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Suppression of Methicillin Resistance in a *mecA*-Containing Pre-Methicillin-Resistant *Staphylococcus aureus* Strain Is Caused by the *mecI*-Mediated Repression of PBP 2′ Production

KYOKO KUWAHARA-ARAI, NORIKO KONDO, SATOSHI HORI, EIKO TATEDA-SUZUKI, AND KEIICHI HIRAMATSU*

Department of Bacteriology, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113, Japan

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The mechanism of methicillin susceptibility was studied in *Staphylococcus aureus* N315P, a pre-methicillin-resistant *S. aureus* strain that is susceptible to methicillin, despite the presence of *mecA* in the chromosome. In the presence of *mec* regulator genes *mecI* and *mecR1*, transcription of the *mecA* gene was not inducible by the addition of methicillin to the culture medium. Inactivation of the *mecI* gene function by replacing it with *tetL* made N315P express heterogeneous-type methicillin resistance. The subclone, in which the *mecI* gene was replaced, subclone PΔI, produced 12 times greater amounts of *mecA* gene transcripts and 8.5 times more PBP 2′ protein than N315P. These data indicate that the *mecI* gene-encoded repression of *mecA* gene transcription is responsible for the apparent methicillin susceptibility phenotype of pre-methicillin-resistant *S. aureus* N315P.

Methicillin-resistant *Staphylococcus aureus* (MRSA) carries the methicillin resistance gene *mecA* on its chromosome (15). The gene encodes the MRSA-specific penicillin-binding protein (PBP) PBP 2′ or PBP 2a; the PBP has reduced affinities of binding to most beta-lactam antibiotics (8, 21, 32). However, a group of clinical staphylococcal strains have the *mecA* gene but test susceptible to methicillin (2, 20, 27). N315, one such strain, produced methicillin-resistant subclones with a frequency of 1 in 10^5 (11, 26). We have proposed the term "pre-MRSA" for the group of *mecA* gene-carrying, methicillin-susceptible *S. aureus* strains (9). In N315, PBP 2′ is not inducible with methicillin in the culture medium (11, 26). Furthermore, this apparent suppression of PBP 2′ production is not eliminated following the loss of a penicillinase plasmid from the strain (26; this study). Subsequent study has revealed that N315 possesses the regulator genes *mecI* and *mecR1*, which are located upstream of the *mecA* gene (10, 11). The genes are so named because their predicted products are homologous with those of *bla* regulator genes (10, 13). A repressor is thought to be encoded by *mecI*, and a signal transducer with antirepressor activity is thought to be encoded by *mecR1* (10, 11, 13, 28).

In most clinical staphylococcal strains classified as methicillin resistant by standard procedures, PBP 2′ production is inducible with methicillin (26, 31; unpublished data). In about 40% of these strains the *mecI* gene is deleted, leaving at least a portion of the *mecR1* structural gene upstream of *mecA* (26). In the rest of the strains, *mecI* genes are present but contain nonsense point mutations (26). Some strains retain intact *mecI* genes, but point mutations are invariably identified in the presumptive operator of the *mecA* gene (9). These observations suggested that *mecI* encodes a repressor of *mecA* transcription and that the loss of *mecI* function either by deletion or by mutation is a necessary step in the production of PBP 2′ and the expression of methicillin resistance (9, 11, 26). This study was designed to provide concrete evidence for the *mecI*-mediated repression of methicillin resistance in pre-MRSA N315.

* Corresponding author. Phone: 81-3-5802-1040. Fax: 81-3-5684-7830. Electronic mail address: hiram@med.juntendo.ac.jp.

MATERIALS AND METHODS

Bacterial strains. Pre-MRSA N315 and its penicillinase plasmid-free derivative, N315P, were used in this study and have been described previously (9–11, 20, 26). PΔI is a *mecI* mutant of N315P obtained by replacing *mecI* with *tetL* (Fig. 1). P::DI-2 is a cointegrate of plasmid pDI-2 (see below) in the *mec* regulator region, in which the *mecI* gene is displaced from its original position (Fig. 1). N315P* is a methicillin-susceptible revertant obtained from P::DI-2 by cultivation in a drug-free medium and by subsequent replica plating on a methicillin-containing plate.

Plasmids. The *Nde*I fragment containing the entire *mecI* gene and a portion of *mecR1* gene from pES1 (Fig. 2A) (10) was subcloned into the pUC18 vector. Two constructs, pMI7 and pMI9, were obtained (Fig. 2B and C). The multiple cloning site was eliminated from pMI7 by *Hin*cII digestion and self-ligation to obtain pMI7′ (Fig. 2D). Most of *mecI* was deleted from the plasmid pMI7′ by *Acc*I and *Pst*I double digestion; this was followed by the insertion of the *tetL* gene derived from pHY300PLK (14) to obtain pDI7Tc (Fig. 2E). The *tetL* gene was obtained by PCR amplification with pHY300PLK as a template by using the synthetic oligonucleotides 5′-ATTGTCGACCTGTTATAAAAAAAGGATC (an introduced *Acc*I site is underlined) and 5′-ATTTCTGCAGTTCAACAAA CGGGCCATA (an introduced *Pst*I site is underlined). For the construction of pDI-2 (Fig. 2F), the 3.9-kb *Eco*RI fragment from PLTV1 (3) containing the pE194 thermosensitive replication origin was ligated into the *Eco*RI site of PDI7Tc. PLTV1 was a kind gift from P. Youngman. Two recombinant plasmids, pRI carrying the intact *mecI* gene (Fig. 2G) and pRdI carrying partially deleted *mecI* (Fig. 2H), were constructed as follows. Plasmid pRI was constructed by inserting the *mecI*-containing *Hin*dIII fragment of plasmid pMI9 into the *Hin*dIII site of a shuttle vector, pRIT5 (carrying a chloramphenicol resistance gene as a selective marker; Pharmacia LKB Biotechnology, Tokyo, Japan). Plasmid pRdI was constructed by deleting *mecI* from pRI by *Pst*I digestion and self-ligation.

Electroporation. N315P was the recipient of *mecI*-gene-directed recombination with pDI-2. An exponentially growing culture of N315P was harvested at an optical density at 600 nm of 0.5 and was washed three times with prechilled 1.1 M sucrose. The pellet was resuspended in 50 μl of EP buffer (1.1 M sucrose, 2 mM $MgCl_2$, 14 mM KH_2PO_4-Na_2HPO_4 [pH 7.4]). A total of 5 μg of the plasmid DNA was added to 50 μl of the cell suspension, and the mixture was kept on ice for 25 min. An electric pulse of 25 μF, 2.5 kV, and 100 Ω was delivered with a Gene Pulser (Nippon Bio-Rad Laboratories, Tokyo, Japan). The cells were then transferred into 0.5 ml of 1.1 M sucrose in brain heart infusion broth, and the mixture was incubated for 2 h at 30°C (permissive temperature for plasmid replication) before aliquots were plated onto DM3 (4) containing 10 μg of tetracycline per ml. After 48 h of incubation at 30°C, mature colonies were picked and subjected to rapid plasmid analysis by digestion with various restriction enzymes to confirm the integrity of the introduced plasmid.

Southern blotting and hybridization. Southern blotting and hybridization were used to analyze the chromosomal DNA recombined with all or part of the temperature-sensitive recombinant plasmid. The method of DNA extraction has been described previously (6, 25). Hybridization took place in a hybridization solution (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2% blocking reagent [Boehringer Mannheim, Mannheim, Germany], 0.1% *N*-lauroylsarcosine, 0.01% sodium dodecyl sulfate [SDS]) containing 20 ng of digoxigenin-labeled DNA probe per ml. The incubation time was 16 h at 42°C.

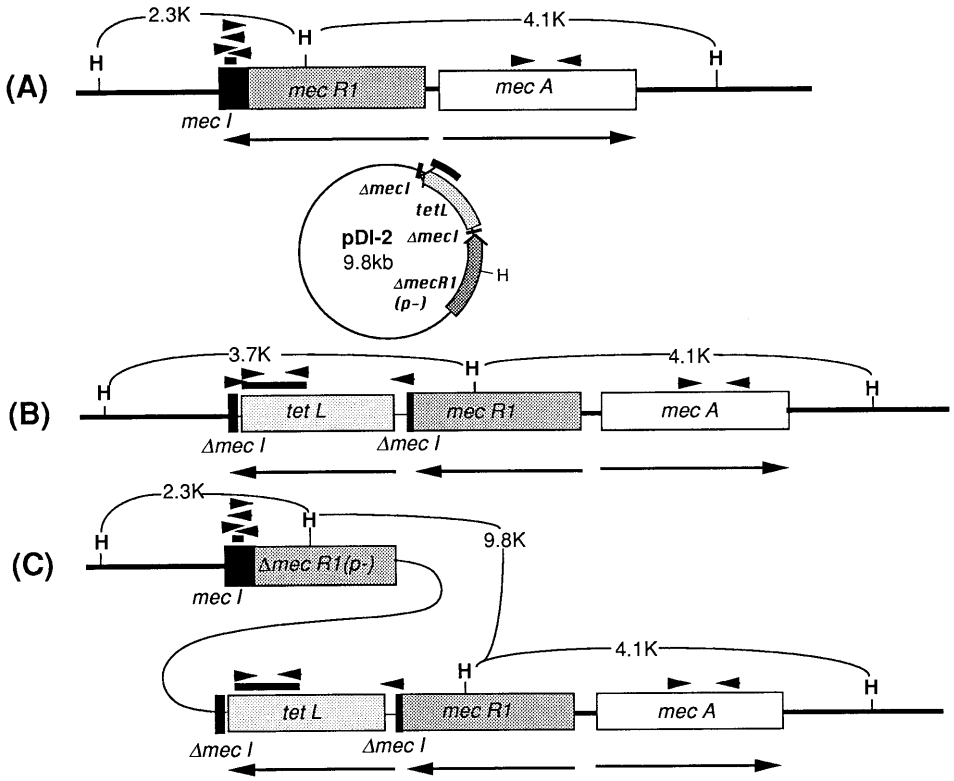

FIG. 1. Restriction maps of the *mec* regulatory region of N315P-derived strains. (A) N315P(pDI-2); (B) PΔI; (C) P::DI-2. The thick bars show the locations of the probes used for Southern blot hybridization. Arrows indicate the direction of transcription of the structural genes *tetL*, *mecA*, *mecR1*, and *mecI*. Arrowheads indicate the location and direction (from the 5′ to the 3′ end) of the PCR primers used for the analysis of the *mec* regulator region. N315P(pDI-2) had an intact *mec* regulatory region and harbored plasmid pDI-2 in the cytoplasm (A). *mecI* of PΔI was replaced with *tetL* (B). The entire plasmid pDI-2 was integrated into the chromosome of P::DI-2, and *mecI* was displaced from its original location (C). Δ*mecI* signifies a fragment remaining after removal of the *Acc*I-*Pst*I portion of the *mecI* structure gene. Δ*mecR1*(*p*−), the promoter region and the 5′ part of the *mecR1* gene are absent; K, kilobases.

The *mecI* gene probe was prepared by using primers 5′-AAAAGGATCCGGA AGTTATGAATATCATTTGGAT (an introduced *Bam*HI site is underlined) and 5′-AAAAGGATCCGATCTATAAAATCCCTTTTTATAC (an introduced *Bam*HI site is underlined), which correspond to the nucleotides from base positions 2020 to 2043 and to the complementary nucleotides from positions 2137 to 2160 of the reported nucleotide sequence of the *mecI* gene, respectively (10). The *tetL* gene probe was prepared by using primers 5′-CCAAAGTTGAT CCCTTAACG and 5′-AGATCCTTTTGTTGATCCCG, which corresponded to the reported nucleotides (14) from positions 78 to 97 and the complementary nucleotides from positions 726 to 745, respectively. These probes were labeled with digoxigenin by using the DNA labeling kit (Boehringer Mannheim). The hybridized filter (Biodyne A; Pall Biosupport Co., Glen Cove, N.Y.) was washed twice in 2× SSC with 0.1% SDS for 15 min at 68°C. Visualization of the signal was achieved with an alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) and the chemiluminescent substrate CSPD (Tropix) by the procedure recommended by the manufacturers.

Population analysis. Analysis of the methicillin-resistant subpopulation of strain N315 and its subclones was performed as follows. A 50-μl aliquot of an undiluted or appropriately diluted overnight culture was spread onto the heart infusion agar plates containing various concentrations of methicillin by using an Autoplate TM model 3000 instrument (Spiral Biotech, Inc., Bethesda, Md.). The plates were then incubated at 37°C for 48 h before the colonies were enumerated. The population curve was drawn by calculating and plotting the number of resistant cells theoretically contained in 50 μl of the undiluted culture.

Reverse transcription PCR. An overnight culture was diluted 1:20 with L broth, and the mixture was incubated at 37°C (with shaking) to a cell density of 7×10^8 CFU/ml. Cells were collected from 1.5 ml of the culture by centrifugation at 15,000 × *g* for 2 min at 4°C, and then the supernatant was removed. The cell pellet was resuspended in 0.25 ml of lysis buffer (100 μg of lysostaphin per ml, 20% sucrose, 20 mM Tris-HCl [pH 6.7], 10 mM EDTA, 50 mM NaCl) and was incubated for 10 min on ice. The RNA was extracted with ISOGEN as described by the manufacturer (Nippon Gene, Toyama, Japan). The concentration of RNA and its purity were determined by measuring the optical densities at 260 and 280 nm. One microgram of RNA was incubated with 1 U of RNase-free DNase I (Boehringer Mannheim) at 37°C for 30 min. The samples were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1); this was followed by ethanol precipitation. The reverse transcription reaction occurred in a reaction volume of 17.5 μl containing 1 μl of 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3]), 4 μl of 25 mM $MgCl_2$, 4 μl of 2.5 mM deoxynucleoside triphosphate (dNTP) mix, 1 μl of the downstream primer (200 ng per 20 mer per μl), 4.5 μl of diethylpyrocarbonate-treated H_2O, and 3 μl of total RNA (100 ng) as a template. After denaturation at 65°C for 15 min and cooling for 5 min at 5°C, 1 μl of 10× reaction buffer, 1 μl of murine leukemia virus reverse transcriptase (5 U), and 0.5 μl of RNase inhibitor (10 units) were added to make the final volume 20 μl. The reaction was performed by using a Gene Amp PCR system 9600 (Perkin-Elmer, Branchburg, N.J.) with 60 min of a reverse transcription reaction at 42°C and then 5 min of denaturation at 95°C and 5 min of cooling at 5°C. PCR amplification of cDNA required 3 μl of 10× reaction buffer, 2.4 μl of 2.5 mM dNTPs, 1 μl of the upstream primer (200 ng per 20mer per μl), 0.3 μl (5 U/μl) of *Taq* polymerase (Perkin-Elmer), and 21.5 μl of diethylpyrocarbonate-treated H_2O to reach a final reaction volume of 50 μl. The thermal cycling was set at 30 cycles, with 30 s of denaturation at 94°C, 30 s of annealing at 53°C, and 45 s of elongation at 72°C; these steps were followed by incubation for 10 min at 72°C. The synthetic oligonucleotides used as upstream primers were 5′-CGTATGAAATATCATC TGCAG-3′ (corresponding to positions 1995 to 2015) for *mecI* detection and 5′-GTTGGTCCCATTAACTCTGAA-3′ (corresponding to positions 766 to 786) for *mecA* detection. The downstream primers were 5′-CAAGTGAATTG AAACCGCCT-3′ (corresponding to the complementary nucleotides at positions 2276 to 2257) for *mecI* detection and 5′-TTACTGCCTAATTCGAGTGCT-3′ (corresponding to the complementary nucleotides at positions 1436 to 1416) for *mecA* detection (10, 24).

RNA blot hybridization. For the slot blot hybridization quantitation of *mecA* gene transcription, 1:3 serial diluents (2.0, 0.66, and 0.22 μg) of RNA samples were blotted onto a sheet of nylon membrane (Biodyne B; Pall Biosupport Co.) with the Bio-Rad slot blot apparatus (Nippon Bio-Rad Laboratories, Tokyo, Japan). RNA samples were prepared as follows. Overnight cultures of strains N315 and N315P were diluted 1:20 in 20 ml of L broth, and the dilutions were incubated at 37°C with shaking to a cell density of 7×10^8 CFU/ml. Then, methicillin or cefoxitin was added to the culture at a concentration of 1 μg/ml. The induction time was ended by transferring 0.7 ml of the culture to a microcentrifuge tube containing 0.7 ml of prechilled (0°C) acetone-ethanol (1:1; vol/

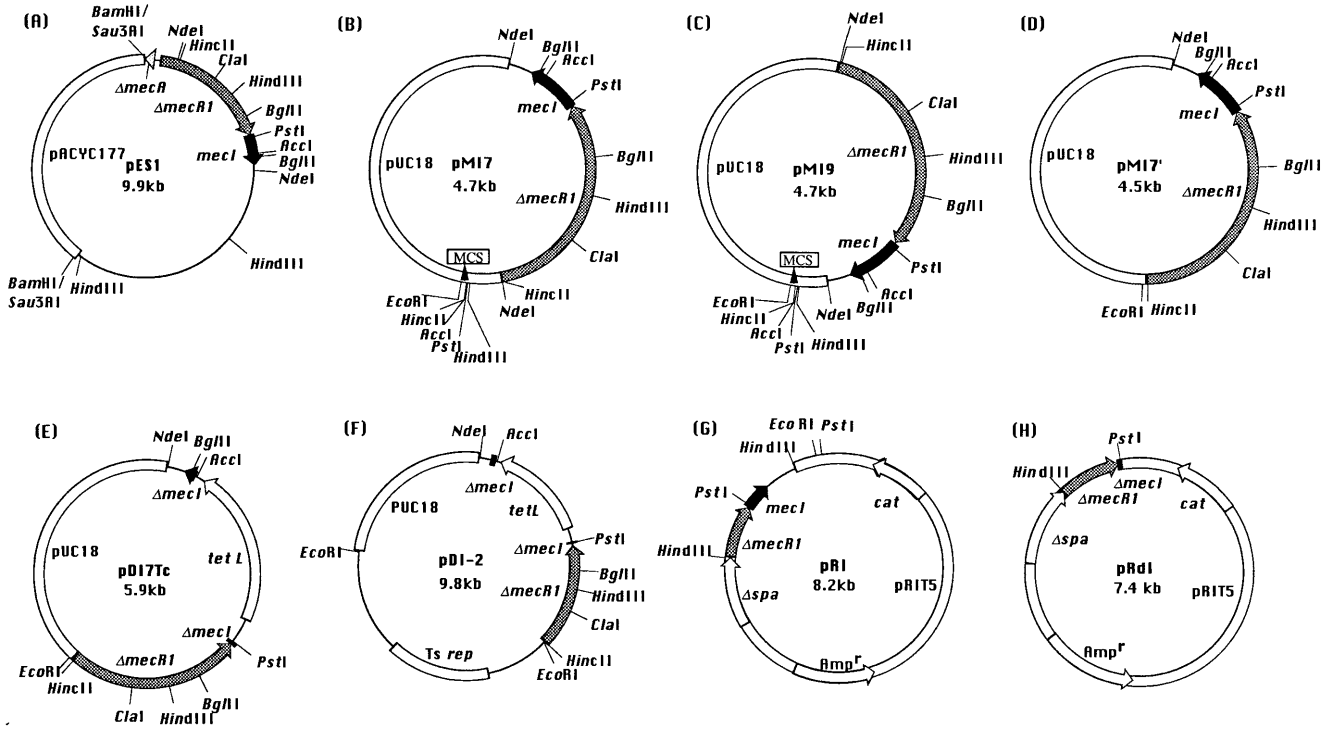

FIG. 2. Structure of the plasmids used in this study. (A to F) Construction of pDI-2. The *Nde*I fragment containing *mecI* and a part of *mecR1* was removed from pES1 (A) (9) and ligated into pUC18 to construct pMI7 (B) and pMI9 (C). The *Hin*cII fragment containing the multiple cloning site of pUC18 was removed from pM17 to obtain pMI7′ (D). The tetracycline resistance gene (*tetL*) was obtained from pHY300PLK (13) with *Acc*I and *Pst*I and inserted into pMI7′, replacing most (the *Acc*I-*Pst*I fragment) of the *mecI* gene, resulting in pDI7Tc (E). The temperature-sensitive replication origin (Ts *rep*) of pE194Ts was removed from pLTV1 (2) by *Eco*RI digestion and was inserted into the unique *Eco*RI site of pDI7Tc to obtain pDI-2 (F). For the construction of pRI (G), the fragment containing *mecI* was removed from pMI9 (C) with *Hin*dIII and ligated into the *Hin*dIII cloning site of *S. aureus-Escherichia coli* shuttle vector pRIT5. To obtain pRdI (H), *mecI* was deleted from pRI by *Pst*I digestion and self-ligation. MCS, multiple cloning site of pUC18; Ts rep, temperature-sensitive replication origin of pE194. Amp^r, ampicillin resistance marker; *cat*, chloramphenicol acetyltransferase gene; Δ*spa*, protein A gene with its 3′ end deleted.

vol). The cells were collected by centrifugation at 15,000 × *g* for 2 min at 4°C, and then the supernatant was removed. The subsequent procedure for the extraction of RNA from the cell pellet was essentially the same as that described above for reverse transcription PCR. The *mecA* probe corresponding to the nucleotides from position 1050 to 1335 of the reported nucleotide sequence of the *mecA* gene (24) was prepared by PCR amplification as described previously (25). Labeling of the probe, hybridization, and detection of the signal were carried out in the same manner as described above for Southern blot hybridization.

Western blotting. For Western blotting (immunoblotting), the cells were grown in 10 ml of L broth overnight, collected by centrifugation, washed once in 0.05 M Tris-HCl (pH 7.4) buffer, and then resuspended in 1 ml of the buffer containing 30 μg of lysostaphin and 10 μg of DNase I. After incubation for 30 min at 37°C, the membrane fraction was obtained by centrifugation at 100,000 × *g* for 30 min. The membrane preparation (20 μg of protein) was subjected to SDS-polyacrylamide gel electrophoresis (with an acrylamide concentration of 10%) and was then transferred to a sheet of nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) with a semidry electrophoresis apparatus (Bio-Rad, Richmond, Calif.). The membrane was probed with anti-PBP 2′ antibody (22, 23), and the signal was visualized with a colorimetric detection kit (AeroC; Abbott laboratories, North Chicago, Ill.). The anti-PBP 2′ antibody was a generous gift from M. Saito.

RESULTS

RNA blot analysis of *mecA* gene transcription. Figure 3 shows the induction of *mecA* gene transcription of strains N315 and N315P with methicillin and cefoxitin. Cefoxitin induced *mecA* gene transcription in N315 after 30 min of incubation (Fig. 3B). *mecA* gene transcription was induced in N315P at essentially the same amount over the same time course as in N315 (Fig. 3D). Thus, elimination of the penicillinase plasmid did not alter the mode of *mecA* gene inducibility of N315. On the other hand, methicillin did not induce *mecA* transcription in N315 even after 60 min of incubation (Fig. 3A). This unresponsiveness of N315 to methicillin was not influenced by the elimination of the penicillinase plasmid of N315 (Fig. 3C).

Isolation of *mecI* gene-replaced strains from N315P. A total of 1.2×10^7 CFU of N315P(pDI-2) was spread onto heart Infusion agar plates containing 10 μg of tetracycline per ml, and the plates were incubated at 43°C (nonpermissive temperature for the replication of plasmid pDI-2). Although incubation at 43°C inhibits replication of the plasmid, 4.7% of the original inoculum appeared to be insensitive to the temperature effect after 18 h of incubation. The colonies were then

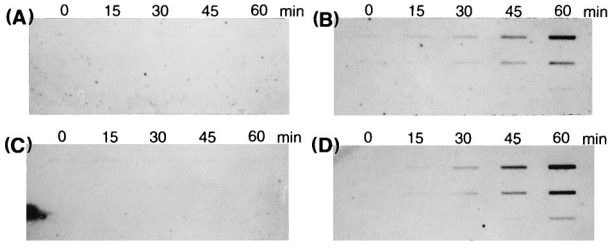

FIG. 3. Mode of *mecA* gene transcription in N315 and N315P. Total RNA extracted from each bacterial strain before and at 15, 30, 45, and 60 min after the addition of methicillin or cefoxitin (at a concentration of 1 μg/ml) to the culture and serially diluted three times (2, 0.66, and 0.22 μg from the top in each panel) was blotted and hybridized with a *mecA* gene probe. (A) N315 induced with methicillin; (B) N315 induced with cefoxitin; (C) N315P induced with methicillin; (D) N315P induced with cefoxitin.

replica plated onto the heart infusion agar plates containing 10 μg of methicillin per ml. The growth of colonies on the replica plates was observed after 18 h of incubation at 37°C. Forty-eight percent of the replicated colonies were methicillin resistant. Since in repeat experiments the rates of spontaneous methicillin-resistant mutants from strain N315P were in the range of 1 in 10^5 to 1 in 10^6, methicillin-resistant subclones appeared at least 5,000 times more frequently from N315P (pDI-2). With repeated replica plating tests of a total of 103 methicillin-resistant strains from N315P(pDI-2), two strains, including PΔI, remained stably methicillin resistant. The rest of the 103 strains represented by P::DI-2 were unstable in their methicillin resistance, and methicillin-susceptible revertants could be obtained by drug-free overnight culture at 37°C. Such methicillin-susceptible revertants represented by N315P* were obtained from P::DI-2 as follows. P::DI-2 was spread onto the heart infusion agar plate containing tetracycline (10 μg/ml), and the plate was incubated at 37°C. After 18 h, the colonies that had grown were replica plated onto the heart infusion agar plate containing 10 μg of methicillin per ml. About 2.9% of the subclones (6 of 208 colonies) were incapable of growth on the plate with methicillin. The methicillin-susceptible subclones were selected from the master plate and were then inoculated into 10 ml of fresh L broth; this was followed by incubation at 43°C for 18 h. Serial dilutions of the culture were then plated onto heart infusion agar without drug, and the plates were incubated overnight at 43°C. The colonies were then replica plated onto the heart infusion agar plates containing 10 μg of tetracycline per ml. Tetracycline-susceptible subclones were picked. The absence of the plasmid in the subclones was confirmed by rapid plasmid analysis and Southern blot hybridization (data not shown).

Genomic organization of the *mec* regulator region of the strains in which *mecI* was replaced. Figure 1 illustrates the genomic organization of the *mec* regulator region on the basis of Southern blot hybridization with *mecI* and *tetL* probes and PCR amplification experiments with various combinations of the primers shown in Fig. 1. Southern blot hybridization was performed with *Hin*dIII-digested genomic DNA. In PΔI, no band was detectable with the *mecI* probe, and the 3.7-kb fragment containing *tetL* and a part of *mecR1* was detected with the *tetL* probe. The data indicated that *mecI* was replaced by *tetL* in PΔI (Fig. 1B). On the other hand, a 2.3-kb fragment was observed with the *mecI* probe in N315P, N315P(pDI-2), and P::DI-2 (Fig. 1A and C). With the *tetL* probe, the 9.8-kb *Hin*dIII fragment corresponding to linearized plasmid pDI-2 was detected in N315P(pDI-2), since a *Hin*dIII-cutting site is unique to plasmid pDI-2 DNA (Fig. 1A). In P::DI-2, cutting with *Hin*dIII and probing with *tetL* probe also detected the characteristic 9.8-kb fragment, which indicated that all of plasmid pDI-2 was integrated into the chromosome of the cell since the strain did not harbor the pDI-2 plasmid, as determined by rapid plasmid analysis (data not shown). Subsequent analysis of P::DI-2 DNA by long PCR amplification with the primers encompassing the *mecI* and *mecR1* genes revealed a characteristic 11-kb band showing the cointegration of whole plasmid DNA into the *mec* regulator genes, displacing the *mecI* gene from its original location (Fig. 1C). The 11-kb band of P::DI-2 was lost in its subclone, N315P*, and instead, the 2.0-kb band reflecting the original *mecI-mecR1* gene structure was observed. This indicated that the cointegrated plasmid had been cut out from the chromosome by a reverse homologous recombination event (data not shown). The organization of the *mec* regulator region in N315P* was the same as that in N315, as far as we could judge from Southern blot hybridization and PCR data.

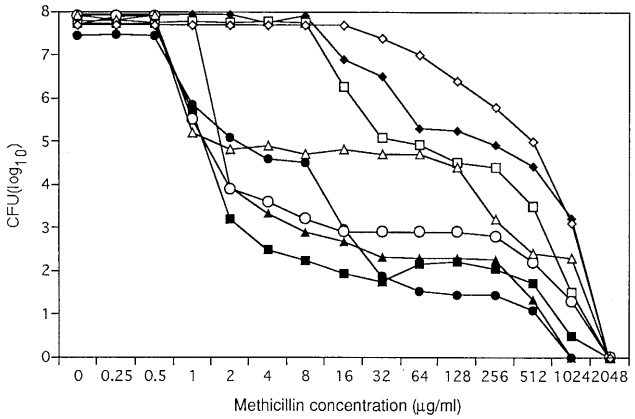

FIG. 4. Population analysis of N315, N315P, and N315P-derived substrains. The number of colonies growing on plates containing various concentrations of methicillin were counted after 48 h of incubation at 37°C and plotted on the graph. Symbols: ▲, N315; ■, N315P; ●, N315P(pDI-2); ◆, PΔI; □, P::DI-2; ○, N315P*; △, PΔI(pRI); ◇, PΔI/(pRdI).

Effect of *mecI* gene replacement on methicillin susceptibility of N315P. The results of analyses of resistant subpopulations of the strains in which the *mecI* gene was replaced compared with the parent strains are presented in Fig. 4. The growth of more than 99.99% of the cells of strain N315P was inhibited on plates containing more than 2 μg of methicillin per ml but the strains had tiny (10^{-6} to 10^{-5}) subpopulations that could grow in the presence of higher concentrations of methicillin. In contrast, an evident increase in the resistant subpopulations was observed in the strain PΔI in which the *mecI* gene was replaced. The patterns of the population curve for the strain showed a typical heterogeneous-type methicillin resistance, and according to the classification proposed by Tomasz et al. (30), it had a pattern of class III heterogeneity. A subpopulation of the parent strain that harbors plasmid pDI-2 [N315P (pDI-2)] also had an evident increase in the number of cells which could grow on plates containing 2 to 16 μg of methicillin per ml, suggesting spontaneous *mecI* gene replacement in the cell population. The resistance level of P::DI-2 was somewhat less than that of PΔI, but P::DI-2 also had a heterogeneous pattern of resistance. PΔI(pRI), carrying the plasmid into which *mecI*, was inserted, showed a substantial recovery of methicillin susceptibility in the presence of the lower concentrations of methicillin, but it still contained a cell subpopulation that could grow in the presence of high methicillin concentrations. Five independent colonies of PΔI(pRI) grown on plates containing 64 μg of methicillin per ml were picked up and analyzed for the integrity of plasmid pRI. For all five colonies, the plasmid's *mecI* gene was deleted (data not shown). We therefore think that the spontaneous loss of *mecI* from pRI was the reason for a resistant subpopulation in PΔI(pRI). The population curve for the methicillin-susceptible revertant N315P* showed a decrease in the resistant subpopulation of P::DI-2. However, the population did not completely revert to the susceptibility of N315P, and there was a greater population of cells which could grow on plates containing high concentrations of methicillin.

PBP 2′ production in N315P and strains in which the *mecI* gene was replaced. Figure 5 shows the results of detection of PBP 2′ by enzyme-linked immunosorbent assay with the specific monoclonal antibody. Only a small amount of PBP 2′ was detectable in N315P (Fig. 5, lane 1), and an increased amount of the protein was produced in PΔI and P::DI-2 (Fig. 5, lanes 2 and 3, respectively). Densitometric comparison of the PBP 2′

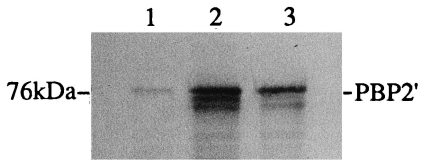

FIG. 5. PBP 2′ protein production in N315P and N315P strains in which the *mecI* gene was replaced. Western blotting and enzyme-linked immunological detection of PBP 2′ were performed with the cytoplasmic membrane preparations from N315P (lane 1), PΔI (lane 2), and P::DI-2 (lane 3).

band intensities showed that PΔI and P::DI-2 produced 8.5 and 7.5 times greater amounts of PBP 2′, respectively, than N315P. The level of PBP 2′ production in N315P* was low and at the same level as that in N315P (data not shown).

N315P and mutants were also tested with by reverse transcription PCR for the levels of transcription of the *mecA* and *mecI* genes. An increased level of *mecA* transcription was observed in PΔI and P::DI-2 compared with that in N315P (12:1 and 13:1, respectively). The level of *mecA* gene transcription in N315P*, which had reverted to methicillin susceptibility, was almost at the same low level as that in N315P. The relative amounts of *mecI* transcription in these strains were in a reverse correlation with those of *mecA* transcription. *mecI* transcription was not detectable in PΔI. The level of the transcription in P::DI-2, as judged from the amount of DNA amplified by reverse transcription PCR, was one-fourth of that in N315P. The level of *mecI* transcription in N315P* was the same as that in N315P. Therefore, the amount of PBP 2′ correlated well with the amount of *mecA* transcription and was inversely correlated with that of *mecI* transcription.

DISCUSSION

The *mecA* gene is responsible for methicillin resistance in practically all clinical MRSA strains, with only a few of exceptions (7, 12, 16, 29, 33). However, with more frequent use of PCR as a tool for the identification of MRSA from clinical samples has drawn attention to a small population of *S. aureus* strains that carry the *mecA* gene and yet for which methicillin MICs are less than the criteria established by the National Committee for Clinical Laboratory Standards for the identification of MRSA (19).

In our retrospective study, up to 16% of *mecA*-carrying clinical *S. aureus* strains at Tokyo University Hospital in 1982 were found to be methicillin susceptible (27), although in 1992 all the *mecA*-carrying *S. aureus* strains in the same hospital had distinctly high levels of methicillin resistance (27). Comparison of the various epidemiological markers of the strains isolated in 1982 and 1992 revealed the presence of the same lineage of strains in both years; one of the strains, strain 82/20-1, isolated in 1982, had an intact *mecI* gene and was methicillin susceptible, while by 1992 the others had mutated *mecI* genes and distinct methicillin resistance (27; unpublished data). This observation indicates that *mecI* gene destruction and the development of methicillin resistance occurred in the hospital environment, just as we observed in vitro by exposing a pre-MRSA strain to selective concentrations of beta-lactam antibiotics (9, 11, 27).

Not all the *mecA*-carrying, methicillin-susceptible clinical isolates conform to the category of pre-MRSA. They are classified into at least two categories. The first group is susceptible to all the beta-lactam antibiotics tested, such as methicillin, oxacillin, imipenem, cefazolin, cefoxitin, ceftizoxime, and moxalactam, and yielded no beta-lactam-resistant subclones when up to 10^9 CFU of the cells were exposed to the selective concentrations of beta-lactam antibiotics (unpublished data). The reason for this beta-lactam susceptibility is unknown, but it may be mutational inactivation of *mecA*. The second category of strains were those susceptible to methicillin but with a substantial level of resistance to some other beta-lactam antibiotics such as moxalactam, ceftizoxime, and cefoxitin (20). Subclones with resistance to practically all beta-lactam antibiotics are easily established from this category of cells by in vitro selection. Nucleotide sequence analysis of the *mec* regulatory regions of strains N315, 82/20-1, and 93/H44, which belong to the second category of strains, revealed that the intact *mecI* gene is in close proximity to the *mecA* gene and is conserved in all three strains (9). This is significant, because in most of the MRSA strains isolated in 20 countries, the *mecI* gene shows deletional loss or nonsense mutations (9, 26). The present study has shown that the *mecI* gene function is the most likely explanation for the apparent methicillin susceptibility in the second category of *mecA*-carrying, methicillin-susceptible strains, for which we have proposed the term pre-MRSA (9).

It is intriguing that although strain N315P* reverted to methicillin susceptibility, it had a substantially larger subpopulation of cells which could grow in the presence of high concentrations of methicillin compared with that for N315P (Fig. 4). One possibility that could account for this phenomenon is that an unexpected mutation that slightly raises the level of methicillin resistance occurred in P::DI-2, e.g., at a gene involved in cell wall synthesis. The derepressed PBP 2′ production in P::DI-2 itself might have served as a selective pressure from within the cell, since PBP 2′ is a rather inefficient PBP compared with the intrinsic PBPs of *S. aureus* (5). Actually, cell growth is slightly dampened when PBP 2′ production is derepressed (9a). A mutant that had overcome this defect in growth rate might have overgrown the culture. Another possibility is that the *mec* regulator region suffered a mutation during the excision of plasmid pDI-2, which partially relieved *mecA* repression in N315P*. Studies are under way to distinguish the two possibilities.

Experimental inactivation of *mecI* in strain N315P led to the production of PBP 2′ and the expression of methicillin resistance. However, it was evident that the level of methicillin resistance in the strains in which the *mecI* gene was replaced was of the heterogeneous type. Subsequent exposure of PΔI to higher concentrations of methicillin (>100 μg/ml) can select homogeneously or highly methicillin-resistant subclones at a high frequency of 0.01 to 0.1% (unpublished data). The amount of PBP 2′ expressed by one of the highly resistant subclones was comparable to that expressed by the parent strain, PΔI (unpublished data). Therefore, it seems likely that the expression of a genetic factor(s) other than *mecA* gene is involved in the expression of homogeneous methicillin resistance, as has previously been proposed by several researchers (1, 17, 18).

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