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Cloning and Nucleotide Sequence Determination of the Entire *mec* DNA of Pre-Methicillin-Resistant *Staphylococcus aureus* N315

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In methicillin-resistant *Staphylococcus aureus*, the methicillin resistance gene *mecA* is localized within a large chromosomal region which is absent in the methicillin-susceptible *S. aureus* chromosome. The region, designated *mec* DNA, is speculated to have originated from the genome of another bacterial species and become integrated into the chromosome of the *S. aureus* cell in the past. We report here cloning and determination of the structure of the entire *mec* DNA sequence from a Japanese *S. aureus* strain, N315. The *mec* DNA was found to be 51,669 bp long, including terminal inverted repeats of 27 bp and a characteristic pair of direct repeat sequences of 15 bp each: one is situated in the right extremity of *mec* DNA, and the other is situated outside the *mec* DNA and abuts the left boundary of *mec* DNA. The integration site of *mec* DNA was found to be located in an open reading frame (ORF) of unknown function, designated *orfX*. Clusters of antibiotic resistance genes were noted in *mec* DNA carried by transposon Tn*554* and an integrated copy of plasmid pUB110. Both the transposon and plasmid were integrated in the proximity of the *mecA* gene, the latter being flanked by a pair of insertion sequence IS*431* elements. Many ORFs other than those encoding antibiotic resistance were considered nonfunctional because of the acquired mutations or partial deletions found in the ORFs. Two ORFs potentially encoding novel site-specific recombinases were found in *mec* DNA. However, there was no ORF that might encode *mec* DNA-specific transposase or integrase proteins, indicating that the *mec* DNA is not a transposon or a bacteriophage in nature.

In 1961, shortly after the introduction of methicillin, methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in England (18). MRSA soon became a serious problem challenging hospital infection control throughout the world (4). MRSA expresses methicillin resistance by producing a specific penicillin-binding protein, PBP2′ (or PBP2a), that has a decreased binding affinity to β-lactam antibiotics (13, 30, 48). The genetic determinant of methicillin resistance (*mec*) has been localized on the chromosome of *S. aureus* (36) and mapped to a locus between the genes encoding protein A (*spa*) and a protein involved in the biosynthesis of purine (*purA*) (19). The *mecA* gene encoding PBP2′ has been cloned from a Japanese MRSA strain by exploiting a tobramycin resistance gene which is closely linked to *mecA* as a selective marker, and its sequence was determined (38). The *mecA* gene is adjoined by a set of regulatory genes, *mecI* and *mecR1*, forming the *mecA* gene complex (*mecI-mecR1-mecA*), though in some strains the set is partially deleted and only the 5′-portion of the *mecR1* gene is left beside the *mecA* gene (Δ*mecR1-mecA*) (2, 14, 20, 24). The *mecA* gene complex (and its deleted version) is widely distributed among *S. aureus* species as well as among other staphylococcal species collectively called coagulase-negative staphylococci (C-NS) (17, 31, 42, 43). Therefore, it has been speculated that *mec* may be freely transmissible among staphylococcal species, crossing the staphylococcal species barrier (3, 42). With classical genetic experiments, it was shown that *mec* is not transferable between *S. aureus* strains by conjugation (21) but is transferable by bacteriophage-mediated generalized transduction (10). Subsequently, Trees and Iandolo reported that *mec* could be mobilized from the chromosome to a penicillinase plasmid, pI524, and suggested the possibility that *mec* may comprise a part of a transposable genetic element (46). In the 1980s, direct chromosome analysis of MRSA strains revealed that a substantial length of the chromosomal DNA segment (greater than 30 kb) carrying *mec* has no allelic equivalence in methicillin-susceptible *S. aureus* (MSSA) strains; the segment was called "additional DNA" or "*mec* DNA" (6, 7, 11, 37). However, the size, structure, and biological properties of *mec* DNA have, until now, long remained unclear.

In this study, we report the first demarcation and determination of the structure of the *mec* DNA of a Japanese *S. aureus* strain, N315.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and media. N315 is a *mecA*-carrying *S. aureus* strain that has been described previously (14, 20). NCTC8325 (BB255), a type strain of *S. aureus*, was a gift from B. Berger-Bächi. *Escherichia coli* MC1061 was used for the propagation of plasmid libraries, and *E. coli* XL1-Blue MRA(P2) was used for the propagation of phage libraries. Plasmid pACYC184 was used for the construction of plasmid libraries, and plasmid vectors pUC118 and pUC119 were used for subcloning. *Bam*HI-cleaved arms of lambda Dash II (Stratagene, La Jolla, Calif.) were used for the construction of phage libraries. L broth and L agar, used for cultivation of *E. coli*, and NZY broth, NZY agar, and NZY soft agar, used for propagation of bacteriophage λ, were prepared as described (33). Heart infusion broth and heart infusion agar (Eiken Kagaku, Tokyo, Japan) were used for cultivation of *S. aureus*. The antibiotics ampicillin (Meiji Seika Co., Tokyo, Japan) and chloramphenicol (Sankyo Co., Tokyo, Japan) were used at the concentrations of 100 and 25 μg/ml, respectively.

Construction of genomic DNA libraries of N315 and NCTC8325. Genomic DNA libraries were prepared from *S. aureus* N315 and NCTC8325. Extraction of DNA from staphylococcal cells has been described previously (14). Partial *Sau*3AI digests and complete *Hin*dIII digests were made, and fragments were separated by agarose gel electrophoresis. Fractions with DNA fragments ranging from 10 to 20 kb in size were extracted by using BIOTRAP (Schleicher & Shuell, Keene, N.H.) and were used for the construction of genomic DNA libraries.

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Plasmid libraries were constructed as follows. Phosphatase-treated linearized pACYC184 (0.3 μg) and partially *Sau*3AI-digested or completely *Hin*dIII-digested *S. aureus* N315 DNAs (1.1 to 1.2 μg) were mixed and ligated by using a DNA ligation kit (Takara Shuzo Co. Ltd., Kyoto, Japan). The ligated DNAs were transformed into *E. coli* MC1061, and transformants were selected with antibiotic resistance by plating on L agar plates containing chloramphenicol. After incubation of the plates at 37°C for 16 h, colonies grown on the plates were lifted onto a nylon filter (BiodyneA; Pall BioSupport, Glen Cove, N.Y.) as described previously (14).

Phage libraries were constructed as follows: 300 ng of partial *Sau*3AI digests of chromosomal DNA of N315 and NCTC8325 (10 to 20 kb in length) and 1 μg of the lambda Dash II arms cleaved with *Bam*HI (Stratagene) were resuspended in 5 μl of ligation buffer containing 1 mM ATP and incubated overnight at 14°C after addition of 2 Weiss units of T4 DNA ligase and packaged by using packaging extract (Giga-puck plus; Stratagene), all as described by the manufacturer. Phages were propagated to produce plaques on *E. coli* XL1-Blue MRA(P2) and were lifted onto a nylon filter (BiodyneA; Pall BioSupport).

DNA hybridization. Colony hybridization was performed by using [α^{32}P]dCTP (3,000 Ci/mmol [NEN Research Products, Du Pont, Boston, Mass.])-labeled probes as described previously (14). Probes 1, 2, 3, and 4 were prepared from 2.6-kb *Hin*dIII DNA fragments of pES1, 2.7-kb *Hin*dIII fragments of pSJ2, 1.9-kb *Hin*dIII fragments of pSJ5, and 1.8-kb *Eco*RI-*Hin*dIII fragments of pSJ7, respectively.

Plaque hybridizations were performed by using digoxigenin-labeled probes (Boehringer, Mannheim, Germany) as described (33). Probe 11A, which was used to clone LD8325, was prepared by PCR amplification with cL2 (5′-ATAG AAAAACAGGACTTGAAC-3′) and M4 (5′-GTTTCCCAGTCACGACGTTG TAA-3′) used as primers and SJ8 used as a template. Probe cR, which was used to clone LN4, was prepared by PCR with cR4 (5′-TTCTAATGTAATGACTG TGGAT-3′) and cR5 (5′-TTATTAGGTAAACCAGCAGTAAGTGAACAAC CA-3′) used as primers and LD8325 used as a template.

DNA manipulation. Large-scale and small-scale preparation of plasmid DNA, purification of phage plaques, extraction of DNA from purified phage particles, and subcloning of DNA fragments into plasmid vector pUC118 or pUC119 were performed by standard techniques (33). All the enzymes for DNA manipulation were purchased from Takara Shuzo Co. Ltd., except for *Taq* DNA polymerase for PCR, which was purchased from Perkin-Elmer, Foster City, Calif.

Nucleotide sequence determination was performed as described previously (14), using a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer). Descriptions of primers synthesized specifically for the sequence determination are given in the figure legends.

PCR amplification. PCR was performed essentially as described previously (42) with a 50-μl reaction volume and by using thermal cycler Gene Amp 9600 (Perkin-Elmer Cetus Instruments, Emeryville, Calif.). In nested PCR, the first round of PCR was carried out for 30 cycles and 1 to 2 μl of the reaction mixture obtained was used as the substrate for the second round of PCR, which was carried out for 10 cycles. Long PCR was performed by using Expand *Taq* polymerase (Boehringer) by the procedure recommended by the manufacturer. Three microliters of the reaction mixture was subjected to agarose gel electrophoresis to detect amplified DNA fragments. PCR products were purified by using a High Pure PCR product purification kit (Boehringer).

Computer analysis of nucleotide and protein sequences. All the analyses were carried out by using programs in The Wisconsin Package (version 9.0; Genetics Computer Group, GCG, Madison, Wis.). Homology searches of the EMBL (release no. 55.0) and GenBank (release no. 107.0) databases were performed by using BLAST and TFastA programs, and the FastA program was used for the homology search of the SWISS-PROT database (release no. 35.0).

Nucleotide sequence accession numbers. The nucleotide sequences of the *mec* DNA of N315 and of pLE1a described in this article have been deposited in the DDBJ, EMBL, and GenBank databases under accession nos. D86934 and AB014440, respectively.

RESULTS

Identification of *mec* DNA on *S. aureus* N315 chromosome. As illustrated in Fig. 1a, overlapping chromosomal DNA fragments were sequentially cloned from the *mecA*-carrying *S. aureus* strain N315 (pre-MRSA strain [14, 16]) and their nucleotide sequences were determined. The procedure was initiated by cloning chromosomal DNA fragments SJ1 and SJ2 which were cross-hybridizable with the previously cloned DNA fragment, ES1, containing a part of the *mecA* gene complex (14). Then the cloning proceeded leftward until the first DNA fragment that hybridized with the chromosomal DNA of the MSSA type strain NCTC8325, SJ8, was obtained. By using probe 11A, which cross-hybridized with the NCTC8325 chromosomal DNA (Fig. 1a), the chromosomal DNA fragment LD8325 was obtained from a phage library of NCTC8325 by plaque hybridization. By comparison of the nucleotide sequences of SJ8 and LD8325, the left chromosome-*mec* DNA junction (*att*L; Fig. 1b) was determined. Then, to clone the DNA fragment containing the right boundary of *mec* DNA, the MSSA-specific probe cR (Fig. 1a), corresponding to a stretch of nucleotides present in LD8325 but absent in SJ8, was used as a hybridization probe to screen a phage library of N315. The chromosomal DNA fragment LN4 was thus obtained (Fig. 1a). By alignment of the nucleotide sequences of LD8325 and LN4, the presumptive right chromosome-*mec* DNA junction (*att*R; Fig. 1b) was identified. The authenticity of *att*R was confirmed by proving that the *mecA* gene and *att*R were closely linked on the chromosome of N315 by using a long PCR amplification. One primer, mA3, for the PCR was prepared inside the *mecA* gene, and the other, cR2, was prepared downstream of the presumptive *mec* DNA boundary (the locations of the primers are indicated in Fig. 1a). The DNA fragment, 12 kb in size, was amplified by long PCR (Fig. 1a). The amplified fragment did not hybridize with NCTC8325 chromosomal DNA in dot blot hybridization, showing that the fragment was extrinsic to MSSA chromosome and was a part of the *mec* DNA. By determining a restriction enzyme map and by subsequent nucleotide sequencing of the 12-kb fragment, the nucleotide sequence of the entire *mec* DNA of N315 was carried out.

Structure of the chromosome-*mec* DNA junction. Figure 1b shows the nucleotide sequences of the regions around the left and right boundaries of *mec* DNA, *att*L and *att*R, together with those of the regions around the *mec* DNA integration site, *att*B (bacterial chromosomal attachment site for *mec* DNA), found on the chromosome of NCTC8325. The left and right chromosome-*mec* DNA junction regions, *att*L and *att*R, were tentatively and operationally defined in this study by using PCR with the primer pair cL1 and mL1 for *att*L and the primer pair mR8 and cR2 for *att*R (Fig. 2a). The *att*B was located at the 3′ end of a novel open reading frame (ORF), *orfX*, whose direction of transcription is shown from right to left in Fig. 1a and b, and its stop codon is indicated in Fig. 1b. The *orfX*, potentially encoding a 159-amino-acid (aa) polypeptide, was modified in N315 by the integration of *mec* DNA at its 3′ end, with its last 15 bases replaced by the *mec* DNA sequence. However, curiously, the modified *orfX*, designated *orfX** (see Fig. 3), had a stop codon generated at the same nucleotide position as that of the unmodified *orfX* and encoded exactly the same last 5 aa, EAYHK, as those of the unmodified *orfX* of NCTC8325. The homology between the deduced amino acid sequences of *orfX* of NCTC8325 and *orfX** of N315 was 100%, and that between the nucleotide sequences was 98.1%. Homology search of the databases revealed homology of the polypeptide encoded by *orfX* with some previously identified polypeptides, but their functions are unknown (see Table 1).

By comparison of the nucleotide sequences of N315 and NCTC8325, the exact integration site of *mec* DNA was inferred to within a maximum ambiguity of four bases (Fig. 1b). The left terminal nucleotide of *mec* DNA was estimated to be one of the four nucleotides TCTG (numbered 1 through 4 in Fig. 1b), and the right terminal nucleotide was estimated to be one of the four nucleotides TTCT (numbered 5 through 8 in Fig. 1b); the set of left and right terminal nucleotides of *mec* DNA was thus estimated to be one of the four nucleotide combinations 1 and 5, 2 and 6, 3 and 7, or 4 and 8. The entire length of the *mec* DNA was estimated to be 51,699 bp. No target duplication typically accompanied by the integration of insertion sequences (IS) or transposable elements was found flanking the *mec* DNA. Instead, characteristic direct repeats of 15 bases each (with 13 identical bases) were found: one was localized outside the *mec* DNA adjacent to its left boundary,

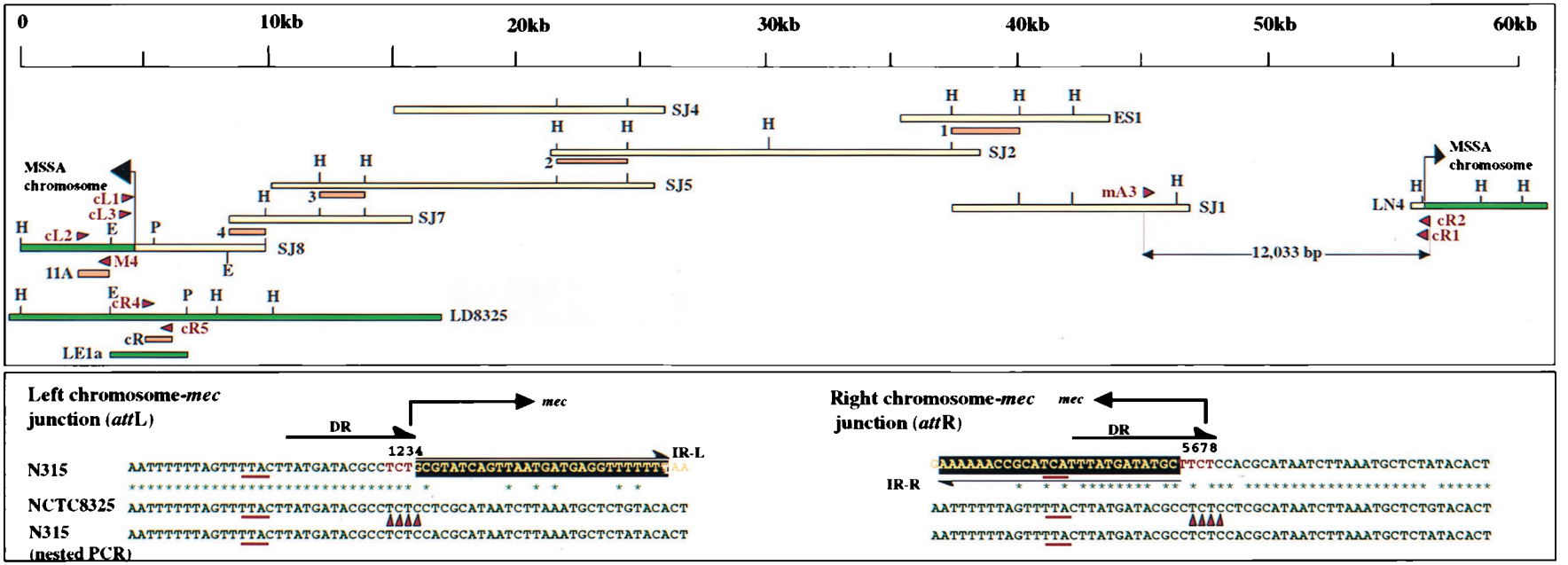

FIG. 1. Genomic structure of *mec* DNA. (a) Cloning strategy of *mec* DNA. The *mec* DNA is shown in light yellow; the chromosomal region found in both the pre-MRSA strain N315 and the MSSA strain NCTC8325 is shown in green. All the hybridization probes are shown in orange. DNA fragments SJ1 and SJ2 were cloned from a N315 plasmid library by colony hybridization by using probe 1 prepared from plasmid pES1 (14). DNA fragments SJ4, SJ5, SJ7, and SJ8 were sequentially cloned by using probes 2, 3, and 4, respectively. All the fragments were cloned from a plasmid library of *Sau*3AI-partial digests except for SJ8, which was cloned from an N315 library of *Hin*dIII digests. LD8325 was cloned from a phage library of NCTC8325 by plaque hybridization by using the probe 11A. LN4 was cloned from a phage library of N315 by plaque hybridization by using cR as a probe. The region downstream of *mecA* was identified and sequenced by using the DNA fragment directly amplified by long PCR with mA3 (5′-ACAACGTTACAAGATATGAAGTGGTAAATGGTA-3′) and cR2 (5′-AAACGACATG AAAATCACCAT-3′) primers and the N315 DNA as a template. Only relevant restriction enzyme sites are indicated (H, *Hin*dIII; E, *Eco*RI; P, *Pst*I), and those in overlapped DNA fragments are omitted. The nucleotide sequence accession numbers of the N315 chromosome containing the entire *mec* DNA (56,939 bp) starting at the first nucleotide of the leftmost *Hin*dIII site and of LE1a, the *Eco*RI-*Pst*I subfragment of LD8325, are given in the Materials and Methods section. (b) Boundary of *mec* DNA. Nucleotide sequences around the left and right boundaries of *mec* DNA, *att*L and *att*R, respectively, are aligned with the sequence around the presumptive integration site, *att*B, on NCTC8325 chromosome. The four possible integration sites are located in the *att*B at the 3′ end of *orfX* (indicated by red arrowheads). The stop codons for *orfX* in NCTC8325 and modified *orfX* (*orfX**) of N315 are indicated by red bars. Inverted complementary repeats, IR-L and IR-R, at both extremities of *mec* DNA are indicated by thin arrows. Direct repeats are indicated by thick arrows. Asterisks indicate identical nucleotides in the two sequences. The numbers on top of the nucleotides indicate possible terminal nucleotides of *mec* DNA. The entire length of *mec* DNA was 51,669 bp; the left-most nucleotide was inferred to be one of the four nucleotides (positions from 4685 to 4688) designated nucleotides 1 to 4 in the figure.

and the other was localized inside the *mec* DNA at its right extremity (Fig. 1b). Degenerate inverted repeats, IR-L and IR-R, composed of 27 bases each (with 22 identical bases) were found at both termini of the *mec* DNA (Fig. 1b).

Genomic organization of *mec* DNA. Figure 2a illustrates the essential structure of *mec* DNA. Transposon Tn*554* (encoding antibiotic resistance genes for spectinomycin [*spc*] and erythromycin [*ermA*]) was found integrated to the left of the *mecA* gene complex (26). To the right of the *mecA* gene complex, an integrated copy of staphylococcal plasmid pUB110 (encoding resistance genes for tobramycin and kanamycin [*aadD*] and for bleomycin [*ble*]) was found flanked by a pair of IS*431* (25). These features of *mec* DNA of N315 were similar to those previously described with other MRSA strains (2, 15, 47).

The *mec* DNA contained 112 ORFs whose locations in six possible reading frames are illustrated in Fig. 2b; 32 of them, listed in Table 1, potentially encoded polypeptides which were similar (having a z score of >400 in the TFastA program) or identical (with greater than 99% identity in amino acids) to the previously reported gene products. Among the 32 ORFs, 7 were associated with drug resistance (Fig. 2b): they were resistance genes for erythromycin (CN026), β-lactam (CN036 or *mecI*, CN037 or *mecR1*, and N058 or *mecA*), spectinomycin (N048), kanamycin and tobramycin (N063), and bleomycin (N064). The *mecA* gene encoded a PBP2′ protein with 668 aa residues. The sequence was different by 24 aa residues from those of two other MRSA strains (reported by Song et al. [38] and Ryffel et al. [31]) but, curiously, was identical to that obtained from a methicillin-resistant *Staphylococcus epidermidis* strain (31). Except for the *mecA* gene complex, drug resistance genes were carried either by the transposon Tn*554* or the integrated copy of plasmid pUB110. No ORF encoding an evident pathogenic factor, such as those for bacterial adherence, invasion, or toxin production, was found. Three ORFs (N045, N046, and N047) encoded the transposases for Tn*554*, and two ORFs (N062 and N070) encoded the transposase for IS*431*. Three other ORFs (N026 through N028) encoded polypeptides homologous to putative *Bacillus anthracis* transposases, but the ORF N026 encoded a polypeptide which was lacking in the C-terminal third of the corresponding *B. anthracis* putative transposase, and the ORFs N027 and N028 encoded polypeptides homologous to the N- and C-terminal halves, respectively, of a putative IS*150*-like transposase of *B. anthracis*, indicating that these three ORFs were the remnants of transposase genes which might have been functional in the past. The ORF N066 corresponded to the *pre* gene, which encodes the recombination enzyme for pUB110 (25). The replication gene, *rep*, of pUB110 was found to be disrupted into two ORFs (N068 and N069) by a nonsense mutation (Fig. 2b and Table 1).

The two ORFs N034 and N037 encoded proteins homologous to site-specific recombinases of the invertase-resolvase family. Among the extant site-specific recombinases, a site-specific integrase of the bacteriophage TP901-1 of *Lactococcus lactis*, which also was distantly related to the invertase-resolvase family of site-specific recombinases, had the best homology to N034 and N037 (8). N037 was also homologous to the site-specific recombinase SpoIVCA of *Bacillus subtilis* (40, 44), a putative integrase of *Bacillus cereus* bacteriophage TP21 ply21 (23), and transposase TnpX of *Clostridium perfringens* (5), all from gram-positive bacterial species. These recombinases have a catalytic motif at the N-terminal domain which is characteristic of recombinases of the invertase-resolvase family (1, 35).

Eight other ORFs were gene homologues presumably involved in the metabolism or physiology of the bacterial cell (Fig. 2b). Six of them (CN009, CN010, CN012, CN013, N021, and N022) formed a cluster which en bloc was homologous to the *kdp* operon (*kdpA* through *kdpE*), whose relative gene order, CBADE, is the same as that in *Mycobacterium tuberculosis* but different from that, ABCDE, in *Clostridium acetobutylicum* and *E. coli*. The *kdp* operon is involved in ATP-dependent potassium transport across the bacterial cell membrane (41). In spite of the difference in the gene order, among various species the *kdp* genes of *C. acetobutylicum* were the closest to those found on *mec* DNA. Comparison with the *C. acetobutylicum kdp* genes revealed that the *kdpA* gene homologue in *mec* DNA was disrupted into two ORFs (CN012 and CN013) by a frameshift mutation, the *kdpC* gene homologue (CN009) was lacking 4 N-terminal aa and 63 C-terminal aa, and the *kdpD* gene homologue (N021) showed a deletional loss of 23 N-terminal aa (Table 1).

The ORF CN034, encoding a polypeptide homologous to xylose repressor, also had a deletion of 4 aa (from residue 12 through 15 at the N terminus), causing a shift of reading frame (Table 1). The ORF CN039 was a homologue of the *E. coli ugpQ* gene. *E. coli* UgpQ is a 247-aa protein with glycerophosphoryldiester phosphotransferase activity. A similar ORF (capable of encoding a 145-aa polypeptide with homology to the N-terminal part of the *E. coli* UgpQ protein) was previously identified by Ryffel et al. downstream of the *mecA* gene in another MRSA strain (32). The comparison of its nucleotide sequence with that of the ORF CN039 showed that the previously identified ORF was a mutated version of CN039 in which the 455th nucleotide (T) was deleted and a premature termination codon was introduced. CN039 itself, however, contained a one-base insertion at around the 508th nucleotide position which, by causing a shift of reading frame, truncated the C-terminal polypeptide, which was otherwise homologous to the C-terminal 28 aa of the *E. coli* UgpQ protein. Four ORFs, N044, N052, CN030, and CN031, were homologous to *B. subtilis* hypothetical proteins with unknown function.

Distribution of G+C content of *mec* DNA. Figure 2c illustrates the regional change of the G+C content in and around *mec* DNA. The overall G+C content of *mec* DNA was 32.79%, which is within the range of variation for the species *S. aureus* (32 to 36%) (34). There were seven regions (two outside of the *mec* DNA and five within the *mec* DNA) having substantially deviant G+C content values (Fig. 2c). The regions might have different evolutionary origins: region 2 contained a group of ORFs homologous to the *kdp* operon, and the adjacent region, region 3, contained remnants of putative transposases (Table 1); region 4 contained *mec* regulator genes, *mecI* and *mecR1* (14, 43), which are proposed to have been derived from a β-lactamase gene complex independently of the *mecA* gene (38); region 5 corresponded to the drug resistance genes *aadD* (conferring tobramycin and kanamycin resistance) and *ble* (conferring bleomycin resistance) carried by the plasmid pUB110. It was noted that the MSSA chromosomal region (region 1) containing *att*L (Fig. 2a) had remarkably low G+C content, as did region 6, at the right extremity of *mec* (Fig. 2a). It was also noted that *orfX*, corresponding to region 7 shown in Fig. 2c, had an unusually high G+C content for an *S. aureus* gene; the content for the entire ORF was 39.41%, and that for the 3′ half (left half) was 43.75%.

Precise excision of *mec* DNA. To test whether *mec* DNA behaves like a mobile genetic element, PCR experiments were designed to monitor spontaneous precise excision of *mec* DNA at the *att*R and *att*L regions. The combinations of primers, cR2 with cL3 and cR1 with cL1, were used for the first and second

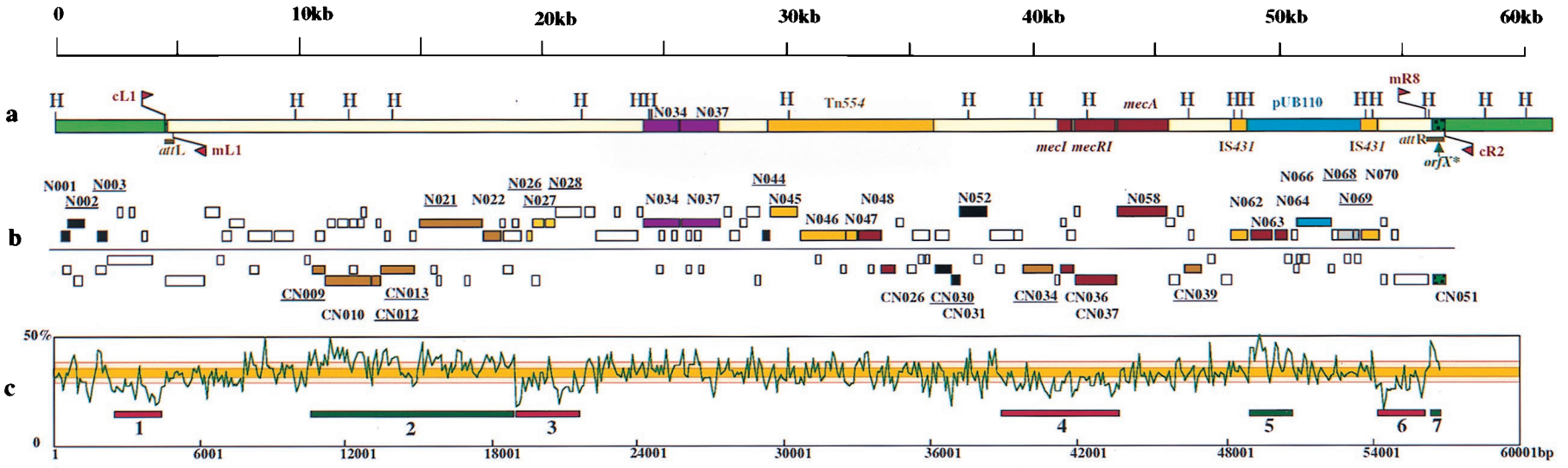

FIG. 2. (a) The essential structure of *mec* DNA. Locations of the essential genes are illustrated. Only restriction sites of *Hin*dIII are indicated. *att*L and *att*R (tentatively defined by the primer pairs cL1 with mL1 and mR8 with cR2) are shown by bars. *orfX** is indicated by an arrow. (b) ORFs in and around *mec* DNA. The ORFs more than 200 bases in size in 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. Colored squares indicate the ORFs whose extant gene homologues were found in the databases, though many of them were considered incomplete ORFs (underlined). These are potentially defective genes or pseudogenes containing either deletion, nonsense mutation, or frameshift mutation. The colors correspond to the inferred functional properties of the ORFs: red, drug resistance; yellow, transposases (for Tn*554*, IS*431*, and putative transposons); gray, plasmid replication (*rep* for pUB110); magenta, site-specific recombinase (N034 and N037); brown, metabolism and physiology; blue, plasmid replication (for pUB110); black, unknown function. CN051 (stippled green) corresponds to *orfX**. Three ORFs, N001 to N003, located outside *mec* DNA have unknown function (black). (c) Regional change of G+C content in and around *mec* DNA. The G+C content was calculated and plotted as a line graph by using the Window program of The Wisconsin Package, with a window size of 100 bases and a shift increment of 100. The yellow bar indicates the range of G+C content of *S. aureus* strains, which is 32 to 36% (34). The range delimited by the orange bars is that of the genus *Staphylococcus*, which is 30 to 39% (34). The regions with substantially lower than average G+C content (regions 1, 3, 4, 6) and with high G+C content (regions 2, 5, 7) are indicated by red bars and green bars, respectively.

TABLE 1. The ORFs in and around *mec* DNA whose deduced products show similarities to extant protein sequences

ORF[a]	Location (nt position[b]) start–stop	Size (bp)	Homology score[c]	% Identity[d]	Gene[e]	Description of gene product	Species[f]
CN009*	10999–10556	444	635.2	42.3	(*kdpC*)	Potassium-transporting ATPase (C chain)	*C. acetobutylicum*
CN010	12996–10975	2,022	4,878.6	56.5	(*kdpB*)	Potassium-transporting ATPase (B chain)	*C. acetobutylicum*
CN012*	13431–13018	414	758.1	45.9	(*kdpA*)	Potassium-transporting ATPase (A chain)	*C. acetobutylicum*
CN013*	14692–13358	1,335	2,195.0	48.7	(*kdpA*)	Potassium-transporting ATPase (A chain)	*C. acetobutylicum*
CN026	34531–33800	732	2,712.2	100	*ermA*	rRNA adenine *N*-6-methyltransferase	
CN030*	36670–36041	630	887.3	40.5	(*yrkJ*)	Protein with unknown function	*B. subtilis*
CN031	36960–36685	276	660.2	52.3	(*yrkD*)	Protein with unknown function	*B. subtilis*
CN034*	40759–39626	1,134	3,134.2	64.2	(*xylR*)	Xylose repressor	*Staphylococcus xylosus*
CN036	41614–41243	372	1,564.3	100	*mecI*	Methicillin resistance protein MecI	*S. aureus*
CN037	43371–41614	1,758	6,779.3	99.8	*mecR1*	Methicillin resistance protein MecR1	*S. aureus*
CN039*	46792–46160	633	657.5	32.4	(*ugpQ*)	Glycerophosphoryldiester phosphodiesterase	*E. coli*
(CN051)	56817–56338	480	1,121.6	59.7	(*yydA*)	Protein with unknown function	*B. subtilis*
(N001)	423–683	261	698.2	50.0	(*yrkD*)	Protein with unknown function	*B. subtilis*
(N002)*	683–1438	756	910.4	39.9	(*yrkJ*)	Protein with unknown function	*B. subtilis*
(N003)*	1863–2198	336	744.7	72.8	(*yfjN*)	Protein with unknown function	*B. subtilis*
N021*	14975–17629	2,655	2,130.1	28.3	(*kdpD*)	Sensor protein KdpD	*C. acetobutylicum*
N022	17604–18299	696	1,782.6	60.8	(*kdpE*)	KDP operon transcriptional regulatory protein KdpE	*C. acetobutylicum*
N026*	19527–19751	225	489.4	55.9	(*orfA*)	Putative transposase	*B. anthracis*
N027*	19826–20179	354	587.6	44.2	(*orfB*)	Putative IS*150*-like transposase	*B. anthracis*
N028*	20180–20578	399	801.3	55.6	(*orfB*)	Putative IS*150*-like transposase	*B. anthracis*
N034	24137–25486	1,350	400.7	26.5		Integrase of bacteriophage TP901-1	*L. lactis*
N037	25508–27136	1,629	720.1	37.4		Integrase of bacteriophage TP901-1	*L. lactis*
N044*	28947–29264	318	517.1	41.6	(*orfB*)	Homologue of *E. coli radC* product	*B. subtilis*
N045	29383–30468	1,086	4,270.5	99.7	*tnpA*	Transposase A (transposon Tn*554*)	
N046	30465–32357	1,893	7,043.1	100	*tnpB*	Transposase B (transposon Tn*554*)	
N047	32364–32741	378	1,282.4	100	*tnpC*	Transposase C (transposon Tn*554*)	
N048	32892–33674	783	2,873.2	100	*spc*	Adenyltransferase (AAD9)	
N052	37081–38145	1,065	879.1	49.4	(*yrkE*)	Protein with unknown function	*B. subtilis*
N058	43471–45477	2,007	7,319.4	100	*mecA*	Penicillin binding protein 2′	
N062	48054–48728	675	2,573.2	100		Transposase for insertion sequence-like element IS*431mec*	
N063	48921–49691	771	3,393.2	100	*aadD*	Kanamycin nucleotidyltransferase	
N064	49914–50312	399	1,629.4	100	*ble*	Bleomycin resistance protein (BRP)	
N066	50819–52081	1,263	3,833.5	100	*pre*	Plasmid recombination enzyme	
N068*	52305–53099	795	3,322.1	99.2	*rep*	Replication protein	
N069*	53100–53309	210	954.5	100	*rep*	Replication protein	
N070	53400–54074	675	2,573.2	100		Transposase for insertion sequence-like element IS*431mec*	

[a] ORFs shown in parentheses were located outside of the *mec* DNA region. Asterisks signify incomplete ORFs that are potentially defective genes or pseudogenes containing either deletion, nonsense mutation, or frameshift mutation.
[b] Nucleotide (nt) position in the nucleotide sequence deposited under accession no. D86934 in the DDBJ, EMBL, and GenBank databases.
[c] Optimal z score in analysis with TFastA.
[d] Identity to the amino acid sequence of the best match revealed in the homology search of the GenBank and EMBL databases with TFastA.
[e] Genes shown in parentheses had deduced ORF with less than 70% amino acid identity to the ORF indicated.
[f] The species whose gene product had the best match to the ORF product.

rounds of PCR, respectively. The primers cR1 and cR2 were located in *orfX* (Fig. 3), and cL1 and cL3 were located in the chromosomal region outside the left boundary of the *mec* DNA (Fig. 1a). Figure 4 shows that the sensitivity of the first round of PCR was such that it could detect the *att*B region of NCTC8325 total DNA which had been mixed with N315 total DNA at the ratio of 1:10,000 (wt/wt). At this level of sensitivity, no amplified band was detected when N315 DNA was used as a template for PCR; the second round of PCR (nested-PCR), however, did amplify a DNA fragment. The DNA fragment was purified and was subjected to nucleotide sequence determination. The sequence contained the *att*B sequence, which was similar to that of NCTC8325 and was identical to the corresponding sequences, *att*L and *att*R, of N315. The data indicated that *att*B was generated from the N315 chromosome as a result of spontaneous precise excision of *mec* DNA. The result suggested that the precise excision of *mec* DNA occurred in N315 at a low frequency: less than 1 in 10^4 cells.

DISCUSSION

The *mecA* gene is known to be located between the markers *nov*, encoding DNA gyrase, and *spa*, encoding protein A, on the same *Sma*I-generated chromosomal fragment in several different strains (19, 27). In this study, we have determined the location and boundaries of *mec* DNA. *mec* DNA was found to be integrated inside *orfX*, which is located on the *Sma*I-G fragment of the *S. aureus* type strain NCTC8325 (data not shown). We have tested strains by PCR with primers cR2 and cR6 (Fig. 3), and we found that the *orfX* is present in all 55 *S. aureus* strains (13 MSSA strains and 42 MRSA strains) tested so far. Therefore, *orfX* seems to have an essential function in the *S. aureus* genome. The preservation of the reading frame of *orfX* even after integration of *mec* DNA in N315 further supports its importance. However, its function cannot be inferred from the amino acid sequence since there is no homologue whose function has been elucidated in the database.

The structural features of the boundaries of *mec* DNA,

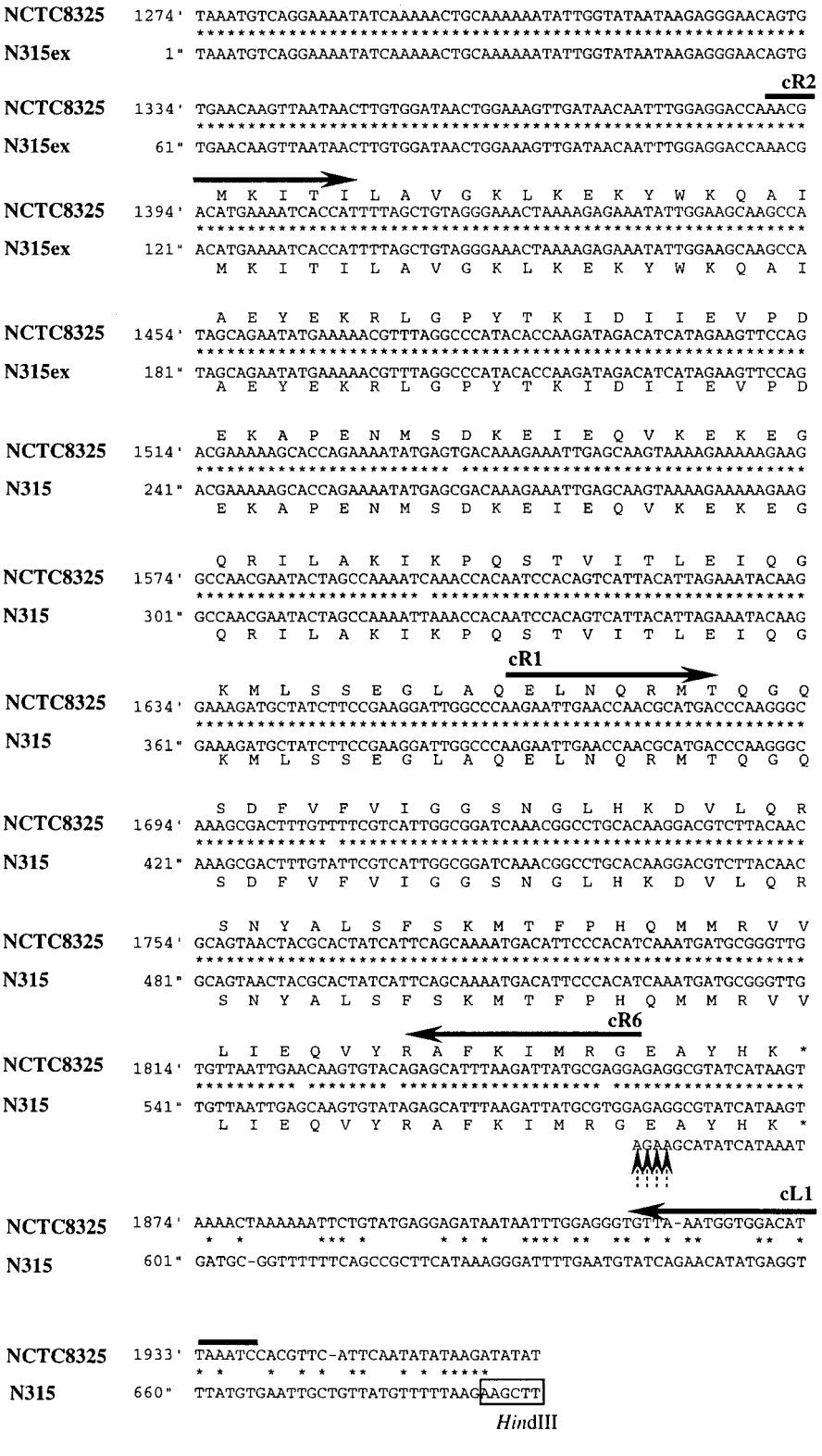

FIG. 3. Nucleotide and predicted amino acid sequences of *orfX* genes of NCTC8325 and *orfX** genes of N315. The nucleotide sequence of the *orfX* gene of NCTC8325 located on the plasmid pLE1a was sequenced and compared to *orfX** of N315. A possible integration site of *mec* DNA is indicated by a dotted arrow; the nucleotide position was ambiguous but within four nucleotides. Arrows indicate the locations and directions of primers used for the PCR assay. The accession number for the sequence of pLE1a is given in the Materials and Methods section.

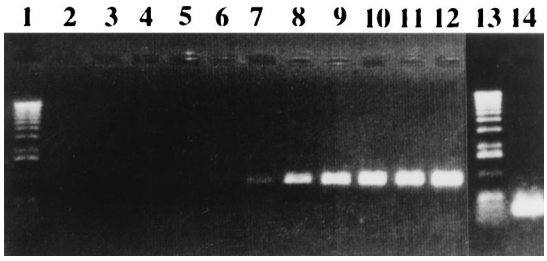

FIG. 4. PCR for the detection of spontaneous precise excision of *mec* DNA. Two sets of primers were used. The primers cR2 (5′-AAACGACATGAAAAT CACCAT-3′) and cL3 (5′-TGAAACTTCATTGGTATATT-3′) were used as the outer primers in (the first round of) PCR to detect generation of a DNA fragment containing *att*B, and the primers cR1 (5′-AAGAATTGAACCAACG CATGA-3′) and cL1 (5′-ATTTAATGTCCACCATTTAACA-3′) were used as the inner primers in (the second round of) PCR to generate a DNA fragment of 275 bp in *att*B. The results of the first round (lanes 2 to 12) and the second round (lane 14) of PCR are shown. Ten nanograms each of chromosomal DNA of N315, NCTC8325, and the mixtures (wt/wt) of N315 and NCTC8325 DNAs at the ratios indicated below were used as templates. Lanes: 1 and 13, 1-kb ladder (Gibco-BRL, Gaithersburg, Md.); 2, N315; 3 to 11, mixtures of N315 and NCTC8325 DNAs at ratios of $1{:}10^{-8}$, $1{:}10^{-7}$, $1{:}10^{-6}$, $1{:}10^{-5}$, $1{:}10^{-4}$, $1{:}10^{-3}$, $1{:}10^{-2}$, $1{:}10^{-1}$, and 1:0.5, respectively; 12, NCTC8325; 14, N315 (nested PCR).

which might reflect the integration mechanism of *mec* DNA were (i) the presence of terminal inverted repeats of 27-bp length and (ii) the presence of direct repeats of 15-bp length: one of the latter was situated in the right extremity of *mec* DNA, and the other was situated outside *mec* DNA and abutting the left boundary. The presence of terminal inverted repeats and duplication of target sequences are characteristic features of most transposons. There were no direct repeats, however, reflecting the target duplication around *mec* DNA. The characteristic deployment of the direct repeats found inside and outside the *mec* DNA is reminiscent of cassette gene integration of an integron (29, 39). However, no ORF possibly encoding integrase, like that characteristically found in the integron machinery, was found in the chromosomal area around *att*B. Therefore, the structural features at the boundaries of *mec* DNA suggested that *mec* DNA is integrated into the chromosome by an as yet unknown recombination process.

As a possible mobile genetic element, the size (52 kb) of *mec* DNA was comparable only to those of bacteriophages, conjugative transposons, or a pathogenicity island (PI). PIs, large (35 to 190 kb) chromosomal DNA segments each carrying a cluster of genes encoding virulence factors, have recently been identified in various gram-negative enteric bacteria (22). However, *mec* DNA did not carry structural genes (or their remnants) encoding head or tail proteins of bacteriophages or any ORFs predictably encoding any virulence factors as far as we could judge from the homology search over extant gene products. It also lacked *tra* gene complexes, which are crucial components for the intercellular transfer of conjugative transposons (9, 28). Therefore, *mec* DNA was distinct from any of the above-described genetic elements.

The *mec* DNA of N315 carried a total of 28 ORFs whose encoded functions were inferable based on the homology search. The seven genes associated with antibiotic resistance, *mecI*, *mecR1*, *mecA*, *spc*, *ermA*, *ble*, and *aadD*, had intact coding frames. Of the rest of the ORFs, however, 13 appeared to be incomplete (Table 1). Prior to the disruption of the coding frames, these ORFs might have encoded products with physiological functions in a bacterial chromosome like those encoded by housekeeping genes; e.g., the *kdp* operon gene homologues might have engaged in ATP-dependent potassium transport, and the *xylR* gene homologue might have been involved in xylose metabolism. It is possible that some of these ORFs were located along with *mecA* gene complex on the chromosome of the bacterium from which the primordial *mec* DNA originated and subsequently were mutated or disrupted during the process of molecular evolution of *mec* DNA into its present structure. While the *mecA* gene complex has remained intact, as it conferred selective advantage to the host cell (i.e., β-lactam resistance), most of the housekeeping genes carried by the primordial *mec* DNA may have been disrupted because they were redundant or even toxic in the foreign physiological milieu of some staphylococcal species to which the *mec* DNA had been transferred.

Besides antibiotic resistance genes, all the three transposase genes, *tnpA*, *tnpB*, and *tnpC*, encoding proteins involved in the movement of Tn*554*, were found to be intact. This indicates that the transposon was integrated later than other pseudogenes during the process of formation of the *mec* DNA in its present form in N315. Tn*554* has been identified not only in *S. aureus* but also in other C-NS species (45). Recently, we found a methicillin-resistant *S. epidermidis* strain, G3, on whose *mec* DNA a copy of Tn*554* is found at exactly the same location relative to the *mecA* gene complex as that in *mec* DNA of N315 (17a). Therefore, it is probable that the copy of Tn*554* is transferable as an integrated member of the *mec* DNA between *S. aureus* and other staphylococcal species.

The plasmid pUB110, also carrying two intact drug resistance genes, may also have been acquired relatively recently as compared to the pseudogenes. The integrated copy of pUB110 was flanked by two IS*431* (25). IS*431* is postulated to serve as a portal of entry of the transposons or plasmids carrying various antibiotic resistance genes into MRSA chromosome. The repertoire of resistance genes accumulated by the IS*431*-mediated recombination differs from strain to strain; for example, plasmid pT181 (carrying tetracycline resistance) and a mercury resistance gene have been found in an Australian MRSA strain (11). However, there also exist MRSA strains with no other antibiotic resistance genes associated with IS*431mec*. NCTC10442, the first MRSA strain isolated in the world, in England in 1961, is one such strain carrying the intact copy of IS*431mec*, but no other resistance gene is associated with it (17b). Therefore, the diversification of *mec* DNA by accumulation of resistance genes via IS*431*-mediated recombination may have occurred rather recently, probably after the establishment of *mec* DNA in the chromosome of *S. aureus*. A mutation was found in the *rep* gene of pUB110. The gene encodes replication of the plasmid. Therefore, the mutation might have served for the stabilization of the integrated copy of pUB110 after its integration into the chromosome of N315.

It is interesting that the *mecA* gene of N315 had quite a few nucleotide sequence differences from those previously reported in other strains (38) but was identical to that in *S. epidermidis* (31). As opposed to other MRSA strains, N315 is a pre-MRSA, in which transcription of *mecA* gene is strongly repressed by the *mecI* gene-encoded repressor function (20). Assuming that the direction of *mecA* gene transfer was from other staphylococcal species to *S. aureus*, as proposed by Archer (2), it is an attractive hypothesis that N315 retains the *mecA* gene in its more original form, which subsequently was mutated in MRSA cells to adapt to the *S. aureus*-specific cell wall synthesis machinery.

The two ORFs, N034 and N037, which showed homology to site-specific recombinases belonging to the invertase-resolvase family were found in the midst of *mec* DNA. Invertases are a well-known cause of DNA rearrangement in bacterial chromosomes or phage genomes resulting in the alteration of gene expression; e.g., Hin causes the phase transition from one

flagellar antigen to another in *Salmonella*, and Gin, Cin, and Pin cause the inversion of specific DNA segments of bacteriophages which control their host ranges (12). Resolvases of the Tn*3* type, also a member of the resolvase-invertase family, bind to the resolution site and accomplish transposition by resolving cointegrate (an intermediate of transposition) into two independent replicons (35). Most DNA invertases and resolvases are 180 to 190 aa in size and have a well-defined two-domain structure (1, 35). Although the predicted sizes of N034 and N037 are about two to three times larger than the site-specific recombinases of the invertase-resolvase family, the N-terminal thirds of the two proteins have substantial homology with the N-terminal domains of recombinases of the invertase-resolvase family; the domains are implicated in the strand-exchange function of site-specific recombination. The nested-PCR and sequencing experiment performed by using primers bracketing the *att*B sequence indicated that a recombination enzyme that can precisely excise *mec* DNA out of the chromosome is encoded on the N315 genome. The enzyme(s) responsible for the spontaneous *mec* DNA excision is thought to be a novel site-specific recombinase. In this regard, the ORFs N034 and N037 are good candidates for the genes responsible for the *mec* DNA-specific excision. Experiments are under way to clarify the function(s) of the products of the two ORFs.

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