototypic *ccrA1* and *ccrB1* genes identified in NCTC10442's SCC*mec* (type I). JCSC6945's SCC*mec* carried *ccrA1*, which has 95% nucleotide identity to that of NCTC10442's SCC*mec*. Furthermore, JCSC6945's *ccrB6* and other constituents of the *ccr* gene complex were more homologous to those carried by *S. saprophyticus* TSU33 and ATCC 15305, with 84 to 97% nucleotide identities, respectively, than to those in NCTC10442's SCC*mec* (9), suggesting that the *ccr* gene complex with *ccrA1-ccrB6* might have evolved by undergoing recombination, although we cannot predict where such recombination has occurred. Such a combination of distinct allotypes of *ccr*, *ccrA3*, and *ccrB5* has been reported in *Staphylococcus pseudintermedius* (5). *S. pseudintermedius* strains are carried mostly by dogs, and two kinds of SCC*mec* elements were identified in the species, the ones identical to those found in human strains and the very diverged-structured SCC*mec*s unique to this species. These data suggested that staphylococcal strains related to animals might carry SCCs or SCC*mec*s that might have evolved in a niche distinct from that of humans. All type V(5C2&5) SCC*mec* strains were from European attendees, while the novel SCC*mec* types were from Canadian and Thai attendees, pointing to a possibly geography-dependent epidemiologic evolution.

In conclusion, we identified two novel SCC*mec* types, IX and X, and present evidence that MRSA CC398 carrying an SCC*mec* of type V(5C2&5)c has been established as the predominant livestock-associated MRSA clone in Europe. How metal and antibiotic resistance genes are linked to SCC or SCC*mec* in animal agriculture, as well as the roles of SCCs and SCC*mec*s in the dissemination and persistence of MRSA strains, are the next questions to be answered.

Nucleotide sequence accession numbers. The nucleotide sequences of SCC*mec* elements from MRSA CC398 strains JCSC6944, JCSC6943, and JCSC6945 have been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB505628 to AB505630. The nucleotide sequence of the *mec* gene complex of JCSC7158 is deposited in GenBank under accession no. HQ157182.

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