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Analysis of *Mec* Regulator Genes in Clinical Methicillin-resistant *Staphylococcus aureus* Isolates according to the Production of Coagulase, Types of Enterotoxin, and Toxic Shock Syndrome Toxin-1

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ABSTRACT

The intrinsic resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) is frequently explained by the production of an additional penicillin-binding protein (PBP), which is encoded by the *mecA* gene. The *mec* regulator genes, *mecR1* and *mecI*, was identified in *mecA*-carrying *Staphylococcus aureus* N315. Between February and March, 1993, 179 clinical MRSA isolates were collected from institutions in Hiroshima prefecture. According to serological types of coagulase, enterotoxins, and toxic shock syndrome toxin-1 (TSST-1) productions, these strains were classified into 6 groups. In 53 strains chosen from all groups, *mec* regulatory gene distributions were divided into two groups; one with whole regulatory genes and another with the lacking region, including 3'-partial region of the *mecR1* gene and *mecI* gene. This same deletion was detected across the different groups, suggesting that the deletion occurred at the ancestral strain before branching according to coagulase or enterotoxin productions. The strains with this lacking region showed a high-level of resistance to methicillin, while the strains with whole regulatory genes consisted of low and high levels of resistant strains. The highly resistant strains with whole regulatory genes were found to harbor a point mutation in the *mecI* gene. The basal levels of *mecA* gene transcription were elevated in the strains with the lacking region or the *mecI* point mutations. These data suggest that deletion or mutation of the *mecI* gene, the repressor on the *mecA* gene, might play an important role in methicillin resistance in clinical isolates of MRSA.

Key words: *Methicillin-resistant Staphylococcus aureus (MRSA), Mec regulator genes, Enterotoxins, Toxic shock syndrome toxin-1 (TSST-1)*

The intrinsic resistance of MRSA has been explained by the production of an additional penicillin-binding protein (PBP), designated PBP2' or PBP2a, that has a low binding affinity for beta-lactam antibiotics[2,5–7,26]. The structural gene *mecA*, encoding PBP2', has been cloned[14]. The *mecA* gene is regulated by the penicillinase plasmid which is present in most MRSA strains[3,24]. This plasmid is thought to produce MRSA strain which has the inducible mode of PBP2' production[18,24]. Recently, another chromosomal regulator region located upstream of the *mecA* gene which down-regulates methicillin-resistance has been identified[8,23]. This regulator region is composed of two regulator genes named *mecR1* and *mecI*[8]. The induction of *mecA* gene transcription by beta-lactams has been shown to be very slow in the strains carrying the intact *mecR1-mecI* gene like *Staphylococcus aureus* (*S. aureus*) N315, and the levels of methicillin resistance of such strains are usually low[19,22]. In clinical isolated MRSA strains with high levels of resistance to methicillin, deletion of *mecI* gene[22] or point mutations on the gene were reported[12,22].

Clinical isolated *S. aureus* strains have been classified into various groups by serological typing of production of coagulase, staphylococcal enterotoxins, and toxic shock syndrome toxin 1 (TSST-1). In this study, we investigated the distribution of *mec* regulator genes and the levels of *mecA* gene transcription as compared to the classified groups using serological typing in clinically isolated MRSA strains collected in Hiroshima prefecture,

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in order to determine the role of these regulator genes in the evolution and epidemiology of MRSA.

MATERIALS AND METHODS

Bacterial strains. A total of 179 MRSA isolates were collected between February and March, 1993 from institutions in Hiroshima prefecture. They were isolated from the clinical specimens of individual patients at three hospitals (Hiroshima University Hospital, A and B hospitals) and at outpatient clinics in Hiroshima prefecture. There was no duplication of patients from whom the strains were isolated. Almost all strains, except for minor groups, were generally classified into 6 groups by coagulase typing and enterotoxins and TSST-1 production by the methods described below. The 53 strains were chosen from all groups and further examination was conducted (Table 1).

Coagulase typing. Isolates were grown in 5 ml of brain heart infusion broth (Nissui, Tokyo, Japan) at 37°C for 20 hr with shaking, and centrifuged at 3000 × *g* for 30 min. Serological coagulase typing was determined immunologically with the supernatant by an inhibition assay kit (Denka Seiken, Tokyo, Japan)[25].

Toxins production assay. TSST-1 and staphylococcal enterotoxins A, B, C, and D productions were determined with the same supernatant as in coagulase typing by the reversed passive latex agglutination (RPLA) test kits (TST-RPLA "SEIKEN", SET-RPLA "SEIKEN": Denka Seiken, Tokyo, Japan)[11,17].

Analysis for β-lactamase. β-lactamase production was evaluated by the nitrocefin disk test (Cefinase®: BBL Microbiology Systems, Cockeysville, U.S.A.)[15].

Determination of minimal inhibitory concentrations (MICs) for methicillin. MIC for methicillin was determined by the plate dilution method with Mueller-Hinton agar (Sensitivity Disk Agar-N, Nissui) plates with an inoculum size of 1–5 × 10^4 CFU of bacteria according to the recommendation of the National Committee for Clinical Laboratory Standards[21]. Growth of the cells was evaluated after incubation for 24 hr at 32°C.

DNA isolation. Each cultured strain was lysed by achromopeptidase (Wako Pure Chemical Co., Osaka, Japan). Chromosomal DNA was extracted using proteinase K (Merk, Darmstadt, Germany) and SDS (sodium dodecyl sulfate) as described by Maniatis et al[13]. The DNA concentration was quantified by spectrophotometer and agarose gel electrophoresis by ethidium bromide staining.

PCR conditions. We used four sets of oligonucleotide primers located in *mecA*, *mecR1*, and *mecI* genes. The location of the four fragments using these primers are indicated in Fig. 1 (A). The *mecA* primers were described previously[9]. Two sets of oligonucleotide primers located on *mecR1* (named as *mecR1* MS and *mecR1* PB) and the primers located on *mecI* were prepared in the manner described previously[22]. The *mecR1* MS and *mecR1* PB fragments are located at the membrane spanning site (near 5' end) and the penicillin-binding site (near 3' end) of *mecR1* gene, respectively. The PCR was performed with 10–100 ng of DNA in a reaction mixture of 50 μl containing 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.25 μM of each primer, and 1 unit of Taq polymerase (Wako Pure Chemical) in a thermal cycler (Thermal Sequencer, IWAKI, Tokyo, Japan). The annealing temperature was 54°C, and the number of cycles was 40. The PCR amplified products were electrophoresed on 2% agarose gels, stained with 2% ethidium bromide for 10 min, and photographed under Ultraviolet.

Probes for hybridization. The four probes used for investigation of *mec* regulatory genes distribution and *mecA* transcription levels were the PCR-amplified fragments of the DNA extracted from N315 strain[22] (Fig. 1).

DNA blot hybridization. Two microgram DNA was digested to completion with restriction endonuclease (*Cla*I, *Mbo*I, *Bgl*II, *Fok*I, or *Pst*I), subjected to electrophoresis on 1% agarose gels, blotted onto nitrocellulose filters, and then hybridized to [α-^{32}P]dCTP labelled each *mec* probe at 65°C. The hybridized filters were washed in 1 × SCC (1 × SCC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS at 65°C, washed twice in

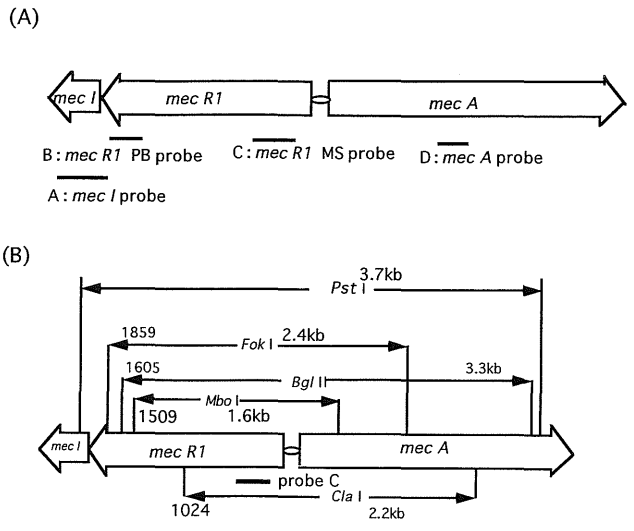

Fig. 1. **(A)** Genomic organization and restriction map of the *mec* region of *S. aureus* N315. The locations of the *mecA*, *mecR1*, and *mecI* loci are indicated by boxes. The thin lines were PCR-amplified fragments (probes): A, *mecI*; B, *mecR1*-PB; C, *mecR1*-MS; D, *mecA*. DNA hybridization was performed using PCR-amplified fragments A, B and C and RNA hybridization was performed by *mecA* probe (D).
(B) The map of restriction sites of enzymes and the location of probe.

0.1 × SCC with 0.1% SDS at 65°C, and then autoradiographed.

RNA blot hybridization. Total cellular RNA was extracted from the strains by the single step method as described by Chomczynski et al[4] using ISOGEN® (Nippon Gene, Tokyo, Japan). ISOGEN® is a solution containing guanidinium thiocyanate and phenol. An overnight culture was diluted 20-fold in 20 ml of LB broth (Sigma, Chemical Co. St. Louis, MO) and was incubated at 37°C with shaking for 3 hr. The stimulation with methicillin was performed by addition of methicillin into the culture at a concentration of 1 μg/ml and stopped by mixing a prechilled (–20°C) acetone-ethanol (1:1;vol/vol) 60 min after the beginning of the induction. Cells were collected by centrifugation at 15,000 × *g* for 2 min at 4°C before and after this stimulation, and the supernatant was removed. The cell pellet was resuspended in 0.1 ml of lysis buffer (100 μg/ml of lysostaphin, 20% sucrose, 20 mM Tris-HCl [pH 7.6], 10 mM EDTA, 50 mM NaCl), and the mixture was incubated for 10 min on ice. The mixture was then frozen in liquid nitrogen and thawed at room temperature. To complete the cell lysis, 0.1 ml of 2% SDS was added. For total RNA extraction, 1 ml of ISOGEN® was added and the mixture was homogenized and stored at room temperature for 10 min followed by the addition of 0.2 ml of chloroform. The suspension was shaken vigorously for 15 sec, stored at room temperature for 3 min, and then centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 0.5 ml of isopropanol and stored at room temperature for 5 min. Sedimentation at 12,000 × *g* for 10 min at 4°C was performed and the resulting RNA pellet was purified in accordance with previous protocols[1]. For these samples, 10 μg of total RNA was electrophoresed on 1.5% agarose gels and blotted onto nitrocellulose filters. These filters were hybridized with ^{32}P-labelled *mecA* probe, at 65°C overnight, washed twice in 1 × SCC with 0.1% SDS at room temperature, washed twice in 0.1 × SCC with 0.1% SDS at 50°C, and then autoradiographed.

Direct sequencing. PCR-amplified *mecI* DNA fragments were used as templates after elution and purification using Ultrafree-3 column (Millipore Co. Bedford, MA). The nucleotide sequence of the *mecI* gene was determined by the dideoxynucleotide termination method described by Sanger et al[20] using a dsDNA Cycle Sequencing System kit (Gibco BRL, Gaithersburg, MD) and synthetic oligonucleotide 5'-ACTTTCTTCTACAAGAGAGT-3' and 5'-ACAAATGCAAAAGGACTGGA-3' as primers. These primers correspond to the nucleotides from positions 2083 to 2102 and the complementary nucleotides from position 2189 to 2208, respectively, in previously described sequence data[8].

RESULTS

Serological Coagulase typing and toxins production of clinical isolated MRSA

Of the 179 MRSA strains collected, serological types of coagulase, staphylococcal enterotoxins, and TSST-1 production are shown in Table 1. In the coagulase typing, 147 were type II, 4 were type III, and 22 were type IV. Among coagulase type II strains, only one strain produced enterotoxin A, 16 produced enterotoxins A and B, 51 produced enterotoxins A, C and TSST-1, 29 produced enterotoxin B, 36 produced enterotoxin C and TSST-1 and 10 had no detectable toxins. None of coagulase type III strains subsequently produced either enterotoxins or TSST-1, and all coagulase type IV strains produced enterotoxin A. According to the data, we classified these strains, except for

Table 1. Classification of isolated MRSA strains according to coagulase typing, enterotoxin, and TSST-1 productions

group	Coagulase*	Enterotoxin*	TSST-1*	Institutions**			
				Hiroshima Univ. Hosp.	Hospital A	Hospital B	outpatient clinic
IIA	II	A	–	0	0	1	0
IIAB	II	AB	–	0	1	14	1
IIACT	II	AC	+	32	14	3	2
IIB	II	B	–	3	11	9	6
IICT	II	C	+	7	12	2	15
II	II	–	–	0	5	2	3
IIT	II	–	+	0	2	2	0
III	III	–	–	0	0	1	3
IVA	IV	A	–	0	3	2	17
Unclassified				1	2	0	3
Total				43	50	36	50

* Coagulase production, enterotoxin, and TSST-1 (toxic shock syndrome toxin 1) production were determined by serological typing.

**institutions where these strains were isolated

Table 2. PCR-based detection of *mecR1* and *mecI* fragments in MRSA

group	(n)	*mecR1*		*mecI*	β-lactamase	MIC for methicillin (μg/ml)
		MS	PB			
IVA	3	+	+	+	+	3.13
	7	+	−	−	+	12.5–100
III	3	+	−	−	+	12.5–50
IIB	10	+	−	−	+	25–800
IIAB	10	+	−	−	−	50–1600
IICT	4	+	+	+	+	25–800
	6	+	+	+	−	800–1600
IIACT	10	+	+	+	−	1600

minor groups, into the following six general groups: IIAB (n = 16), IIACT (n = 51), IIB (n = 29), IICT (n = 36), III (n = 4), and IVA (n = 22).

Distribution of the *mec* regulator genes in clinical isolated MRSA strains

Among the six groups, we randomly chose 3 strains from group III and 10 strains from each of the other groups, except for group III. Within these 53 strains, we examined the genomic existence of *mecA*, *mecR1* MS, *mecR1* PB, and *mecI* sequence using PCR, and analyzed β-lactamase production and the susceptibility against methicillin. As shown in Table 2, the results of PCR indicated two patterns of genomic *mec* regulatory gene distribution: the strains with whole *mec* regulatory genes as N315 strain, and those with *mec* regulatory genes which had the deleted regions containing *mecR1* PB and *mecI* fragments. All of the group IICT and IIACT strains had whole *mec* regulatory genes, whereas all of groups III, IIB, and IIAB strains showed a deleted pattern. Interestingly, group IVA strains showed these two patterns. β-lactamase production was detected in all strains of group IVA, III, and IIB, but not in group IIAB and IIACT. Strains in group IICT were divided into β-lactamase producing strains and non-producing strains.

According to the eight subgroups classified in Table 2, three strains in each subgroup with the same pattern of the *mec* regulator genes, β-lactamase production, in addition to coagulase-toxins groups, and the N315 strain were used for detailed analysis of *mec* regulatory gene distribution using DNA blot hybridization (Table 3). Fig. 1 (B) indicates a map of the restriction sites of enzymes and the location of the probe. To identify the breakpoint of the *mecRI* gene, we examined DNA blot hybridization of DNAs digested by several restriction enzymes using the *mecRI* MS probe. The hybridized bands of DNA digested by restriction enzyme *Cla*I were about 2.2 kb in all DNA samples. In the DNA samples in which the three fragments (*mecI*, *mecR1* PB and *mecR1* MS) were amplified by the PCR analysis, the hybridized bands with *Mbo*I digestion were 1.6 kb (Fig. 2) and those with *Bgl*II digestion and *Fok*I digestion were 3.3 kb and 2.4 kb, respectively. The lengths of these digested bands were consistent with the expected sizes according to the sequences of *mec* regulator genes of the N315 strain. In the DNA samples in which *mecI* and *mecR1* PB fragments were not amplified by PCR, the hybridized bands with *Mbo*I digestion were about 1.4 kb (Fig. 2) and those with *Bgl*II, *Fok*I and *Pst*I digestion also showed aberrant bands for the same sizes (data not shown). Thus, the break point was considered to have occurred at the same site in all strains which lacked *mecI* and *mecR1* PB regions, and to be located between *Cla*I and *Mbo*I restriction sites in *mecR1* (Fig. 2).

These results revealed that the deletion of *mecI* and *mecR1* PB regions has been conserved among several MRSA coagulase and toxins types, also suggesting two patterns of *mec* regulatory gene distribution in these MRSA strains. Almost all strains which lacked *mecI* gene, except for No. 21, 24 strains, showed a high resistance to methicillin (MIC ≥ 25 μg/ml). In these strains, the resistance to methicillin was likely to have been caused by the loss of *mecA*-repressing function of the *mecI* gene. However, among the strains with all *mec* regulatory genes, only 3 showed low MIC for methicillin (MIC ≤ 3.13 μg/m) like N315, and the others were methicillin-resistant (MIC ≥ 25 μg/m).

Point mutation of *mecI* genes in highly resistant MRSA strains

In order to explain the different levels of methicillin-resistance among the strains with *mecI* gene, we analyzed the *mecI* sequences in N315 and the following representative three strains: highly resistant group IICT strain with *mecI* gene (No. 4 strain), highly resistant group IIACT strain with *mecI* gene (No. 10 strain), and low resistant group IVA strain with *mecI* gene (No. 16 strain). The *mecI* gene sequence of No. 16 strain was identical to that of N315, whereas No. 4 strain showed a T-to-A single nucleotide change at position 260 of the 372-bp *mecI* coding sequence, resulting in an Ile-to-Asn change at position 86 in the predictable *mecI* amino acid sequence, and No. 10 strain, showed a G-to-T single nucleotide change at position 43, resulting in a Val-to-Phe change at position 13 in the *mecI* amino acid sequence. Thus, it is likery that high methicillin-resistant MRSA strains with the whole *mecI* gene lost *mecI* function due to point mutation of the *mecI* gene locus.

mecA gene transcription in clinical isolated MRSA

MecA mRNA expression levels were examined in

Table 3. DNA blot analysis of *mecA* and *mec* regulatory genes in representative strains

strain No.	group	isolated hospital*	DNA blot hybridization				β-lactamase	MIC for methicillin
			mecA	*mecR1* MS	*mecR1* PB	*mecI*		
1	IIB	H	+	+	−	−	+	800
2	IIB	H	+	+	−	−	+	200
3	IIB	H	+	+	−	−	+	100
4	IICT	H	+	+	+	+	−	1600
5	IICT	H	+	+	+	+	−	800
6	IICT	A	+	+	+	+	−	800
7	IICT	H	+	+	+	+	+	400
8	IICT	C	+	+	+	+	+	400
9	IICT	C	+	+	+	+	+	25
10	IIACT	H	+	+	+	+	−	1600
11	IIACT	H	+	+	+	+	−	1600
12	IIACT	A	+	+	+	+	−	1600
13	IIAB	A	+	+	−	−	−	1600
14	IIAB	B	+	+	−	−	−	100
15	IIAB	B	+	+	−	−	−	50
16	IVA	C	+	+	+	+	+	3.13
17	IVA	C	+	+	+	+	+	3.13
18	IVA	C	+	+	+	+	+	3.13
19	IVA	C	+	+	−	−	+	100
20	IVA	C	+	+	−	−	+	25
21	IVA	C	+	+	−	−	+	12.5
22	III	C	+	+	−	−	+	50
23	III	C	+	+	−	−	+	25
24	III	C	+	+	−	−	+	12.5
25	II	N315	+	+	+	+	+	3.13

* These strains were isolated from H, Hiroshima University Hospital; A, Hospital A; B, Hospital B, or C, outpatient clinic.

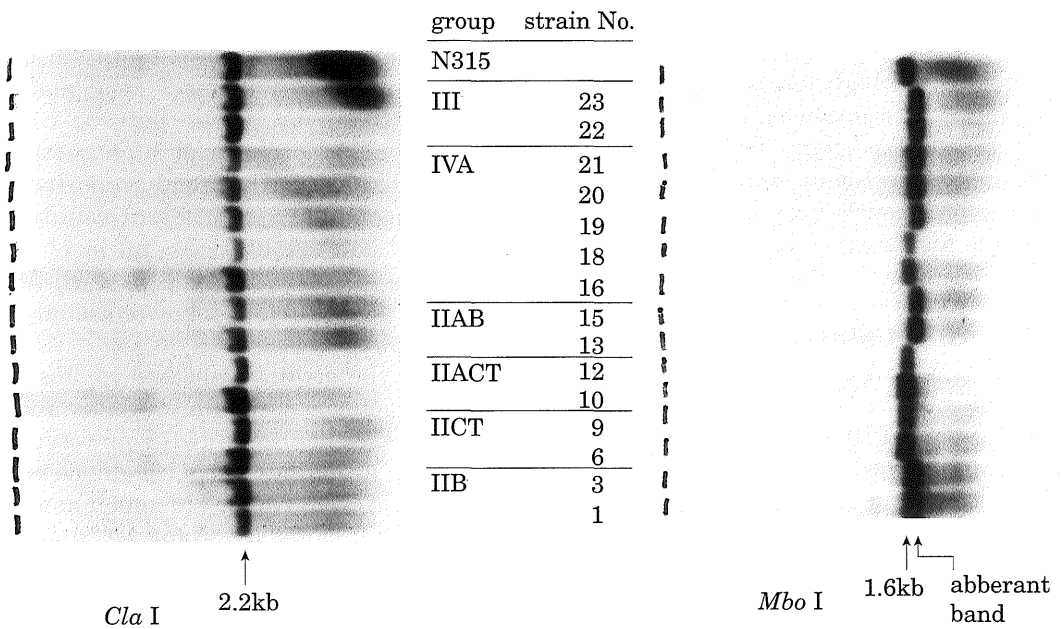

Fig. 2. DNA hybridization of *mecR1* regions. DNAs isolated from MRSA strains were digested by restriction enzymes *Cla*I or *Mbo*I and then hybridized with ^{32}P-labelled *mecR1*-PB probe. All DNA samples showed 2.2 kb bands with *Cla*I digestion. In the DNA samples in which the three fragments (*mecI*, *mecR1* PB and *mecR1* MS) were amplified by the PCR analysis, the hybridized bands with *Mbo*I digestion were 1.6 kb, expected from the sequence of *S. aureus* N315. In the DNA samples in which *mecI* and *mecR1* PB fragments were not amplified by PCR, the hybridized bands with *Mbo*I digestion were about 1.4 kb. The DNA samples with aberrant bands by *Mbo*I digestion also showed aberrant bands by *Bgl*II or *Fok*I digestion (data not shown).

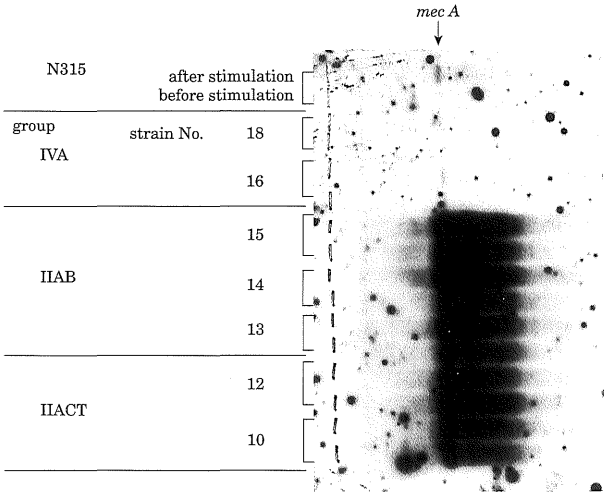

Fig. 3. RNA blot hybridization of *mecA* expression. *MecA* mRNA expression levels were compared before and after stimulation by methicillin. The strains in group IIACT with mutated *mecI* and strains in group IIAB with deleted *mecI* gene, showed high and constitutive expression of *mecA* transcript before and after stimulation. On the other hand, No. 16, 18 strain (group IVA) and N315, which had the intact *mecI* gene, showed undetectable *mecA* gene expression before stimulation and slightly detectable expression after stimulation.

the strains with or without *mecI* genes using RNA blot analysis. *MecA* mRNA expression levels were also compared before and after stimulation by methicillin. The strains in groups IIACT and IIAB, in which *mecI* gene was deleted or mutated, constitutively expressed high levels of *mecA* transcript before and after stimulation. On the other hand, No. 16, 18 strains (group IVA) and N315, which had the intact *mecI* gene, was undetectable for *mecA* gene expression before stimulation and slightly detectable for its expression after stimulation (Fig. 3).

DISCUSSION

The resistance of MRSA has been explained by the production of a characteristic penicillin-binding protein, designated PBP2' or PBP2a, that has a decreased binding affinity for beta-lactam antibiotics[2,5–7,26]. The structural *mecA* gene encoding PBP2' has been cloned[14] and its regulators, *mecR1* and *mecI*, were identified[8] in *mecA* carrying *Staphylococcus aureus* N315. N315 is the strain for which the methicillin MIC is low and is characteristic for the mode of PBP2' production induced by the stimulation of latamoxef or cefoxitin[16]. The presence of *mecI* and *mecR1* regulator genes in some *Staphylococcus aureus* strains have shown a strong repression of the *mecA* gene. Among the MRSA strains isolated in various countries, including Japan, Suzuki et al[22] reported two distribution patterns of *mec* regulator genes: those with whole *mec* regulator regions and those with deleted regions, including the *mecI* gene and 5' end of the *mecR1* gene. Highly methicillin-resistant MRSA strains showed both of these patterns. Those with the whole *mecI* gene were reported to have point mutations of the *mecI* gene. Moreover, Hurlimann-Dalei et al[10] reported that these two patterns had already been detected in prevalent strains in the 1960s.

In the present study, clinical strains isolated from patients in Hiroshima prefecture also showed the same two distribution patterns of *mec* regulator genes: one with whole *mec* regulatory genes and another with the deletion of *mecI* and the 5' partial region of *mecR1*. Detailed analysis by DNA blot using several restriction enzymes revealed that a deleted locus existed in the same portion between the *Cla*I site (#1052) and *Mbo*I site (#1509). Unexpectedly, this deletion occurred across the strains with different coagulase types and with enterotoxin types. This suggested that this deletion in the *mec* regulatory locus had occurred at the ancestral strain before branching, according to coagulase or enterotoxin production. The distribution pattern of *mec* regulatory genes was the same between groups IIB and IIAB and between groups IIAC and IIACT, suggesting that enterotoxin A production has evolved recently.

Analysis of the *mecI* gene sequence revealed that the point mutation of T to A at position 260 was same as that reported by Suzuki et al[22], and the other point mutation of G to T at position 43 was same as that reported by Kobayashi et al[12]. These results indicate that point mutation may occur in various nucleotide of *mecI* gene and the strains with mutated *mecI* gene may be conserved and spread widely because of high methicillin resistance. Suzuki et al[22] already reported that MRSA strains isolated from different countries shared a common point mutations in *mecI* gene. Thus, sequence of the *mecI* gene in each MRSA strain may be a useful tool for evolutionary as well as epidemiological study.

Among the strains analyzed in this study, those with intact *mecA* regulator genes showed as low MIC for methicillin as N315 prototype strain, but showed a different type of coagulase or enterotoxin production from N315. This phenomenon indicates that these strains, as prototype N315, were established before the differentiation of coagulase or enterotoxin typing.

The present study has revealed that two types of *mec* regulator gene distribution which had been reported in the 1960s' strains, existed in MRSA strains isolated from patients in Hiroshima prefecture. Although the levels of antibiotic-susceptibility could not be explained only by *mecI* gene expression, the deletion or mutation of *mecI* gene is likely to play a major role in antibiotic-susceptibility of MRSA strains.

By means of analysis of coagulase and toxins production, it was determined that *mec* regulatory genes had already existed before acquirements of coagulase and toxins productions and the deletion in the *mec* regulatory genes also occurred before acquirement of enterotoxin A production. Thus, *mec* regulatory genes are considered to play a major role in the regulation of antibiotic-susceptibility, and the deletion of this locus or point mutation of *mecI* gene is likely to be highly conserved in MRSA strains which show resistance for antibiotics.

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REFERENCES

1. **Ausbel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.A., Smith, J.A. and Struhl, K.** 1991. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Brown, F.J. and Reynolds, P.E.** 1980. Intrinsic resistance to beta-lactam antibiotics in *Staphylococcus aureus*. FEBS Lett. **122:** 275–278.
3. **Chambers, H.F., Hartman, B.J. and Tomasz, A.** 1985. Increased amounts of a novel penicillin-binding protein in a strain of methicillin-resistant *Staphylococcus aureus* exposed to nafcillin. J. Clin. Invest. **76:** 325–331.
4. **Chomczynski, P. and Sacchi, N.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159.
5. **Georgopapadakou, N.H., Smith, S.A. and Bonner, D.P.** 1982. Penicillin-binding proteins in a *Staphylococcus aureus* strain resistant to specific beta-lactam antibiotics. Antimicrob. Agents Chemother. **22:** 172–175.
6. **Hartman, B.J. and Tomasz, A.** 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158:** 513–516.
7. **Hayes, M.V., Curtis, N.A.C., Wyke, A.W. and Ward, J.B.** 1981. Decreased affinity of a penicillin-binding protein for beta-lactam antibiotics in a clinical isolate of *Staphylococcus aureus* resistant to methicillin. FEMS Microbiol. Lett. **10:** 119–122.
8. **Hiramatsu, K., Asada, K., Suzuki, E., Okonogi, K. and Yokota, T.** 1992. Molecular cloning and nucleotide sequence determination of the regulator region of *mecA* gene in methicillin-resistant *Staphylococcus aureus*. FEBS Lett. **298:** 133–136.
9. **Hiramatsu, K., Kihara, H. and Yokota, T.** 1992. Analysis of borderline-resistant strains of methicillin-resistant *Staphylococcus aureus* using polymerase chain reaction. Microbiol. Immunol. **36:** 445–453.
10. **Hurlimann-Dalei, R.L., Ryffel, C., Kayser, F.H. and Berger-Bachi, F.H.** 1992. Survey of the methicillin-associated genes *mec A*, *mec R1-mec I*, and *fem A-fem B* in clinical isolates of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36:** 2617–2621.
11. **Igarashi, H., Fujikawa, H., Shingaki, M. and Bergdoll, M.S.** 1986. Latex agglutination test for staphylococcal Toxic shock syndrome toxin 1. J. Clin. Microbiol. **23:** 509–512.
12. **Kobayashi, N., Taniguchi, K. and Urasawa, S.** 1998. Analysis of diversity of mutations in the *mec I* gene and *mec A* promoter / operator region of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **43:** 717–720.
13. **Maniatis, T., Fritsch, E.F. and Sambrook, J.E.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Matsuhashi, M., Song, M.D., Ishino, F., Wachi, M., Doi, M., Inoue, M., Ubukata, K., Yamashita, N. and Konno, M.** 1986. Molecular cloning of the gene of a penicillin-binding protein supported to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. J. Bacteriol. **167:** 975–980.
15. **O'Callaghan, C.H., Morris, A.M., Kirby, S. and Shingler, A.H.** 1972. Novel method for detection of β-lactamase by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:** 283–288.
16. **Okonogi, K., Noji, Y., Kondo, M., Imada, A. and Yokota, T.** 1989. Emergence of methicillin-resistant clones from cephamycin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. **24:** 637–645.
17. **Park, C.E. and Szabo, R.** 1986. Evaluation of the reversed passive latex agglutination (RPLA) test kits for detection of staphylococcal enterotoxins A, B, C, and D in foods. Can. J. Microbiol. **32:** 723–727.
18. **Rossi, L., Tonin, E., Cheng, Y.R. and Fontana, R.** 1985. Regulation of penicillin-binding protein activity:description of a methicillin-inducible penicillin-binding protein in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **27:** 828–831.
19. **Ryffel, C., Kayser, F.H. and Berger-Bachi, B.** 1992. Correlation between regulation of *mecA* transcription and expression of methicillin resistance in staphylococci. Antimicrob. Agents Chemother. **36:** 25–31.
20. **Sanger, F., Nicklen, S. and Coulson, A.R.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.
21. **Standards., N.C.f.C.L.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. National Committee for

Clinical Laboratory Standards, Villanova, Pa.
22. **Suzuki, E., Kuwahara-Arai, K., Richardson, J.F. and Hiramatsu, K.** 1993. Distribution of *mec* regulator genes in methicillin-resistant *Staphylococcus* clinical strains. Antimicrob. Agents Chemother. **37:** 1219–1226.
23. **Tesch, W., Ryffel, C., Strassle, A., Kayser, F.H. and Berger-Bachi, B.** 1990. Evidence of a novel staphylococcal *mec*-encoded element (*mecR*) controlling expression of penicillin-binding protein2'. Antimicrob. Agents Chemother. **34:** 1703–1706.
24. **Ubukata, K., Yamashita, N. and Konno, M.** 1985. Occurrence of a beta-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. Antimicrob. Agents Chemother. **27:** 851–857.
25. **Ushioda, H., Terayama, T., Sakai, S., Zen-Yoji, H., Nishiwaki, M. and Hidano, A.** 1981. Coagulase typing of *Staphylococcus aureus* and its application in routine work. Zbl. Bakt. Suppl. 10 ed. Gustav Fischer Verlag, Stuttgart, New York. 77–83.
26. **Utsui, Y. and Yokota, T.** 1985. Role of an altered penicillin-binding protein in methicillin-and cephem-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **28:** 397–403.