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Structural Comparison of Three Types of Staphylococcal Cassette Chromosome *mec* Integrated in the Chromosome in Methicillin-Resistant *Staphylococcus aureus*

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The β-lactam resistance gene *mecA* of *Staphylococcus aureus* is carried by a novel mobile genetic element, designated staphylococcal cassette chromosome *mec* (SCC*mec*), identified in the chromosome of a Japanese methicillin-resistant *S. aureus* (MRSA) strain. We now report identification of two additional types of *mecA*-carrying genetic elements found in the MRSA strains isolated in other countries of the world. There were substantial differences in the size and nucleotide sequences between the elements and the SCC*mec*. However, new elements shared the chromosomal integration site with the SCC*mec*. Structural analysis of the new elements revealed that they possessed all of the salient features of the SCC*mec*: conserved terminal inverted repeats and direct repeats at the integration junction points, conserved genetic organization around the *mecA* gene, and the presence of cassette chromosome recombinase (*ccr*) genes responsible for the movements of SCC*mec*. The elements, therefore, were considered to comprise the SCC*mec* family of staphylococcal mobile genetic elements together with the previously identified SCC*mec*. Among 38 epidemic MRSA strains isolated in 20 countries, 34 were shown to possess one of the three typical SCC*mec* elements on the chromosome. Our findings indicated that there are at least three distinct MRSA clones in the world with different types of SCC*mec* in their chromosome.

β-Lactam resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) is determined by the function of penicillin-binding protein 2′ (PBP2′) encoded by the methicillin resistance gene *mecA* (20, 29). PBP2′ binds to β-lactam antibiotics with much lower affinity than the intrinsic set of PBPs of *S. aureus* do (7, 23, 35). By nucleotide sequence determination of an MRSA-specific chromosomal region of strain N315 (isolated in Japan in 1982), we have found that the *mecA* gene is carried by a novel genetic element, designated staphylococcal cassette chromosome *mec* (SCC*mec*), inserted into the chromosome (14, 17).

SCC*mec* is a mobile genetic element characterized by the presence of terminal inverted and direct repeats, a set of site-specific recombinase genes (*ccrA* and *ccrB*), and the *mecA* gene complex (14, 17). The element is precisely excised from the chromosome of N315 and integrates site and orientation specifically into an *S. aureus* chromosome through the function of a unique set of recombinase genes, *ccrA* and *ccrB*. SCC*mec* was distributed widely in Japanese MRSA strains isolated in the 1990s (11). However, most of the MRSA strains isolated in other countries did not possess SCC*mec*, as judged by dot-blot hybridization of extracted chromosomal DNA with probes covering various parts of the SCC*mec* of N315. By cloning and nucleotide sequence determination of the DNA region surrounding the *mecA* gene from two representative MRSA strains, NCTC 10442 (the first MRSA isolate in England in 1961) and 85/2082 (the 1985 isolate in New Zealand), we found two novel genetic elements that shared similar structural features of SCC*mec*. We designated them type I (NCTC 10442) and type III (85/2082) SCC*mec*, and we designated that of N315 type II SCC*mec* in the order of the year of isolation of the strains. Here, we report a detailed structural comparison of the three types of SCC*mec*.

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MATERIALS AND METHODS

Bacterial strains and media. All of the MRSA or pre-MRSA strains used in this study are listed in Table 1. Pre-MRSA, represented by N315, is an *S. aureus* strain that has a *mecA* gene, but is susceptible to methicillin because of a strong repression of *mecA* gene transcription exerted by *mecI*-encoded repressor function (18). Two strains, ATCC 25923 isolated in 1945 and NCTC 8325 (a kind gift from B. Berger-Bachi), were used as methicillin-susceptible *S. aureus* (MSSA) standard strains.

Escherichia coli strain XL1-Blue MRA(P2) was used for the propagation of phage libraries. *Bam*HI-cleaved arms of lambda Dash II (Stratagene, La Jolla, Calif.) were used for the construction of phage libraries. L broth and L agar, used for cultivation of *E. coli*, and NZY broth, NZY agar, and NZY soft agar, used for the propagation of bacteriophage λ, were prepared according to the method of Sambrook et al. (25). Heart infusion agar, heart infusion broth, brain heart infusion (BHI) broth, and BHI agar (Eiken Kagaku, Co., Ltd., Japan) were used for cultivation of *S. aureus*.

The following antibiotics were freshly prepared and used at the indicated concentrations: ampicillin (Meiji Seika Co., Tokyo, Japan), 100 μg/ml; tetracycline (Sigma Co., St. Louis, Mo.), 10 μg/ml; latamoxef (Shionogi Pharmacy Co., Osaka, Japan), 15 μg/ml; ceftizoxime (Fujisawa Pharmacy Co., Osaka, Japan), 25 μg/ml.

Cloning and determination of the nucleotide sequence of the SCC*mec* of NCTC 10442. Phage libraries were prepared from *S. aureus* strain NCTC 10442 with partial *Sau*3A1 digests of chromosomal DNA of NCTC 10442 and lambda Dash II arms cleaved with *Bam*HI (Stratagene) as described previously (14). Phages were propagated to produce plaques on *E. coli* XL1-Blue MRA(P2) and were lifted onto a nylon filter (Biodyne A; Pall BioSupport, East Hills, N.Y.). Plaque hybridization was performed with digoxigenin-labeled probes (Boehringer, Mannheim, Germany) as described previously (25). Chromosomal DNA fragments containing the left and right boundaries of SCC*mec* were cloned with

TABLE 1. Characteristics of 38 MRSA strains isolated worldwide

MRSA strain[a]	Country of isolation	Yr of isolation	Coagulase isotype[b]	Designation of probe(s) with positive hybridization			PCR result for localization of representative gene or structure[d]						Reference
				NCTC 10442	N315	85/2082	*ccr* gene type[e]	IS*1272*	*mecI*	*mecRI* PB/MS	*mecA*	MREP typing[f]	
NCTC 10442	United Kingdom	1961	3	1–6	2, 3, 10	2–5	1	+	−	−/+	+	i	32
61/6219	United Kingdom	1961	3	1–6	3, 4, 10	2–5	1	+	−	−/+	+	i	32
64/3846	United Kingdom	1964	3	1–6	2–4, 10	2–5	1	+	−	−/+	+	i	32
64/4176	United Kingdom	1964	3	1–6	1–3, 10	2–5	1	+	−	−/+	+	i	32
86/4372(DNH)	United Kingdom	1986	3	1	1–3, 10	2, 3, 5	−	+	−	−/+	+	ii	32
KL3	Malaysia	1987	3	1–6	2–4, 10	2–5	1	+	−	−/+	+	i	38
KL50	Malaysia	1989	4	1, 5	1–3	1–7	3	−	+	+/+	+	iii	38
86/961	United Kingdom	1986	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
86/560	United Kingdom	1986	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
85/1340	Yugoslavia	1985	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
85/1762	Hungary	1985	4	1, 5	1–4	1–7	3	−	+	+/+	+	iii	32
85/2082	New Zealand	1985	4	1, 5	1–4	1–7	3	−	+	+/+	+	iii	32
85/2111	Norway	1985	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
85/1836	Germany	1985	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
85/2147	Hong Kong	1985	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
85/3907	Germany	1985	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
86/2652	United Kingdom	1986	4	1, 2, 4	1–4	1–7	3	−	+	+/+	+	iii	32
85/5495	Saudi Arabia	1985	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
85/5328	Portugal	1985	4	None	1–4	1–7	3	−	+	+/+	+	−	32
85/3619	Austria	1985	4	5	1–4	1–7	3	−	+	+/+	+	iii	32
85/3566	Holland	1985	4	None	1–4	1–7	3	−	+	+/+	+	iii	32
82/20-1	Japan	1982	2	1, 2, 5	1–10	1, 2, 4, 5	2	−	+	+/+	+	ii	33
85/2235	United States	1985	2	5	1–10	1, 2, 4, 5	2	−	+	+/+	+	ii	32
86/JO60	Japan	1985	2	1, 5	1–10	1, 2, 4, 5	2	−	+	+/+	+	ii	32
86/BB5918	Japan	1986	2	1	1–10	1, 2, 4, 5	2	−	+	+/+	+	ii	32
87/27	Japan	1987	2	1, 2, 5	1–10	1, 2, 4, 5	2	−	+	+/+	+	ii	32
N315	Japan	1982	2	None	1–10	2	2	−	+	+/+	+	ii	10
87/20	Japan	1987	2	1, 2, 5	1–10	1, 2, 4, 5	2	−	+	+/+	+	ii	32
87/25 (DNH)	Japan	1987	2	1, 5	1–4, 6–10	1, 2, 4, 5	2	−	+	+/+	+	ii	32
84/9580	South Africa	1984	2	1–6	1–4, 10	2–5, 7	1	+	−	−/+	+	i	32
86/9302	United Kingdom	1986	2	1–6	1–4, 10	2–5	1	+	−	−/+	+	i	32
85/1774	Italy	1985	2	1–6	1–4, 10	2–5	1	+	−	−/+	+	i	32
85/1940	France	1985	2	1–6	2, 3, 5	2–5	1	+	−	−/+	+	i	32
85/4231	Canada	1985	4	None	1–10	2–5	2	−	+	+/+	+	ii	32
85/2232	United States	1985	4	None	1–10	2–5	2	−	+	+/+	+	ii	32
81/108 (DNH)	Japan	1981	4	1, 5	2–4	2–5	2	+	−	−/+	+	ii	12
93/H44	Japan	1993	4	1, 5	1–3	1–7	3	−	+	+/+	+	i	11
85/4547 (DNH)	Israel	1985	7	1, 3	3, 4	2, 5	2	+	−	−/+	+	ii	32

[a] DNH, strain whose chromosomal DNA did not typically hybridize to any of the three sets of the typing probes.
[b] See the article by Ushioda et al. (34) for details of coagulase isotyping.
[c] See Fig. 1 for the locations of the probes.
[d] Localizations of the essential genes in SCC*mec* were estimated by PCR. The *mecA* gene and its regulator genes *mecI* and *mecRI* of both the penicillin-binding region (PB) and membrane spanning region (MS) are identified by using the primers described by Suzuki et al. (31). Localization of IS*1272* in SCC*mec* was identified with the set of primers mA2, corresponding to the nucleotide sequence of the *mecA* gene, and iS-4, corresponding to the nucleotide sequence of IS*1272* (2). A minus sign indicates that no DNA fragment was amplified by the set of primers described above.
[e] The type of *ccr* complex was identified with PCR by combining primer β2, which was common to three *ccrB* genes, and three primers specific for each *ccrA* gene, α2(*ccrA1*), α3 (*ccrA2*), and α4 (*ccrA3*).
[f] MREP typing is a method to amplify the right extremity region of SCC*mec* by using the primer sets bracketing the right SCC*mec*-chromosome junction point. The right PCR primer was cR4, and the left primers for each type of SCC*mec* were mR2 (types I and II) and mN16 (type III). For the locations of primers, see Fig. 1.

the probes 11A and cR, respectively. The preparation of the two probes was described previously (14). With probe 11A, which corresponded to the chromosomal region flanking the left extremity of type II SCC*mec* or the chromosomal region upstream of *att*Bscc of methicillin-susceptible strain NCTC 8325, lambda clone LO2 containing the left boundary of SCC*mec* was obtained. With probe cR, which corresponded to the chromosomal region downstream of *att*Bscc of strain NCTC 8325, lambda clone LO21 containing the right boundary of SCC*mec* was obtained. With probe MA, which was prepared by PCR amplification with a set of primers (mA1 and mA2) based on the nucleotide sequence of the *mecA* gene (29), lambda clone LO5 containing *ccr* genes and the *mecA* gene was obtained.

Lambda clone LO7 containing the region upstream of the *ccr* genes was cloned by using an *Xba*I fragment 4.3 kb in size located at the left end of LO5 as a probe. The region downstream of *mecA* was sequenced with a long-range PCR-amplified DNA fragment 6.6 kb in size amplified by using mA3 and cR2 primers and chromosomal DNA as a template. Similarly, the region between the right end of LO2 and the left end of LO7 was identified by using a 7.5-kb DNA fragment amplified by long-range PCR with primers mE1 (based on the nucleotide sequence of LO2) and mE2 (based on the nucleotide sequence of LO7). The nucleotide sequences of the primers used in these experiments are listed in Table 3, except for the primers described in the previous reports (14, 17). The nucleotide sequence of the entire SCC*mec* of NCTC 10442 was determined by using the DNA fragments of lambda clones LO2, LO21, LO5, and LO7 and DNA fragments amplified by PCR.

Amplification of DNA fragments by PCR and determination of the entire nucleotide sequence of the SCC*mec* of 85/2082. We have cloned the regions containing both boundaries of SCC*mec* of MRSA 85/3907 (11) and determined their nucleotide sequences (DDBJ/EMBL/GenBank accession no. AB047088 and AB047089). The regions containing both boundaries of the SCC*mec* of 85/2082 were similar to those of 85/3907, and the surrounding region of the *mecA*

gene was similar to that of MRSA strain ANS46 described previously by Dubin's group (4, 6). Taking advantage of this similarity, we could successfully amplify DNA fragments covering the entire SCC*mec* of 85/2082 by long-range PCR with several sets of primers. The DNA fragment corresponding to the chromosomal region upstream to the left extremity of SCC*mec* was amplified by long-range PCR with a set of primers, cLt1 and cLt4. The region spanning from the left extremity of SCC*mec* to transposon ΨTn*554* was amplified with two sets of primers (cLt2 and mN2; mN1 and Tn*554*[171–146]). By using nine sets of primers, we could amplify the region spanning from transposon ΨTn*554* located upstream of *mecA* to transposon Tn*554* located downstream of *mecA*. The sets of primers used were as follows: mN3 and mN5 (the region in and around ΨTn*554*); cad1 and mN6 plus mN4 and mN7 (the region from ΨTn*554* to downstream of *mecA* gene); mA3 and tetK1 (the region from *mecA* to plasmid pT181); tetK4 and merA1 (the region from pT181 to the mercury operon); is-1 and merN plus merA2 and mN9 (the mercury operon flanked by a pair of IS*431* sites); mN8 and Tn*554*R; and mN11 and TnpA636 (the region spanning from IS*431* to transposase A of Tn*554*).

The region spanning from Tn*554* to the right extremity of SCC*mec* was amplified by long-range PCR with two sets of primers, TnpA1016 and mN13 and mN12 and cR1.

By using these DNA fragments amplified by long-range PCR, the nucleotide sequence of the entire SCC*mec* of 85/2082 was determined.

The primer cLt4 was designed based on the nucleotide sequence of pSJ9 cloned from 85/3907 (11). Primers cLt1, cLt2, cLt3, mN1, and mN2 were designed on the basis of the nucleotide sequence of the left extremity of SCC*mec* and its flanking chromosomal region of 85/3907 (DDBJ/EMBL/GenBank accession no. AB047088). The primers mN4, mN5, mN6, and mN7 were designed on the basis of the type II SCC*mec* sequence (DDBJ/EMBL/GenBank accession no. D86934). The following primers were designed on the basis of the previously reported sequences: Tn*554*(171–146), Tn*554*R, TnpA636, and TnpA1016, Tn*554* (DDBJ/EMBL/GenBank accession no. X03216); cad 1, ΨTn554 (DDBJ/EMBL/GenBank accession no. L10909); is-1, IS431*mec* (DDBJ/EMBL/GenBank accession no. X53818); tetK1 and tetK4, plasmid pT181 (DDBJ/EMBL/GenBank accession no. JO1764); and merA2 and merN, mer operon of pI258 (DDBJ/EMBL/GenBank accession no. L29436).

The primers mN12 and mN13 were designed on the basis of the nucleotide sequence of the right extremity of SCC*mec* of 85/3907 (DDBJ/EMBL/GenBank accession no. AB047089).

DNA manipulation. Colony hybridization and plaque hybridization were performed by using cellular DNA extracted from MRSA strains and digoxigenin-labeled probes (Boehringer Mannheim Biochemica, Mannheim, Germany) as described previously (31).

Large-scale and small-scale preparation of plasmid DNA, purification of phage plaques, extraction of DNA from purified phage particles, and subcloning of DNA fragments into plasmid vector pUC118 or pUC119 were performed by standard techniques (25). All of the enzymes for DNA manipulation were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan, except for *Taq* DNA polymerase for PCR, which was purchased from Perkin-Elmer, Foster City, Calif.

Nucleotide sequence determination was performed as described previously (10), with a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer). A description of primers synthesized specifically for the primer extension sequence determination was omitted from the text.

Isolation of SCC*mec*-excised strains. The method of obtaining SCC*mec*-excised strain N315ex from N315 has been described previously (17). The type I SCC*mec*-excised strain 85/1940ex was obtained by the method used for the preparation of N315ex, with a slight modification. Briefly, the recombinant plasmid, pSR, which carries *ccrA* and *ccrB* genes, was introduced into 85/1940 cells by electroporation. After overnight cultivation of the transformant strain 85/1940(pSR) on BHI agar containing tetracycline (10 μg/ml), cells were resuspended in saline and plated onto BHI agar containing tetracycline (10 μg/ml) for replica plating. The loss of methicillin resistance was examined first by replica plating on the agar with and without latamoxef (15 μg/ml). Latamoxef-susceptible colonies were selected and examined further to determine whether precise excision of type I SCC*mec* occurred by PCR with the primer set cR2 and cL1. Plasmid pSR was eliminated by serial cultivation in drug-free BHI broth at 43°C to obtain 85/1940ex.

The SCC*mec*-excised strain 85/2082ex was obtained by spontaneous excision of SCC*mec* from 85/2082. Cells were cultivated in BHI broth at 37°C for 2 to 3 days, and then a 0.1-ml portion of the cell suspension was inoculated into new BHI broth. After 38 days of serial passages, cells were plated onto BHI agar. The strains that had lost β-lactam resistance were identified by replica plating onto BHI agar containing ceftizoxime (25 μg/ml), and precise excision of SCC*mec* of these strains was confirmed by PCR with the primer set cLt3 and cR2 (Fig. 1).

PCR amplification. PCR was performed essentially as described previously (31) with a 50-μl reaction volume and with thermal cycler Gene Amp 9600 (Perkin-Elmer Cetus Instruments, Emeryville, Calif.). Long-range PCR was performed with Expand *Taq* (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the procedure recommended by the manufacturer. PCR products were purified with High Pure PCR product purification kit (Boehringer Mannheim Biochemica). The nucleotide sequences of the primers used in this experiment are described in Table 1 or were reported previously (14, 17).

Computer analysis of nucleotide and protein sequences. All of the analyses were carried out with programs in the Wisconsin Package (version 9.0; Genetics Computer Group, Madison, Wis.). A homology search was performed with the BLAST and TFastA programs for the EMBL (release no. 55.0) and GenBank (release no. 107.0) databases, and the FastA program for the SWISS-PROT database (release no. 35.0). Tree View software was obtained from the web site http://taxonomy.zoology.gla.ac.uk/rod/treview.html.

RESULTS

Two *mecA*-carrying elements are new members of the SCC*mec* family. Initially, we tested whether the previously identified SCC*mec* on the chromosome of Japanese *S. aureus* strain N315 was also distributed in MRSA strains around the world. Thirty-eight representative epidemic strains listed in Table 1 were analyzed by dot-blot hybridization with 10 probes prepared in the N315 SCC*mec*. (See Fig. 1 for the location of the probes). A typical positive hybridization pattern was observed with the DNAs extracted from 9 of the 38 MRSA strains, but with others, only a few probes reacted positively, or the hybridization signal intensities were weak even if they reacted positively (Table 1). Since all of the listed strains possessed the *mecA* gene, the observation indicated that there were other types of genetic elements carrying the *mecA* gene on their chromosome.

From the strains with an atypical hybridization pattern to N315 SCC*mec* probes, two strains, NCTC 10442 and 85/2082, which represented two different (incomplete) hybridization patterns to N315 SCC*mec* probes, were chosen for cloning of the DNA region surrounding the *mecA* gene. With a strategy of cloning described in Materials and Methods, we identified from these strains new genetic elements that were inserted at the *attB* site of SCC*mec* (14). The boundaries of the element of NCTC 10442 were identified by comparing its nucleotide sequence with that of *mecA*-negative *S. aureus* type strain NCTC 8325 (Fig. 2). The boundaries of the element of 85/2082 were determined by comparing its nucleotide sequence with that of strain 85/2082ex, a spontaneous SCC*mec*-excised strain (Fig. 2). (We have previously reported that some MRSA strains spontaneously generate SCC*mec* excisant strains when cultivated in drug-free medium [14, 17]. Strain 85/2082 was one of the strains generating spontaneous excisants.)

The two elements were found integrated at exactly the same nucleotide position in open reading frame (ORF) X gene *orfX* as that N315 SCC*mec* utilizes for integration (17). Both elements had characteristic 15-bp direct repeat sequence (DRscc-R) at the right extremity and its counterpart (DRscc-L) in the chromosomal region abutting the left terminus of the element (Fig. 2), although direct repeat sequences of 15 bp were incomplete (with 13 identical bases) in the case of the SCC*mec* of NCTC 10442. Curiously, another copy of 15-bp sequence similar to that of N315 SCC*mec* (Fig. 2) was found on the 85/2082 element between the second and the third IS*431* copies (shown by an arrowhead in Fig. 1). Degenerate inverted repeats charac-

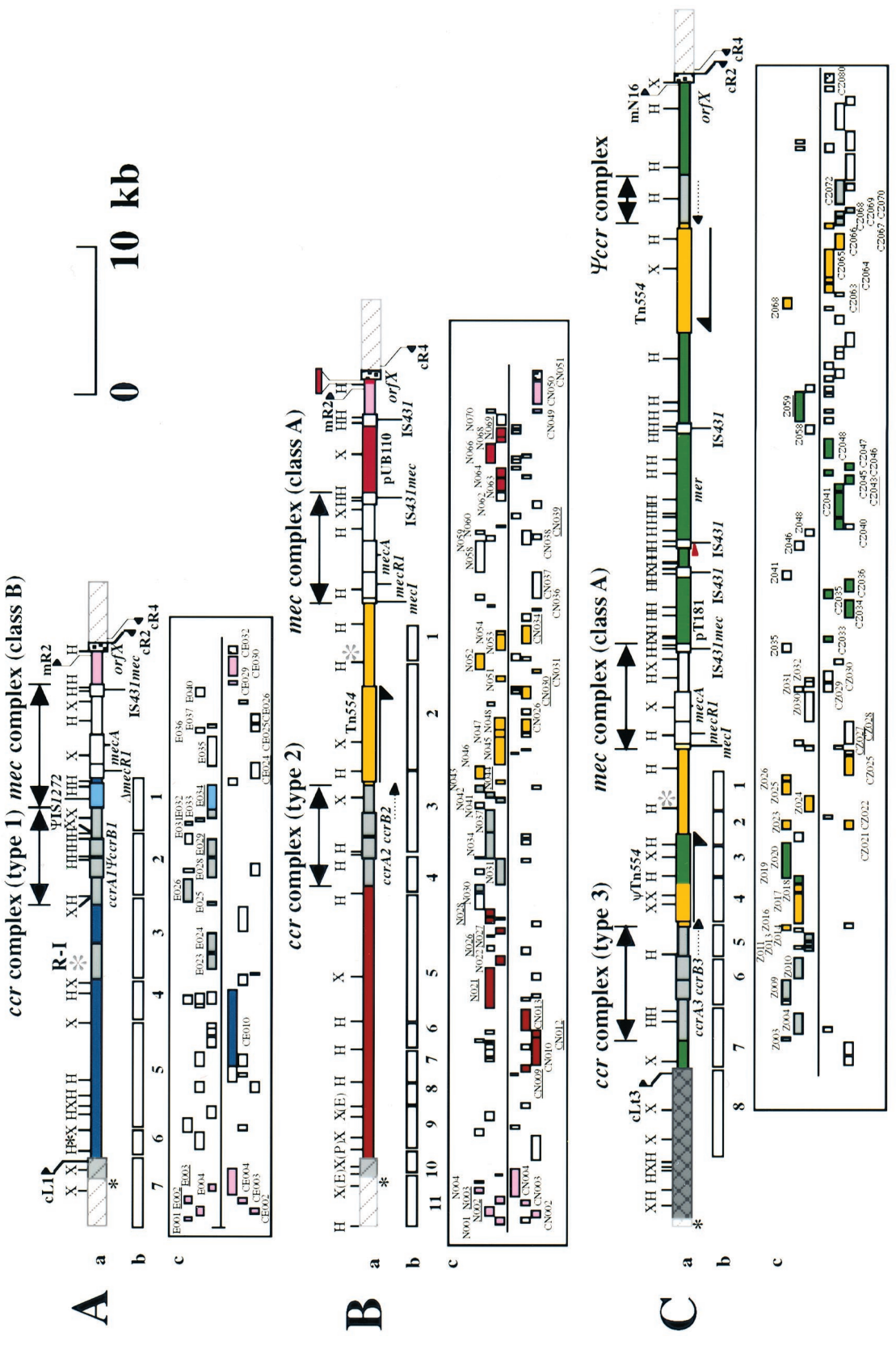
A
ccr complex (type 1) mec complex (class B)
0 10 kb
cL1
R-I
mR2
ΨIS1272
ccrA1ΨccrB1
ΔmecR1
mecA
orfX
IS431mec
cR2
cR4
B
ccr complex (type 2)
mec complex (class A)
ccrA2 ccrB2
Tn554
mecA
mecR1
mecI
pUB110
IS431mec
IS431
C
Ψccr complex
mec complex (class A)
ccr complex (type 3)
cL13
ΨTn554
ccrA3 ccrB3
Tn554
mN16
pT181
mer
IS431mec
IS431

FIG. 1. Structures of three types of SCC*mec*. The structures of type I SCC*mec* (A), type II SCC*mec* (B), and type III SCC*mec* (C) are illustrated based on the nucleotide sequences deposited in the DDBJ/EMBL/GenBank databases under accession no. AB033763 (type I), D86934 (type II), and AB037681 (type III). (a) Essential structure of SCC*mec*. Locations of the essential genes are illustrated. The restriction sites of *Hin*dIII and *Xba*I are indicated. Only the *Eco*RI (E) and *Pst*I (P) restriction sites, which are inevitable for the description of probes of type II SCC*mec*, are indicated (in parentheses). The essential genes as well as other regions are colored based on the color of the ORFs described in panel C. The regions common to MSSA are shown in white with gray-striped bars. The regions found in *S. aureus* NCTC 8325 and not found in ATCC 25923 are shown in gray with striped bars. The regions common to *S. aureus* ATCC 25923 and not found in NCTC 8325 are shown in dark gray with cross-hatched bars. Small black asterisks signify the locus common to all three strains. Large gray asterisks signify the region common to three SCC*mec*s. An arrowhead signifies the location of a 15-bp sequence similar to that of N315 found between the second and the third IS*431* copies of type III SCC*mec*. Arrows indicate the direction of Tn*554* or ΨTn*554*. Dotted arrows indicate the transcription of direction of the ORFs located downstream of *ccrB* or CZ072. (b) Probes used for dot-blot hybridization. (c) ORFs in and around SCC*mec*. The ORFs >200 bases in size in six possible reading frames are indicated by squares. Those above the line are the ORFs that have transcription directed to the right, and those below the line have transcription directed to the left. Colored squares are the ORFs the extant gene homologues of which were found in the databases, although many of them were considered incomplete (underlined). The color corresponds to the difference in the conservation of the ORFs (or regions) in three types of SCC*mec* white, ORFs in three types of SCC*mec* with identity of more than 99%: gray, conserved in three types of SCC*mec* with on identity score of 47 to 92%; magenta, ORFs common to type II and type III SCC*mec*; yellow, ORFs common to type II and type III SCC*mec*; blue, ORFs unique in type I SCC*mec*; red, ORFs unique in type II SCC*mec*; green, ORFs unique in type III SCC*mec*. ORFX is indicated in a stippled box.

teristic of SCC*mec* were also found in the extremities of all three elements.

Shown in Fig. 1 are the genomic organizations of the two novel elements identified from NCTC 10442 and 85/2082 in comparison with that of N315 SCC*mec*. The regions conserved in all three elements are illustrated in white (amino acid identities among the corresponding ORFs were equal to or greater than 99%) and in gray (identities equal to or greater than 47%). Two classes of *mec* gene complex, either with a complete structure (*mecI-mecR1-mecA*-IS*431* [class A *mec* complex]) or with a deletion and integration of an insertion sequence (IS*1272*-Δ*mecR1-mecA*-IS*431* [class B *mec* complex]), were created with the entire SCC*mec*. Another common structure was a *ccr* complex (composed of *ccrA*, *ccrB* homologue genes, and surrounding ORFs) (Table 2 and Fig. 1). The *ccrA* and *ccrB* genes encoding putative site-specific recombinases of SCC*mec* are known to be responsible for the movement (excision and integration) of N315 SCC*mec* from and into the *S. aureus* chromosome (17). The corresponding ORFs found in the two other elements had a substantial homology to the *ccr* genes of N315, although the *ccrB* gene of NCTC 10442 had a frameshift mutation (Table 2). Based on the structural similarities described above, these elements were considered to be new members of the SCC*mec* family. Accordingly, the two elements found in NCTC 10442 and 85/2082 were designated type I and type III SCC*mec*, respectively, and that of N315 was designated type II SCC*mec*. The *ccr* gene homologues found in each SCC*mec* were designated *ccrA* and *ccrB* genes with an Arabic numeral suffix to show the type of SCC*mec* with which they were associated. It was noted that the ORFs adjacent to each type of *ccr* gene were also conserved (amino acid identities of the corresponding ORFs were equal to or greater than 47%) among the three types of SCC*mec*; thus, they were unified as a *ccr* complex together with the *ccr* genes.

Another region, called R-I, 3.5 kb in size was identified in type I SCC*mec* that had a substantial similarity to the intervening region between Tn*554* (or ΨTn*554*) and *mecI* of type II and type III SCC*mec* (indicated by large asterisks in Fig. 1). Two ORFs of unknown function were contained in the R-I and corresponding regions of type II and type III SCC*mec*, the amino acid identities of the deduced polypeptides of which were greater than 52% (Table 2).

Structure outside the integration site of SCC*mec*. Nucleotide sequencing of the regions around the left and right chromosome-SCC*mec* junctions revealed that the *orfX* genes of three MRSA strains, NCTC 10442, N315, and 85/2082, were extremely well conserved. All of the *orfX* genes were composed of 480 nucleotides with greater than 99% identity, and their encoded polypeptides were identical (Table 2). Thus, the nucleotide sequences of the chromosomal regions abutting the right junction point of SCC*mec* were extremely well conserved. In contrast, the nucleotide sequences abutting the left boundary of type III SCC*mec* differed substantially from those abutting type I and type II SCC*mec*.

Other genetic components of SCC*mec*. The size of type I SCC*mec* (NCTC10442) was 34,364 bp, and that of type III SCC*mec* (85/2082) was 66,896 bp. In contrast, the size of type II SCC*mec* (N315) was 53,017 bp. These differences in size were due to the presence of a type-specific DNA region in addition to the essential structures of SCC*mec*. The regions

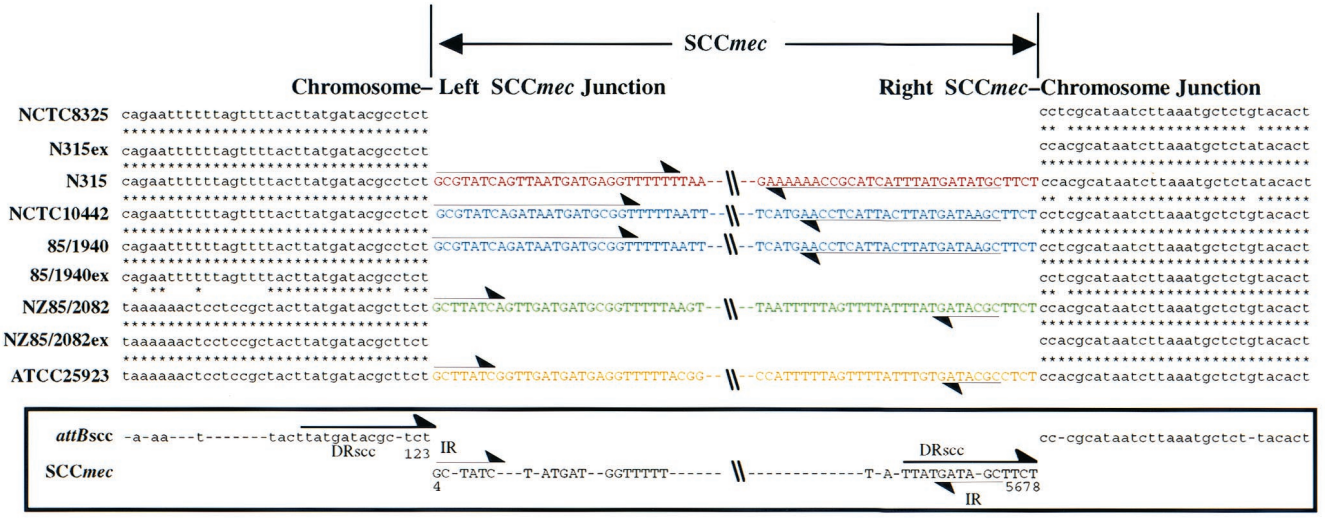

FIG. 2. Boundaries of SCC*mec*. Nucleotide sequences around the left and right boundaries of SCC*mec*s of N315, NCTC 10442, 85/1940, and 85/2082 (*att*L and *att*R respectively) are aligned with the sequence around the presumptive integration site, *att*B, on the chromosome of MSSA strains (NCTC 8325, ATCC 25923, N315ex, 85/1940ex, and 85/2082ex). The boundary sequences of an inserted element, IE25923 (DDBJ/EMBL/GenBank accession no. AB047239), identified in an MSSA type strain, ATCC 25923, is also shown in comparison with those of SCC*mec*. Inverted complementary repeats IR-L and IR-R at both extremities of SCC*mec*s are indicated by thin arrows. Direct repeats are indicated by thick arrows. Asterisks indicate identical nucleotides in the two sequences. Consensus sequences of *att*Bscc and inverted repeats of SCC*mec* are indicated below. The left- and rightmost nucleotides were each inferred to be one of the four nucleotides numbered 1 to 4 and 5 to 8, respectively, in the figure. The entire lengths of three SCC*mec*s were 34, 364, 53,017, and 66,896 bp. We have revised the nucleotide sequence of type II SCC*mec* (17). We found that 931- and 344-bp deletions had occurred in the cloned DNA fragment and a sequencing error. The 931 bp segment was deleted upstream of ORF N026, and 344 bp was deleted downstream of ORF N028 (Fig. 1). Consequently, the size of type II SCC*mec* changed from 51,669 bp to 53,017 bp.

commonly shared by type I and type II SCC*mec* are shown in magenta; they were located at the right extremities of the elements between the rightmost IS*431* copy and the right junction point (Fig. 1). Two ORFs of unknown function were contained in the region, and they were identical between the two types of SCC*mec* (Fig. 1). The nucleotide sequence of the region was also extremely well conserved between the two types (only 1 base substitution in 2, 120 bases), but type II SCC*mec* possessed an additional 102 bases of unique sequence in the very end of SCC*mec* (shown in red in Fig. 1).

The regions common to type II and type III SCC*mec* are shown in yellow; these regions were located between the *ccr* and *mec* complexes (Fig. 1). In the case of type III SCC*mec*, another copy of Tn*554* was found downstream (to the right) of the *mec* complex, which was also associated with a *ccr*-complex-like structure (designated the Ψ*ccr* complex in Fig. 1), just as Tn*554* in the mid-part of the element was associated with *ccr* complex. The Ψ*ccr* complex was composed of a *ccrB* homologue and three adjacent ORFs whose deduced amino acid sequences had greater than 30% identity to the corresponding ORFs in the type II *ccr* complex (Fig. 1 and Table 2).

The regions unique to each type of SCC*mec* are illustrated in Fig. 1 in either blue (type I), red (type II), or green (type III). No antibiotic resistance gene except for *mecA* was found in type I SCC*mec*. In contrast, type III SCC*mec* contained multiple antibiotic resistance genes. They were transposon ΨTn*554* encoding cadmium resistance inserted between *ccr* and *mec* complexes, an integrated copy of plasmid pT181 encoding tetracycline and mercury resistance, and another transposon, Tn*554*, encoding erythromycin and spectinomycin resistance. The latter three were found downstream (to the right) of the *mec* complex, and pT181 and the *mer* operon were found bracketed by a pair of IS*431* copies.

The ORFs contained in the left half of type I SCC*mec* were type specific (shown in blue in Fig. 1), but mostly unknown with regard to their function, except for one. The CE010 ORF potentially encoded a polypeptide belonging to the Shine-Dalgarno repeat multigene family (16), which was nearly identical to a large surface protein of *S. aureus* designated plasmin-sensitive surface protein (Pls) (8, 26). It was a repeat-rich protein characteristically having unusual dipeptide repeats composed of serine and aspartate residues. Another unique ORF of type III SCC*mec* was Z059, the deduced amino acid sequence of which showed a high similarity to HsdR of *Klebsiella pneumoniae* and *Salmonella enterica*, which was flanked by IS*431* (Fig. 1). HsdR is a catalytic subunit of the restriction-modification system (19). However, the N-terminal portion of the ORF Z059 appeared to be deleted by approximately 300 amino acid residues compared to the intact HsdR.

Distribution of the three types of SCC*mec* in clinical MRSA strains around the world. The 38 MRSA strains listed in Table 1 were reanalyzed with dot-blot hybridization with the probe sets prepared in each of the three types of SCC*mec* (see Fig. 1 for the locations of probes). Now, 34 strains showed typical hybridization patterns to either one of the three sets of probes (Table 1). In the 34 strains, additional PCR typing experiments of various genes and right extremity polymorphism (REP; see below) listed in Table 1 agreed with the dot-blot hybridization results. For example, all of the strains with type I SCC*mec* reacted positively to PCR detection of IS*1272* and negatively to

TABLE 2. ORFs in and around type I SCC*mec* (NCTC 10442) and type III SCC*mec* (85/2082) with deduced products showing similarities to extant protein sequences

ORF[a]	Location[b]	Size (bp)	Homology to ORFs in database			Homology to ORFs of N315		Homology to ORFs of 85/2082	
			% Identity[c]	Gene	Description of gene product	% Identity	Corresponding ORF(s) (size in bp)	% Identity	Corresponding ORF(s) (size in bp)
Type I (NCTC 10442)									
ORFs with homologues found in type II and III SCC*mec*									
E023	17197–18264	1,068				69.9	N052 (1065)	69.9	Z024 (1065)
E024	18283–19611	1,329				74.0 and 60.1	N053 (861)* and N054* (396)	74.0 and 61.1	Z025* (861) and Z026* (396)
E025	21439–21735	297				78.6	N030 (561)	52.2	Z003 (288)
E026	21735–23504	1,770				75.6	N031 (1793)	65.1	Z004 (1569)
E028	23693–25041	1,350		*ccrA*	Cassette chromosome recombinase A	74.8	*ccrA2* (1350)	72.5	Z009 (1347)
E029*	25063–26214	1,152		*ccrB*	Cassette chromosome recombinase B	80.4	*ccrB2* (1629)	86.5	Z010 (1629)
E031	27153–27503	351				88.8	N041 (351)	54.3	Z011 (351)
E032	27575–27901	327				89.4	N042 (315)	48.1	Z013 (396)
E033	27913–28422	510				91.7	N043 (507)	61.3	Z014 (520)
CE024*	Complement (30304–31290)	987	100	*mecR1*	Methicillin resistance protein MecR1	100	$mecR1_{N315}$ (1758)	99.6	CZ028* (1068)
E035	31390–33396	2,007	100	*mecA*	Penicillin-binding protein 2′	95.9	$mecA_{N315}$ (2007)	99.9	Z030 (2007)
CE025	Complement (33442–33870)	429				99.3	CN038 (429)	99.3	CZ029 (429)
E036*	33455–33772	31.8				100	N059 (384)	100	Z031 (384)
E037	33700–33906	207				100	N060 (207)	100	Z032 (207)
CE026	Complement (33967–34710)	744	35.4	(*ugp*)	Glycerophosphoryldiester phophodiesterase	99	CN039 (744)	100	CZ030 (744)
E040	36014–36688	675	100	*tnp*	Transposase for insertion sequence-like element IS*431mec*	100	N062 and N070 (675)	100, 99.6, 99.1, 99.1	Z035, Z041, Z046, and Z058
(CE032)		480		*orfX*	ORFX	100	*orfX* (480)	100	CZ080 (480)
ORFs with homologues found in type II but not in type III SCC*mec*									
(E001)	417–683	267	50	(*yrkD*)	Protein with unknown function	98.9	N001 (267)		
(E002)	683–1438	756	39.9	(*yrkI*)	Protein with unknown function	100	N002 (756)		
(CE002)	Complement (931–1269)	339				100	CN002 (339)		
(E003)	1653–2105	453	71.1	(*yfiN*)	Protein with unknown function	87.6	N003 (453)		
(CE003)	Complement (1634–1948)	315				79.6	CN003 (276)		
(CE004)	Complement (2199–3944)	1,746				100	CN004 (1746)		
(E004)	2581–2805	225				100	N004 (225)		
CE029	36720–36959	240				100	CN049 (240)		
CE030	37374–38669	1,296				100	CN050 (1296)		
ORFs unique in type I SCC*mec*									
E034*	28423–30081	1,659	75.2	*tnp*	Putative transposase of IS*1272*				
CE010	Complement (10734–15827)	5,097	79		Fibrinogen binding protein				

Continued on following page

TABLE 2—*Continued*

ORF[a]	Location[b]	Size (bp)	Homology to ORFs in database			Homology to ORFs of N315		Homology to ORFs of 85/2082	
			% Identity[c]	Gene	Description of gene product	% Identity	Corresponding ORF(s) (size in bp)	% Identity	Corresponding ORF(s) (size in bp)
Type III(85/2082) ORFs with homologues found in type I and II SCC*mec*									
Z003	2796–3083	288				53.3	N030 (297)		
Z004	3083–4651	1,569				65.1	N031 (1783)		
Z009	5430–6776	1,347		*ccrA*	Cassette chromosome recombinase A	72.5	*ccrA2* (1350)		
Z010	6797–8425	1,629		*ccrB*	Cassette chromosome recombinase B	86.5	*ccrB2* (1629)		
Z011	8896–9246	351				48.7	N041 (351)		
Z013	9247–9642	396				47.2	N042 (351)		
Z014	9661–10181	520				61.3	N043 (507)		
Z024	18193–19257	1,065	49.4	(*yrkE*)	Protein with unknown function	100	N052 (1065)		
Z025*	19368–20228	861				100	N053 (861)		
Z026*	20229–20624	396				100	N054 (396)		
CZ028*	Complement 22730–24334	1,068	99.8	*mecR1*	Methicillin resistance protein MecR1	99.8	$mecR1_{N315}$ (1758)		
Z030	24421–26427	2,007	99.9	*mecA*	Penicillin-binding protein 2′	99.7	$mecA_{N315}$ (2007)		
Z031	26420–26803	384				100	N059 (384)		
CZ029	Complement (26473–26901)	429				100	CN038 (429)		
Z032	26731–26937	207				100	N060 (207)		
CZ030	Complement (26998–27742)	744	15.4	(*ugp*Q)	Glycerophosphoryl-diester phophodiesterase	99	CN039 (633)		
Z035	29043–29717	675	100	*tnp*	Transposase for insertion sequence-like element IS*431mec*	100	N062 and N070 (675)		
Z041	34281–34955	675	99.6	*tnp*	Transposase for insertion sequence-like element IS*431mec*	99.6	N062 and N070 (675)		
Z046	36122–36796	675	99.1	*tnp*	Transposase for insertion sequence-like element IS*431mec*	99.1	N062 and N070 (675)		
Z058	43846–44520	675	99.1	*tnp*	Transposase for insertion sequence-like element IS*431mec*	99.1	N062 and N070 (675)		
CZ068	Complement (57975–58481)	507				66.7	N043		
CZ069	Complement (58497–58808)	312				46.7	N042		
CZ070	Complement (58904–59245)	342				49.1	N041		
CZ072	Complement (59472–61025)	1,554	33.3		Integrase of bacteriophage TP901-1	49.1	N041		
(CZ080)	Complement (67777–68256)	480		*orfX*	ORFX	100	*orfX* (480)		
ORFs with homologues found in type II but not in type I SCC*mec*									
Z016	10383–10706	324				73.1	N044 (318)		
Z017	10901–12793	1,893	99.5	*tnpB*	Transposase B (transposon Tn*554*)	96.5	*tnpB* (1893)		
Z018	12800–13177	378	96.8	*tnpC*	Transposase C (transposon Tn*554*)	96.8	*tnpC* (378)		
Z023	17130–17624	522				100	N051 (522)		
CZ021	Complement (17153–17798)	645	40.5	(*yrkf*)	Protein with unknown function	100	CN030* (630)		

Continued on following page

TABLE 2—*Continued*

ORF[a]	Location[b]	Size (bp)	Homology to ORFs in database			Homology to ORFs of N315		Homology to ORFs of 85/2082	
			% Identity[c]	Gene	Description of gene product	% Identity	Corresponding ORF(s) (size in bp)	% Identity	Corresponding ORF(s) (size in bp)
CZ022	Complement (17797–18063)	267	52.3	(*yrkD*)	Protein with unknown function	100	CN031 (267)		
CZ025	Complement (20738–21886)	1,140	38.6	(*xylr*)	Xylose repressor	100	*xylr* (1134)		
CZ027*	Complement (22527–22730)	204	100	*mecI*	Methicillin resistance protein Mecl	100	$mecI_{N315}$ (372)		
Z068	52369–53110	732	100	*ermA*	rRNA adenine N-6 methyltransferase	100	*ermA* (732)		
CZ063*	Complement (53485–54009)	525	100	*spc*	Adenyltransferase AAD9	99.4	*spc* (783)		
CZ064	Complement (54160–54537)	378	100	*tnpC*	Transposase C (transposon Tn*554*)	100	*tnpC* (378)		
CZ065	Complement (54544–56436)	1,893	100	*tnpB*	Transposase B (transposon Tn*554*)	100	*tnpB* (1893)		
CZ066	Complement (56433–57518)	1,086	100	*tnpA*	Transposase A (transposon Tn*554*)	100	*tnpA* (1086)		
CZ067	Complement (57637–57954)	318				98.1	N044 (318)		
ORFs unique in type III SCC*mec*									
Z019	13415–13780	366	100	*cadC*	Cadmium resistance protein C				
Z020	13773–16187	2,415	99.9	*cadA*	Cadmium resistance protein A				
CZ033	Complement (29806–30750)	945	99.6	*rep*	RepC protein				
CZ034	Complement (31277–32518)	1,242	98.5	*pre*	Preprotein (plasmid recombination)				
CZ035	Complement (32704–33252)	549	99.4		Polypeptide D				
CZ036	Complement (33197–34084)	888	96.5	*tetK*	Polypeptide B				
Z048	36949–37362	414	99.3		ORF1				
CZ040	Complement (37557–38207)	651	100	*merB*	Organomercurial lyase				
CZ041	Complement (38289–39932)	1,644	100	*merA*	Mercuric reductase				
CZ043*	Complement (39990–40328)	339	100	*merT*	Transport protein				
CZ045	Complement (40463–41143)	681	99.1		ORF4				
CZ046	Complement (41140–41493)	354	94.9		ORF3				
CZ047	Complement (41510–41917)	408	97.8		ORF2				
CZ048	Complement (42217–43566)	1,350	98.9	*merR*	Regulatory protein				
Z059*	44552–46795	2,244	30.5	*hsdR*	Catalytic subunit of restriction-modification system				

[a] ORFs shown in parentheses were located outside of SCC*mec*. Asterisks signify incomplete ORFs that are potentially defective genes or pseudogenes containing either a deletion, nonsense mutation, or frameshift mutation.
[b] Nucleotide position in the nucleotide sequence deposited under DDBJ/EMBL/GenBank accession no AB033763 (type I) and AB0376701 (type III).
[c] Identity to the amino acid sequence of the best match revealed in the homology search of the GenBank and EMBL databases with TFastA.
[d] Genes shown in parentheses had a deduced ORF with less than 70% amino acid identity to the ORF indicated.

PCR detection of the *mecI* gene (Table 1). The results of *ccr* complex typing also agreed with the SCC*mec* typing results (Table 1).

We also used the PCR typing method designated *mec* right extremity polymorphism (MREP) typing (11). MREP typing is a quick SCC*mec* typing method that takes advantage of the polymorphism among the three types of SCC*mec* in the right extremity: type III had a unique nucleotide sequence, and type II SCC*mec* had additional 102-bp nucleotides to the right terminus of type I SCC*mec* (Fig. 1). PCR primers were prepared

TABLE 3. Primers used in this study

Primer	Nucleotide sequence
Localization of IS*1272*	
mA2	5′-AACGTTGTAACCACCCCAAGA-3′
iS-4	5′-ACAATCTGTATTCTCAGGTCGT-3′
ccr gene type	
β2	5′-ATTGCCTTGATAATAGCCITCT-3′
α2	5′-AACCTATATCATCAATCAGTACGT-3′
α3	5′-TAAAGGCATCAATGCACAAACACT-3′
α4	5′-AGCTCAAAAGCAAGCAATAGAAT-3′
MREP typing	
cR4	5′-GTTCAAGCCCAGAAGCGATGT-3′
mR2	5′-GATAGACTAATTATCTTCATC-3′
mN16	5′-ATATTCTAGATCATCAATAGTTG-3′
Amplification of DNA fragments for nucleotide sequences	
NCTC10442	
mE1	5′-AACTTCACTGTTGACGACTTA-3′
mE2	5′-AACAGCATTAAGAGCAGCTGCACAA-3′
85/2082	
cLt1	5′-AGAAGCGTATCATAAGTAGCGGA-3′
cLt2	5′-ATCTTCTGAAGGATAATTCGCA-3′
cLt3	5′-TCCGCTACTTATGATACGCTTCTG-3′
cLt4	5′-CAATTGGCATGACACTAAATGGCA-3′
mN1	5′-TGGAGAATATGAAGATTACATTCA-3′
mN2	5′-TTTTGACGATGAAGGTCTTCA-3′
mN3	5′-AGTAACGCAACGGGTATGATTA-3′
Tn*554*(171–146)	5′-TACGGCTTATTCTCCACTTCTATCCT-3′
Tn*554*R	5′-AAGCTGTGGCTTTGAAAAGTTGA-3′
TnpA1016	5′-TGTGATGTAAATTCTATTCCAGT-3′
TnpA636	5′-TGAGATCAAAGGAAGTTAAGCAAATTATTGATG-3′
cad1	5′-TGTAATTGGCGGATATTCACTAT-3′
mN4	5′-TAGCAACATAATAGTCATATTTGCT-3′
mN5	5′-TTGCTTCGGGACTTACCTCTAGT-3′
mN6	5′-ACCTCTAACGTTAACAATATTC-3′
mN7	5′-TAATCAATACAAATCTATCGACTTCT-3′
tetK1	5′-TTCGATAGGAACAGCAGTATAT-3′
tetK4	5′-ATATTACTATACACTCCAGAAGA-3′
merA1	5′-AGGCTAAGCAAAATATTTCGGCA-3′
merA2	5′-TCTTCACAGCCTGTGCATGTCATGCCT-3′
merN	5′-ACGGATTGCTGTACGCCTCCAGA-3′
mN8	5′-ACTCTGTACCTCATCCACAGTTTGA-3′
mN9	5′-ACCAGACCGTCCTTTCGATTTAACAA-3′
mN10	5′-GACAACATGATTTAGAAGTAGAGGT-3′
mN11	5′-ATCGTCCGAGGACTTGTATCGAGTTCTA-3′
mN12	5′-TACCATTCTTAGCTCCACCATAT-3′
mN13	5′-ACAACTTGCGAATTATGACGA-3′

to bracket the right SCC*mec*-chromosome junction point to detect the polymorphism of the three types of SCC*mec*. MREP typing results of the 34 strains also agreed with those of SCC*mec* typing, but with two exceptions: strain 85/5328 did not react positively to any set of primers, and strain 93/H44, having type III SCC*mec* by dot-blot hybridization, was judged as having type I MREP (typically associated with type I SCC*mec* of NCTC 10442) (Table 1). Four strains, 81/108, 85/4547, 86/4372, and 87/25, did not hybridize typically to any of the three sets of SCC*mec* probes. However, strain 87/25 hybridized with all of the probes of type II SCC*mec*, except for probe 5. The other three strains appeared to carry the class B *mec* complex (IS*1272*-Δ*mecR1-mecA*-IS*431*) characteristically found in type I SCC*mec*. However, strains 81/108 and 85/4547 responded to the primer set of the type II *ccr* complex. In the case of strain 86/4372, a PCR using the three sets of *ccr* primers did not amplify any DNA fragment (see Discussion).

Experimental precise excision of type I SCC*mec* with type 2 *ccr* genes. Spontaneous precise excision of type II SCC*mec* in the culture of MRSA strains can be detected by the PCR amplification method by using the extracted DNA from the strain as a template and by using a primer set of cR2 and cL1 bracketing the integration site for SCC*mec* (designated *att*B PCR) (see Fig. 1 for the location of primers, and see Table 3 for their sequences) (14, 17). In this study, a set of primers, cR2 and cLt3 (see Fig. 1 for the location of primers), was prepared to detect spontaneous excision of type III SCC*mec*. DNA extracted from an overnight culture of strain 85/2082 was positive with the set of primers, which coincided with the fact that the spontaneously excised strain 85/2082ex was obtained after se-

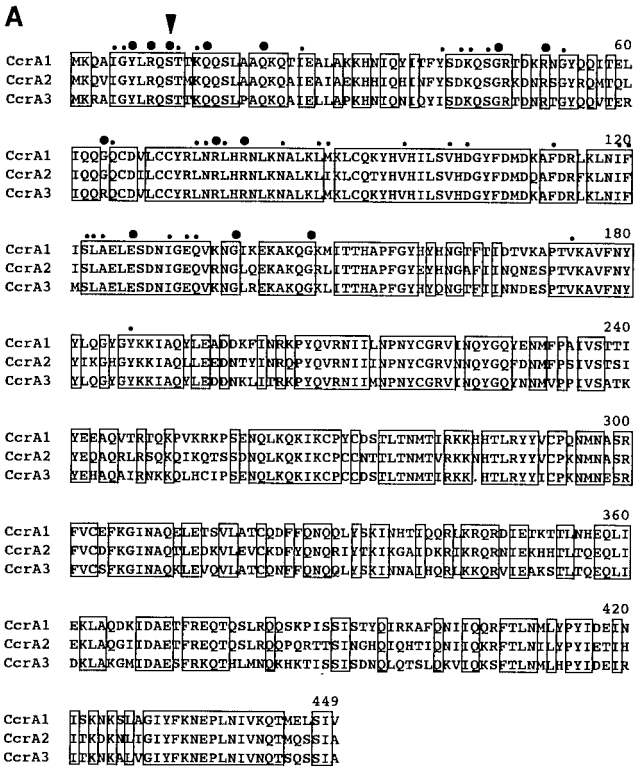

FIG. 3. Deduced amino acid sequences of *ccr* genes. (A) Deduced amino acid sequences of *ccrA1, ccrA2*, and *ccrA3*. (B) Deduced amino acid sequences of *ccrB1*, ccrB2*, and *ccrB3*. Amino acid sequences of *ccr* genes were aligned by using the Pile-Up program with a GCG default scoring matrix in the Wisconsin Package (version 9.0; Genetics Computer Group). Amino acids shared by the three peptides are boxed. Large bullets indicate amino acid residues of Ccr proteins shared by site-specific recombinases of the invertase-resolvase family. Small bullets indicate amino acid substitution within the same class of amino acids. A black arrowhead indicates the presumptive serine involved in the phosphoseryl linkage of the recombinase of DNA conserved in the NH_2-terminal catalytic domain of site-specific recombinases of the invertase-resolvase family (27). A white arrowhead indicates the locus of the first amino acid residue changed by an addition of adenine in the nucleotide sequence of type I SCC*mec* as described in the text.

rial passages of the strain in drug-free medium. In contrast, the set of primers cR2 and cL1, which theoretically detect excision of type I as well as type II SCC*mec*, could not amplify DNA from the culture of NCTC 10442. This was in agreement with the fact that *ccrB1* of NCTC 10442 had a frameshift mutation (Table 2). Moreover, all nine strains with type I SCC*mec* were negative with the *att*B PCR, and the mutation found in the *ccrB1* of NCTC 10442 was commonly found in all nine of the *ccrB1* genes. Therefore, we could not obtain a spontaneous excisant strain from any of the strains having type I SCC*mec*. To reconfirm the boundary of type I SCC*mec*, therefore, we introduced type 2 *ccr* genes into the type I SCC*mec*-carrying strain 85/1940. The strain was used instead of NCTC 10442, because the latter strain was resistant to tetracycline; tetracycline resistance was used as a marker for the selection of the transformants. The culture of a transformant strain 85/1940(pSR) generated β-lactam-susceptible cells with a high frequency. The nucleotide sequences around the *att*B region of the excisant strain 85/1940ex thus obtained are shown in Fig. 2. The type I SCC*mec* integration site on the chromosome of 85/1940 coincided with that inferred by comparing the corresponding regions of NCTC 10442 and NCTC 8325 strains (Fig. 2).

Molecular evolutionary relationship of members of the *ccr* gene family and *mecA* genes. Comparisons of the deduced amino acid sequences of *ccrA* and *ccrB* genes of three types of SCC*mec* are shown in Fig. 3. Since *ccrB1* seemed to be disrupted by a deletion of a single base, we have reconstituted a potential *ccrB1* gene (*ccrB1**) by an addition of adenine at the position where it seemed to be deleted and used its deduced product for the comparison. All of the Ccr proteins were highly basic, with pI values of 10.07 to 10.49, and shared the motifs of the site-specific recombinases of the invertase-resolvase family in their N-terminal domains (27). The catalytic serine residue of the recombination active site was also conserved in all Ccr proteins. Figure 4 illustrates the phylogenetic relationship among the *ccrA* and *ccrB* genes of three types of SCC*mec*. We also compared them with the CcrB-like product of ORF CZ072 found in type III SCC*mec* and with several site-specific recombinases of gram-positive bacteria as well. The latter were the site-specific integrase of bacteriophage TP901-1 of *Lactococcus lactis*; a site-specific recombinase (5), *spoIVCA*, of *Bacillus subtilis* (30); and a transposase, *tnpX*, of *Clostridium per-*

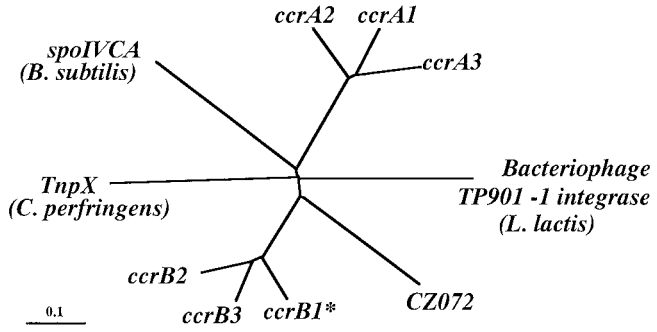

FIG. 4. Phylogenetic relationships among *ccrA* genes, *ccrB* genes the ORF CZ072, and three site-specific recombinases. Three site-specific recombinases that showed a high similarity to *ccr* genes were selected to investigate phylogenetic relationships. They were the integrase (*int*) of bacteriophage TP901-1 found in *L. lactis* (1,458 bp; DDBJ/EMBL/GenBank accession no. X85213), the site-specific recombinase (*spoIVCA*) found in *B. subtilis* (1,503 bp; DDBJ/EMBL/GenBank accession no. D32216), and the transposase (*tnpX*) found in the conjugative transposon Tn*4451* of *C. perfringens* (2,124 bp; DDBJ/EMBL/GenBank accession no. U15027). The nucleotide sequences of the *ccrA* genes (*ccrA1, ccrA2*, and *ccrA3*), *ccrB* genes (*ccrB1*∗, *ccrB2*, and *ccrB3*), ORF CZ072, *int, spoIVCA*, and *tnpX* were aligned by using the Pile-Up program with a GCG default scoring matrix. Phylogenetic relationships were developed with the Paupsearch program by the neighbor-joining method. The tree was visualized with Tree View software. The branch length indicates the distance, which is expressed as the number of substitutions per 100 bases.

fringens (3), which had comparable sizes and substantial amino acid similarities to *ccr* genes. The phylogenetic tree showed that *ccrA* and *ccrB* genes form separate subfamilies. The CZ072 ORF was more closely related to the *ccr* genes of subfamily B than those of subfamily A. The three site-specific recombinases of other bacterial species were distantly related to either one of the two *ccr* subfamilies (Fig. 4).

The phylogenetic relationships among the *mecA* genes were also compared by using the nucleotide sequences of the *mecA* genes in three types of SCC*mec* and previously reported ones in coagulase-negative staphylococcal species *S. epidermidis* WT55 (DDBJ/EMBL/GenBank accession no. X59592) (24) and *S. sciuri* K8 (DDBJ/EMBL/GenBank accession no. Y13069) (37) (data not shown). All five *mecA* genes were 2,007 bp in size. The *mecA* gene of N315 was identical to that of *S. epidermidis* WT55, whereas it differed from those of *S. aureus* NCTC 10442 and *S. sciuri* K8 by 1 bp and from that of *S. aureus* 85/2082 by 2 bases. Therefore, they were very well conserved, in contrast to the *ccrA* and *ccrB* genes.

DISCUSSION

We have shown that there are at least three distinct types of SCC*mec* in the chromosome of MRSA worldwide. SCC*mec* was defined as the DNA element on the MRSA chromosome demarcated by a pair of direct repeats and inverted repeats, having *ccr* genes required for its movement and carrying the *mecA* gene (14, 17). As far as we could judge from the structure of the two elements newly identified in this study, they seem to constitute a family of SCC*mec* together with N315-type SCC*mec*.

The *mecA* gene is considered to have originated in some coagulase-negative staphylococcus species (36) and was then transferred into *S. aureus* to generate MRSA (1, 13, 32). It is likely that SCC*mec* serves as the carrier of the *mecA* gene moving across staphylococcal species, since *mecA* genes in other staphylococcal species have never been found without the accompaniment of SCC*mec*-like structure (T. Ito and Y. Katayama, unpublished observation). Since both *ccrA* and *ccrB* genes are required for the integration event, we considered that the *ccrB1* gene must have been intact when SCC*mec* was introduced into the recipient *S. aureus* cell to produce NCTC 10442 or its precedent strain (17). However, so far, our search for the intact *ccrB1* in MRSA as well as in methicillin-resistant coagulase-negative staphylococcus (MRC-NS) strains isolated in Japan in 1980s and 1990s have not been successful (K. Tsutsumimoto, unpublished observation). Successful excision of type I SCC*mec* by type II *ccr* genes raised another related question of whether the type 1 and type 2 *ccr* genes were present as distinct types before or even soon after the integration of SCC*mec* into the *S. aureus* chromosome. We cannot rule out the possibility that the type 1 *ccr* genes were derived from type 2 *ccr* genes after establishment of type II SCC*mec* in the MRSA chromosome as a result of sequential accumulation of mutations. To finally clarify the question, it would be necessary to find intact type 1 *ccr* genes retaining recombination function or, alternatively, to test the functional integrity of *ccrA1* in combination with the *ccrB1* gene artificially reconstructed from Ψ*ccrB1* by eliminating the frameshift mutation. Study in this direction is under way.

It is also noteworthy that type III SCC*mec* contained another unit of *ccr* complex (Ψ*ccr* complex). Moreover, the 15-bp direct repeat sequence present at the right end of SCC*mec* was also found between the two IS*431* copies in type III SCC*mec*, as illustrated in Fig. 1. These findings indicate that the type III SCC*mec* is composed of two separate SCC*mec* or SCC (cassette chromosome without *mec* complex) elements that were sequentially integrated in the chromosome of 85/2082. We have observed that an experimental SCC plasmid carrying type 2 *ccr* genes, *att*SCC, and *tetL* as a selective marker can integrate into N315 chromosome side-by-side with the type II SCC*mec* (H. Yuzawa, unpublished data). In light of this, the putative right-part element of type III SCC*mec* may be an SCC carrying a transposon, Tn*554*, the erythromycin and spectinomycin resistance of which serves as a selective marker. Alternatively, it may be another copy of SCC*mec* in which the *mec* complex had been deleted together with the *ccrA* gene after integration into the chromosome.

In contrast to the old strain, NCTC 10442, recent MRSA isolates are resistant to many antibiotics besides β-lactam antibiotics (9). Such a multiple resistance of MRSA is attained by the activity of IS*431* copies downstream of *mec* complex (Fig. 1). IS*431* is known to serve as a chromosomal deposit site for multiple resistance genes (21). The integrated copies of pUB110 (in type II SCC*mec*) and pT181 (in type III SCC*mec*) were flanked by two copies of IS*431* (6, 22). Direct repeats were present at both ends of these integrated plasmids. This suggests that these plasmids were accumulated by homologous recombination events across two copies of IS*431*: one present on the chromosome and the other present on the plasmid (28).

We referred to the nucleotide sequences of two other MRSA strains by using the BLAST search program of The

Institute for Genomic Research (TIGR) (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=s_aureus) and the Sanger Centre (http://www.sanger.ac.uk/Projects/S_aureus/). When we compared the nucleotide sequence of type I SCC*mec*, we found that strain COL, which is being sequenced by the genome project of TIGR, carried a DNA region corresponding to the element. The overall nucleotide identity of the region corresponding to type I SCC*mec* was 98% over 34,364 bases of alignment. The minor difference was found in the CE010 ORF potentially encoding *pls*, a polypeptide belonging to the SD repeat multigene family. On the other hand, strain 252 (EMRSA-16), which is being sequenced by the Sanger Centre, carried DNA regions divided into three contigs, which corresponded to type II SCC*mec*. There was a greater than 95% nucleotide identity between the two elements.

Thirty-four of 38 MRSA strains carried one of the three types of SCC*mec*. However, four strains reacted atypically to any of the three sets of probes. One of them, 85/25, may be interpreted simply as a carrier of type II SCC*mec* with a minor deletion in it: the chromosomal DNA of the strain hybridized with 9 of 10 type II probes positively and served as a positive template for the PCR detecting type 2 *ccr* genes (Table 1). The other three strains, however, were much more complex. The results of a PCR experiment suggested that they carried type II *ccr* complex (except for 85/4372), and the structure of the type ii right extremity (MREP type corresponding to type II SCC*mec*). However, the strains possessed the class B *mec* complex typically carried by type I SCC*mec*. There are several possible explanations for these cases. (i) The class B *mec* complex was introduced into a type II SCC (presumptive primordial element without a *mec* complex) located in a staphylococcus chromosome. (ii) Two SCC (or SCC*mec*) elements of type I and type II were cointegrated in the strains at the *attB* site, and, subsequently, homologous recombination between the two elements took place, leaving a chimeric SCC*mec*. In either case, the negative hybridization reaction of these strains with most probes of type I or type II SCC*mec* should be explained by the deletion of the region upstream of the *ccr* complex subsequent to their integration in the chromosome. If this was not the case, and there were unique DNA regions not hybridizable with either set of SCC*mec* probes, the elements carried by the strains may be referred to as other types of SCC*mec*.

With regard to strain 85/4372, no DNA fragment was amplified by PCR with a set of primers used for the *ccr* typing or those detecting the common nucleotide sequences of the three types of *ccrA* and *ccrB* genes (T. Ito, unpublished observation). Therefore, the *ccr* genes of this strain might have either been deleted or composed of a different nucleotide sequence from those of the three *ccr* genes described in this paper. Further experiments are required to clarify the complex structure of SCC*mec* of these strains.

There is no reason to limit the putative SCC to being only the conveyer of methicillin resistance alone. It might be serving as a vehicle for exchange of useful genes for the better survival of staphylococci in various environments. For example, plasmin-sensitive surface protein is found in type I SCC*mec*, and the Kdp operon, encoding potassium-dependent ATPase and its regulators, is carried by type II SCC*mec*. They, although mostly found as pseudogenes, might have been useful for the host cells to survive in their particular environment. Our proposal that SCC is a general genetic information exchange system of staphylococci and is not confined to antibiotic resistance also comes from our finding with an MSSA strain, ATCC 25923. The *S. aureus* type strain, isolated in 1945, long before the first isolation of MRSA (in 1961), carried a DNA fragment 5,877 bp in size (designated IE25923) inserted at exactly the same nucleotide position in *orfX* as that utilized by three SCC*mec*s for their integration. (The entire sequence of IE25923 is available under DDBJ/EMBL/GenBank accession no. AB047239.) Moreover, it had similar structural characteristics of SCC*mec*s at both ends, i.e., incomplete inverted repeats and direct repeats of 15 bp (Fig. 2). The nucleotides of both the left and right extremities showed high similarity to those of type III SCC*mec*: 92.6% (25 identical bases in 27 nucleotides at the left extremity) and 93.1% (27 identical bases in 29 nucleotides at the right extremity) (Fig. 2). However, the other region of IE25923 did not show any significant similarity to three types of SCC*mec*. No drug resistance gene was found in it. Unfortunately, no ORFs with inferable function based on the search of extant gene products or *ccr* genes were found in it. Therefore, IE25923 seems to be a remnant of SCC or SCC*mec* that was integrated in its complete form and then afterwards deleted with *ccr* as well as with *mec* complexes. Substantiation of our proposal will await finding a complete form of SCC carrying *ccr* genes, but with no *mec* complex on it.

In conclusion, we have found that the *mec* complex, conferring methicillin resistance to *S. aureus*, was conveyed by a novel family of the mobile genetic element SCC*mec*. SCC*mec* is defined by its characteristic structures at the extremities and by carriage of *ccr* as well as *mec* complexes. At least three distinct members make up the family. SCC*mec* may have evolved from a primordial mobile element, SCC, into which the *mec* complex was inserted. Exploration of staphylococcal genomes of more strains will find more diversified members of SCC*mec* as well as presumptive SCC, which will enable us to understand how staphylococcal species are exchanging genetic information to cope with antibiotics as well as the physiological selective pressure of the environment.

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