

# Genetic Organization of the Chromosome Region Surrounding *mecA* in Clinical Staphylococcal Strains: Role of IS431-Mediated *mecI* Deletion in Expression of Resistance in *mecA*-Carrying, Low-Level Methicillin-Resistant *Staphylococcus haemolyticus*

YUKI KATAYAMA, TERUYO ITO, AND KEIICHI HIRAMATSU\*

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

Received 5 September 2000/Returned for modification 26 December 2000/Accepted 6 April 2001

**We report on the structural diversity of *mecA* gene complexes carried by 38 methicillin-resistant *Staphylococcus aureus* and 91 methicillin-resistant coagulase-negative *Staphylococcus* strains of seven different species with a special reference to its correlation with phenotypic expression of methicillin resistance. The most prevalent and widely disseminated *mec* complex had the structure *mecI-mecR1-mecA-IS431R* (or IS431*mec*), designated the class A *mecA* gene complex. In contrast, in *S. haemolyticus*, *mecA* was bracketed by two copies of IS431, forming the structure IS431*L-mecA-IS431R*. Of the 38 *S. haemolyticus* strains, 5 had low-level methicillin resistance (MIC, 1 to 4 mg/liter) and characteristic heterogeneous methicillin resistance as judged by population analysis. In these five strains, IS431*L* was located to the left of an intact *mecI* gene, forming the structure IS431*L-class A mecA-gene complex*. In other *S. haemolyticus* strains, IS431*L* was associated with the deletion of *mecI* and *mecR1*, forming the structure IS431*L-ΔmecR1-mecA-IS431mec*, designated the class C *mecA* gene complex. Mutants with the class C *mecA* gene complex were obtained in vitro by selecting strain SH621, containing the IS431*L-class A mecA* gene complex with low concentrations of methicillin (1 and 3 mg/liter). The mutants had intermediate level of methicillin resistance (MIC, 16 to 64 mg/liter). The *mecA* gene transcription was shown to be derepressed in a representative mutant strain, SH621-37. Our study indicated that the *mecI*-encoded repressor function is responsible for the low-level methicillin resistance of some *S. haemolyticus* clinical strains and that the IS431-mediated *mecI* gene deletion causes the expression of methicillin resistance through the derepression of *mecA* gene transcription.**

Despite the introduction of *mecA* into the chromosome, some clinical strains are found to remain susceptible to methicillin (pre-methicillin-resistant *Staphylococcus aureus* [MRSA] status). In those strains, *mecA* gene transcription is strongly repressed by the function of the accompanying regulator genes, *mecI* and *mecR1* (9, 10). Transcription of *mecA*, encoding PBP2' (or PBP2a), is essential for the phenotypic expression of methicillin resistance (23, 28). Since *mecI* encodes a strong repressor of *mecA* gene transcription, pre-MRSA strains have only a marginal level of resistance to methicillin (MIC < 8 mg/liter) (9, 12). Experimental inactivation of *mecI* causes derepressed production of PBP2' and makes the cell express methicillin resistance (22, 27). This requirement of *mecI* inactivation for the phenotypic expression of methicillin resistance coincides well with the observation with clinical MRSA isolates in which the *mecI* gene is either mutated or completely deleted in the tested strains (19, 27, 32).

In pre-MRSA strain N315, *mecA* is flanked by the intact set of *mec* regulator genes on the left-hand side, and by a DNA region of about 3 kb, called the hypervariable region on the right-hand side (26). The right boundary of the latter region is demarcated by a copy of insertion sequence IS431*mec* (or

IS431*R*) (3). The *mecA* locus of pre-MRSA strains thus forms a prototypic structure of *mecI-mecR1-mecA-IS431R* that is designated the class A *mecA* gene complex (*mec* complex). In other clinical MRSA strains, the entire *mecI* gene and the 3' part of *mecR1* are deleted from the class A *mec* complex and a fragment of the insertion sequence IS1272 ( $\psi$ IS1272) has been associated with the deletion point (1, 2). We designate this structure,  $\psi$ IS1272- $\Delta$ *mecR1-mecA-IS431R*, the class B *mec* complex.

Recently, it has been recognized that some methicillin-resistant coagulase-negative *Staphylococcus* (MRC-NS) strains express a low-level methicillin resistance in spite of their carriage of *mecA* (15). We have previously shown that *mecI* is widely disseminated in C-NS species (29). Dickinson and Archer have demonstrated that intact *mecI* is found in some *S. epidermidis* clinical strains with low-level oxacillin resistance (6). They have shown that allelic replacement inactivation of *mecI* raises the oxacillin resistance of these strains by severalfold, indicating a role for *mecI*-mediated repression in the low-level beta-lactam resistance in certain *S. epidermidis* strains. On the other hand, Kobayashi et al. have found some *S. epidermidis* clinical strains in a Japanese hospital that carry intact *mecI* in spite of their overt beta-lactam resistance expression (18).

We have been studying the genetic organization of the chromosomal region surrounding *mecA* in clinical C-NS strains to trace the evolutionary makeup of the *mec* complex. During the course of this study, we found a few *S. haemolyticus* strains with

\* Corresponding author. Mailing address: Department of Bacteriology, Faculty of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo, Japan 113-8421. Phone: 81-3-5802-1040. Fax: 81-3-5684-7830. E-mail: hiram@med.juntendo.ac.jp.

TABLE 1. Synthetic oligonucleotides primers used for the analysis of *mec* complex structure

| Primer designation | Sequence <sup>a</sup>            | Localization     | Positions | Reference(s) |
|--------------------|----------------------------------|------------------|-----------|--------------|
| mA2                | 5'-AACGTTGTAACCCACCCAAGA-3'      | <i>mecA</i>      | 1461-1441 | 11,28        |
| mA4                | 5'-AGTGTATGATGAGCTATATGAGA-3'    | <i>mecA</i>      | 1959-1981 | 28           |
| mA5                | 5'-CGCTCAGAAATTTGTTGTGC-3'       | <i>mecA</i>      | 212-180   | 28           |
| mA6                | 5'-TATACCAAACCCGACAAC-3'         | <i>mecA</i>      | 83-66     | 28           |
| mA7                | 5'-ATCGTTGACGATAATAGCAATACA-3'   | <i>mecA</i>      | 907-930   | 28           |
| mA8                | 5'-ATGTTTGGATTATCTTTATCATAT-3'   | <i>mecA</i>      | 1863-1886 | 28           |
| iS-1               | 5'-ACATTAGATATTTGGTTGCGT-3'      | IS431 <i>mec</i> | 566-596   | 3            |
| iS-2               | 5'-TGAGGTTATTCAGATATTTTCGATGT-3' | IS431 <i>mec</i> | 767-743   | 3            |
| iS-3               | 5'-TCGGATGCTATCATTAAAGCAT-3'     | IS1272           | 1668-1688 | 2            |
| iS-4               | 5'-ACAATCTGTATTCTCAGTTCGT-3'     | IS1272           | 1722-1701 | 2            |
| mI-1               | 5'-AATGGCGAAAAAGCACACA-3'        | <i>mecI</i>      | 1923-1942 | 10, 30       |
| mI-2               | 5'-GACTTGATTGTTTCTCTGT-3'        | <i>mecI</i>      | 2403-2283 | 10, 30       |
| mCR2               | 5'-CGCTCAGAAATTTGTTGTGC-3'       | <i>mecR1</i>     | 1954-1935 | 10, 30       |
| mCR3               | 5'-ATCTCCACGTTAATCCATT-3'        | <i>mecR1</i>     | 357-376   | 10, 30       |

<sup>a</sup> The location of each primer is shown in Fig. 1.

low-level methicillin resistance and carrying a copy of IS431 (*IS431L*) inserted upstream of and close to the class A *mec* complex. Using a representative strain, SH621, in vitro experiments were performed to evaluate the role of IS431L in the expression of methicillin resistance in *S. haemolyticus*.

#### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Thirty-eight epidemic strains of MRSA isolated from patients in 20 countries in 1961 to 1985 have been described previously (12). Ninety-one MRC-NS strains of seven staphylococcal species (38 *S. haemolyticus*, 34 *S. epidermidis*, 9 *S. sciuri*, 5 *S. caprae*, 2 *S. hominis*, 2 *S. capitis*, and 1 *S. warneri* strains) were isolated from patients in five hospitals in Japan in 1981 to 1993. The species were determined using API Staph-Ident strips (Analytical Profile Index System S.A., Montalieu-Vercieu, France) and Staphyogram (Terumo Co., Tokyo, Japan). All bacterial strains were grown in Luria-Bertani broth with gentle shaking at 37°C (22). Pre-MRSA strain N315 has been described previously (9). *S. haemolyticus* strain SH621, used for the derivation of resistant mutants in this study carried a beta-lactamase plasmid (7) and had a low-level methicillin resistance (MIC = 2 mg/liter).

**PCR for the analysis of *mec* complex structure.** The preparation of chromosomal DNA has been described previously (11, 23). PCR amplification was performed using 1 unit of AmpliTaq (Perkin-Elmer Cetus, Foster City, Calif.) in 50 µl of reaction mixture (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.001% [wt/vol] gelatin, 50% [vol/vol] glycerol, 1.5 mM MgCl<sub>2</sub>, 200 mM each deoxynucleoside triphosphate, 1.0 mM each primer, and template DNA). The reaction was carried out by using a Gene Amp PCR system 9600 (Perkin-Elmer). Thermal cycling was set at 30 cycles (30 s for denaturation at 94°C, 1 min for annealing at 50°C, and 2 min for elongation at 72°C).

Long-range PCR amplification was performed using 2.6 U of Expand high-fidelity PCR system enzyme mix as recommended by the manufacturer (Boehringer Mannheim Biochemica, Mannheim, Germany). A 5-µl portion of the reaction volume was subjected to electrophoresis in a 0.8% agarose gel containing 1 µl of ethidium bromide per ml to detect the amplified DNA fragment.

**PCR primers.** The primers used for the detection of *mec* complexes are listed in Table 1. The locations and direction of the primers are indicated in Fig. 1. Technical detection of the class A *mec* complex was based on the positive PCR test results for both sets of primers, mI-1 plus mI-2 and mCR2 plus mCR3 (Fig. 1). These sets of primers amplified DNA fragments of 0.5 and 1.6 kb, respectively. They included part of *mecI* and the penicillin-binding (PB) and membrane-spanning (MS) domains of *mecR1* (10). The class B *mec* complex was detected by long-range PCR using two sets of primers encompassing  $\psi$ IS1272 and *mecA* genes: iS-3 plus mA5 and iS-4 plus mA5 (Fig. 1). The latter primer set (iS-4 plus mA5) was used to explore the possible presence of IS1272 inserted in the opposite orientation to the *mecA* gene.

**Nucleotide sequencing.** Nucleotide sequence determination was performed as described previously (22), using the Dye Terminator cycle-sequencing FS ready reaction kit (Perkin-Elmer). Description of primers synthesized specifically for the sequence determination is omitted from the text.

**Computer analysis of nucleotide and protein sequences.** All the analyses were carried out using programs in the Wisconsin Package (version 9.0; Genetics

Computer Group Madison, Wis.). A homology search was performed using BLAST and TFAST programs over the EMBL (release 55.0) and GenBank (release 107.0) databases and the FASTA program over the SWISS-PROT database (release 35.0).

**Cloning of the region upstream of *mecA* by IPCR.** Using inverse PCR (IPCR), DNA fragments corresponding to the region upstream (left-hand side) of *mecA* complex were amplified and then sequenced by primer walking. The experimental procedure for IPCR has been described previously (33). The chromosomal DNAs extracted from *S. haemolyticus* strains SH518, SH631, and JB16, *S. epidermidis* strain JK8, and *S. caprae* strain JA186 were digested with *SphI*. That of *S. haemolyticus* strain SH621 was digested with *EaeI*. The restriction enzymes were purchased from Takara Shuzo Co., Ltd., Shiga, Japan. Self-ligation of the *SphI*-digested DNA and the *EaeI*-digested DNA was performed at a DNA concentration of 1 to 2 µg/ml for 10 min using a ligation kit (Boehringer Mannheim Biochemica). The ligation mixtures were then used as template DNAs for PCR amplification. The primer sets were mI2 plus mA4 for the *SphI* self-ligates and mA4 plus mA6 for the *EaeI* self-ligates (Fig. 1).

**Detection of PBP2'.** A rapid slide latex agglutination assay kit was used to evaluate expression of PBP2' by the procedure recommended by the manufacturer (8, 24) (Denka Seiken Co., Ltd., Niigata, Japan).

**Selection of resistant mutants from a low-level methicillin-resistant *S. haemolyticus* strain, SH621.** *S. haemolyticus* SH621 was cultivated in 5 ml of brain heart infusion (BHI) broth (Becton Dickinson Microbiology Systems). The overnight culture was diluted in the same medium to make a cell suspension with an optical density at 540 nm of 0.3. The 100-µl portions of the dilutions were spread onto BHI agar plates containing 1, 3, or 8 mg of methicillin per liter. For the enumeration of inoculum size (in CFU), the original cell suspension and its 10-fold serial dilutions were spread on BHI plates without antibiotic. The plates were incubated overnight at 37°C, and the mature colonies were enumerated. Six colonies were picked from the agar plate containing 1 mg of methicillin per liter, and 10 colonies each were picked from those containing 3 and 8 mg of methicillin per liter. Picked colonies were subjected to colony purification on plates containing the respective concentration of methicillin before being established as mutant strains.

**MIC determination and population analysis.** The MIC of methicillin was determined by the plate dilution method as described previously (14). The plates were incubated for 24 h at 37°C before the inspection of cell growth.

Analysis of methicillin-resistant subpopulations of strain SH621 and its derivative strains was performed as described previously (14). Briefly, aliquots (100 µl) of an undiluted or appropriately diluted overnight culture were spread onto BHI agar plates containing various concentrations of methicillin. The plates were then incubated at 37°C for 48 h. The population curve was drawn by calculating and plotting the number of resistant cells contained in 100 µl of the undiluted overnight culture. Methicillin was obtained from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan.

**Total RNA isolation for RT-PCR from *S. haemolyticus*.** Total RNA extraction was performed as described previously (21). For methicillin induction, methicillin was added to the cultures to a final concentration of 8 mg/liter and cells were collected after a 30-min incubation at 37°C. Contaminating DNAs were degraded by digesting the RNA samples with RNase-free DNase (22). The yield and purity of total RNA were determined by measuring the absorbance of an

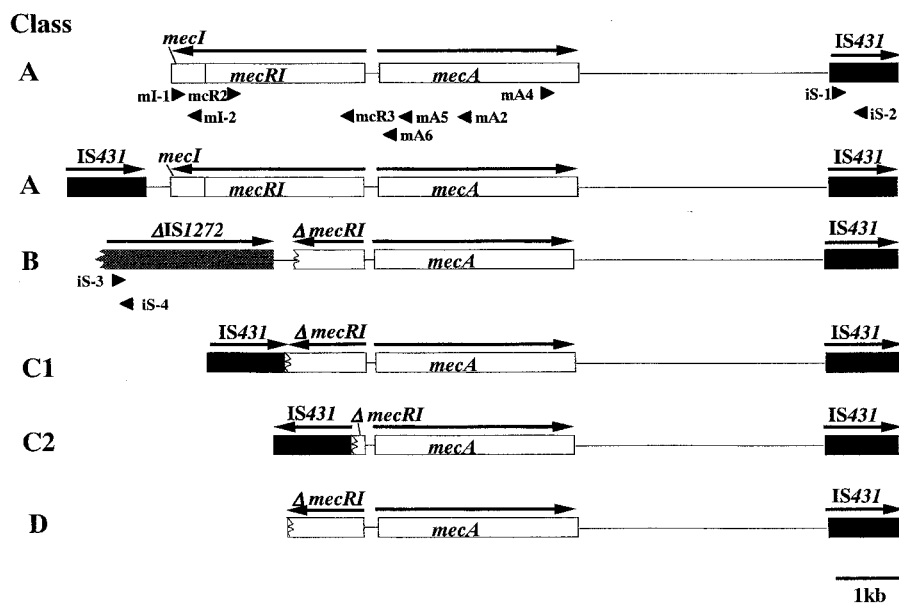


FIG. 1. Genetic organization of the *mecA* gene complex in staphylococci and the PCR primers for the detection of IS431L and IS1272 upstream of *mecA*. The structures of five classes of *mec* complexes found in MRSA and MRC-NS are shown. In all five classes, IS431R was present downstream of *mecA*. The class A *mec* complex has the structure *mecI-mecRI-mecA-IS431mec*. In *S. haemolyticus*, the class A *mec* complex was accompanied by an upstream IS431 copy (IS431L). The class B *mec* complex has the structure  $\psi$ IS1272- $\Delta$ *mecRI-mecA-IS431. Class C1 and C2 *mec* complexes have a deletion in the left side of the class A *mec* complex associated with an insertion of IS431. In class C1, IS431L is inserted in the same orientation as *mecA*, and in class C2, IS431L is inserted in the opposite orientation relative to *mecA*. Class D has a deletion without any insertion sequence adjacent to the deletion point. Arrows above the genes indicate the direction of transcription. The arrowheads indicate the location and direction of primers (see Materials and Methods).*

aliquot at 260 and 280 nm using a DO 400 spectrophotometer (Beckman Instruments, Palo Alto, Calif.).

**Preparation of the internal standard for comparative RT-PCR.** The internal standard DNA was synthesized by PCR amplification using pUC119 as a template. The primers used were 5'-AAAA-mA7-ATACGCCTATTTTATAGGT TAATGTCAT-3' and 5'-AAAA-mA8-AGTTGCTCTTGCCCGGCGTCAATA CGGGAT-3', where the underlined sequences corresponded to nucleotides 1179 to 1207 and 1598 to 1627 of pUC119, respectively (31). The set of primers amplified the DNA fragment of 0.5 kb. The amplified PCR product was subjected to agarose gel electrophoresis and was purified from the gel using the Suprec-01 purification system (Takara Shuzo co. Ltd., Shiga, Japan). The integrity of the purified DNA was confirmed by nucleotide sequence determination. The concentration of DNA was determined by measuring the absorbance at 260 nm.

**Quantitation of *mecA* gene transcripts by RT-PCR.** Reverse transcription-PCR (RT-PCR) was carried out using the Geneamp RT-PCR system (Perkin-Elmer Cetus). RT and PCR were carried out as described previously (22), except that serially diluted competitor DNAs were added to 0.0015 to 200 pM in 50  $\mu$ l of PCR mixture. Preliminary experiments were performed to confirm the linear relationship between the amounts of input RNA and the amounts of PCR product for the entire set of experiments (25). The quantity of *mecA* mRNA in the RNA samples was calculated by the procedure described by Köhler (20).

## RESULTS

**Distribution of class A and class B *mec* complexes among C-NS strains.** Figure 1 illustrates the genomic structures around the *mecA* gene in 38 MRSA and 91 MRC-NS strains. The PCR amplification using primers mA4 plus iS-2 (shown in Fig. 1) demonstrated that all the strains possessed a copy of IS431R at about 2.5 kb downstream of *mecA*. The distance between *mecA* and IS431R varied slightly among the strains, ranging from 2.3 to 2.6 kb as judged from the differences in the electrophoretic mobility of PCR-amplified fragments in aga-

rose gel. This minor difference in size was explained by the difference in the number of direct repeat units in the region between *mecA* and IS431R (reference 26 and data not shown). Another set of primers, mA4 plus iS-1, was used for the detection of an IS431 copy inserted in the opposite orientation relative to *mecA* gene. However, no DNA fragment was amplified, showing that IS431 and *mecA* were directed in the same orientation in all the strains.

Significant structural differences were present upstream of *mecA* gene. Four classes of *mec* complexes were distinguished (Fig. 1). The class A *mec* complex has an apparently intact structure *mecI-mecRI-mecA-IS431R*, and the class B *mec* complex has the structure  $\psi$ IS1272- $\Delta$ *mecRI-mecA-IS431R. Both structures have been identified previously in MRSA strains (1, 29). The *mec* complexes of all *S. aureus* strains were classified into either class A (26 strains [68.4%]) or class B (12 strains [31.6%]) *mec* complexes (Table 2). Among the MRC-NS strains, 42 strains (46.2%) carried the class A *mec* complex, which was the most common *mec* complex distributed in all the seven species tested (Table 2). The class B *mec* complex was found in 16 strains of two C-NS species: 14 in 34 *S. epidermidis* strains (41.2%) and 2 in 38 *S. haemolyticus* strains (5.3%) (Table 2). The nucleotide sequences of the region between the right boundary of  $\psi$ IS1272 and the deletion point of  $\Delta$ *mecRI* were determined for all the 28 strains of three staphylococcal species (*S. aureus*, *S. haemolyticus*, and *S. epidermidis*) (Fig. 2). All had an identical *mecRI* deletion point (nucleotide 976 of *mecRI*), and the right boundary of IS 1272 was located 267 nucleotides away from the deletion point. The 267-nucleotide sequences were 100% identical among 28 strains. The nucle-*

TABLE 2. Distribution of various classes of *mec* complex among C-NS species

| <i>mec</i> complex class <sup>a</sup> | No. (%) of isolates of:   |                                 |                                |                          |                          |                           |                           |                           |
|---------------------------------------|---------------------------|---------------------------------|--------------------------------|--------------------------|--------------------------|---------------------------|---------------------------|---------------------------|
|                                       | <i>S. aureus</i> (n = 38) | <i>S. haemolyticus</i> (n = 38) | <i>S. epidermidis</i> (n = 34) | <i>S. sciuri</i> (n = 9) | <i>S. caprae</i> (n = 5) | <i>S. hominis</i> (n = 2) | <i>S. capitis</i> (n = 2) | <i>S. warneri</i> (n = 1) |
| A                                     | 26 (68.4)                 | 5 (13.2)                        | 19 (55.9)                      | 9                        | 4 (80.0)                 | 2                         | 2                         | 1                         |
| B                                     | 12 (31.6)                 | 2 (5.3)                         | 14 (41.2)                      | 0                        | 0                        | 0                         | 0                         | 0                         |
| C1                                    | 0                         | 1 (2.6)                         | 0                              | 0                        | 0                        | 0                         | 0                         | 0                         |
| C2                                    | 0                         | 30 (78.9)                       | 1 (2.9)                        | 0                        | 0                        | 0                         | 0                         | 0                         |
| D                                     | 0                         | 0                               | 0                              | 0                        | 1 (20.0)                 | 0                         | 0                         | 0                         |

<sup>a</sup> Corresponds to the *mec* complex shown in Fig. 4.

otide sequence of  $\psi$ IS1272 revealed that the inverted repeat at the left end was missing and that there was a 23-base deletion at the 5' end of *orf1*. This deletion was identical to that previously reported in MRSA strain COL (the GenBank accession number is U35635 [2]).

**The class C *mec* complex is prevalent in *S. haemolyticus*.** A total of 31 of 38 *S. haemolyticus* strains and 1 strain each of *S. epidermidis* and *S. caprae* strains had their *mecI* and *mecR1* genes deleted, but there was no IS1272 copy associated with the deletion. Five of the strains (three *S. haemolyticus* strains [SH621, SH631, and JB16], one *S. epidermidis* strain [JK8], and one *S. caprae* strain [JA186]) were analyzed for the nucleotide sequences in the upstream region of *mecA* by using IPCR. A copy of IS431, designated IS431L, was found closely associated with the deletion point of  $\Delta$ *mecR1* in all strains except the *S. caprae* strain (see below). The structure of the *mec* complex, IS431L- $\Delta$ *mecR1*-*mecA*-IS431R, was designated the class C *mec* complex (Fig. 1 and 2; Table 2).

To find the prevalence of the class C *mec* complex, we

proceeded to test all the other 126 staphylococcal strains for the presence of IS431 upstream of *mec* complex by using long-range PCR. Two primer sets were used: iS-1 plus mA5 and iS-2 plus mA5 (for the detection of an IS431L copy in possible reverse orientation) (Fig. 1). Positive PCR results were obtained with all 30 *S. haemolyticus* strains that did not carry either class A or class B *mec* complexes. However, no strain of other species except for one *S. epidermidis* strain JK8 was found to carry the class C *mec* complex.

Based on the extent of *mecI* and *mecR1* deletion and orientation of IS431L, the class C *mec* complex was subgrouped into class C1 and class C2 as illustrated in Fig. 1. Curiously, it was found that all the five *S. haemolyticus* strains having the class A *mec* complex also possessed IS431L located 192 bp to the left of an intact *mecI* gene (Fig. 1). In the strains tested by us, IS431L was found only in species of *S. haemolyticus* and *S. epidermidis*. In other species, IS431L was not found within a ca 12-kb upstream region of the *mecA* gene, the distance that was covered by our long-range PCR.

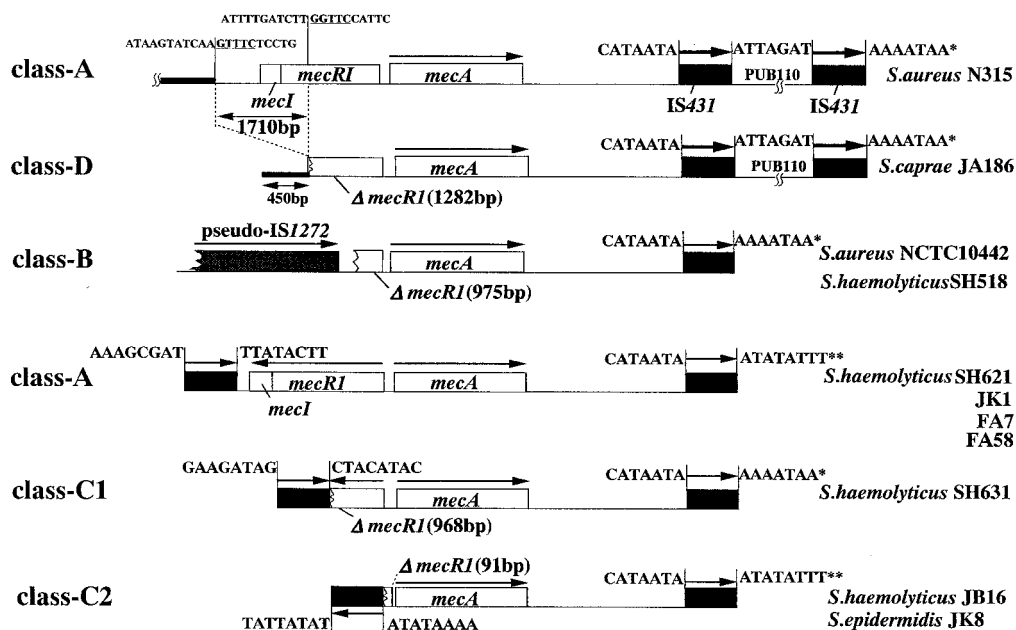


FIG. 2. Nucleotide sequencing analysis of *mec* complexes and their surrounding regions. The arrows indicate the direction of transcription of genes. The region upstream of  $\Delta$ *mecR1* of *S. caprae* strain JA186 (class D) was compared with SCC*mec* of N315 (carrying the class A *mec* complex). Eight nucleotides flanking the IS431 copies are also shown to infer a possible relationship of individual IS copies. The single asterisk and the double asterisks indicate pairs of the same sequence. No conserved direct repeats indicating target duplication were found in the 8 bp flanking the IS431-*mecA*-IS431 structures (5).



TABLE 3. Correlation between methicillin MIC and the class of *mec* complex carried by C-NS clinical strains

| Species                | Total no. of strains tested | <i>mec</i> complex class <sup>a</sup> | No. of strains for which the MIC (mg/liter) is: |   |   |    |    |    |      |
|------------------------|-----------------------------|---------------------------------------|---|---|---|----|----|----|------|
|                        |                             |                                       | 2   | 4 | 8 | 16 | 32 | 64 | ≥128 |
| <i>S. epidermidis</i>  | 16                          | A                                     | 1   | 4 |   | 5  |    |    | 6    |
|                        | 5                           | B                                     |   |   |   | 1  | 1  |    | 3    |
|                        | 1                           | C2                                    |   |   | 1 |    |    |    |      |
| <i>S. haemolyticus</i> | 5                           | A                                     | 3   | 2 |   |    |    |    |      |
|                        | 2                           | B                                     | 1   | 1 |   |    |    |    |      |
|                        | 1                           | C1                                    | 1   |   |   |    |    |    |      |
|                        | 30                          | C2                                    |   | 2 | 7 | 10 | 1  | 1  | 9    |
| <i>S. sciuri</i>       | 7                           | A                                     |   |   |   | 2  | 4  |    | 1    |
| <i>S. caprae</i>       | 4                           | A                                     |   |   |   |    |    |    | 4    |
|                        | 1                           | D                                     |   |   |   |    |    |    | 1    |
| <i>S. hominis</i>      | 2                           | A                                     |   |   |   | 2  |    |    |      |
| <i>S. capitis</i>      | 2                           | A                                     |   |   |   |    |    |    | 2    |
| <i>S. warneri</i>      | 1                           | A                                     |   |   |   | 1  |    |    |      |

<sup>a</sup> Corresponds to the *mec* complex shown in Fig. 4.

**Class D *mec* complex in *S. caprae* strain JA186.** The *mecR1* gene of *S. caprae* strain JA186 was partially deleted at its 3' portion from nucleotide 1283 to the end. Neither IS431 nor IS1272 was identified in the vicinity of the deletion point. Therefore, the structure was designated the class D *mec* complex. Nucleotide sequencing showed that the region flanking the 3' deletion point of  $\Delta$ *mecR1* was identical to a stretch of DNA sequence in the type II SCC *mec* (carrying a class A *mec* complex [16]) found in pre-MRSA strain N315 (Fig. 2). When the two sequences were aligned, there was a deletion of 1,710 bp from JA186 chromosomal DNA, which involved the *mecI* gene and the 3' one-third of the *mecR1* gene. The nucleotide sequences of the deletion endpoints of JA186 had a modest homology to each other (4 bases identical in the 5 bases [GNTTC] [underlined in Fig. 2]).

**Detection of IS431L movements in methicillin-resistant mutant strains derived from SH621.** Methicillin MICs for 28 MRC-NS strains of seven species representing all classes of *mec* complexes (37 of 42 class A strains, 8 of 16 class B strains, class C1 strain SH631, 31 class C2 strains, and the unique class D strain JA186) were determined (Table 3). It was evident that the MICs for some of the class A strains were relatively low (<8 mg/liter). Strain SH621, for which the methicillin MIC was 2 mg/liter, was selected with various concentrations of methicillin to obtain mutants with raised methicillin resistance. A total of 6, 10, and 10 mutant strains were isolated from each of the three experiments using 1, 3, and 8 mg of methicillin, respectively, as selective concentrations. The 10 mutants obtained from the above three experiments were mixed, cultured overnight, and subjected to DNA extraction. Using three DNA templates thus prepared, PCR was performed to see possible movements of IS431L in any one of the 6 or 10 mutants, using primers iS-1 (detecting IS431L) plus mA2 (detecting the 5' part of *mecA*). A single 4.1-kb DNA fragment was amplified by PCR performed on the template DNAs extracted from the mutants selected with 8 mg of methicillin per liter (Fig. 3A).

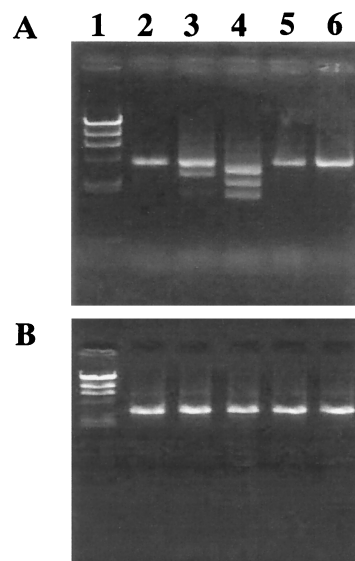


FIG. 3. Detection of IS431L-mediated deletion in resistant mutants derived from *S. haemolyticus* SH621. Portions (3  $\mu$ l) of the PCR mixture were applied to an agarose gel and subjected to electrophoresis. Lanes 1,  $\lambda$ -HindIII digest as a DNA molecular mass standard marker (the sizes were 23.1, 9.5, 6.6, 4.3, 2.3, and 2.2 kb from top to bottom); 2, SH621; 3 to 5, SH621-derived mutants with increased methicillin resistance. Mutants were selected with 1 (lane 3), 3 (lane 4), or 8 (lane 5) mg of methicillin per liter, respectively. (A) Primers iS-1 plus mA2 were used to detect the region between IS431L and *mecA*. (B) Primers mA4 plus iS-2 were used to detect the region between *mecA* and IS431R (IS431R). Note that there are varied band sizes only in lanes 3 and 4 in panel A.

On the other hand, several DNA fragments of different sizes were observed when PCR was performed on the template DNAs extracted from the mutants selected with 1 and 3 mg of methicillin per liter (Fig. 3A). The sizes ranged from 1.0 to 2.0 kb and were equal to or smaller than the 4.1-kb band amplified from the parent strain SH621. This indicated that IS431L was relocated to sites closer to *mecA* gene in some of the mutant strains. The control PCR experiment was performed using a set of primers, mA4 plus iS-2, intended for the detection of possible movements of IS431R relative to the *mecA* gene (Fig. 3B). This experiment did not show any DNA product with altered electrophoretic mobility; a single band of 2.8 kb was observed (Fig. 3B).

Table 4 lists the methicillin MICs for the 26 mutant strains. It was noted that methicillin resistance was raised in all 26 mutants. Three different levels of resistance were noted among the strains. Thirteen mutants selected with 3 and 8 mg of methicillin per liter had high of methicillin resistance (MIC > 512 mg/liter). Eleven mutants selected with methicillin at 1 or 3 mg/liter expressed an intermediate level of methicillin resistance (MIC = 16 to 64 mg/liter). Two strains selected with 1 mg of methicillin per liter showed marginal or low-level methicillin resistance (MIC = 4 and 8 mg/liter for strains SH621-15 and SH621-16, respectively) (Table 4).

**IS431L-mediated *mecI* gene deletion.** To correlate the movements of IS431L and the level of methicillin resistance expressed by each mutant strain, the 26 isolates were subjected to

TABLE 4. Structure of *mec* complex of and methicillin MIC for mutants derived from *S. haemolyticus* strain SH621

| Strain    | PBP2 production <sup>a</sup> | Selective methicillin concn (mg/liter) <sup>b</sup> | Class of <i>mec</i> complex <sup>c</sup> | PCR <sup>d</sup> structure of: |              |    | MIC of methicillin (mg/liter) |
|-----------|------------------------------|---|--|--------------------------------|--------------|----|-------------------------------|
|           |                              |   |  | <i>mecI</i>                    | <i>mecR1</i> |    |                               |
|           |                              |   |  |                                | PB           | MS |                               |
| SH621     | –                            |   | A  | +                              | +            | +  | 2                             |
| SH621-15  | +                            | 1   | A  | +                              | +            | +  | 8                             |
| SH621-16  | +                            | 1   | A  | +                              | +            | +  | 4                             |
| SH621-32  | +                            | 3   | A  | +                              | +            | +  | 512                           |
| SH621-34  | +                            | 3   | A  | +                              | +            | +  | <1,024                        |
| SH621-310 | +                            | 3   | A  | +                              | +            | +  | 512                           |
| SH621-81  | +                            | 8   | A  | +                              | +            | +  | <1,024                        |
| SH621-82  | +                            | 8   | A  | +                              | +            | +  | <1,024                        |
| SH621-83  | +                            | 8   | A  | +                              | +            | +  | 1,024                         |
| SH621-84  | +                            | 8   | A  | +                              | +            | +  | 1,024                         |
| SH621-85  | +                            | 8   | A  | +                              | +            | +  | <1,024                        |
| SH621-86  | +                            | 8   | A  | +                              | +            | +  | <1,024                        |
| SH621-87  | +                            | 8   | A  | +                              | +            | +  | 1,024                         |
| SH621-88  | +                            | 8   | A  | +                              | +            | +  | 1,024                         |
| SH621-89  | +                            | 8   | A  | +                              | +            | +  | <1,024                        |
| SH621-810 | +                            | 8   | A  | +                              | +            | +  | 512                           |
| SH621-17  | +                            | 1   | C1a                                      | Δ                              | +            | +  | 16                            |
| SH621-37  | +                            | 3   | C1a                                      | Δ                              | +            | +  | 16                            |
| SH621-38  | +                            | 3   | C1a                                      | Δ                              | +            | +  | 16                            |
| SH621-39  | +                            | 3   | C1a                                      | Δ                              | +            | +  | 16                            |
| SH621-13  | +                            | 1   | C1b                                      | –                              | Δ            | +  | 32                            |
| SH621-35  | +                            | 3   | C1b                                      | –                              | Δ            | +  | 64                            |
| SH621-31  | +                            | 3   | C1b                                      | –                              | Δ            | +  | 32                            |
| SH621-33  | +                            | 3   | C1b                                      | –                              | Δ            | +  | 16                            |
| SH621-36  | +                            | 3   | C1b                                      | –                              | Δ            | +  | 16                            |
| SH621-11  | +                            | 1   | C1c                                      | –                              | –            | Δ  | 16                            |
| SH621-12  | +                            | 1   | C1c                                      | –                              | –            | Δ  | 16                            |

<sup>a</sup> Judged by the rapid slide latex agglutination assay kit (24).

<sup>b</sup> The concentration of methicillin used for the selection of mutants.

<sup>c</sup> Corresponds to the *mec* complex shown in Fig. 4.

<sup>d</sup> + indicates that the size of the DNA fragment amplified by PCR was indistinguishable from that of SH621. Δ indicates that the amplified DNA fragment contained a deletion.

DNA extraction individually. Each chromosomal DNA was used as a template for PCR amplification with primers is1 plus mA5. Using the PCR product, nucleotide sequence of the region between IS431L and *mecA* gene was determined for each strain. The sequences are shown schematically in Fig. 4. The structure, IS431L-class A *mec* complex, remained intact in 15 mutants, and their *mecI* genes were intact as well. The rest of the mutants (11 strains), however, carried class C *mec* complex; i.e. their *mec* regulator genes were partially deleted. In these mutants, the right boundary of IS431L was found inside *mecR1* or *mecI* (Fig. 4; Table 4). The boundary was in the 3' part of *mecI* in four mutant strains (subclass C1a). In five mutant strains, the boundary was found in the 3' part of *mecR1* encoding the PB domain of *mecR1* (subclass C1b). In two mutant strains, the boundary of IS431L was found in the central part of *mecR1* encoding the MS domain (subclass C1c). On the other hand, all 10 mutant strains selected with 8 mg of methicillin per liter and 5 mutants selected with 1 and 3 mg of methicillin per liter retained the class A *mec* complex of parent strain SH621.

**The *mecI* deletion is correlated with heterogeneous methicillin resistance expression.** Comparison between methicillin resistance and the *mec* complex structure showed that all the mutants with class C *mec* complex had intermediate level of resistance (Table 2). In contrast, either high- or low-level resistance was associated with the class A *mec* complex. In this

case, a selection concentration of 1 mg/liter was correlated with low-level methicillin resistance (Table 4). Figure 5 illustrates the patterns of methicillin-resistant subpopulations (population curves) of representative mutant strains in comparison with that of the parent strain, SH621. SH621-37 (carrying class-C1 *mec* complex) had a typical heterogeneous methicillin resistance. In contrast, two strains carrying the class A *mec* complex, SH621-34 and SH621-81, contained substantial sizes of cell subpopulations with high (>512 mg/liter) methicillin resistance. They expressed homogeneous methicillin resistance.

**Derepressed transcription of *mecA* in the *mecI*-deleted strain SH621-37.** We analyzed the quantitative expression of *mecA* in SH621 and its derivative strains SH621-31 and SH621-810 before and after methicillin induction (Table 5). The amount of *mecA* transcripts in SH621-810 was slightly larger than but almost comparable to that in SH621. The transcript increased after 30-min induction with 8 mg of methicillin per liter. This was in contrast to the parent strain SH621, in which methicillin could not induce increased *mecA* gene transcription. On the other hand, the amount of *mecA* gene transcripts in SH621-31 was increased by approximately 10-fold compared to the parent strain (Table 5), signifying that the *mecA* gene transcription was derepressed in the strain. Methicillin induction further increased the transcription level of *mecA* gene in SH621-31 (Table 5).

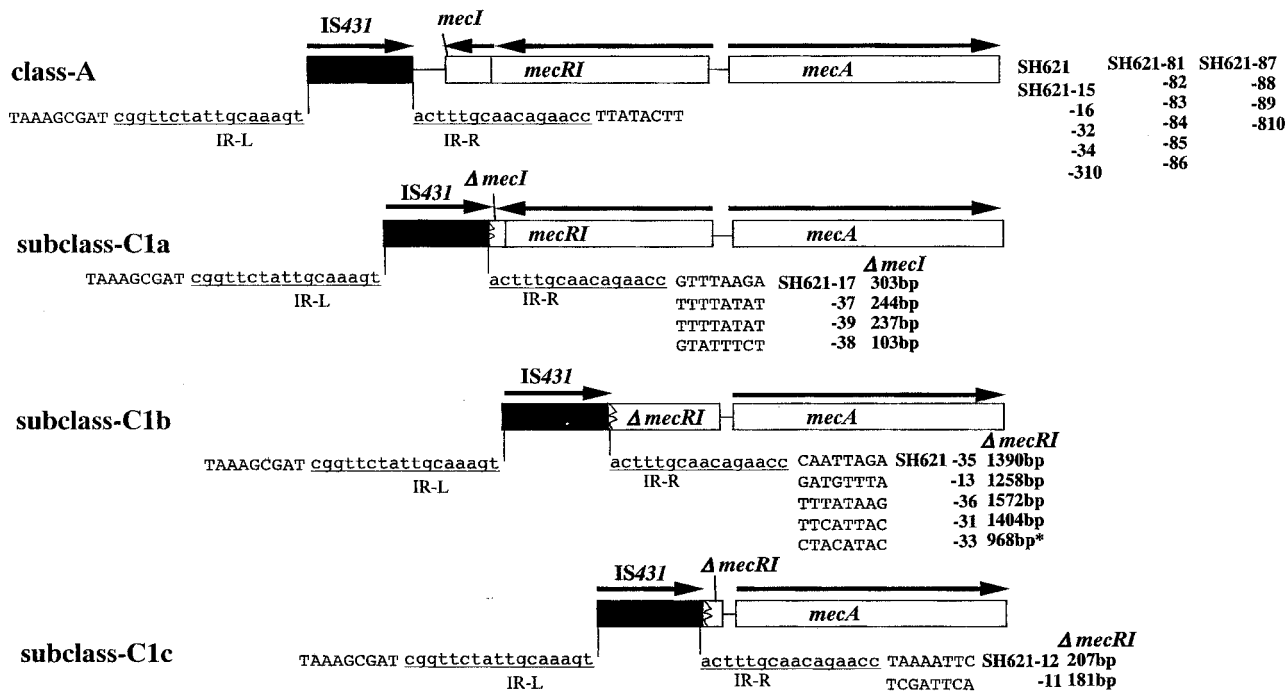


FIG. 4. *IS431L*-mediated deletion of the class A *mec* complex. The structures of three subclasses of the class C1 deletion of the *mec* complex in the mutants are shown as follows: subclass C1a (*IS431-ΔmecI-mecRI-mecA*), subclass C1b (*IS431-ΔmecRI* [retaining the MS domain]-*mecA*), and subclass C1c (*IS431-ΔmecRI* [retaining only the 5' end]-*mecA*). The flanking sequences of *IS431L* and inverted repeats (IR-L; underlined) are presented to show the deletion points in detail. The bold type indicates the names of the (mutant) strains and the sizes of the deletion. The arrows indicate the direction of transcription. A total of 15 mutants including all 10 mutants obtained by selection with 8 mg of methicillin per liter retained the class A *mec* complex. Others were classified as either one of the three class C subtypes. The asterisk in the mutant strain 33 shows that the position of *IS431L* was identical to that found in clinically isolated strain SH631.

DISCUSSION

Song et al. have proposed that the *mecA* gene was fused with *mec* regulator genes in the past to form the present operon structure of *mec* complex (28). Besides the prototypic class A

*mec* complex thus formed, class B *mec* complexes have been found in MRSA (18, 23, 29, 30) as well as in MRC-NS strains (2, 6, 19, 29, 34). However, as this study and previous reports showed, at least a 5' portion of *mecRI* is identified adjacent to *mecA* in all the tested staphylococcal strains having various deletion patterns in *mec* complexes. Close linkage of *mecA* with *IS431R* was also demonstrated in C-NS strains, although there were minor size differences in the hypervariable region between the two genes (26). So far no *mec* complex without *IS431R* or a part of *mec* regulator genes has been found. Thus, it seems likely that the class A prototypic *mec* complex was formed in much early evolutionary times before the complex was transmitted into various staphylococcal species carried by a mobile genetic element, the staphylococcal cassette chromosome (SCC) (16, 17).

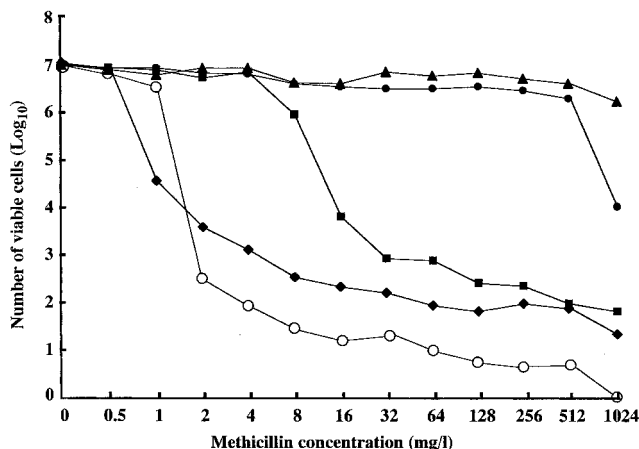


FIG. 5. Population analysis of mutant strains SH621-37, SH621-34, and SH621-81 in comparison with the parent strains SH621 and N315. See Materials and Methods for the procedure of population analysis. Symbols: ◆, SH621 (class A); ■, SH621-37 isolated with 3 mg of methicillin per liter (class C1); ●, SH621-34 isolated with 3 mg of methicillin per liter (class A); ▲, SH621-81 isolated with 8 mg of methicillin per liter (class A); ○, *S. aureus* N315 (class A).

TABLE 5. Quantitative analysis of *mecA* transcripts in SH621 and its derivative strains with and without methicillin induction

| Strains   | MIC of methicillin (mg/liter) | Class of <i>mec</i> complex | Amt of <i>mecA</i> mRNA (amol) <sup>a</sup> |                      |
|-----------|-------------------------------|-----------------------------|---|----------------------|
|           |                               |                             | Noninduced                                  | Induced <sup>b</sup> |
| SH621     | 2                             | A                           | 1.48  | 2.66                 |
| SH621-31  | 32                            | C1b                         | 17.1  | 63.9                 |
| SH621-810 | 512                           | A                           | 1.79  | 39.8                 |

<sup>a</sup> Average of two experiments.

<sup>b</sup> Induced with methicillin (8 mg/liter) for 30 min at 37°C.

*S. haemolyticus* strains were characteristic in their possession of IS431L upstream of *mec* complex. The overall structure of IS431-*mecA*-IS431 reminded us of the structure of composite transposon (4, 5). However, analysis of nucleotide sequences flanking this structure revealed no conserved target duplication (Fig. 2). Thus, there is no evidence to support the possibility that the *mec* complex was transferred as a composite transposon (Fig. 2). It seems more likely that the structure (IS431L-*mec* complex) was constructed by the insertion of an IS431 copy upstream of the *mec* complex after the *mec* complex was integrated in SCC to form SCC*mec*. Although the structure was distributed preferentially in *S. haemolyticus* strains in our study, this structure is not confined to *S. haemolyticus*, since it has been identified recently in one *S. sciuri* strain as well (34).

The most common *mec* complex was class A, the prototype *mec* complex, which was found in practically all staphylococcal species including *S. aureus* (10). Therefore, it would be reasonable to assume that some *S. haemolyticus* strains had also acquired class A *mec* complex in the past. However, the observed high prevalence of the class C *mec* complex or IS431L-class A *mec* complex in *S. haemolyticus* requires explanation. The most plausible explanation is that an *S. aureus* clone, in which IS431L was inserted upstream of the class A *mec* complex, significantly expanded in the hospital environment. In this case, however, we have to assume some advantage for the strain with a class C *mec* complex to survive preferentially in the hospital environment (see below).

It has been postulated that the class B *mec* complex was transmitted from C-NS to *S. aureus* based on its carriage of  $\psi$ IS1272; the insertion sequence (IS1272) is widely disseminated in *S. haemolyticus* strains but not in *S. aureus* strains (1). Extensive nucleotide sequencing of various *mec* complexes in this study provided further concrete evidence for the active transmission of *mec* complexes across staphylococcal species. The nucleotide sequence determination of the regions between IS1272 and  $\Delta$ *mecR1* unequivocally showed identity of the structures across three staphylococcal species. For example, the class C2 *mec* complex found in *S. haemolyticus* and *S. epidermidis* strains had an identical deletion point of the  $\Delta$ *mecR1*, clearly showing that the two *mec* complexes have the same evolutionary origin. In *S. haemolyticus* strain FA83 carrying the class A *mec* complex, IS431L was located 190 bp to the left of the 3' end of the intact *mecI* gene. The position coincided with the location of IS431 in *S. sciuri* strain SK8, which was previously identified by others (34). The data indicated that the structure, IS431L-class A *mec* complex, also has been transmitted across species barriers.

The  $\psi$ IS1272 copies identified in three species, *S. aureus*, *S. haemolyticus*, and *S. epidermidis*, suffered an identical partial deletion, indicating that the insertion sequence was not capable of transposition (2). Therefore, neither of the insertion sequences IS431 (see above) or IS1272, found closely associated with *mecA*, seem to play a role in interspecies transfer of the *mecA* gene complex. On the other hand, nucleotide sequence analysis of four classes of *mec* complexes revealed that each complex was a component of SCC*mec*, a staphylococcal mobile genetic element (16, 17). For example, nucleotide sequencing of 450 bp of DNA upstream of  $\Delta$ *mecR1* of the class D *mec* complex in *S. caprae* JA186 revealed that it was identical to a part of type II SCC*mec* identified in *S. aureus* strain N315

(16, 17). Therefore, it is likely that the diversification of the *mec* complex occurred (presumably from the prototypic class A *mec* complex) after its establishment as a component of SCC-*mec* in a C-NS strain. Diversified *mec* complexes then seem to have been transmitted to *S. aureus* and other staphylococcal species.

As demonstrated in pre-MRSA strain N315, the presence of an intact *mecI* gene causes repression of *mecA* gene transcription and makes the cell susceptible to methicillin (10, 12, 13, 22). *S. haemolyticus* strain SH621 had a class A *mec* complex whose *mecI* gene was intact, and only a small level of PBP2' production was observed (Table 4). Population analysis of the strain showed a typical heterogeneous methicillin resistance, and the MIC of methicillin for this strain was 2 mg per liter. By selecting the strain with 8 mg of methicillin per liter, only the mutants with high-level methicillin resistance were obtained (MIC  $\geq$  512 mg/liter) (Table 4). On the other hand, mixture of mutants with intermediate-level (seven mutants) and high-level (three mutants) methicillin resistance were obtained by the selection with 3 mg of methicillin per liter. It was noted that in all seven mutants with intermediate resistance, *mecI* was partially or completely deleted by the IS431L-mediated deletion (Fig. 4). This coincides with the previous observation with N315 (10): when the strain is selected with 3 mg of methicillin per liter, heterogeneous methicillin-resistant mutants are obtained for which the methicillin-MICs are comparable to those for the seven mutants in this study. However, in N315, the inactivation of *mecI* function is caused by mutations incorporated either in the structural gene or at the *mecA* gene operator to which the MecI repressor is supposed to bind (10, 27). The observation presented in this study indicates that the IS431L-mediated *mecI* gene inactivation in SH621 occurs at least several times more frequently than the mutational inactivation of *mecI*. This coincides with the difference in the population curves between SH621 and N315 (Fig. 5): SH621 contains about 10-fold more subpopulations resistant to 2 and 4 mg of methicillin per liter than does N315 (Fig. 5). This high rate of generation of mutants with the *mecI* gene deleted may confer a survival advantage to the strain. This may explain the clonal dominance of IS431L-carrying *S. haemolyticus* strains in the hospital environment.

The mutants listed in Table 4 possessed three different levels of methicillin resistance. Intermediate methicillin resistance seems to be caused by the inactivation of the *mecI* gene. On the other hand, except for the two strains with a marginal level of resistance, the mutant strains retaining intact *mecI* showed homogeneous methicillin resistance (Fig. 5). In the representative strain SH621-810, *mecA* transcription was inducible with methicillin despite the presence of intact *mecI* (Table 5). Considering the frequency of occurrence of the mutants with this property (appearance rate,  $9.8 \times 10^{-6}$  [Fig. 5]), a mutation would be postulated in a regulatory gene involved in *mecA* gene transcription. However, such a gene explaining this phenomenon is totally unknown at this moment. It is also unknown why a rapid slide latex agglutination assay for PBP2' could discriminate between SH621 and its derivatives represented by SH621-810 even though the amount of *mecA* gene transcripts was almost comparable between the two (Table 4). This result was reproducible in repeated experiments, suggesting that some factors other than the level of transcript, such as mRNA



stability, are involved with the phenomenon. Further studies are required to solve these questions and elucidate the regulatory system for methicillin resistance in *S. haemolyticus*.

#### ACKNOWLEDGMENTS

We thank T. Watanabe for her excellent technical assistance.

This work was supported by a Core University Program under Japan Society for the Promotion of Science (JSPS), coordinated by the University of Tokyo Graduate School of Medicine and University Sains Malaysia School of Medical Sciences; by a Specially Designated Research Promotion of Monbusho, Japan; and by a Grant for International Health Cooperation Research (11C-4) from the Ministry of Health and Welfare.

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