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Identification in Methicillin-Susceptible *Staphylococcus hominis* of an Active Primordial Mobile Genetic Element for the Staphylococcal Cassette Chromosome *mec* of Methicillin-Resistant *Staphylococcus aureus*

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We previously reported that the methicillin resistance gene *mecA* is carried by a novel type of mobile genetic element, SCC*mec* (staphylococcal cassette chromosome *mec*), in the chromosome of methicillin-resistant *Staphylococcus aureus* (MRSA). These elements are precisely excised from the chromosome and integrated into a specific site on the recipient chromosome by a pair of recombinase proteins encoded by the cassette chromosome recombinase genes *ccrA* and *ccrB*. In the present work, we detected homologues of the *ccr* genes in *Staphylococcus hominis* type strain GIFU12263 (equivalent to ATCC 27844), which is susceptible to methicillin. Sequence determination revealed that the *ccr* homologues in *S. hominis* were type 1 *ccr* genes (*ccrA1* and *ccrB1*) that were localized on a genetic element structurally very similar to SCC*mec* except for the absence of the methicillin-resistance gene, *mecA*. This genetic element had mosaic-like patterns of homology with extant SCC*mec* elements, and we designated it SCC_{12263} and considered it a type I staphylococcal cassette chromosome (SCC). The *ccrB1* gene identified in the *S. hominis* strain is the first type 1 *ccrB* gene discovered to retain its function through the excision process as judged by two criteria: (i) SCC_{12263} was spontaneously excised during cultivation of the strain and (ii) introduction of the *S. hominis ccrB1* into an MRSA strain carrying a type I SCC*mec* whose *ccrB1* gene is inactive generated SCC*mec* excisants at a high frequency. The existence of an SCC without a *mec* determinant is indicative of a staphylococcal site-specific mobile genetic element that serves as a vehicle of transfer for various genetic markers between staphylococcal species.

The emergence and worldwide spread of methicillin-resistant *Staphylococcus aureus* (MRSA) since the early 1960s has long posed serious problems for antimicrobial chemotherapy. The genetic basis of methicillin resistance in MRSA is the acquisition of the *mecA* gene (30, 39) that encodes the low-affinity penicillin-binding protein 2′ (PBP 2′ [see references 9, 37, and 46]). The *mecA* gene is carried by an exogenous DNA element inserted in the *Staphylococcus aureus* chromosome. Ito et al. sequenced the entire element (17, 18), and Katayama et al. showed that it is a novel class of mobile genetic element, designated SCC*mec* (staphylococcal cassette chromosome *mec*) (20). The SCC*mec* element is integrated in the *S. aureus* chromosome at the $attB_{SCC}$ site located in *orfX*, an open reading frame (ORF) of unknown function (17, 18, 20). The SCC*mec* element has both inverted repeats (IR) and direct repeats (DR) at its termini and carries a set of site-specific recombinase genes, cassette chromosome recombinase genes *ccrA* and *ccrB*, which are responsible for the integration and excision of SCC*mec*, and the *mecA* gene complex that encodes inducible methicillin resistance (17, 18, 20). So far, four distinct types of SCC*mec* have been identified in MRSA strains isolated from around the world (12, 13, 18). The type I SCC*mec* group includes the older MRSA strains prevalent in the 1960s and is exemplified by NCTC 10442, the first MRSA strain isolated in 1960 in England. The type II SCC*mec* group first identified by studies of a Japanese pre-MRSA strain, N315, isolated in 1982 (14, 17), is ubiquitous among recent MRSA isolates from Japan, Korea, and the United States (12). The type III SCC*mec* group is found in strains prevalent in the countries that have close geographical or historical ties to England, such as European countries, Saudi Arabia, India, Singapore, Hong Kong, Australia, and New Zealand (18). Recently, Ma et al. have identified a fourth type of SCC*mec* from community-acquired MRSA strains (29, 36).

The type of an SCC*mec* element is defined by the allotype of the *ccr* genes and the structural class of the *mecA* gene complex it possesses. Three allotypes of *ccr* genes (type 1, type 2, and type 3) and four classes of *mecA* gene complex (class A, class B, class C, and class D) have been identified (21). The class A *mecA* gene complex maintains the prototypic structure, *mecI-mecR1-mecA*-IS*431mec*, where *mecI* and *mecR1* encode a repressor and a signal transducer of beta-lactam-induced *mecA* gene transcription, respectively (11, 16, 27, 31). In the other classes, the *mecI* and *mecR1* genes are either partially or completely deleted in presumable association with an integrated copy of IS*1272* (class B) or IS*431* (class C). The class D complex found in *Staphylococcus caprae* JA186 has a deleted

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TABLE 1. Synthetic oligonucleotide primers

Genetic element(s) and primer designation	Sequences[a]	Nucleotide positions	Source or reference
ccr genes[b]			
βc(β2)	5′-ATTGCCTTGATAATAGCCTTCT-3′	25987–25967	Okuma et al. (35)
αc	5′-ATCTATTTCAAAAATGAACCA-3′	25424–25444	Okuma et al. (35)
SCC_{12263}[c]			
hL-1	5′-TCACCTTCACATACTTTTCTCA-3′	12–33	This study
hL-2	5′-TCGTATGCGGTATAACCTCCGTCT-3′	3361–3338	This study
hL-3	5′-ACCTTTATCGATAAACTCAT-3′	4851–4870	This study
hL-4	5′-AAGTTCCTTCTTGTGTAAATGGA-3′	507–529	This study
hR-1	5′-ATGAAGATTACTATTTTAGCT-3′	22820–22800	This study
hR-2	5′-TTACTTTGCTTCCAATCTATTAAT-3′	22140–22163	This study
hR-3	5′-ATGTAGAATATAAAGAAGGCAA-3′	21795–21774	This study
hR-4	5′-AACGTATGACACAAGGACAAA-3′	22540–22520	This study
Hom1	5′-AAAAGGATCCTTGAAGAAATTAATCATGCTGA-3′	11940–11951	This study
Hom2	5′-AAAAGGATCCTCATTGTTAGATAGTTGGAATA-3′	15584–15563	This study

[a] Introduced *Bam*HI sites are underlined.
[b] Primers listed are specific for *ccr* genes of strain N315 (DDBJ/EMBL/GenBank accession no. D86934 [see reference 17]).
[c] Primers listed are specific for SCC_{12263} of strain GIFU12263 (DDBJ/EMBL/GenBank accession no. AB063171).

mecR1, but adjacent to the deletion point neither IS*1272* nor IS*431* was identified. The region abutting the deletion point of Δ*mecR1* was identical to a stretch of sequences in the type II SCC*mec* element in the region distant from *mecA* gene complex, and we speculate that a homologous recombination might have caused the deletion (21). Type I SCC*mec* carries a type 1 *ccr* (*ccr-1*) and a class B *mecA* gene complex, type II SCC*mec* carries a type 2 *ccr* (*ccr-2*) and a class A *mecA* gene complex, type III SCC*mec* carries a type 3 *ccr* (*ccr-3*) and a class A *mecA* gene complex, and type IV carries a type 2 *ccr* (*ccr-2*) and a class B *mecA* gene complex. Among the three types of *ccr* genes, only the *ccrB1* gene of type I SCC*mec* is inactive because of a frame-shift mutation (thus, the gene is generally designated *ccrB1*∗). Because both *ccrA* and *ccrB* genes are required for the excision and integration events of SCC*mec* (20), the *ccrB1* gene must have been intact prior to its integration into *S. aureus* chromosome (18). However, the search for the intact *ccrB1* in MRSA strains as well as in strains of methicillin-resistant coagulase-negative staphylococci (C-NS) has been unsuccessful (20).

Since the class B *mecA* gene complex found in MRSA strains contains a truncated copy of IS*1272* that is prevalent in *Staphylococcus haemolyticus* but not in *S. aureus* (2, 4), Archer et al. (2) and others (44, 48) proposed that the direction of interspecies transfer of the *mecA* gene complex is from C-NS to *S. aureus.* Various types of SCC*mec* are indeed widely distributed among staphylococcal species other than *S. aureus.* SCC*mec* elements are typically present in methicillin-resistant C-NS, and we have observed several methicillin-susceptible strains that harbor staphylococcal cassette chromosome (SCC)-like elements on their chromosomes. Structural comparison of extant SCC*mec* elements revealed that SCC*mec* is made up of two mutually independent genetic components (the *ccr* and *mecA* gene complexes) (18). We hypothesized, therefore, the existence of a family of mobile genetic elements (SCCs) that serve for interspecies transfer of any useful genetic information among staphylococcal species and that SCC*mec* is one of the SCCs that is specialized for the transfer of methicillin resistance (17). In this study, in order to prove this hypothesis, we looked for a staphylococcal strain carrying *ccr* genes but lacking the *mecA* gene. By screening methicillin-susceptible staphylococcus type strains, we identified a type I SCC element that carries an intact set of type 1 *ccr* genes (*ccrA1* and *ccrB1*) but no *mecA* gene in the genome of *Staphylococcus hominis* type strain GIFU12263.

MATERIALS AND METHODS

Bacteria and growth conditions. The *S. hominis* type strain GIFU12263 (equivalent to ATCC 27844) used in this study was a kind gift from Takayuki Ezaki (22). Bacteria were cultivated in brain heart infusion (BHI) broth as described previously (20). The antibiotics tetracycline (Sigma Chemical Co., St. Louis, Mo.) and ceftizoxime (Fujisawa, Pharmaceutical Co. Ltd., Osaka, Japan) were used at concentrations of 10 and 25 μg/ml, respectively. MRSA strain 85/1940 carrying a type I SCC*mec* and susceptible to tetracycline was used for the transformation experiment (10).

DNA preparation, PCR amplification, and nucleotide sequencing. The DNA extraction and PCR, long-range PCR, and nested PCR amplification steps were performed as previously described (17, 20). To detect *ccr* genes among staphylococcus type strains, we carried out PCR amplifications with a set of primers (αc and βc) that we designed by using the sequences common to the three *ccr* genes. The *ccr* genes and their surrounding chromosomal regions were amplified by long-range PCR (20), using seven primers (see Fig. 2b), followed by nucleotide sequence determination. The seven primers were designed based on the nucleotide sequences of type I SCC*mec* in strain NCTT10442 (DDBJ/EMBL/GenBank accession no. AB033763 [see reference 18]). By an inverse PCR (IPCR) procedure, DNA fragments corresponding to the regions upstream and downstream of the *ccr* genes were amplified, and the products were then sequenced by primer walking. The experimental procedure for IPCR was as follows. The chromosomal DNA of GIFU12263 was digested with *Eco*RI for cloning the right side and with *Hin*dIII for cloning the left side of the SCC_{12263} element (Takara Shuzo Co., Ltd., Shiga, Japan). Self-ligation of the *Eco*RI- and the *Hin*dIII-digested DNA was performed (DNA concentrations of 1 to 2 μg/ml) for 10 min with a ligation kit (Boehringer Mannheim Biochemica, Mannheim, Germany). The ligation mixtures were then used as the template DNAs for PCR amplification. The primer sets were hR-2 and hR-3 for the *Eco*RI self-ligation and hL-2 and hL-3 for the *Hin*dIII self-ligation (see Fig. 2b). Sequence analysis was performed with the big dye terminator cycle sequencing kit (Applied Biosystem Inc., Foster City, Calif.) on a DNA analyzer (model 3100; Applied Biosystem Inc.). The nested PCR procedure was performed as described previously (17). We used two sets of primers: hL1 and hR1 for the first round of PCR amplification and hL-4 and hR4 for the second round of PCR amplification. All primers used in this study are listed in Table 1.

Computer analysis of nucleotide and protein sequences. An initial set of ORFs likely to encode proteins was identified with the GAMBLER computer program,

and those shorter than 30 codons were eliminated. ORF prediction and gene homology search and identification had been reported by Kuroda et al. previously (26). A total of 26 ORFs including *ccr* genes were compared to those of three types of SCC*mec*s (type I in strain NCTT10442 [DDBJ/EMBL/GenBank accession no. AB033763], type II in strain N315 [DDBJ/EMBL/GenBank accession no. D86934], and type III in strain 85/2082 [DDBJ/EMBL/GenBank accession no. AB037671] [see reference 18]). The homologies between the nucleotide sequences of SCC_{12263} and SCC*mec* types I, II, III, and IVa in strain CA05 (DDBJ/EMBL/GenBank accession no. AB063172) and IVb in strain 8/6-3P (DDBJ/EMBL/GenBank accession no. AB063173) (29) were studied as follows. First, the homologous regions were computed using b12seq, i.e., BLAST analysis for two sequences (45), for each pair of sequences. Then, the BLAST results were processed by a script written in Perl (Larry Wall, script in Perl, version 5, 1999, available at website http://www.perl.com/), and visualized with commercial software (Mathematica, version 4, 1999; Wolfram Research, Inc. Champaign, Ill.).

Finally, major matching regions were extracted by combining neighboring shorter matching regions as follows. First, for a pair of nucleotide sequences of SCCs, fragments of homologous regions were enumerated by using bl2seq (45). Second, neighboring fragments were connected to each other to form homologous regions. For this, for each segment *A,* a segment *B* was defined to be in its right neighbor if (i) the segments were matches in the same direction; (ii) the left end point of *B* was to the right of the right end point of *A,* but within 1,000 bp; (iii) the vertical distance between the two parallel lines including *A* and *B*, respectively, was at most 100 bp; and (iv) among such segments, *B* had the closest left end point to the right end point of *A.* The distance between end points was measured as the sum of the difference between the horizontal and vertical axes. Finally, maximal collections of neighboring segments with slopes between the two very end points determined to be between −1.1 and −0.9 or between 0.9 and 1.1 were chosen as homologous regions.

Several types of BLAST analyses were carried out with software available at the NCBI site (http://www3.ncbi.nlm.nih.gov/BLAST/). TransQ analysis was carried out with software available at brown.ims.u-tokyo.ac.jp:8118/html /transq.html.

Construction of recombinant plasmid and excision assay. Two recombinant plasmids, $pSR1_{hom}$ and $pSR1_{aur}$, harboring *ccrA* and *ccrB* genes of GIFU12263 and NCTC10442, respectively, were constructed by cloning the *Bam*HI-digested DNA fragments containing *ccr* genes into the unique *Bam*HI site of plasmid vector pYT3 (7), as described previously (20). The two primers Hom1 and Hom2 were used for the amplification and cloning of the *ccrAB* gene complexes from the chromosomal DNAs of the above-mentioned strains. The fidelity of the nucleotide sequences of the *ccr* genes harbored by the two plasmids $pSR1_{hom}$ and $pSR1_{aur}$ was confirmed before use. Electroporation was performed as described previously (20).

Nucleotide sequence accession number. The sequence of SCC_{12263} of strain GIFU12263 has been assigned DDBJ/EMBL/GenBank accession number AB063171.

RESULTS

Identification of *ccr* genes among methicillin-susceptible *Staphylococcus* type strains. Using primers common to three types of *ccr* genes, we performed PCR amplification, with DNAs extracted from 27 C-NS type strains as the templates (18). Positive PCR results were obtained with *S. hominis* type strain GIFU12263 (38), *Staphylococcus arlettae* GIFU10765, and *Staphylococcus auricularis* GIFU10395. The 0.5-kb amplified PCR fragment from *S. hominis* type strain GIFU12263 was sequenced and compared with three types of *ccr* gene in an *S. aureus* strain. The partial nucleotide sequences of *ccr* genes in the *S. hominis* type strain were identical to the corresponding regions of type 1 *ccr* genes. Then the whole nucleotide sequences of the *ccr* genes were determined, and the deduced amino acid sequences were compared to those of *ccr1*∗ of NCTC10442 (Fig. 1a). The Ccr proteins encoded by the *ccr* genes in *S. hominis* GIFU12263 were highly basic proteins with 99 and 84% amino acid identities to CcrA1 and CcrB1* of NCTC10442, respectively. For the sake of comparison, we reconstituted the putative intact CcrB1 polypeptide of NCTC10442 by adding back an adenine to the deletion point of the mutated Ψ*ccrB1* gene (Fig. 1a). The N-terminal domain of about 20 residues, containing presumptive catalytic active sites of CcrA and CcrB (6, 18, 19, 21), were well conserved between the two polypeptides.

To obtain a further insight about the evolutionary relationship among *ccr1* genes and some related site-specific recombinases in the databases, we constructed a phylogenetic tree with CcrA and CcrB proteins, the integrases of *Enterococcus faecalis* bacteriophage phi-FC1 and *Lactococcus lactis* bacteriophage TP901-1 (5), and site-specific recombinases of *Clostridium acetobutylicum* ATCC 824 (34) and *Streptococcus pneumoniae* (15), all of which have substantial amino acid similarities to the Ccr recombinases (Fig. 1b). Figure 1 clearly shows that GIFU12263 Ccr and NCTC10442 Ccr constitute the type 1 Ccr subfamily.

***S. hominis ccr* genes are carried by an element similar to SCC*mec*.** Figure 2 shows the *S. hominis* chromosomal region around the *ccr* genes bounded by the characteristic direct repeats (DRscc) of 14 bases (with 12 identical bases) found 11.3 kb upstream and 7.3 kb downstream of *ccr* genes, (Fig. 2b and c). Incomplete inverted repeats of 28 bases, IR-L (left) and IR-R (right), also found in typical SCC*mec* elements were localized at both extremities. The region designated SCC_{12263} was 21,589 bp in length and contained a total of 26 ORFs whose characterization is given in Table 2. Furthermore, the homologue of *S. aureus* $orfX_{aur}$, designated $orfX_{hom}$, was identified to the right of IR-R. *orfX* is an ORF of unknown function located near the origin of replication of the *S. aureus* chromosome, which contains the integration site ($attB_{scc}$) of SCC*mec* (17, 26). The $orfX_{hom}$ was 82% identical to $orfX_{aur}$ in nucleotide sequences, and their deduced amino acid sequences were 91% identical to each other (Fig. 1c).

The ORFs similar to those of SCC*mec* elements (amino acid identities between the corresponding ORFs were equal to or greater than 48%) are shown in Fig. 2a. The *ccr* gene complex (composed of *ccrA* and *ccrB* genes plus surrounding ORFs) was also well conserved in SCC_{12263}. Four ORFs (*orf4* to *-7*) located near the left extremity of the element were well conserved among SCC_{12263} and three types of SCC*mec.* The 3.5-kb region, called R-I (17), containing *orf6* and *-7*, was also well conserved among three types of SCC*mec.* The ORF11 shown in Fig. 2 had homology to ORFN029 of the type II SCC*mec* (17).

Certain ORFs shown in Fig. 2 were specific to SCC_{12263}. However, most of them did not show homology with any of the extant genes (see "Cluster of restriction-modification enzyme gene homologues in right end of SCC_{12263}"). It was also significant that there was no antibiotic resistance gene or mobile element found in SCC_{12263}. The G+C content of SCC_{12263} was 29.52%, which, though not in itself significant, was lower than the range of G+C content reported for species *S. hominis* (30 to 36% [38]). There were nine regions (eight ORFs within SCC_{12263} and one ORF outside SCC_{12263}) having substantially deviant G+C content values (under 29%). The most notable examples were *orf22* (*Sts*I methylase [M.*Sts*I] homologue), *orf23*, and *orf25*, whose G+C contents were 23.77, 23.93, and 24.21%, respectively.

Further analysis of GC3, the G+C content of the third

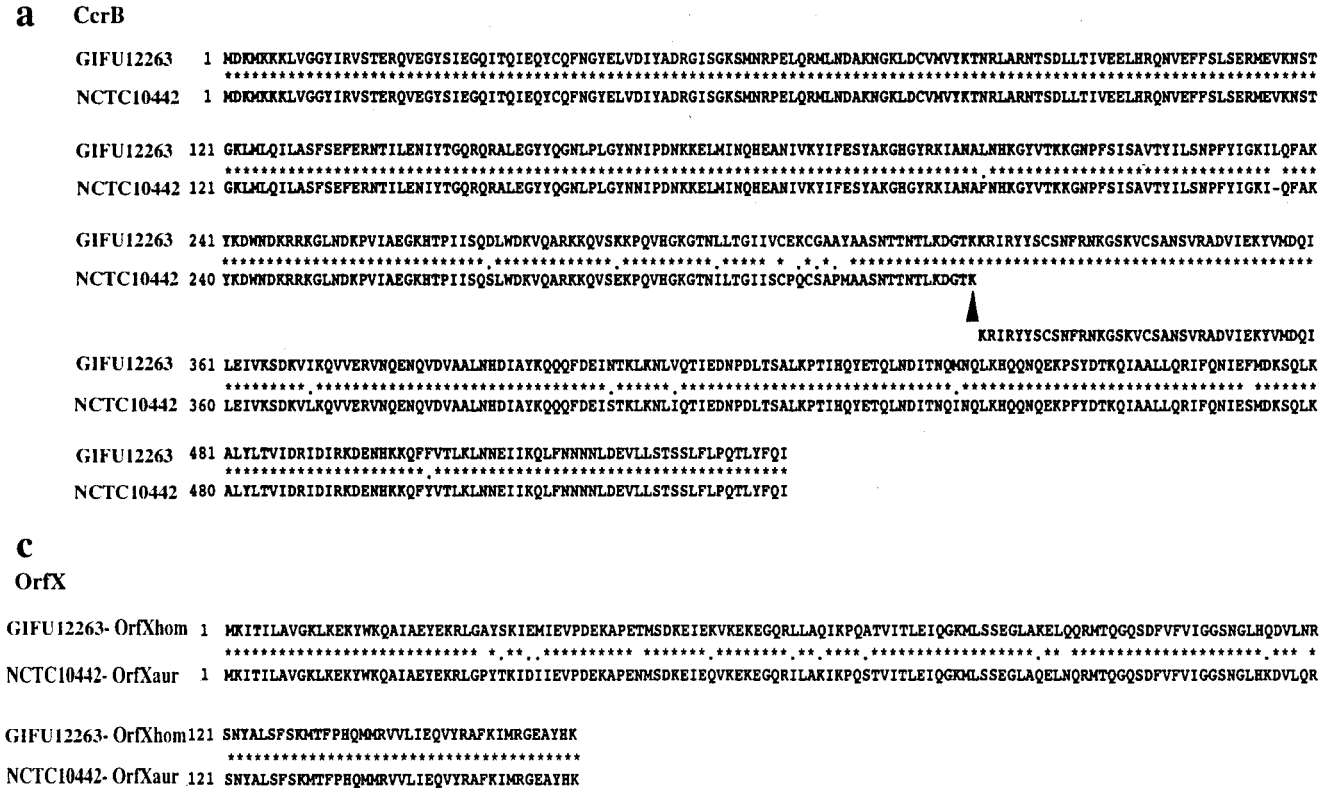

FIG. 1. (a) The deduced amino acid sequences of CcrB1 of GIFU 12263 *S. hominis* were aligned with those of NCTC 10442 MRSA. A black arrowhead indicates the locus of the first amino acid residue changed by an addition of adenine in the nucleotide sequence of *ccrB1∗* as described in the text. (b) Phylogenetic relationships among *ccrA* genes, *ccrB* genes, and four site-specific recombinases. The four site-specific recombinases that show high similarity to *ccr* genes and that were selected to investigate phylogenetic relationships are the integrase (int) of bacteriophage phi-FC1 found in *E. faecalis* (1,216 bp; DDBJ/EMBL/GenBank accession no. AF124258), the integrase of bacteriophage TP901-1 found in *L. lactis* (1,458 bp; DDBJ/EMBL/GenBank accession no. X85213), the site-specific recombinase found in *C. acetobutylicum* ATCC 824 (1,635 bp; DDBJ/EMBL/GenBank accession no. AE007636), and the site-specific recombinase found in *S. pneumoniae* R6 (1,680 bp; DDBJ/EMBL/GenBank accession no. AE008466). The nucleotide sequences of six *ccrA* genes, six *ccrB* genes, and four site-specific recombinases were aligned by using the PileUp program with a Genetics Computer Group default scoring matrix. Phylogenetic relationships were examined with the Paupsearch program by the neighbor-joining method by creating 1,000 bootstrap replicates and by selecting 70% of consensus. The tree was visualized with Tree View software, which was obtained from the Tree View 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. (c) The alignment of deduced amino acid sequences between OrfXaur representative from NCTC 10442 MRSA strain and OrfXhom in GIFU12263.

codon letter, whose deviation from the average value of the entire chromosome may be associated with genes acquired by horizontal transfer (26), revealed GC3 values lower than 24% for *orf2* to *-11* and *-22* to *-25.* Especially low were the values of *orf22* to *-25,* which were 13 to 15%. It was also noted that $orfX_{hom}$, shown as ORF26, had a significantly high G+C content of 37.5%, as in the case with $orfX_{aur}$ in MRSA strains (39.41% [see reference 17]).

Figure 3 compares nucleotide sequences of SCC_{12263} and SCC*mec* types I, II, III, IVa, and IVb. Two regions, B (from nucleotide positions 1,117 to 4,890) and D (from nucleotide positions 8,600 to 16,198), of SCC_{12263} were found to be highly homologous to the extant SCC*mec*s. Another three regions, A (from nucleotide positions 1 to 1,116), C (from nucleotide positions 4,891 to 8,599), and E (from nucleotide positions 16,199 to 21,589), did not show significant homology. The extents of homology of the B and D regions with the corresponding regions of the SCC*mec* elements are indicated in Table 3. Region D contained *orf11* to *-19* that corresponded to the *ccr* gene complex (18). On the other hand, region B encompassed *orf2* to *-7*, whose functions are unknown.

Both regions B and D had the highest similarity (96% identical) to the corresponding regions of type I SCC*mec*, indicating that not only the *ccr* gene complex, but also at least one other region of the SCC_{12263} element, is phylogenetically associated with type 1 SCC *mec.*

It was also remarkable that the regions similar to B and D regions were located in reverse order in type II and III SCC*mec*. Furthermore, the region B was very similar to the corresponding region of type IVa (97% identity), whereas the region D was not (79%) (Table 3). These observations suggest that recombination occurred between the two regions during molecular diversification of the SCC elements. Phylogenic relationship was inferred by computing multiple alignments of the core homologous regions, shared by five SCCs for region B and six SCCs for region D (data not shown). The two phylogenetic trees CcrA and CcrB computed as described above were different from each other. As mentioned above, the GC3 of the

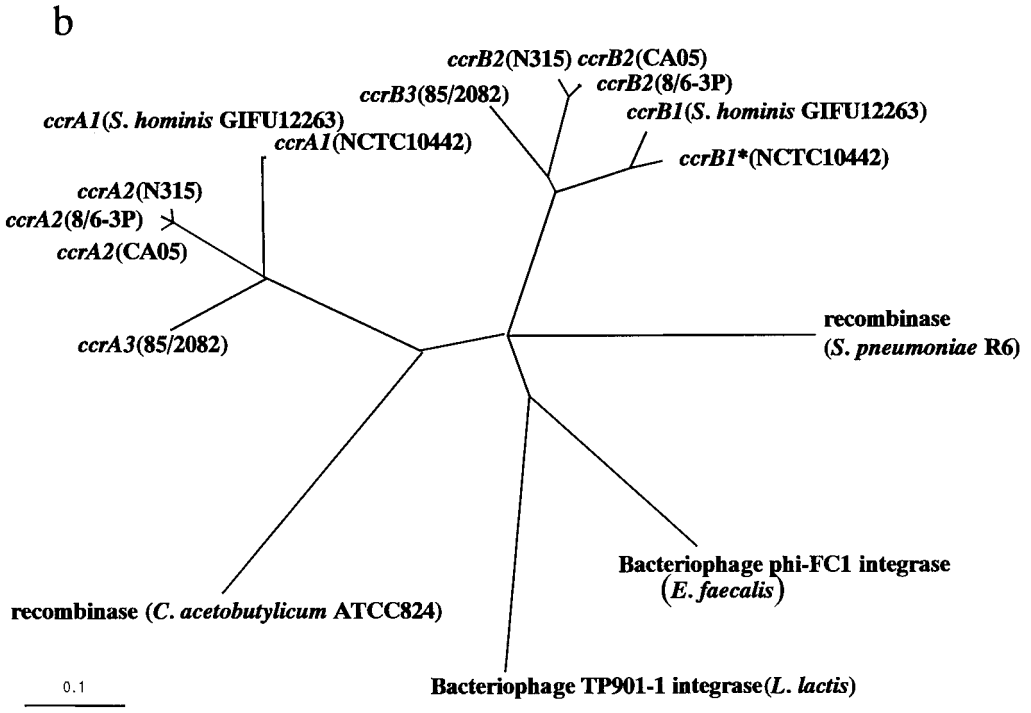

FIG. 1—*Continued.*

ORFs in the two regions of SCC_{12263} was also discrepant: low in region B but not in region D. Therefore even SCC_{12263} might have been formed by recombination between two evolutionary unrelated DNA fragments. A very weakly homologous region was observed between the rightmost part of type III SCC*mec* and the region from 14.6 to 16.0 kb of SCC_{12263} in reverse direction. Another one is between the leftmost part of type VIa SCC*mec* and the region from 20.7 to 21.2 kb of SCC_{12263}. We know that the rightmost part of the type III SCC*mec* contains a pseudo-*ccr* gene complex containing a *ccrA2*-like gene (18). However, because of the paucity of homology between *ccrA* gene of the SCC_{12263} (which should be classified as a *ccrA1* gene) and a degenerate *ccrA2*-like gene, no apparent homology line was visualized with the set for the computation. Regions A, C, and E of SCC_{12263} did not have corresponding regions in four SCC*mec* types. However, the nucleotide sequence from positions 771 to 1474 in region A showed significant homology (98% identical) to that in the chromosome of *S. epidermidis* strain RP62A. Several restriction-modification enzyme gene homologues were identified in region E.

Cluster of restriction-modification enzyme gene homologues in right end of SCC_{12263}. Several nucleotide sequences homologous to restriction-modification genes were identified in region E of SCC_{12263}. ORF22 (the deduced peptide encoded by *orf22*) showed high homology to type IIS modification enzymes, M.*Sts*I (the DNA methyltransferase of the *Sts*I restriction-modification system) and M.*Fok*I, that recognize an asymmetric sequence 5′-GGATG-3′ and generate 6-mA on both the strands (23, 24). A motif search with pfam indicated that ORF22 carries two alpha-type domains (motif I [AdoMet binding]-target recognition domain [TRD]-motif IV [DPPY]) in tandem. As for M.*Fok*I (41), this putative protein appears to be formed by fusion of two methyl transferase proteins, each methylating one strand of the recognition sequence.

In the upstream of ORF22, there was a 174-bp nucleotide stretch homologous to type III SCC*mec* in strain 85/2082. TransQ search revealed weak similarity of this sequence stretch with that encoding a part of *Hga*I, a type IIS restriction enzyme that recognizes 5′-GACGC-3′ (40). Its two cognate modification enzymes methylate its internal C5 on the two strands (42). This region may represent a remnant of a type IIS restriction enzyme gene. We cannot conclude whether this was part of a once-active gene forming a restriction-modification system with *orf22* or not. The carboxy end of ORF25 showed homology with several restriction enzymes (by TransQ and PSI-BLAST analyses). These included McrA, a C5 methyl-specific endonuclease; ml17993 in *Mesorhizobium loti* found adjacent to a DNA methylase homologue; and HP0052, a gene in a cluster of restriction-modification homologues in *Helicobacter pylori* (strain 26695) (1). A BLASTx search with ORF23 as a query criterion revealed weak homology with a putative NTPase/helicase of a virus (gi|9631396|ret|NP_048295.1|) and a gene in *Sulfolobus solfataricus* (gi|13815496|gb|AE006826.1| AE006826). PSI-BLAST analysis detected similarity of the *Sulfolobus* gene with SAM-dependent O-methyltransferases (eg., gi:13399467) (49). It is not clear whether ORF23 represents a remnant of a novel type of DNA methyltransferase that is homologous to O-methyltransferases.

Spontaneous precise excision of SCC_{12263}. To test whether SCC_{12263} behaves like SCC*mec* as a mobile element, PCR experiments were designed to monitor spontaneous precise excision of SCC_{12263} occurring in the *attL* and *attR* regions. The two combinations of primers, hL-1 and hR-1 and hL-4 and

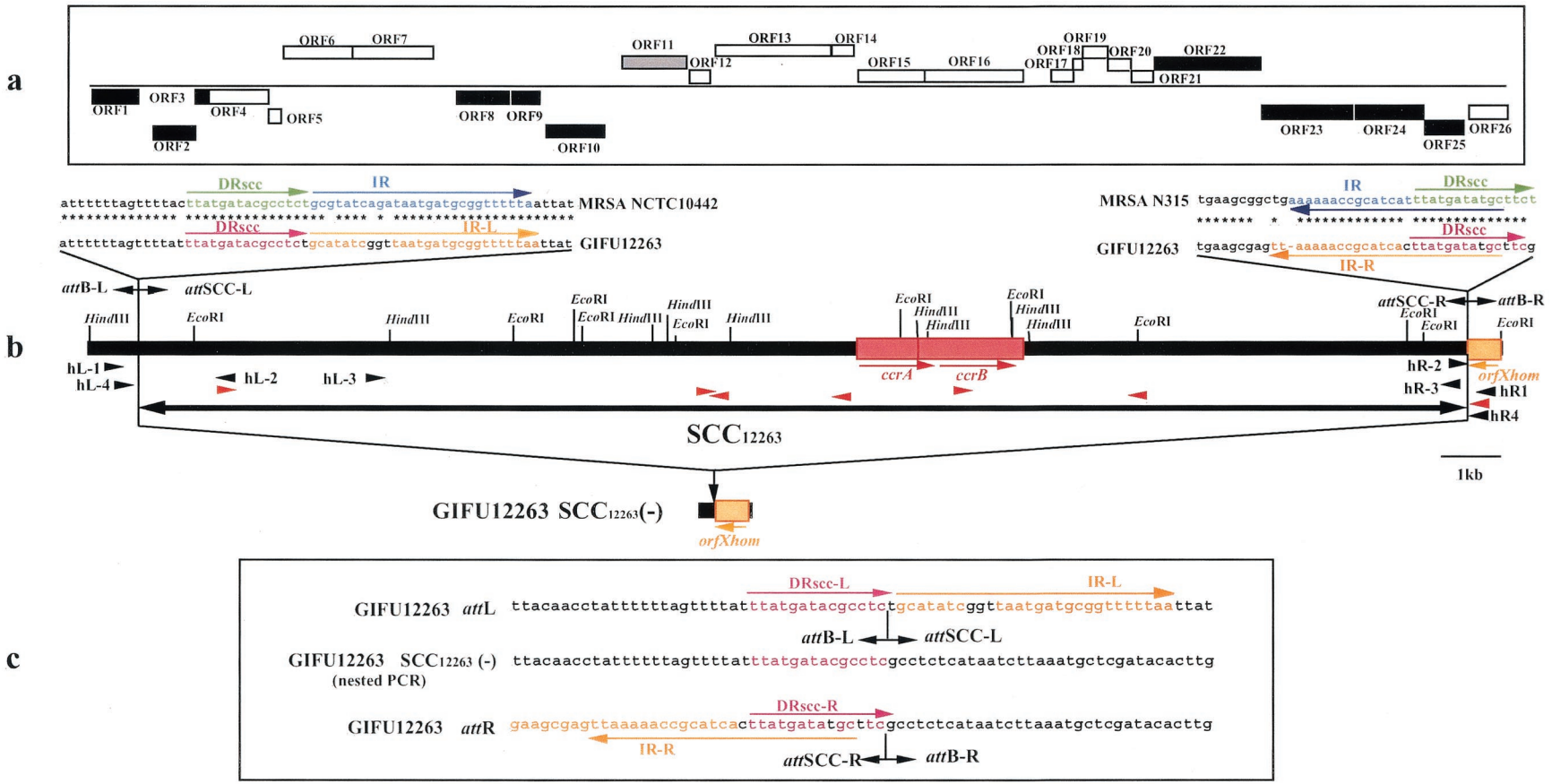

FIG. 2. Genomic structure of SCC_{12263}. (a) ORFs in and around SCC_{12263}. The ORFs corresponding to sequences more than 90 amino acids in size and twenty-six possible reading frames are indicated by squares. Those above and those below the line are the ORFs whose transcription is directed to the right and those whose transcription is directed to the left, respectively. The white squares indicate the ORFs that were conserved among three SCC*mec* elements. The black squares indicate the ORFs that showed no identity to the four SCC*mec* elements. The gray square indicates the ORF with identity to type II SCC*mec*. (b) The essential structure of SCC_{12263}. Locations of the essential genes are illustrated. The only restriction sites indicated are those of of *Hin*dIII and *Eco*RI. $orfX_{hom}$ is shown in orange, the *ccr1* genes are shown in red, and the arrow indicates the direction of transcription. Nucleotide sequences of the left and right boundaries of *attL* and *attR* in SCC_{12263} were aligned with those of *attL* in NCTC10442 and *attR* in N315, respectively. The orange arrows indicate inverted complementary repeats, IR-L and IR-R, at both extremities of SCC_{12263}, and the blue arrows indicate those of MRSA. The direct repeats in SCC_{12263} are indicated by magenta arrows, and those of MRSA are indicated by green arrows. The sequences that are colored are identical to each other. (c) Boundary of SCC_{12263}. Nucleotide sequences around the *attL* and *attR,* respectively, are aligned with the sequence of the presumptive integration site, *att*B, on the GIFU12263 $SCC_{12263}(-)$ chromosome. The orange arrows indicate inverted complementary repeats, IR-L and IR-R, at both extremities of SCC_{12263}. The red arrows indicate direct repeats. The orange and red sequences are identical to each other.

TABLE 2. ORFs in and around *S. hominis* SCC_{12263} with deduced products showing similarities to extant proteins

ORF[a]	Value for CDS[b]				Gene	Product	Data for homologue in the database		NCTC10442		Data indicating homology to ORF of strain[a]: N315		85/2082	
	Starting nucleotide	Ending nucleotide	Size (bp)	Length (aa)			% Identity[c]	Description of gene product (size [bp])[d]	% Identity[c]	Corresponding ORF (size [bp])	% Identity[c]	Corresponding ORF (size [bp])	% Identity[c]	Corresponding ORF (size [bp])
(ORF1)	708	52	657	218		Hypothetical protein	40	Hypothetical protein, i.e., partial ORF 59 (216) of *Staphylococcus aureus* bacteriophage phl PVL						
ORF2	1519	974	546	181		Hypothetical protein	52	Hypothetical protein YdhK (205) of *Bacillus subtilis*						
ORF3*	1716	1537	180	59		Hypothetical protein	83	Partial copper-transporting ATPase CopB (745) of *Enterococcus hirae*						
ORF4	2676	1918	759	252		Hypothetical protein			92	CE014 (252)	71	CN030 (21)	71	CZ021 (214)
ORF5	2915	2673	243	80		Hypothetical protein			97	CE015 (80)	88	CN031 (88)	88	CZ022 (88)
ORF6	3072	4145	1074	357		Hypothetical protein		RI	95	E023 (355)	69	N052 (354)	69	Z024 (354)
ORF7	4164	5492	1329	442		Hypothetical protein		RI	96	E024 (442)	74	N054 (131)	74	Z025 (286)
ORF8	6744	5815	930	309		Hypothetical protein					68	N053 (287)	68	Z026 (396)
ORF9	7230	6760	471	156		Hypothetical protein								
ORF10	8239	7319	921	306		Hypothetical protein								
ORF11	8585	9610	1026	341		Hypothetical protein					54	N029 (348)		
ORF12	9802	10098	297	98		Hypothetical protein			91	E025 (98)	77	N030 (98)	53	Z003 (95)
ORF13	10098	11867	1770	589		Hypothetical protein			98	E026 (589)	75	N031 (597)	65	Z004 (522)
ORF14	11937	12152	216	71		Hypothetical protein			98	E027 (70)	50	N033 (61)	55	Z008 (70)
ORF15	12055	13404	1350	449	*ccrA1*	Cassette chromosome recombinase A1			99	*ccrA1* (449)	78	*ccrA2* (448)	78	*ccrA3* (448)
ORF16	13426	15054	1629	542	*ccrB1*	Cassette chromosome recombinase B1			84	*ccrB1* (542)	80	*ccrB2* (542)	85	*ccrB3* (542)
ORF17	15520	15870	351	116		Hypothetical protein			93	E031 (116)	87	N0410 (116)	53	Z011 (116)
ORF18	15863	15955	93	30		Hypothetical protein								
ORF19	15957	16268	312	103		Hypothetical protein			91	E032 (108)	97	N042 (103)	48	Z013 (131)
ORF20*	16285	16518	234	77		Hypothetical protein			90	E033 (169)	90	N043 (168)	63	Z014 (173)
ORF21*	16583	16789	207	68		Hypothetical protein			92	E033 (169)	95	N043 (168)	58	Z014 (173)
ORF22	17135	19078	1944	647	M.*Sts*I	Modification methylase	58	*Sts*I methylase (653) of *Streptococcus sanguis*						
ORF23	20489	19086	1404	467		Hypothetical protein								
ORF24	21398	20523	876	291		Hypothetical protein								
ORF25	22105	21470	636	211		5′-Methylcytosine-specific restriction enzyme	35	5′-Methylcytosine-specific restriction enzyme A of *Methanosarcima mazei*						
(ORF26)	22820	22341	480	159	$orfX_{hom}$	Conserved hypothetical protein OrfX			91	*orfX* (159)	91	*orfX* (159)	91	*orfX* (159)

[a] ORFs shown in parentheses were located outside of SCC_{12263}. Incomplete ORFs that are potentially defective genes or pseudogenes containing frame-shift mutations are annotated with asterisks.
[b] Nucleotide positions given are from the nucleotide sequence deposited under DDBJ/EMBL/GenBank accession no. AB063171, and they were measured in the 5′ (starting nucleotide) to 3′ (ending nucleotide) direction. CDS, coding sequence.
[c] Identity to the amino acid sequence of the best match revealed in homology search of the GenBank and EMBL databases with TFastA.
[d] Gene product sizes are numbers of amino acids. PVL, Panton-Valentine leukocidin; RI, region I.

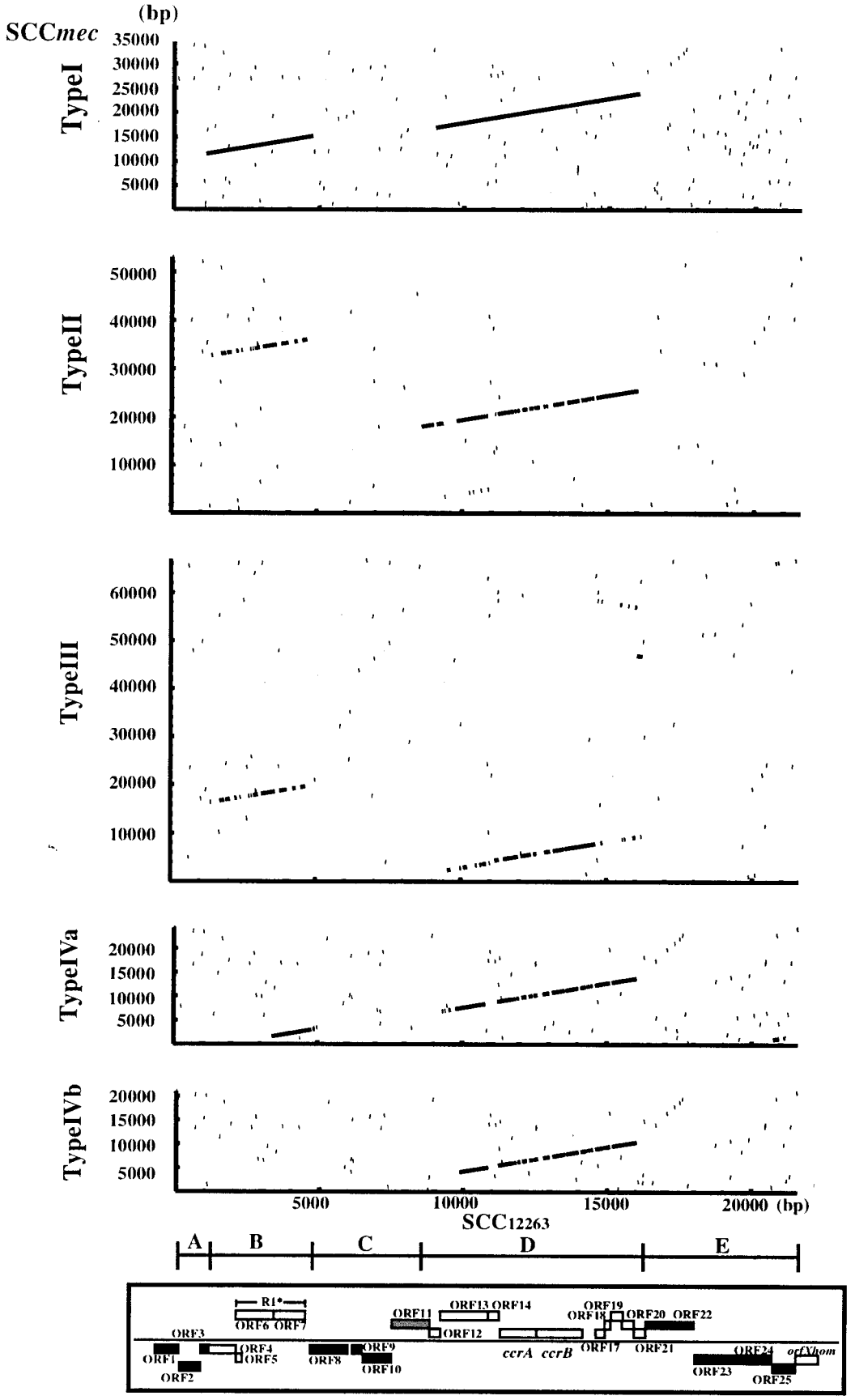
SCCmec
(bp)
TypeI
35000
30000
25000
20000
15000
10000
5000
TypeII
50000
40000
30000
20000
10000
TypeIII
60000
50000
40000
30000
20000
10000
TypeIVa
20000
15000
10000
5000
TypeIVb
20000
15000
10000
5000
5000
10000
15000
20000 (bp)
SCC12263
A
B
C
D
E
R1*
ORF6 ORF7
ORF3
ORF1
ORF2
ORF4
ORF5
ORF8
ORF9
ORF10
ORF11
ORF13 ORF14
ORF12
ccrA ccrB
ORF19
ORF18
ORF20 ORF22
ORF17
ORF21
ORF24
orfXhom
ORF23
ORF25

TABLE 3. Corresponding regions of SCC_{12263} and SCC*mec*[a]

SCC*mec* type	Region B							Region D						
	SCC*mec*			SCC_{12263}			% Identity	SCC*mec*			SCC_{12263}			% Identity
	Starting nucleotide	Ending nucleotide	Length (nt)	Starting nucleotide	Ending nucleotide	Length (nt)		Starting nucleotide	Ending nucleotide	Length (nt)	Starting nucleotide	Ending nucleotide	Length (nt)	
Type I	11508	15164	3,657	1117	4787	3,671	96	16921	23914	6,994	9018	16018	7,001	96
Type II	32763	36040	3,278	1369	4644	3,276	73	17968	25508	7,541	8600	16017	7,418	80
Type III	16364	19641	3,278	1369	4644	3,276	73	2395	9434	7,040	9542	16198	6,657	72
Type IVa	1617	3110	1,494	3411	4890	1,480	97	6870	13699	6,830	9306	16018	6,713	79
Type IVb[b]								4109	10371	6,263	9876	16018	6,143	80

[a] Nucleotide positions were measured in the 5′ (starting nucleotide) to 3′ (ending nucleotide) direction.
[b] For region B of this SCC*mec* type, no corresponding sequence was found.

hR-4, were used for the first and second rounds of PCR, as described previously (17). The primers hR-1 and hR-4 were located in $orfX_{hom}$, and hL-1 and hL-4 were located in the chromosomal region outside the left boundary of SCC_{12263} (Fig. 2b). It has been reported that the sensitivity of the first round of PCR was such that it could detect $attB_{scc}$ sequence generated in 1 in 10,000 cells of the culture by precise excision of SCC element (17). At this level of sensitivity, no amplified band was detected when GIFU12263 DNA was used as a template for PCR. However, the second round of PCR (nested PCR) did amplify a 0.8-kb DNA fragment. The DNA fragment was purified and was subjected to nucleotide sequence determination. By comparing it with the nucleotide sequences of chromosome-SCC junction sequences, we could confirm occurrence of precise excision of the SCC and infer the precise cutting site as shown in Fig. 2. The result indicated that spontaneous precise excision of SCC_{12263} presumably mediated by *ccr* genes occurred in *S. hominis* strain GIFU12263, though at a low frequency of less than 1 in 10^4 cells (17).

***ccr* genes from SCC_{12263} promote excision of SCC*mec*.** In order to examine if the *ccr* genes on SCC_{12263} encode active recombination or not, the *ccrA1* and *ccrB1* genes of *S. hominis* GIFU12263 and *S. aureus* NCTC 10442 were cloned into a shuttle vector, pYT3 (7). The recombinant plasmids obtained were $pSR1_{hom}$ and $pSR1_{aur}$, respectively. Four plasmids; pYT3 (plasmid vector), $pSR1_{hom}$, $pSR1_{aur}$, and pSR2 (pYT3 harboring *ccr2* genes was formerly designated pSR [20]) were introduced by electroporation into strain 85/1940, which has a type I SCC*mec* with a mutated *ccrB1* gene that is indistinguishable from that of NCTC10442 (18), followed by selection of transformants on the tetracycline plates. (The reason that we did not use NCTC10442, a representative MRSA strain carrying type I SCC*mec* (17), was that the strain was tetracycline resistant). The strains 85/1940, 85/1940(pYT3), 85/1940(pSR2), 85/1940($pSR1_{hom}$), and 85/1940($pSR1_{aur}$) were then cultivated in drug-free broth for 9 days at 30°C with one passage per day, after which the proportion of the cells that had lost SCC*mec* was evaluated by replicating them onto agar plates with and without ceftizoxime. On the 9th day, the cultures of strains 85/1940, 85/1940(pYT3), and 85/1940($pSR1_{aur}$) yielded essentially equal numbers of colonies on the BHI agar plates with or without ceftizoxime (Table 4). On the other hand, the 9 days passage of the strains 85/1940(pSR2) and 85/940($pSR1_{hom}$) generated ceftizoxime-susceptible cells, which constituted 6 and 4% of the entire cell population, respectively (Table 4). This demonstrates that the *ccrB1* of the *S. hominis* strain were active in excising the type I SCC *mec.* To determine the site of excision mediated by the *ccr* genes of the *S. hominis* SCC_{12263}, we amplified the $attB_{scc}$ region on the chromosome in 85/1940($pSR1_{hom}$) and N315($pSR1_{hom}$) by PCR with cR2 and cL3 as the primers (17). As controls, we used the chromosomes of 85/1940, 85/1940(pSR2), 85/1940($pSR1_{aur}$), N315, N315(pSR2), and N315($pSR1_{aur}$) strains. All strains were cultivated in drug-free broth for 18 h, and then their chromosomal DNAs were extracted. Figure 4 shows that bands were amplified with 85/1940(pSR2), 85/1940($pSR1_{hom}$), N315(pSR2), and N315 ($pSR1_{hom}$), but not with 85/1940($pSR1_{aur}$) or N315($pSR1_{aur}$). The DNA fragments were purified and were subjected to nucleotide sequence determination, and we compared the sequences with those of 85/1940 and N315. The nucleotide sequences in 85/1940($pSR1_{hom}$) and N315($pSR1_{hom}$) were identical to those in 85/1940(pSR2) and N315(pSR2), respectively. The identity of the excision mediated by pSR2 and $pSR1_{hom}$ indicated that the *ccr* genes on SCC_{12263} encoded the recombinases of the same specificities of those of the extant *ccr* gene products of type I and type II SCC *mec.*

DISCUSSION

The SCC_{12263} carried several homologues of restriction-modification genes. Among them, *orf22* may encode an active type IIS DNA methyltransferase. Many kinds of mobile genetic elements, such as bacteriophages, integrons, and transposons (4, 19), etc., carry a restriction-modification gene complex (25),

FIG. 3. Homologous regions of nucleotide sequences of SCC_{12263} and SCC*mec* type I, II, III, IVa, and IVb and their identities. The nucleotide positions are relative to the SCCs. The SCC region of SCC_{12263} corresponds to the region from nucleotide 770 to 22358 in GIFU12263, the type I SCC*mec* corresponds to the region from nucleotide 4504 to 38867 in AB033763, the type II SCC*mec* corresponds to the region from nucleotide 4687 to 57653 in D86934, the type III SCC*mec* corresponds to the region from nucleotide 899 to 67794 in AB037671, the type IVa SCC*mec* corresponds to the region from nucleotide 975 to 25222 in AB063172, and type IVb SCC*mec* corresponds to the region from nucleotide 301 to 21219 in AB063173. The regions A through E (see text for description of location) and how they correspond to the structure presented in Fig. 2a are shown at the bottom.

TABLE 4. Excision of SCC*mec* mediated by plasmids carrying *ccr* genes

Strain[a]	Day of passage	No. of CFU on[b]:			No. of CZX-resistant colonies (%)[c]
		Master plates	TET plates	TET/CZX plates	
85/1940(pYT3)	1	159	159	159	100
	9	278	278	278	100
85/1940(pSR2)	1	188	188	187	99.4
	9	188	188	12	6.38
85/1940(pSR1$_{hom}$)	1	218	218	215	98.6
	9	294	294	12	4.08
85/1940(pSR1$_{aur}$)	1	225	225	225	100
	9	224	224	224	100

[a] Plasmids (pSR2, pSR1$_{hom}$, pSR1$_{aur}$, and pYT3) that carried *ccr* genes from N315, the *S. hominis* strain, NCTC 10442, and none, respectively, were introduced into strain 85/1940 (SCC*mec* type I).

[b] Total number of CFU on three plates. Abbreviations: TET, tetracycline; CZX, ceftizoxime.

[c] Values are percentages calculated by the following formula: [(number of CFU on the TET- and CZX-supplemented plates) ÷ number of CFU on the TET-supplemented plates] × 100.

which can stabilize maintenance of a plasmid (32) and of genomic islands in the chromosome (8). This is because the cells that have lost the restriction-modification gene complex are supposed to be killed by the attack of the restriction enzyme at the recognition sites of the chromosome that are not protected by a sufficient amount of the methyltransferase. The restriction-modification genes identified on SCC$_{12263}$ may be responsible at least partially for the stable maintenance of SCC$_{12263}$ despite its carriage of active *ccr* genes that encode activity to precisely excise the element from the chromosome. The modification enzyme gene (*orf22*) appears to be orphan in the sense that it is not adjacent to a cognate restriction enzyme gene in an active form. However, its stabilization effect is expected if its cognate restriction enzyme gene in an active form is present elsewhere in the genome, as has been previously described for the maintenance of the genomic islands in *S. aureus* chromosome (25, 26). Another likely role of the methyltransferase is defense of SCC$_{12263}$ DNA upon entry into restricting host bacteria. This role is reminiscent of the role of orphan methylase (not paired with a restriction enzyme) on bacteriophage genomes. The restriction-modification gene homologues were present in a region E that is unique to SCC$_{12263}$ but lying next to the region D, which is shared by other SCC elements. This type of linkage between macroscopic genome polymorphism with restriction-modification genes has been reported for other genomes as well (1, 33), which is considered supportive of the hypothesis that some restriction-modification systems represent mobile genetic elements (25). The deviation in GC content in this area also supports this hypothesis.

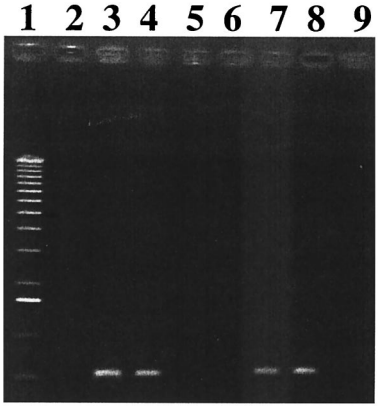

FIG. 4. PCR for detection of *attB*$_{scc}$ region on the chromosome. Amplification was carried out with a set of primers cL3 and cR2 specific for the regions to the left and right of the chromosome-SCC*mec* junction point. Lanes: 1, 1-kb ladder as a molecular weight marker; 2, N315; 3, N315(pSR2); 4, N315(pSR1$_{hom}$); 5, N315(pSR1$_{aur}$); 6, 85/1940; 7, 85/1940(pSR2); 8, 85/1940(pSR1$_{hom}$); 9, 85/1940(pSR1$_{aur}$).

This study was done as a part of our pursuit of a molecular predecessor of the SCC*mec* elements, which are distributed widely among clinical strains of various staphylococcal species (21). Sequencing analysis of the chromosome regions of the *S. hominis* strain surrounding the *ccr* genes identified a genomic island at the boundaries of which were found DR and IR sequences and *orfX*$_{hom}$ having high homology to *orfX*$_{aur}$ of *S. aureus* in which an attachment site for SCC*mec* is located. The structure of the boundaries indicated that the island was integrated at the attachment site *attB*$_{scc}$ found in the 3′ end of *orfX*$_{hom}$, whose sequence was indistinguishable from the *attB*$_{scc}$ into which SCC*mec* is integrated site specifically. Thus, we designated the island SCC as the representative of hypothesized SCC family in which the staphylococcal mobile elements share the same structural and functional features with extant SCC*mec* elements (possession of *ccr* genes, as well as DR and IR sequences, and site-specific integration into the *attB*$_{scc}$) except for carriage of *mecA* gene complex.

The SCC$_{12263}$ appears to be a type I SCC element (i.e., it is part of a subgroup of the SCC family as defined by the possession of type 1 *ccr* gene complex). An SCC belonging to this subfamily should have served as a recipient of class B *mecA* gene complex in the E region to generate type I SCC*mec* in the past. However, since strong homologies between SCC$_{12263}$ and type I SCC*mec* were confined only to regions B and D, SCC$_{12263}$ may not be the immediate precursor SCC for the generation of type I SCC *mec.* It may be that the SCC family is more diverse than we have expected, and continued search may eventually identify an SCC more closely related to the precursor SCC, with a homology extending, for example, to region A as well. The precursor SCCs for type II and type III SCC*mec* may also be identified in C-NS strains, since some *mecA*-negative strains that hybridize these types of *ccr* genes have been identified (Y. Katayama et al., unpublished data).

Besides the structural diversities in SCC elements, there seem to be significant diversities in the biological functions which they carry. We have started to observe that SCC is not confined to the transmission of methicillin resistance alone; that it is a well-developed vehicle for genetic exchange of any useful genes among staphylococcal species is also supported by other recent observations. We have previously described a genetic element, IE25923, integrated at the *attB*$_{scc}$ in the chromosome of methicillin-susceptible *S. aureus* type strain ATCC 25923 (18). Although it is considered as a remnant of SCC (it lacks *ccr* genes), neither the *mecA* gene complex nor any other antibiotic resistance gene was found among dozens of the

ORFs present on it (18). Even extant SCC*mec* contains some genes whose functions are unrelated to antibiotic resistance: e.g., type I SCC*mec* contains SD repeat (a repeat domain comprising mainly aspartate and serine)-rich surface proteins that interfere the host cell's capacity to bind to fibronectin and fibrinogen (47), and type II SCC*mec* contains KDP operons that are involved in the ATP-dependent potassium transfer (43). A recent illustrative case in this line of observation is that found in the genome of an MRSA strain MW2 the sequencing of whose whole genome revealed the presence of an SCC-like structure integrated in tandem with a copy of SCC *mec,* and in association with this SCC-like structure a staphylococcal enterotoxin gene *seh* was identified (3). This genetic organization suggests the possibility that the *seh* gene, which is not present at the corresponding locus in any of the other five sequenced *S. aureus* chromosomes, might have been acquired by SCC-mediated lateral gene transfer (3). Recently, an SCC without the *mecA* gene complex has also been identified in *S. aureus* strain M, which, designated SCC*cap1*, carries a *cap* operon instead of antibiotic resistance genes (28). Therefore, it would be quite plausible to consider SCC a ubiquitous mobile genetic element whose function in staphylococcal species is interspecies transfer of genetic information useful to the host cells living in various stressful environments. The environmental selective pressure is not limited to antibiotics alone but could be any number of things, such as competition with other microorganisms, assault from the human immune system, poor or biased nutritional resources, and even pollutants such as cadmium and mercury, against which specific resistance genes in type III SCC*mec* have been identified (18). In this regard, detection of SCC and identification of the genes it carries would be useful not only for us to learn a bacterium's capability to survive antibiotic chemotherapy but also to track the individual life history of the staphylococcal strain (i.e., the series of environmental stresses that the given organism has undergone).

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