

Novel Type V Staphylococcal Cassette Chromosome *mec* Driven by a Novel Cassette Chromosome Recombinase, *ccrC*

Teruyo Ito,* Xiao Xue Ma, Fumihiko Takeuchi, Keiko Okuma,
Harumi Yuzawa, and Keiichi Hiramatsu

Department of Bacteriology, Faculty of Medicine, Juntendo University, Tokyo, Japan

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Staphylococcal cassette chromosome *mec* (SCC*mec*) is a mobile genetic element composed of the *mec* gene complex, which encodes methicillin resistance, and the *ccr* gene complex, which encodes the recombinases responsible for its mobility. The *mec* gene complex has been classified into four classes, and the *ccr* gene complex has been classified into three allotypes. Different combinations of *mec* gene complex classes and *ccr* gene complex types have so far defined four types of SCC*mec* elements. Now we introduce the fifth allotype of SCC*mec*, which was found on the chromosome of a community-acquired methicillin-resistant *Staphylococcus aureus* strain (strain WIS [WBG8318]) isolated in Australia. The element shared the same chromosomal integration site with the four extant types of SCC*mec* and the characteristic nucleotide sequences at the chromosome-SCC*mec* junction regions. The novel SCC*mec* carried *mecA* bracketed by IS431 (IS431-*mecA*- Δ *mecRI*-IS431), which is designated the class C2 *mec* gene complex; and instead of *ccrA* and *ccrB* genes, it carried a single copy of a gene homologue that encoded cassette chromosome recombinase. Since the open reading frame (ORF) was found to encode an enzyme which catalyzes the precise excision as well as site- and orientation-specific integration of the element, we designated the ORF cassette chromosome recombinase C (*ccrC*), and we designated the element type V SCC*mec*. Type V SCC*mec* is a small SCC*mec* element (28 kb) and does not carry any antibiotic resistance genes besides *mecA*. Unlike the extant SCC*mec* types, it carries a set of foreign genes encoding a restriction-modification system that might play a role in the stabilization of the element on the chromosome.

The spread of antibiotic resistance among *Staphylococcus aureus* strains is of great concern in the treatment of staphylococcal infections, since *S. aureus* has quickly acquired resistance to all antibiotics introduced for clinical use. The first clinical isolate of methicillin-resistant *S. aureus* (MRSA) was reported in 1961, only 1 year after the introduction of methicillin (18). MRSA produces penicillin-binding protein 2' (PBP 2'), which has a reduced affinity for β -lactam antibiotics (11, 34, 42). The *mecA* gene, which encodes PBP 2', and its regulatory genes, *mecl* and *mecRI*, were cloned and sequenced in the 1980s (27, 38). Those genes are located on the chromosome, and they have become widely distributed among many staphylococcal species (1, 12, 14, 23, 35, 37, 40, 41).

In the last few years, understanding of the genetic basis for methicillin resistance has advanced significantly. MRSA is produced when methicillin-susceptible *S. aureus* (MSSA) acquires a genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*).

SCC*mec* is a genomic island (Gisland) that is inserted at the 3' end of *orfX* and that is located near the replication origin of *S. aureus* (3, 24). Since our discovery of the first SCC*mec* element from pre-MRSA strain N315 in 1999, several types of SCC*mec* elements have been identified by determining their entire nucleotide sequences (16, 17, 26, 39). Pre-MRSA is a *mecA* gene-carrying MSSA strain in which *mecA* gene expression is strongly repressed by the presence of an intact *mecl*

gene. The SCC*mec* element contains the *mec* gene complex (the *mecA* gene and its regulators) and the *ccr* gene complex, which encodes site-specific recombinases responsible for the mobility of SCC*mec* (22).

Four classes of the *mec* gene complex have been identified by PCR, using chromosomal DNA from methicillin-resistant coagulase-negative staphylococci as templates. The different *mec* gene complexes are structured as follows: class A, IS431-*mecA*-*mecRI*-*mecI*; class B, IS431-*mecA*- Δ *mecRI*-IS1272; class C, IS431-*mecA*- Δ *mecRI*-IS431; and class D, IS431-*mecA*- Δ *mecRI* (21).

The *ccr* gene complex contains two site-specific recombinase genes, *ccrA* and *ccrB*, which are responsible for the mobility of SCC*mec* (16, 22). There are four allotypes in each of the *ccrA* and *ccrB* genes: *ccrA1*, *ccrA2*, *ccrA3*, and *ccrA4* for *ccrA* and *ccrB1*, *ccrB2*, *ccrB3*, and *ccrB4* for *ccrB*. SCC*mec* is classified into allotypes according to the combination of the *mec* gene complex class and the *ccr* gene complex type that it possesses (16, 26), as follows: type I SCC*mec*, class B *mec* gene complex and type 1 *ccr* gene complex; type II SCC*mec*, class A *mec* gene complex and type 2 *ccr* gene complex; type III SCC*mec*, class A *mec* gene complex and type 3 *ccr* gene complex; and type IV SCC*mec*, class B *mec* gene complex and type 2 *ccr* gene complex. The region other than the *mec* and *ccr* gene complexes is designated the J (junkyard) region. Each SCC*mec* type is further classified into subtypes on the basis of the J-region sequence (13).

SCC is a basic mobile genetic element that serves as the vehicle for gene exchange among staphylococcal species; it has been reported in some coagulase-negative staphylococci as well as in *S. aureus* (15, 20, 25). SCC*mec* is a member of the

* Corresponding author. Mailing address: Department of Bacteriology, Faculty of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo, Japan. Phone: 81-3-5802-1041. Fax: 81-3-5684-7830. E-mail: teruybac@med.juntendo.ac.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Description	Reference(s) or source
Strains		
MRSA		
WIS [WBG8318]	C-MRSA strain isolated in Australia	31 (provided by W. B. Grubb)
81/0342	NORSA strain isolated in Australia	32 (provided by J. D. Turnidge)
91/2574	NORSA strain isolated in Australia	32 (provided by J. D. Turnidge)
N315	Pre-MRSA strain isolated in Japan in 1982	12
N315ex	SCCmec excised from strain N315	22
Plasmids		
pYT3	Temperature-sensitive shuttle vector (tetracycline resistant)	9a
pSR2	pYT3 into which the <i>ccrA2</i> and <i>ccrB2</i> genes were cloned	22
pSR5 _w	pYT3 into which <i>ccrC</i> _{Wis} was cloned	This study
pSR5 _E	pYT3 into which <i>ccrC</i> ₀₃₄₂ was cloned	This study
pYTattII	pYT3 into which <i>attII</i> (the presumptive attachment-sequence in type II SCCmec) was cloned	22
pYTattV	pYT3 into which <i>attV</i> (the presumptive attachment sequence in type V SCCmec) was cloned	This study
pSR2attII	pYTattII into which the <i>ccrA2</i> and <i>ccrB2</i> were genes cloned	22
pSR5 _E attV	pYTattV into which <i>ccrC</i> ₀₃₄₂ was cloned	This study

SCC family, the members of which specialize as carriers of methicillin resistance.

Both SCC₁₂₂₆₃ (found in *S. hominis* GIFU12263) and SCC₄₇₆ (found in MSSA strain 476 [the seventh *S. aureus* strain whose whole genome sequence is being determined]) carried *ccrA* and *ccrB* genes, but they did not carry *mecA* or any other antibiotic resistance gene (20) (http://www.sanger.ac.uk/Projects/S_aureus/). SCCcap1 encodes a capsule gene cluster that confers a mucoid appearance because of overexpression of the capsule in *S. aureus* and is also a member of the SCC family (25).

MRSA has been a major causative agent of nosocomial infections (2). Recently, however, MRSA has become increasingly isolated from patients with community-acquired infections (4, 5, 9, 28, 30, 31). The SCCmec typing system that we described above has turned out to be an important marker for distinguishing these two categories of MRSA. Namely, by using the SCCmec typing system, we have provided strong evidence

for the independent derivation of health care-associated MRSA (H-MRSA) and community-acquired MRSA (C-MRSA) clones (32).

The majority of H-MRSA strains carry one of the three types of SCCmec (type I, II, or III) (6, 16), whereas well-defined American C-MRSA and nonmultiresistant oxacillin-resistant *S. aureus* (NORSA) strains carry type IV SCCmec (32). Then, Vandenesch et al. (43) reported that majority of C-MRSA strains in France also carry type IV SCCmec. Type IV SCCmec is a small element that does not carry antibiotic resistance genes other than *mecA* and has multiple subtypes. The extreme heterogeneity of the chromosome genotypes in C-MRSA strains suggests that type IV SCCmec is highly transmissible. However, we have also noted several strains whose SCCmec elements are nontypeable (32). We propose that clarifying the unknown structures of these SCCmec elements is indispensable for increasing the typeability of strains for SCCmec-

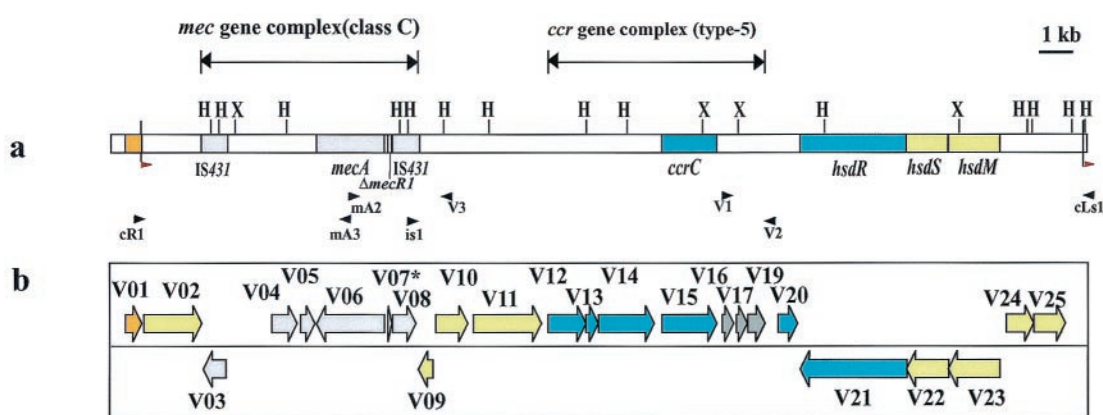


FIG. 1. Genetic structure of the type V SCCmec element of strain WIS. The structure of the type V SCCmec element illustrated is based on the nucleotide sequence deposited in the DDBJ/EMBL/GenBank databases under accession no. AB121219. (a) Essential structure of type V SCCmec. The locations of the essential genes are illustrated. The colors of the essential genes are based on those for the ORFs described for panel b. The HindIII (H) and XbaI (X) restriction sites are indicated. Black arrowheads indicate primer-specific locations. The nucleotide sequences of primers cR1, mA2, mA3, is1, and cLs1 have been described previously (17, 26). The nucleotide sequences of primers V1, V2, and V3 are listed in Table 2. The locations of direct repeats are indicated by red arrowheads. (b) ORFs in and around type V SCCmec. The ORFs corresponding to sequences more than 100 bp are indicated by arrows, which also indicate the directions of the ORFs. Light gray arrows, ORFs conserved in the five types of SCCmec elements with identities of more than 99%; gray arrows, ORFs conserved in the five types of SCCmec elements with identities of 48.1 to 93.4%; blue arrows, ORFs commonly found in both the type V SCCmec element and the J region of type III SCCmec; yellow arrows, ORFs unique to type V SCCmec; orange arrow, *orfX*.

TABLE 2. Primers used in this study

Genetic element(s) or purpose and primer	Nucleotide sequence	Expected product size (kb)	Reference or source
Type V SCC _{mec} of WIS			
V1	5'-TACCACTTTTACCACTTAGCTTT-3'		This study
V2	5'-ATGAGGCTTTAACATTTCCATCA-3'		This study
is1	5'-ACATTAGATATTTGGTTGCGT-3'		21
V3	5'-TATCATTACACTCTTGAGTCTCT-3'		This study
mA2	5'-AACGTTGTAACCACCCCAAGA-3'		21
mA3	5'-TGCTATCCACCCTCAAACAGG-3'		21
Detection of <i>ccrC</i>			
γF	5'-CGTCTATTACAAGATGTTAAGGATAAT-3'	0.52	This study
γR	5'-CCTTTATAGACTGGATTATTCAAAATAT-3'		This study
Construction of recombinant plasmids with <i>ccrC</i>			
Vcc1	5'-AAAAGGATCCAAGTTGTTTGCTTAGCGTCATTA-3'	1.93	This study
Vcc2	5'-AAAAGGATCCTAGTACTCATATGATTAAGTGGT-3'		This study
<i>att</i> SCC-V (<i>att</i> SCC of type V SCC _{mec})			
mVR2	5'-AAAAAGTCGACTACCGTCGATATCAATTGCTTTTT-3'	0.90	This study
mVL2	5'-AAAAAGTCGACTGGAGACGTAGTATAAATATAGCT-3'		This study
Detection of precise excision and closed circular DNA formation			
<i>att</i> B _{sc} (N315)			
cL1	5'-ATTTAATGTCCACCATTAAACA-3'	0.28	17
cR1	5'-AAGAATTGAACCAACGCATGA-3'		17
<i>att</i> B _{sc} (WIS)			
cLs1	5'-TGCCAATCACAGTTCAATCAATT-3'	0.31	26
cR1	5'-AAGAATTGAACCAACGCATGA-3'		17
<i>att</i> SCC (N315)			
mL1	5'-GAATCTTCAGCATGTGATTTA-3'	0.46	22
mR8	5'-ATGAAAGACTGCGGAGGCTAACT-3'		22
<i>att</i> SCC (WIS)			
mVL1	5'-TACTTTGGTTTCATATTAATAGCACT-3'	0.29	This study
mVR1	5'-TCACTAGTGTAATTATCGAATGAT-3'		This study
Generation of chromosome-SCC _{mec} (mini-SCC) junction			
α	5'-TTTCACACAGGAAACAGCTATGAC-3'		22
β	5'-ATCACGATATTGCTTATAAGCA-3'		22
γ	5'-ATGTTATTAAGCAGATTGCGTCAA-3'		This study

based epidemiology and, most importantly, for obtaining a better understanding of the role of SCC in the evolution of *S. aureus*, including the acquisition of multidrug resistance.

In this study, we determined the entire nucleotide sequence of the unknown element found in Australian C-MRSA strain WIS to characterize this SCC_{mec} element and to relate it phylogenetically to other known SCC_{mec} types. It turned out to be a distinct type of SCC_{mec} with a distinct *ccr* gene homologue and with the *mecA* gene characteristically bracketed by two insertion sequences.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in the experiments described here are shown in Table 1. Brain heart infusion (BHI) broth and agar (Becton Dickinson, Sparks, Md.) were used as culture media for

S. aureus. Tetracycline (Sigma Chemical Co., St. Louis, Mo.), tobramycin (Shionogi Co., Osaka, Japan), and ceftizoxime (Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan) were used where appropriate at the concentrations indicated in the text.

DNA manipulation and nucleotide sequencing. The DNA fragments encompassing the entire SCC_{mec} nucleotide sequence of strain WIS were amplified by long-range PCR with several sets of primers, as follows: the region from the *orfX* gene to the *mecA* gene was covered by primers cR1 and mA3, and the region from the *mecA* gene to the chromosomal region flanked to the left end of SCC_{mec} was amplified by PCR with primers is1 and cLs1. The latter region was later amplified by PCR with primer sets mA2 and V3, is1 and V2, and V1 and cLs1, as indicated in Fig. 1. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and their nucleotide sequences were determined. The amplification steps for PCR, long-range PCR, and nested PCR were performed as described previously (17).

Construction of recombinant plasmids and excision assay. The DNA fragments containing the *ccrC* gene of strain WIS (*ccrC*_{WIS}) and the *ccrC* gene of strain 81/0342 (*ccrC*₀₃₄₂) were amplified by PCR with primers Vcc1 and Vcc2 and

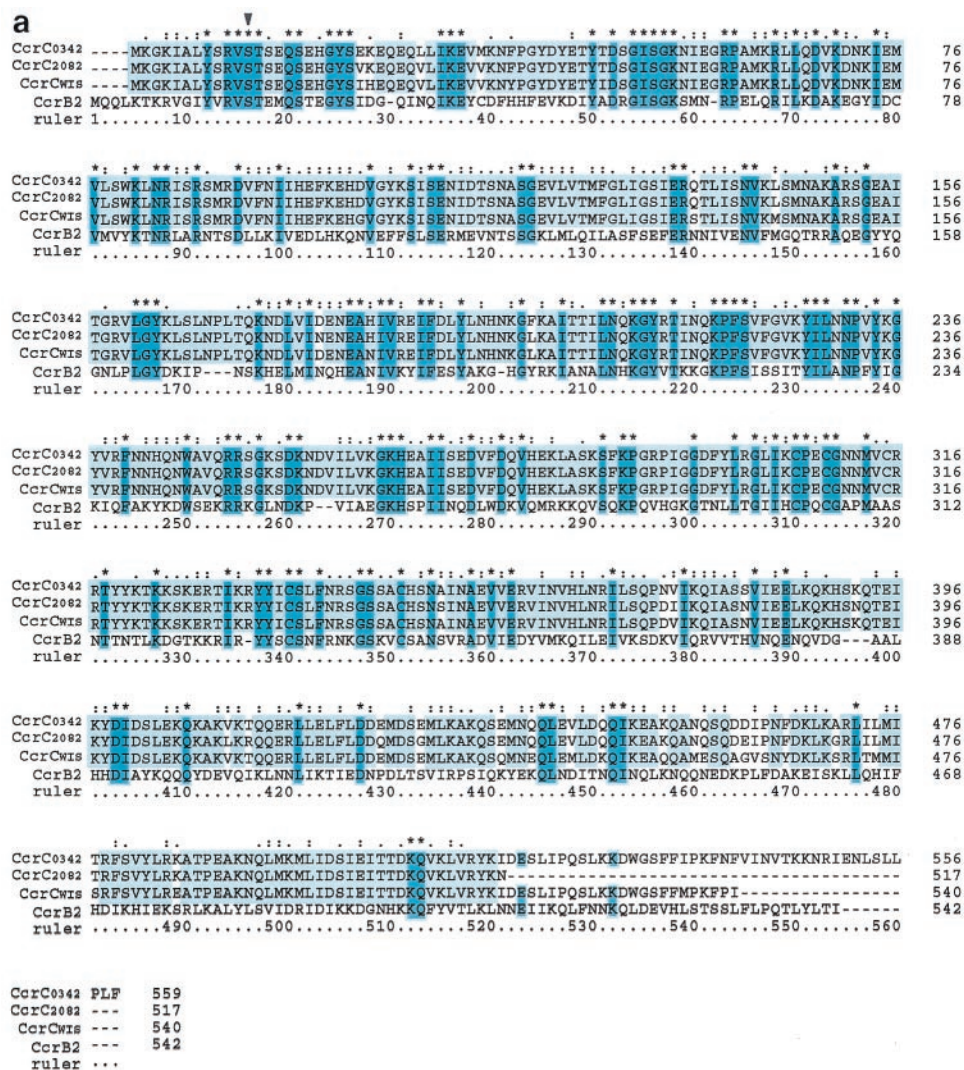


FIG. 2. Characteristics of deduced amino acid sequences of CcrC. (a) The deduced amino acid sequences of the three *ccr* genes were aligned with that of *ccrB2* of strain N315. The alignment was performed by using ClustalX software with the protein weight matrix PAM series. The three Ccr proteins were CcrC_{WIS} (CcrC of WIS), CcrC₀₃₄₂ (CcrC of strain 81/0342; the sequence of the strain 91/2674 CcrC was exactly identical to that of CcrC₀₃₄₂), and CcrC₂₀₈₂ (CcrC of strain 85/2082). The three Ccr proteins were highly homologous. Of the 517 amino acids corresponding to CcrC₂₀₈₂, 484 amino acids were conserved in the three Ccr proteins (pale blue) and 30 amino acids were conserved in two of three Ccr proteins. One hundred thirty-eight amino acids were conserved in CcrC₀₃₄₂, CcrC_{WIS}, and CcrB2 (blue). The consensus sequence shown above the amino acid sequences indicates conserved amino acids, as follows: asterisks, identical or conserved residues in all sequences in the alignment; colons, conserved substitutions; dots, semiconserved substitutions; hyphens, gaps. A black arrowhead indicates the presumptive serine involved in the phosphoserine linkage conserved in the NH₂-terminal catalytic domain of site-specific recombinases of the invertase-resolvase family. (b) Phylogenetic relationships among *ccrA* genes, *ccrB* genes, *ccrC* genes, and two site-specific recombinases. The two site-specific recombinases used were the integrase (*int*) of bacteriophage ϕ FC1 found in *E. faecalis* (1,216 bp; DDBJ/EMBL/GenBank accession no. AF124258) and the site-specific recombinase found in *C. acetobutylicum* ATCC 824 (1,635 bp; DDBJ/EMBL/GenBank accession no. AE007636). The *ccrA* and *ccrB* genes used were as follows: *ccrA1* and *ccrB1**, respectively, found in strain NCTC10442 (DDBJ/EMBL/GenBank databases accession no. AB033763); *ccrA2* and *ccrB2*, respectively, found in strains N315 (DDBJ/EMBL/GenBank databases accession no. D86934) and CA05 (DDBJ/EMBL/GenBank databases accession no. AB063172); *ccrA3* and *ccrB3*, respectively, found in strain 85/2082 (DDBJ/EMBL/GenBank databases accession no. AB037671); and *ccrA4* and *ccrB4**, respectively, found in strain HDE288 (DDBJ/EMBL/GenBank databases accession no. AF411935). Since *ccrB1* of NCTC10442 and *ccrB4* of HDE288 were truncated, we reconstituted large ORFs of 1,626 bp (*ccrB1**) and 1,629 bp (*ccrB4**) by adding an adenine and used them for the comparison. The nucleotide sequences of five *ccrA* genes, five *ccrB* genes, four *ccrC* genes, and two site-specific recombinases 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 Tree View software, which was obtained from the website <http://taxonomy.zoology.gla.ac.uk/rod/treeview.htm>. The branch length indicates the distance, which is expressed as the number of substitutions per 100 bases.

digested with BamHI. The fragments were cloned into the BamHI site of temperature-sensitive shuttle vector pYT3 to construct recombinant plasmids pSR5_W and pSR5_E, respectively. The fidelity of the cloning was ascertained by determining the nucleotide sequence of the cloned DNA fragment. Recombinant plasmids were introduced into *S. aureus* cells by electroporation, as described previously (22).

For the excision assay, approximately 10³ cells of transformants and their parent strains were inoculated into 10 ml of BHI broth containing tetracycline (10 mg/liter), and the culture was incubated at 30°C with shaking. After 20 h, an aliquot of 0.8 ml was transferred to an Eppendorf tube and cells were collected by centrifugation. DNAs were extracted from the cells and used as templates for

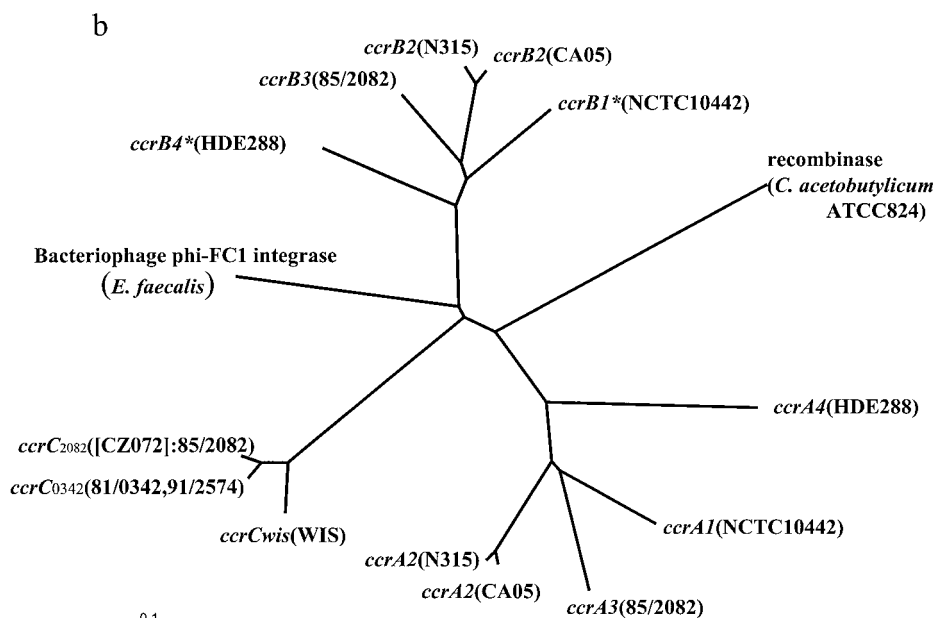


FIG. 2—Continued.

subsequent PCR experiments to identify the *attB*sc and *attSCC* sequences generated in the cells with the primer sets listed in Table 2.

To investigate the strains from which DNA was excised for the generation of SCCmec, cells were further cultivated in BHI broth with tetracycline at 30°C, with one passage per day for 10 days. The cultures were diluted, and approximately 10³ cells were inoculated for each passage. The cells were evaluated for the loss of SCCmec by replicating them onto agar plates with and without tobramycin at 10 mg/liter (for strain N315) and ceftizoxime at 5 mg/liter (for strain WIS).

Construction of a recombinant plasmid carrying the *ccrC* gene and *attSCC* formed in WIS(pSR5_E) and integration assay. Recombinant plasmid pSR5_EattV, which carried *ccrC*₀₃₄₂ and *attSCC* formed in WIS(pSR5_E), was constructed as a model of the closed circular form of type V SCCmec (mini-SCC). Briefly, the DNA fragment containing *attSCC* of type V SCCmec was amplified by PCR with primers mVR2 and mVL2 (Table 2). DNA extracted from WIS(pSR5_E) was used as the template for the amplification of type V *attSCC*. A SalI-digested DNA fragment carrying *attSCC* type V was cloned into pYT3 to produce pYT3attV. Plasmid pSR5_EattV was constructed by cloning a BamHI-digested DNA fragment carrying *ccrC*₀₃₄₂ into pYT3attV.

For the integration assay, recombinant plasmids and control plasmids were introduced into N315 by electroporation, and the cells were grown on BHI agar plates containing tetracycline at a concentration of 10 mg/liter at 30°C for 46 to 47 h. The colonies that grew on each tetracycline plate were resuspended in 0.5 ml of BHI broth, spread onto the BHI agar plates with or without tetracycline (10 mg/liter), and incubated at 30°C (permissive temperature for the replication of plasmid pYT3) and 43°C (a temperature nonpermissive for the replication of plasmid pYT3) for 18 h.

Computer analysis. Open reading frames (ORFs) of more than 100 bp were identified with the GAMBLER software, and their functions were predicted by a search homology with the BLAST program. All the ORFs in and around type V SCCmec were compared to those of the four known types of SCCmec (type I in strain NCTC10442 [DDBJ/EMBL/GenBank accession no. AB033763], type II in strain N315 [DDBJ/EMBL/GenBank accession no. D86934], type III in strain 85/2082 [DDBJ/EMBL/GenBank accession no. AB037671], and type IVa in strain CA05 [DDBJ/EMBL/GenBank accession no. AB063172]). The homologies between the nucleotide sequence of the type V SCCmec element of strain WIS and those of the type I, II, III, and IVa SCCmec elements were studied as described previously (20).

Several types of analyses were carried out with the BLAST program at the website of the National Center for Biotechnology Information (<http://www3.ncbi.nlm.nih.gov/BLAST/>). The codon usage values for prokaryotes were taken from a database (<http://www.kazusa.or.jp/codon/>). Codon usage was tabulated from international DNA sequence databases (sequence status for the year 2000) (29). The similarity of codon usage was evaluated by codon bias analysis (19).

Nucleotide sequence accession number. The sequence of the type V SCCmec element of strain WIS has been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB121219.

RESULTS

Type V SCCmec as a new member of the SCCmec family.

Our purpose was to investigate the genetic organization of the unknown element carrying the class C *mec* gene complex that was found in three strains, a C-MRSA strain (strain WIS) and two NORSA strains (strains 81/0342 and 91/2574). For that reason, we selected strain WIS and determined the nucleotide sequence of the region in and around the *mec* gene complex. The overall organization of the element and the strategy used to amplify the DNA fragment by long-range PCR with different sets of primers are shown in Fig. 1a.

The element carried the class C *mec* gene complex, which is composed of a copy of insertion sequence IS431, *mecA*, truncated *mecR1* (Δ *mecR1*), and another copy of IS431 inserted in the opposite direction (21). From the size of Δ *mecR1* and the direction of another copy of IS431, it was judged that the element carried the class C2 *mec* gene complex (21).

Neither the *ccrA* gene nor the *ccrB* gene, both of which are responsible for the mobility of SCCmec, was found in the element. Instead, a novel ORF sequence, V15, encoding a protein similar to site-specific recombinases was found. The deduced amino acid sequence of V15 showed the highest degree of similarity to that of a type III SCCmec ORF, CZ072, with sequence identity of 93.2%.

We examined by PCR whether the novel ORF is commonly found in the other two NORSA strains. By amplifying DNA fragments with the primer set listed in Table 2 and determining the nucleotide sequences of the two DNA fragments, we found that the other two strains also carried identical ORFs. These ORFs were actually highly similar to that of WIS (ORF V15), with a nucleotide sequence identity of 91.4%.

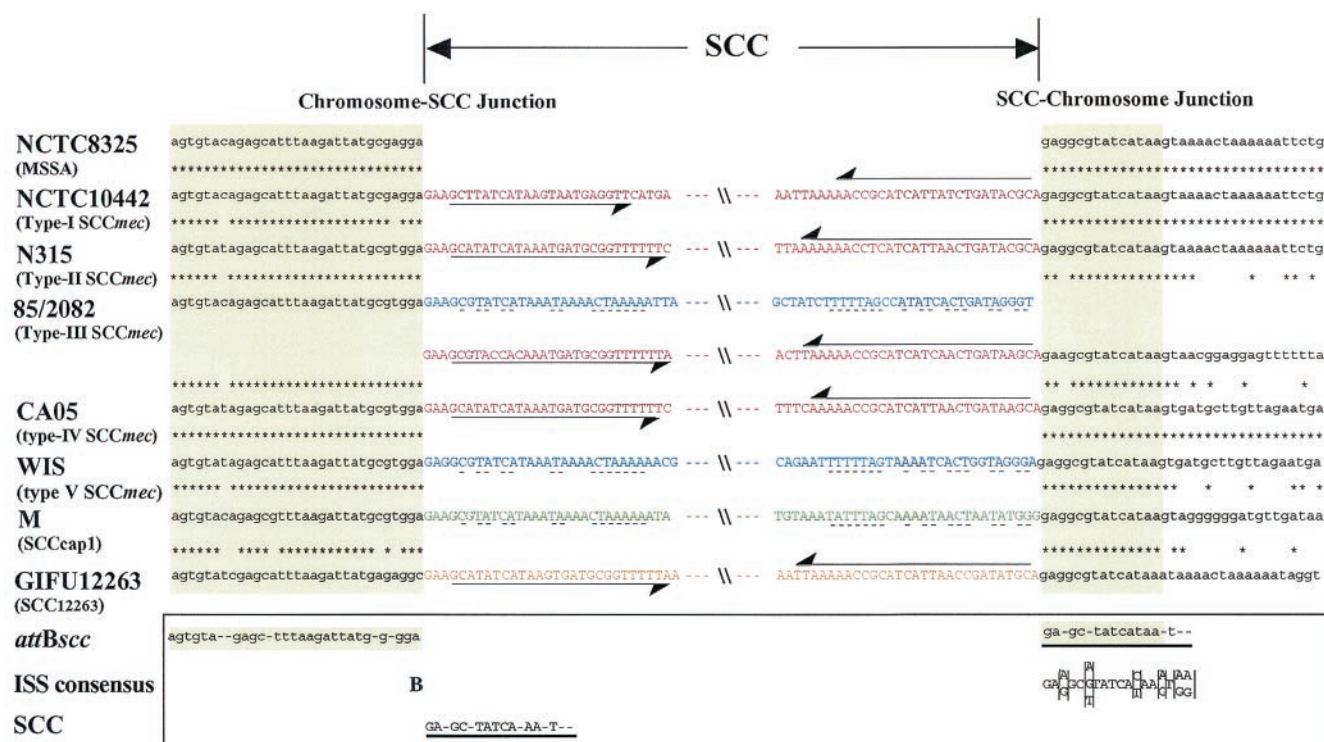


FIG. 3. Chromosome-SCC junction sequences. The nucleotide sequences at the left and right boundaries of the SCCmec element of strain WIS are aligned with those of six previously reported SCC elements: type I SCCmec of *S. aureus* NCTC10442 (DDBJ/EMBL/GenBank databases accession no. AB033763), type II SCCmec of *S. aureus* N315 (DDBJ/EMBL/GenBank databases accession no. D86934), type III SCCmec of *S. aureus* 85/2082 (DDBJ/EMBL/GenBank databases accession no. AB037671), type IV SCCmec of *S. aureus* CA05 (DDBJ/EMBL/GenBank databases accession no. AB063172), SCCcap1 of *S. aureus* M (DDBJ/EMBL/GenBank databases accession no. U10927), and SCC₁₂₂₆₃ of *S. hominis* GIFU12263 (DDBJ/EMBL/GenBank databases accession no. AB063171). Two sets of nucleotide sequences are listed for the type III SCCmec of *S. aureus* 85/2082. One set is composed of the nucleotide sequence of the left extremity (direct repeat 1) and the nucleotide sequence in the midst of the element flanked by direct repeat 2. The other set is composed of the nucleotide sequence containing direct repeat 2 and the right extremity of the element flanked by direct repeat 3 (15). Thin arrows indicate inverted repeats at both extremities of SCCmec elements carrying the *ccrA* and *ccrB* genes. Dotted lines indicate the characteristic nucleotide sequence conserved at both extremities of type V SCCmec or SCC elements carrying the *ccrC* gene. The 3' ends of *orfX* are indicated with light green shading and lowercase bases. The consensus sequences of *attBscs* and ISS are boxed. Direct repeat sequences in ISS and the left end of the SCC element are indicated by thick lines. Nucleotide sequences of both extremities of SCC or SCCmec elements are colored as follows: red, type I, II, and IV SCCmec elements and the region between direct repeat 2 and direct repeat 3 in type III SCCmec element; orange, SCC₁₂₂₆₃; blue, type V SCCmec and the region between direct repeat 1 and direct repeat 2 in type III SCCmec element; green, SCCcap1.

The deduced amino acid sequences of the four ORFs, ORF V15 of strain WIS, the corresponding ORFs of strains 81/0342 and 91/2574, and ORF CZ072 of strain 85/2082, showed high degrees of similarity, with identities of 93.2 to 96.9%, although their C-terminal portions were dissimilar. Figure 2a shows the alignments of the deduced amino acid sequences of four ORFs: ORF V15, the corresponding ORF of 81/0342, CZ072 of 85/2082, and *ccrB* of N315. All four ORFs have a catalytic motif at the N-terminal domain, which is characteristic of recombinases of the invertase-resolvase family. They were basic proteins with pI values of 9.68 to 9.85. These features are similar to those of the CcrA and CcrB proteins that have been reported previously. We tried to relate the newly found *ccrC* genes phylogenetically to previously reported *ccr* genes: the three types of each of the *ccrA* and *ccrB* genes and another set of *ccrA* and *ccrB* genes (16, 33). In order to investigate the phylogenetic relations of those *ccr* genes, we reconstituted the putative *ccrB1* (*ccrB1**) and *ccrB4* (*ccrB4**) genes of 1626 and 1,629 bp, respectively, by adding back an adenine to the de-

leted point of the truncated *ccrB1* and *ccrB4* genes, since *ccrB1* of NCTC10442 and *ccrB4* of HDE288 are truncated. Figure 2b illustrates the phylogenetic relations of those ORFs, extant Ccr proteins, and other site-specific recombinases such as the integrase of *Enterococcus faecalis* bacteriophage ϕ FC1 and the site-specific recombinase of *Clostridium acetobutylicum* ATCC 824 (Fig. 2b). A phylogenetic tree showed that the four ORFs constitute a novel group of *ccr* genes distinct from the *ccrA* and *ccrB* genes. Accordingly, we designated the ORFs cassette chromosome recombinase C (*ccrC*) as a new putative site-specific recombinase and the elements as type V SCCmec. Type V SCCmec is defined by the carriage of the class C2 *mec* gene complex and the *ccrC* gene, which is located in the type 5 *ccr* gene complex, as described below.

The boundaries of type V SCCmec. Both the left and right boundaries of type V SCCmec were determined by comparing their nucleotide sequences with those of previously reported SCCmec and SCC elements. Determination of the nucleotide sequence of *attBscs* on the chromosome and *attSCC* on closed

circular SCC, generated by precise excision of the element, supported the predicted positions of the boundaries. Type V SCC*mec* was integrated at exactly the same nucleotide position at the 3' end of *orfX* (shown as a yellow square in Fig. 3), where the four types of SCC*mec* were integrated (Fig. 3). Two SCC elements which did not carry the *mecA* gene, SCC*cap1* and SCC₁₂₂₆₃, were also integrated at the same position. SCC*cap1*, which is found in *S. aureus* M, is an element that carries a capsule gene cluster (25). SCC₁₂₂₆₃ was found in *S. hominis* GIFU12263, and it is an element that carries active *ccrA1* and *ccrB1* genes (20). Previously, we could not judge the exact extremities of the SCC*mec* elements only by comparison of the nucleotide sequences of SCC*mec* elements found in *S. aureus* strains. This was possible because of the availability of new data from the nucleotide sequences of the *attB**scc* elements from strains M and GIFU12263, generated by precise excision of the SCC element, and they were estimated to be at the positions shown in Fig. 3 (20, 25). The integration site sequence for SCC (ISS), which is uniquely present at the 3' end of *orfX* in MSSA NCTC8325, is conserved in all strains examined so far. The ISS contains the consensus sequence 5'-BGA (A/G)GC(A/G/T)TATCA(C/T)AA(A/G)T(A/G)(A/G)-3' (where the cutting site is between the B [B signifies A, C, or G] and the G at the 5' end). The nucleotide sequences of the ISSs and the nucleotide sequences of the left extremities of the elements were nearly identical and constitute directly repeated sequences. These directly repeated sequences at the chromosome-SCC junction were found in all the elements, as shown in Fig. 3. In contrast, the degenerate inverted repeats found at both extremities of the four types of SCC*mec* and SCC₁₂₂₆₃ were not found at the extremities of type V SCC*mec* or SCC*cap1* (Fig. 3).

Structure of type V SCC*mec*. Judging from the nucleotide sequences at both extremities of the type V SCC*mec* shown in Fig. 3, type V SCC*mec* was estimated to be 27,624 bp. This is slightly larger than the type IV SCC*mec* elements (21 to 25 kb) but smaller than the type I SCC*mec* element of NCTC10442 (34 kb), the type II SCC*mec* element of N315 (53 kb), and the type III SCC*mec* element of 85/2082 (67 kb).

A total of 23 ORFs larger than 100 bp were found in the type V SCC*mec* element (Fig. 1b and Table 3). No antibiotic resistance gene other than *mecA* was found in the element. Figure 4 compares the nucleotide sequences of the five types of SCC*mec* elements obtained with the BLAST program. Type V SCC*mec* is composed of the regions conserved in other types of SCC*mec* and the regions unique to type V SCC*mec*. The regions in type V SCC*mec* similar to extant SCC*mec* elements are denoted A, B, and C in Fig. 4. Region A contained seven ORFs conserved in all five types of SCC*mec* elements with very high degrees of identity. The identities between region A1 of type V SCC*mec* and the corresponding regions in other SCC*mec* elements were more than 99.7%. Region A1 contained six ORFs in the *mec* gene complex, a transposase for IS431 (V03), a glycerophosphoryldiester phosphodiesterase homologue (V04), a hypothetical protein (V05), *mecA* (V06), and Δ *mecR1* (V07) (shown in light gray in Fig. 1b). Δ *mecR1* (V07) was smaller than Δ *mecR* in the class B *mec* gene complex. Region A2 corresponded to IS431, which was found to be closely associated with the deletion point of Δ *mecR1*. This insertion of a copy of IS431 whose direction is opposite that of

IS431 *mec* is characteristic of the class C2 *mec* gene complex. The transposases V03 and V08 encoded by two IS431 copies were not identical. The transposase V03 was nearly identical to that of IS431*mec* elements of four other types of SCC*mec* elements, whereas the transposase V08 showed slightly lower identity to that of IS431*mec* elements.

Region B represents the region conserved in type I, II, and IV SCC*mec* elements, with nucleotide sequence identities of 79.7 to 82.6%. Two ORFs, V17 and V19, were located in this region of type V SCC*mec* (Table 3). Two ORFs showed high degrees of similarity to the ORFs in the *ccr* gene complex of type I, II, and IV SCC*mec* elements.

The sequence of region C of type V SCC*mec* was homologous only to the corresponding region of the type III SCC*mec* element of strain 85/2082. Two regions of the type V SCC*mec* sequence, C1 and C2, showed high degrees of similarity to the corresponding region of the type III SCC*mec* element of strain 85/2082, with nucleotide sequence identities of 86.6 and 94.4%, respectively. Region C1 contained four ORFs (V12 to V15) whose sequences showed high degrees of similarity to the sequences of CZ072 (*ccrC*), CZ073, CZ074, and CZ075 located downstream of *orfX* in 85/2082. Although the region corresponding to region B was not identified in the type III SCC*mec* element, two sets of three ORFs whose sequences were similar to those of V16, V17, and V19, respectively, and which had amino acid identities ranging from 48.1 to 67.1% were found in the element. The first set of three ORFs (Z011, Z013, and Z014) was located downstream of the *ccrA3* and *ccrB3* genes, and these three ORFs were constituents of the type 3 *ccr* gene complex. The other set of three ORFs (CZ070, CZ069, and CZ068) was located downstream of *ccrC* (CZ072). It was noted that seven ORFs (three ORFs upstream of *ccrC*, *ccrC*, and three ORFs downstream of *ccrC*) were conserved in type V SCC*mec* and the J region of the type III SCC*mec* of 85/2082 (the amino acid identities of the corresponding ORFs were greater than or equal to 48.1%).

Thus, they were unified as a *ccr* gene complex together with the *ccr* gene, similar to the constructions of the type I to IV SCC*mec* elements.

Region C2 of type V SCC*mec* contained two ORFs (V20 and V21) which showed high degrees of similarity to CZ053 and CZ059 (truncated *hsdR*), respectively, in type III SCC*mec*. Although two regions, C1 and C2, were located near each other in type V SCC*mec*, the regions homologous to C1 and C2 in type III SCC*mec* were separated (Fig. 4).

The deduced amino acid sequence of ORF V21 (*hsdR*) showed a high degree of similarity (identity, 98.0%) to that of ORF Z059 (truncated *hsdR*) in the type III SCC*mec* of 85/2082, whereas it showed a very low degree of similarity (identity, less than 21%) to HsdR proteins encoded by *hsdR* genes in the *S. aureus* genome (*hsdR*_{aur}).

The regions other than those described above are unique in type V SCC*mec*. Two ORFs, V22 (*hsdS*) and V23 (*hsdM*), encoding the restriction-modification system were located at the right end of the element. Two type I restriction-modification DNA specificity domains, one of which is known as the target recognition domain and the other of which is the region conserved in the *hsd* subunit, were identified in ORF V22. The deduced amino acid sequence of ORF V23 showed the highest degree of similarity to the putative HsdM protein of *Lactococ-*

TABLE 3. ORFs in and around type V SCCmec of WIS with deduced products showing similarities to extant proteins

ORF ^a	Value for CDS ^b				Gene	Product
	Starting nucleotide	Ending nucleotide	Size (bp)	Length (amino acids)		
V01	418	897	480	159	<i>orfX</i>	Conserved hypothetical protein orfX
V02	952	2652	1,701	566		Hypothetical protein
V03	3367	2693	675	224	<i>tnp</i>	Transposase for IS431
V04	4709	5452	744	247		Glycerophosphoryl diester phosphodiesterase homolog
V05	5549	5977	429	142		Conserved hypothetical protein
V06	8029	6023	2,007	668	<i>mecA</i>	PBP 2'
V07*	8129	8239	111	36	<i>ΔmecR1</i>	Truncated signal transducer protein MecR1
V08	8277	8951	675	224	<i>tnp</i>	Transposase for IS431
V09	9439	9011	429	142		Hypothetical protein
V10	9520	10449	930	309		Hypothetical protein
V11	10611	12599	1,989	662		Hypothetical protein
V12	12794	13903	1,110	369		Hypothetical protein
V13	13896	14264	369	122		Hypothetical protein
V14	14264	15907	1,644	547		Hypothetical protein
V15	16132	17754	1,623	540	<i>ccrC</i>	Cassette chromosome recombinase C
V16	17915	18253	339	112		Hypothetical protein
V17	18332	18658	327	108		Hypothetical protein
V19	18674	19177	504	167		Hypothetical protein
V20	19563	20129	567	188		Hypothetical protein
V21	23325	20206	3,120	1039	<i>hsdR</i>	Type I restriction-modification system endonuclease
V22	24547	23309	1,239	412	<i>hsdS</i>	Specificity subunit of type I restriction-modification system
V23	26051	24537	1,515	504	<i>hsdM</i>	Modification subunit of type I restriction-modification system
V24	26242	27054	813	270		Hypothetical protein
V25	27054	27986	933	310		Hypothetical protein

^a ORFs in parentheses were located outside of the type V SCCmec. Incomplete ORFs that are potentially defective genes or pseudogenes containing frameshift mutations are designated with asterisks.

^b CDS, coding sequence. The nucleotide positions are given from the nucleotide sequence deposited in the DDBJ/EMBL/GenBank databases under accession no. AB121219, and the sizes were measured from the 5' (starting nucleotide) to the 3' (ending nucleotide) direction.

^c Identity of the amino acid sequence to each ORF.

cus sakei, with an identity of 65.3%. On the other hand, both the HsdS (V22) and the HsdM (V23) proteins showed low levels of similarity to the HsdS and HsdM proteins encoded by the corresponding genes in staphylococcal G islands, with identities of less than 26 and 29%, respectively (3, 24). Figure 5 shows the phylogenetic relationships of the HsdR proteins (Fig. 5a), the HsdS proteins (Fig. 5b), and the HsdM proteins (Fig. 5c). Phylogenetic trees clearly showed that three ORFs, V21 (HsdR), V22 (HsdS), and V23 (HsdM), belonged to a group distinct from those found in the *S. aureus* genome or the G island.

The G+C content of type V SCCmec was 30.5%. This value is lower than that for *S. aureus* (32.8 to 32.9%).

Further analysis of the G+C content of the third nucleotide in the codon (GC3) revealed that the GC3 values for the ORFs in the region unique to type V SCCmec were very low (range, 14.2 to 19.7%; average, 18.6%). When we applied the definition of the criteria that we used for *S. aureus* genome analysis, the ORFs were regarded as possible alien genes; i.e., their GC3 values differed by more than 1.5 standard deviations from the average GC3 values for all ORFs longer than 150 bp in the *S. aureus* genome (3, 24). They were transposases for IS431 (V02 and V08), *mecA* (V07), *ΔmecR1* (V08), V12, V17, V18, V19, V20, and *hsdS* (V22).

CcrC-mediated precise excision and closed circular SCCmec formation. To investigate whether CcrC can catalyze the precise excision of type V SCCmec, we constructed two recombinant plasmids, pSR5_W and pSR5_E, which carried *ccrC*_{WIS} and

*ccrC*_{N315}, respectively, and introduced them into WIS and N315, respectively, by electroporation.

As controls, plasmids pSR2 (a plasmid formerly called pSR that carries the *ccrA2* and *ccrB2* genes) and pYT3 were also each introduced into WIS and N315. After cultivation of the eight purified transformants, WIS(pSR5_W), WIS(pSR5_E), WIS(pSR2), WIS(pYT3), N315(pSR5_W), N315(pSR5_E), N315(pSR2), and N315(pYT3), as well as recipient strains, for 20 h at 30°C, the DNAs were extracted from the cultures. We tested by PCR whether *attBsc* was generated and whether the closed circular form of SCCmec was formed in each strain. The strategy and the primer sets used for the detection of *attBsc* and the closed circular form of SCCmec, which carries *attSCC*, are shown in Fig. 6c and Table 2, respectively.

When we used the chromosomal DNAs of WIS(pSR5_W) and WIS(pSR5_E) as templates, a 0.31-kb DNA fragment containing *attBsc* and a 0.29-kb DNA fragment containing *attSCC* were successfully amplified, whereas no DNA fragment was amplified with DNAs extracted from WIS and WIS(pYT3) (Fig. 6a). By nucleotide sequencing of the amplified DNA fragments, we have verified that a novel nucleotide sequence, *attSCC* of type V SCCmec, is generated by the head-to-tail ligation of both termini and that *attBsc* is generated by the precise excision of type V SCCmec (Fig. 6b). These results show that CcrC serves as a site-specific recombinase responsible for the precise excision and formation of the closed circular form of type V SCCmec. Since no visible DNA fragment was amplified with DNAs extracted from N315(pSR5_W) and N315

TABLE 3—Continued

Data indicating homology to ORF in SCCmec of:

NCTC10442 (type I)		N315 (type II)		85/2082 (type III)		CA05 (type IV)	
% Identity ^c	Corresponding ORF (size [bp])	% Identity	Corresponding ORF(s) (size [bp])	% Identity	Corresponding ORF(s) (size [bp])	% Identity	Corresponding ORF (size [bp])
100	<i>orfX</i> (480)	100	<i>orfX</i> (480)	100	<i>orfX</i> (480)	100	<i>orfX</i> (480)
99.10	E040 (675)	99.10	N062, N070 (675)	99.1, 98.7, 98.2, 98.2	Z035, Z041, Z046, Z058 (675)	99.10	Q012 (675)
100	CE026 (744)	99.00	CN039 (633)	100	CZ030 (744)	100	CQ007 (744)
99.30	CE025 (429)	100	CN038 (429)	100	CZ029 (429)	100	CQ005 (429)
99.70	<i>mecA</i> ₁₀₄₄₂ (2007)	99.90	<i>mecA</i> _{N315} (2007)	99.60	<i>mecA</i> ₂₀₈₂ (2007)	99.90	<i>mecA</i> _{CA05} (2007)
100	Δ <i>mecRI</i> ₁₀₄₄₂ (987)	100	<i>mecRI</i> _{N315} (1758)	88.60	Δ <i>mecRI</i> ₂₀₈₂ (114)	100	Δ <i>mecRI</i> _{CA05} (987)
97.80	E040 (675)	97.80	N062, N070 (675)	97.8, 97.3, 97.8, 97.8	Z035, Z041, Z046, Z058 (675)	97.80	Q012 (675)
				76.70	CZ075 (1101)		
				84.30	CZ074 (372)		
				82.10	CZ073 (1644)		
				93.60	CZ072 (1554)		
54.50	E031 (351)	53.60	N041 (351)	50.9, 48.6	Z011 (351), CZ070 (342)	55.90	Q007 (351)
91.70	E032 (327)	89.30	N042 (312)	48.1, 48.1	Z013 (395), CZ069 (312)	87.40	Q008 (312)
91.60	E033 (510)	92.80	N043 (318)	61.7, 67.1	Z014 (522), CZ068 (507)	93.40	Q009 (510)
				97.20	CZ053 (323)		
				98.00	Z059* (2244)		

(pSR5_E), we could not be certain whether CcrC works on other types of SCCmec elements.

In contrast, DNA fragments of 0.28 and 0.31 kb containing *attB*_{sc} were amplified with DNAs extracted from N315(pSR2) and WIS(pSR2), respectively. Furthermore, DNA fragments of 0.46 and 0.29 kb containing *att*SCC were amplified with DNAs extracted from the two strains, respectively. Subsequent nucleotide sequencing showed that the fragments contained *attB*_{sc} and *att*SCC. The data indicate that the sets of *ccrA* and *ccrB* genes cause precise excision and formation of the closed circular forms of both type II SCCmec and type V SCCmec.

CcrC catalyzes site- and orientation-specific integration of type V SCCmec. To examine whether the putative closed circular form of type V SCCmec serves as a substrate for integration similar to that of type II SCCmec, we constructed experimental plasmid pSR5_EattV, in which *ccrC*₀₃₄₂ and the presumptive attachment sequence of type V SCCmec (*attV*) are subcloned, and used the plasmid as a model of the closed circular form of type V SCCmec (mini-SCC). Recombinant plasmids pSR2attII (a plasmid formerly called pSRatt that carries *ccr2* genes and the presumptive attachment sequence of type II SCCmec [*att*SCC-II]), pYTattII (a plasmid carrying *att*SCC-II), pYTattV (a plasmid that carries the presumptive attachment sequence of type V SCCmec [*att*SCC-V]), pSR2, pSR5_E, and pYT3 were used as controls. The recombinant plasmids were introduced into N315ex by electroporation, followed by selection on plates with tetracycline at 30°C. A colony of each transformant was respread onto BHI agar plates containing tetracycline (10 mg/liter) and incubated for 18 h at 30 and 43°C (the latter of which is nonpermissive for plasmid replication). The numbers of colonies that grew on each plate were counted. Two strains, N315ex(pSR5_EattV) and N315ex(pSR2attII), generated significant number of colonies at 43°C

compared with the numbers that grew at 30°C (2.2 and 4.4%, respectively), whereas the other strains did not generate colonies when they were grown at 43°C. The result shows that integration of the plasmids into the chromosome occurs when the *att*SCC and the *ccr* genes are present on the plasmids and that CcrC may mediate the integration of pSR5_EattV (a model of the closed circular form of type V SCCmec) at an efficiency nearly equal to that for the integration of pSR2attII (a model of the closed circular form of type II SCCmec) mediated by a set of CcrA and CcrB proteins.

The integration sites of the plasmids and their directions in the chromosome were examined by PCR experiments with primers as follows: cL1 and cR1, whose sequences flank that of the *attB* region on the chromosome of N315ex; α (RV in pUC119) and β (in the *ccrB* gene), whose sequences flank that of the *att*SCC-II region of plasmid pSR2attII; and γ (in the *ccrC* gene), whose sequence flanks the *att*SCC-V region of plasmid pSR5_EattV (Fig. 6d and Table 2). The results show that two plasmids, pSR2attII and pSR5_EattV, generated plasmid-chromosome integration junctions which were detected by two combinations of primers: primers cL1- β and cR1- α and primers cL1- γ and cR1- α , respectively. By determining the nucleotide sequences of the amplified DNA fragments, we found that the fragment contains sequences which are identical to those of the chromosome-SCCmec junction regions of N315 or artificially constructed chromosome (N315)-type V SCCmec junction regions.

These results indicate that the recombinant plasmid is integrated in the chromosome only when it carries the *ccrA* and *ccrB* genes and *att*SCC-II or the *ccrC* gene and *att*SCC-*attV* and that *ccrC* mediates the site- and orientation-specific integration of type V SCCmec in a way similar to that for a set of *ccrA* and *ccrB* genes.

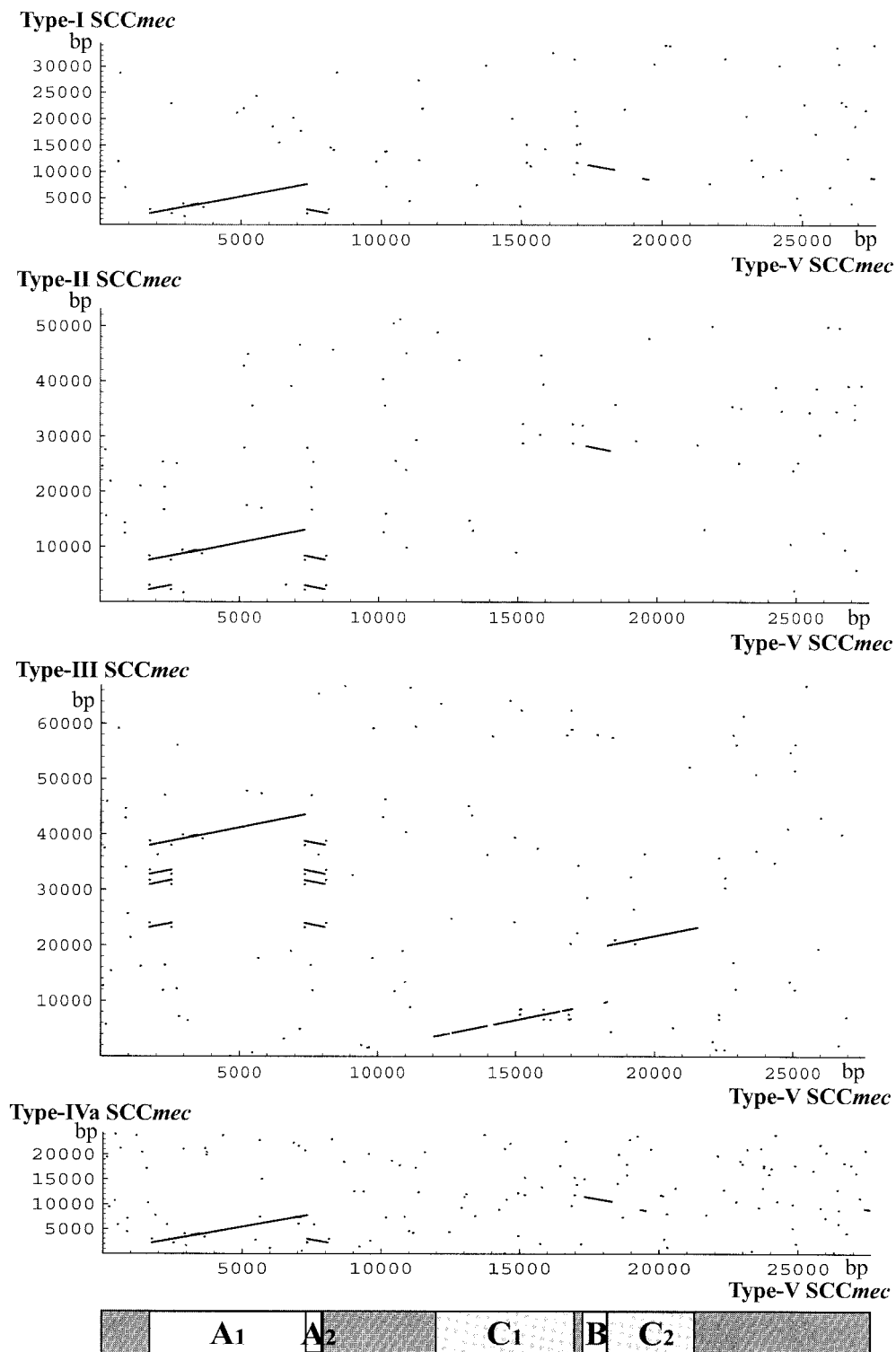


FIG. 4. Homologous regions in nucleotide sequences of the type V SCCmec and extant SCCmec elements. The nucleotide positions of the type V SCCmec element are indicated on the horizontal axes, and those of extant SCCmec elements (types I, II, III, and IVa) are indicated on the vertical axes. The following regions of the nucleotide sequences of the SCCmec elements were used: type V SCCmec, nucleotides 880 to 28503 of the nucleotide sequence with DDBJ/EMBL/GenBank databases accession no. AB121219; type I SCCmec, the region from nucleotides 4504 to 38867 of the nucleotide sequence with DDBJ/EMBL/GenBank databases accession no. AB033763; type II SCCmec, nucleotides 4687 to 57653 of the nucleotide sequence with DDBJ/EMBL/GenBank databases accession no. D86934; type III SCCmec, nucleotides 899 to 67794 of the nucleotide sequence with DDBJ/EMBL/GenBank databases accession no. AB037671; and type IVa SCCmec, nucleotides 975 to 25222 of the nucleotide sequence with DDBJ/EMBL/GenBank databases accession no. AB063172. The regions showing the high degrees of similarity between two SCCmec elements are indicated with lines. Rising lines signify that homologous regions between two elements are located in the opposite direction. Short homologous regions appear as dots. The regions through A (A1 and A2), B, and C (C1 and C2) were designated by the extent of nucleotide sequence identity described in the text. The locations of the regions in the nucleotide sequence of type V SCCmec are as follows: A1, nucleotide positions 1778 to 7341; A2, nucleotide positions 7342 to 8131; B, nucleotide positions, 16964 to 18296; C1, nucleotide positions 12041 to 17040; and C2, nucleotide positions 18309 to 21540.

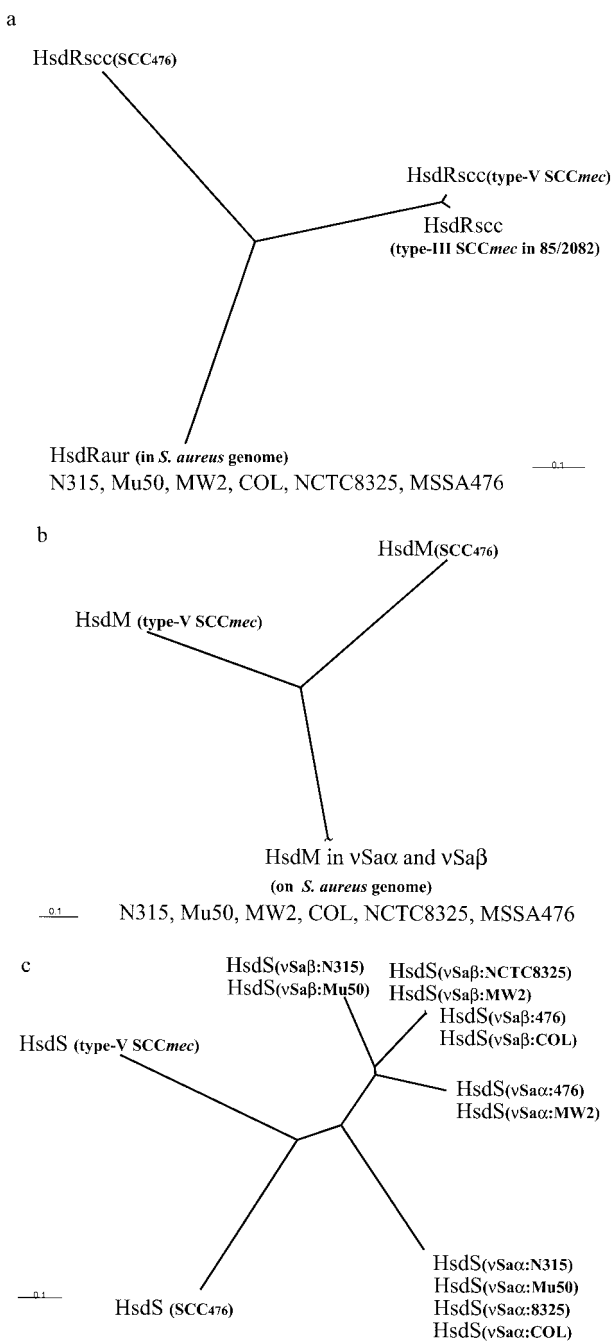


FIG. 5. Phylogenetic relations of the constituents of the *hsd* system. Type V SCCmec carries three genes, *hsdR*, *hsdM*, and *hsdS*, which encode a type I restriction-modification system. To look for the derivations of those genes, the phylogenetic relationships among the genes found in the type V SCCmec element and previously reported *hsd* genes were investigated by creating phylogenetic trees for the *hsdR* (a), *hsdM* (b), and *hsdS* (c) genes. Phylogenetic trees were generated as described in the legend to Fig. 2b. The nucleotide sequence of the truncated *hsdR* gene in type III SCCmec was obtained from the DDBJ/EMBL/GenBank databases (accession no. AB037671). All other nucleotide sequences of the *hsd* genes analyzed here were obtained from websites that provide the whole genome sequences of the following *S. aureus* strains: N315 and MW2 (<http://www.bio.nite.go.jp/>), Mu50 (<http://w3.grt.kyushu-u.ac.jp/VRSA/>), MSSA 476 and strain 252 (http://www.sanger.ac.uk/Projects/S_aureus/), COL (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>), and NCTC8325 (<http://www.genome.ou.edu/staph.html>).

DISCUSSION

Characteristics of a new site-specific recombinase, CcrC.

We demonstrated in this study that type V SCCmec carries a recombinase gene, *ccrC*, which can mediate type V SCCmec recombination events (integration and excision), whereas a set of *ccrA* and *ccrB* genes was previously found to be required for this function in other types of SCCmec elements. It was curious that both *ccrC* gene variants, *ccrC*_{WIS} (1,623 nucleotides) and *ccrC*₀₃₄₂ (1,680 nucleotides), have been demonstrated to be active in the precise excision function. Approximately one-fourth of the deduced amino acid sequence from the NH₂ terminus, which contains the serine residue that catalyzes DNA strand exchange (36), was well conserved among the CcrB variant and two CcrC variants (Fig. 2a). Therefore, a lack of some amino acids in the COOH-terminal domain may not much affect the activities of Ccr proteins. However, when we compared the frequency of precise excision by quantitative PCR, we observed that *attB*_{SCC} was present in slightly larger amounts in WIS(pSR5_E) than in WIS(pSR5_W) (X. X. Ma, unpublished data). That was why we used *ccrC*₀₃₄₂ to construct the recombinant plasmid used for the integration experiment.

Mini-SCC plasmid pSR5_EattV was integrated in the N315ex chromosome at exactly the same nucleotide position at which pSR2attII integrated. The data indicate that CcrC recognizes the ISS in a way similar to that in which a set of CcrA and CcrB proteins in combination does.

Interestingly, when *ccr* genes were introduced into host cells carrying preexisting SCCmec to test *ccr* gene-mediated excision, the frequency of excision differed depending on the combination of the types of SCCmec elements and *ccr* genes. The types of SCCmec elements greatly influenced the efficiency of excision mediated by *ccrC*. The recombinase encoded by *ccrC* appeared to be inactive in excising type II SCCmec from the N315 chromosome, while it was active in the excision of type V SCCmec from the WIS chromosome. It is tempting to speculate that the difference resides in the nucleotide sequences at the extremities of SCCmec. Characteristic inverted repeat sequences were located at the extremities of the extant types of SCCmec carrying *ccrA* and *ccrB* genes, whereas they were not found at the extremities of type V SCCmec. They have also not been found in the extremities of SCCcap1, which carries a broken homologue of the *ccrC* gene (25). A similar preference between the types of *ccr* and SCCs was also observed in the integration experiment. The efficiency of integration decreased when the recombinant plasmids carried the *att*SCC sequences of different types of SCCmec elements. The efficiency of integration observed with N315ex(pSR5_EattII) and N315ex(pSR2attV) was smaller than that observed with N315ex(pSR5_EattV) and N315ex(pSR2attII). Those data suggest that both *ccr* proteins have specificity for the recognition of the nucleotides located at the extremities of the SCC elements.

Origin of *hsd* system and its role in maintenance of the element. The restriction-modification system seems to play an important role in the stability of certain regions of the *S. aureus* chromosome (3, 24). A set of *hsdS* and *hsdM* genes is located in each of the two *Gislands*, vSaα and vSaβ, in the genomes of all seven *S. aureus* isolates that have been completely sequenced (isolates COL, MRSA 252, MSSA 476, MW2, Mu50,

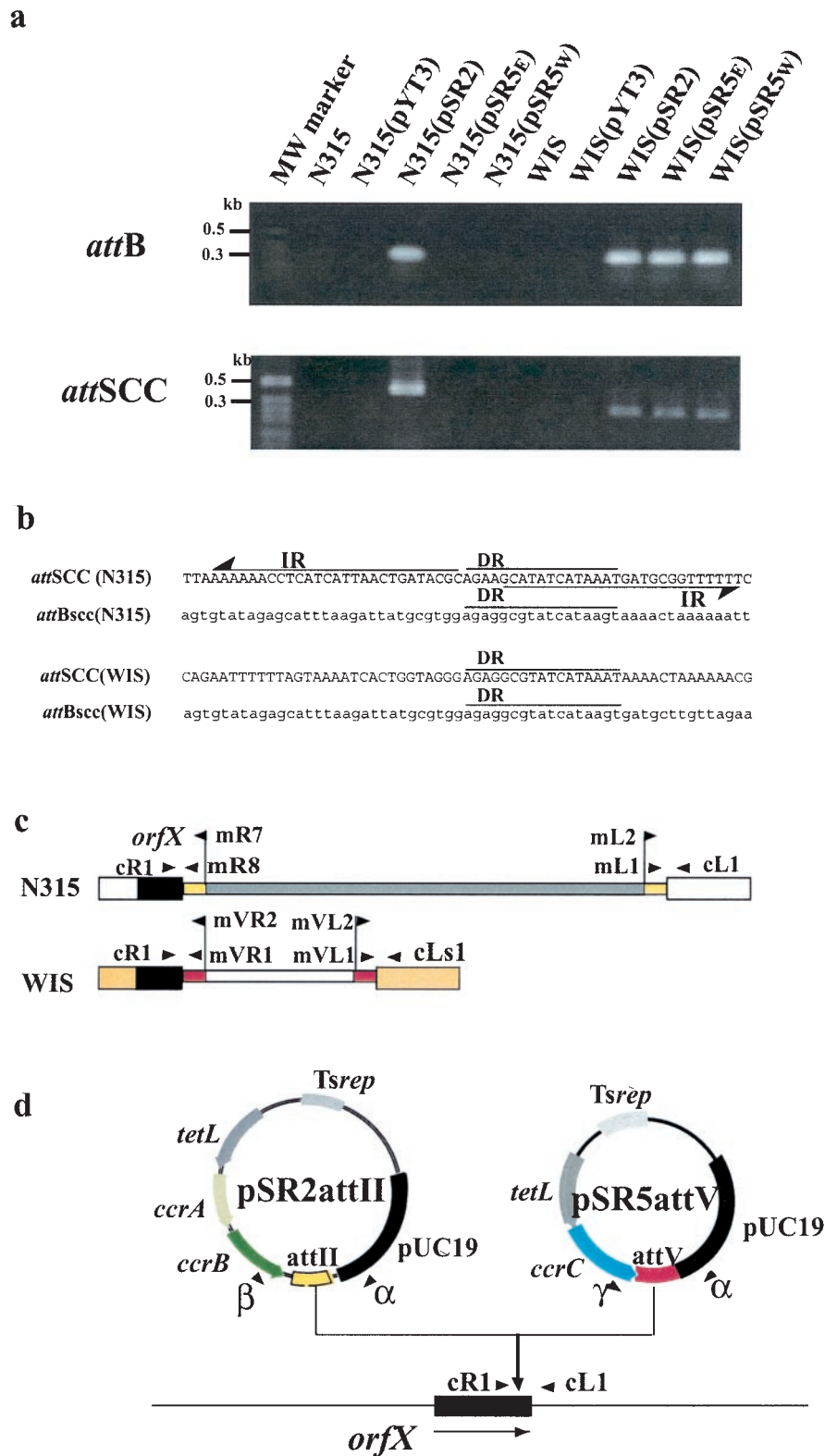


FIG. 6. Precise excision and site- and orientation-specific integration of *SCCmec*. (a) Detection of *ccr*-mediated *SCCmec* excision and appearance of *attSCC*. Template DNAs for PCR were extracted from a culture incubated in BHI broth with tetracycline (10 mg/liter) for 20 h. Four sets of primers were used to detect the precise excision and the closed circular form of *SCCmec* in strain N315 or WIS cells. The locations of the primers used are shown in panel b. For N315, primers cL1 and cR1 were used to detect *attBsc* (276 bp), and primers mL1 and mR8 were used to detect *attSCC* (456 bp). For WIS, primers cLs1 and cR1 were used to detect *attBsc* (314 bp), and primers mVL1 and mVR1 were used to detect *attSCC* (292bp). Lane MW, molecular size marker (1-kb ladder; Invitrogen, Carlsbad, Calif.), and only the relevant sizes are indicated. (b) Generation of *attBsc* and *attSCC*. The nucleotide sequences of *attBsc* in the DNA fragments amplified from N315ex and N315(pSR2) and

N315, and NCTC8325). The sequences of the HsdM proteins encoded by the two Gislands are nearly identical to each other, whereas those of the HsdS proteins in the target recognition domain regions differ significantly from each other. The *hsdR_{aur}* gene (the *hsdR* gene commonly found on the genomes of the seven *S. aureus* strains) encodes the restriction function and is found on the *S. aureus* genome at the same locus, which is far from the two Gislands. The sequences of the HsdR proteins of the seven strains were nearly identical. It is speculated that the Gislands, which carry enterotoxins and exotoxin-like genes, are protected from deletional loss by the copresence of the modification function (3).

Unlike the other G islands, the type V SCC_{mec} is unique in that it carries a complete set of the genes involved in the type I restriction-modification system, composed of *hsdR*, *hsdS*, and *hsdM*. A complete set of the genes involved in the restriction-modification system is also found on an SCC in MSSA strain 476 (SCC₄₇₆) (http://www.sanger.ac.uk/Projects/S_aureus/). The restriction-modification system encoded by those elements was judged to be distinct from that encoded by the *hsdR_{aur}*, *hsdM*, and *hsdS* genes on the *S. aureus* chromosome, according to data from phylogenetic trees as well as codon usage patterns. This may signify that those mobile genetic elements were formed in a species different from *S. aureus* and that *S. aureus* acquired those elements by horizontal transfer. We tried to excise SCC_{mec} elements from strain WIS. Although we could not obtain them from either WIS(pSR5_E) or WIS(pSR5_w), large amounts of DNA fragments containing *attB_{sc}* and *attSCC* were amplified by PCR from cultures of both strains. It is reported that carriage of type II restriction-modification system genes contributes to plasmid stability (7, 8). Handa et al. (10) showed that *Escherichia coli* cells harboring a plasmid that carries the type II restriction-modification system die due to restriction cleavage of the chromosome after they lose the plasmid. This postsegregational killing phenomenon may explain why we could not excise SCC_{mec} elements, despite the PCR amplification results indicating that an apparent excision event occurred in cultured cells of strain WIS. The restriction enzymes (encoded by either *hsdR_{aur}* or *hsdR_{sc}*) that remain in the cells after the loss of type V SCC_{mec} might have cleaved the chromosome and killed the cells from which the element was excised. Thus, the type I restriction-modification system may serve as a stabilizer of the type V SCC_{mec} element integrated in the *S. aureus* chromosome.

Type V SCC_{mec} as an element found in C-MRSA. MRSA clones are defined by the types of SCC_{mec} and the genotypes of the MSSA chromosomes into which the SCC_{mec} element is

integrated. Multiple MRSA clones carrying type IV SCC_{mec} were identified in C-MRSA strains from both the United States and Australia by multilocus sequence typing for chromosome genotyping and the PCR technique for SCC_{mec} typing (32). On the basis of that observation, it was proposed that C-MRSA strains have been generated de novo from *S. aureus* populations more diverse than H-MRSA strains (32). The identification of a new type of SCC_{mec} in C-MRSA strains in the present study further supports that proposal. Type V SCC_{mec} was structurally similar to type IV SCC_{mec}, in that it contains *mecA* as the only gene encoding antibiotic resistance. Its size (28 kb) was also comparable to that of type IV SCC_{mec} and was much smaller than those of the type I to III SCC_{mec} elements (34 to 67 kb) found in H-MRSA strains. Although the type V SCC_{mec} element was slightly larger than the smallest subtype of type IV SCC_{mec} (type IVb; 21 kb), the only difference was the presence of a restriction-modification system in type V SCC_{mec}.

The most unique feature of type V SCC_{mec} is the carriage of a type 5 *ccr* gene complex composed of the *ccrC* gene and its surrounding ORFs (Fig. 1). After sequence determination, we realized that the type 5 *ccr* gene complex is also found in SCC elements other than the type V SCC_{mec}. SCC_{cap1}, which encodes capsule formation but which has no *mec* gene, carried a gene complex similar to the type 5 *ccr* gene complex, although most of the ORFs in SCC_{cap1} were truncated. In addition, the Ψ *ccr* gene complex that we previously reported in the type III SCC_{mec} element was closely related to the type 5 *ccr* gene complex: ORF CZ072 was a *ccrC* gene homologue, and the sequences of the surrounding ORFs were highly homologous to those of the corresponding ORFs in the type 5 *ccr* gene complex (Table 3). A copy of type 3 *ccr* gene complex and three ISS copies, in addition to a copy of type 5 *ccr* gene complex, are present in the type III SCC_{mec} element (13, 15). This configuration now indicates that the long DNA region downstream of *orfX*, previously designated the J3 region of type III SCC_{mec}, is in fact an SCC element independent of the rest of the region. We now consider the latter region to constitute a true type III SCC_{mec}. If this is the case, the SCC—in which a truncated copy of *hsdR*, the mercury resistance operon, and Tn554, in addition to the type 5 *ccr* gene complex, are carried—must have been integrated prior to or in succession with the type III SCC_{mec} element.

The class C2 *mec* gene complex, which, along with the type 5 *ccr* gene complex, defines the type V SCC_{mec} element, is distributed among coagulase-negative staphylococcal species, especially *S. haemolyticus*. Among 38 *S. haemolyticus* strains

from WIS(pSR2), WIS(pSR5_w), and WIS(pSR5_E) are indicated *attB_{sc}*(N315) and *attB_{sc}*(WIS), respectively. The nucleotide sequences of *attSCC* in DNA fragments amplified from N315(pSR2) and from WIS(pSR2), WIS(pSR5_w), and WIS(pSR5_E) are indicated *attSCC*(N315) and *attSCC*(WIS), respectively, above the nucleotide sequence of *attB_{sc}*. (c) Locations of the primers used to detect *attB_{sc}* generated from N315 (primers cR1 and cL1) and WIS (primers cR1 and cL1) and *attSCC* generated from N315 (primers mR8 and mL1) and WIS (primers mVR1 and mVL1). The locations of the primers used to clone *attSCC*-II (primers mR7 and mL2) and *attSCC*-V (primers mVR2 and mVL2) are also illustrated. The drawings are not to scale. (d) Identification of site- and orientation-specific integration of recombinant plasmids (mini-SCCs) into the chromosome. The recombinant plasmids carrying *ccr* gene and *attSCC* of each type, pSR2attII and pSR5_EattV, were introduced into N315ex by electroporation as models of SCC_{mec} elements. DNAs were extracted from cultures of the transformants grown at 43°C on BHI agar plates containing tetracycline (10 mg/liter). The locations and directions of the four primers (primers α , β , cL1, and cR1) used to detect the integration of plasmid pSR2attII as well as four primers (primers α , γ , cL1, and cR1) used to detect the integration of plasmid pSR5attV are illustrated. We could amplify DNA fragments of the expected sizes by the site- and orientation-specific integration of the plasmids. The expected sizes of the DNA fragments are 1,255 bp (cL1- β) and 735 bp (cR1- α) for pSR2attII and 826 bp (cL1- γ) and 719 bp (cR1- α).

tested, 30 strains carried the class C2 *mec* gene complex and the other 8 strains carried *mec* gene complexes of class A (5 strain), class B (2 strain), or class C1 (1 strain) (21). The deletion point of $\Delta mecR1$ in the class C2 *mec* gene complex of the type V SCC*mec* element was identical to that in the class C2 *mec* gene complex found in *S. haemolyticus* strain JB16 and *S. epidermidis* strain JK8 (21). The data suggest the horizontal transfer of a certain molecular version of the class C2 *mec* gene complex among staphylococcal species. On the other hand, only 20 of 30 *S. haemolyticus* strains carrying the class C2 *mec* gene complex had the *ccrC* gene. Three other strains carried *ccrA2* and *ccrB2* genes, and the remaining seven did not carry any of the type 1 to 4 *ccr* genes (X. X. Ma, H. Yuzawa, and M. Kapi, unpublished observations). Thus, class C2 may not always be linked with the type 5 *ccr* gene complex, and additional types of SCC*mec* elements may be existent in coagulase-negative staphylococci. We have also reported that many subtypes defined by J1-region polymorphisms exist in the type IV SCC*mec* elements prevalent in C-MRSA strains. Type IV SCC*mec* elements with diverse J1 regions are distributed in more than 30% of community-acquired *S. epidermidis* isolates in Japan (K. Hisata, K. Hiramatsu et al., unpublished data). We think that it is quite likely that the *mec* gene complex and the *ccr* gene complexes (of diverse SCC elements) go through complex recombination and rearrangement processes in the genomes of coagulase-negative staphylococci; thus, novel types of SCC*mec* elements are incessantly generated, and only a fraction of them are transferred to *S. aureus* strains in the community. Thus, we may be witnessing only the tip of the iceberg of the SCC*mec* element diversity displayed by C-MRSA isolates. The type IV and type V SCC*mec* elements that have come onto the scene, however, may be the most refined molecular products and may have high competitive capabilities in terms of their transferability among the cells of staphylococcal species and strains.

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