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Genomic diversity of *mec* regulator genes in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*

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SUMMARY

Low-affinity penicillin-binding protein PBP-2a encoded by *mecA* is closely related to methicillin resistance in staphylococci, and expression of PBP-2a is controlled by regulator elements encoded by *mecR1* and *mecI* which are located adjacent to *mecA* on the chromosome. Deletion or mutation which occurred in *mec* regulator gene is considered to be associated with constitutive production of PBP-2a. The distribution of the *mec* regulator genes in 176 strains of *Staphylococcus aureus* and 33 strains of *S. epidermidis* isolated from a single hospital was studied by polymerase chain reaction amplification. Most clinical isolates of methicillin-resistant *S. aureus* (MRSA) (94·3%) and *S. epidermidis* (MRSE) (83·9%) possessed both *mecI* and *mecR1* genes (type I), whereas no *mec* regulator genes were detected in *mecA*-negative isolates. In contrast, 7 MRSA and 5 MRSE isolates were found to have incomplete regulator genes, and they were classified into three groups; strains which lacked only *mecI* gene (type II), strains which lacked *mecI* and 3′-end of *mecR1* gene (type III), and strains which lacked both regulator genes (type IV). Analysis of *mecI* gene from all the strains having *mecI* by restriction fragment length polymorphism after *Mse* I digestion indicated that three MRSA strains possessed one of the known point mutations identified previously. These findings indicated the predominance of a single type of MRSA possessing both *mecI* and *mecR1* in the study period and also suggested a high genomic diversity in *mec* regulator region of staphylococci.

INTRODUCTION

Methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) are recognized as pathogens that can cause serious nosocomial infection and contribute significantly to mortality and morbidity in hospitals around the world [1, 2]. Resistance to methicillin in staphylococci is primarily mediated by penicillin-binding protein (PBP-2a) which is a unique cell wall-synthesizing enzyme [3–6]. Due to the low affinity for beta-lactam antibiotics, PBP-2a can compensate for the transpeptidation function of all other PBPs in staphylococci and enable the organism to survive in the presence of lethal concentrations of beta-lactams [7]. PBP-2a is encoded by the chromosomal structural gene *mecA* which is highly conserved in clinical isolates of methicillin-resistant staphylococci [8–10]. However, the level of methicillin resistance is affected by other chromosomal factors (*fem* or *aux* factors) encoded by *femA*, *femB*, *femC*, and *femD* genes which are not linked to the *mec* locus [11–13].

Expression of PBP-2a is considered to be controlled by regulator elements which are putative trans-membrane-signalling protein and repressor protein encoded by *mecR1* and *mecI* genes, respectively [14–16]. These regulator genes, *mecR1* and *mecI* are located

upstream of the *mecA* gene on the chromosome. The expressions of these regulators strongly repress *mecA* transcription in the absence of inducer [15, 17]. Hence MRSA possessing intact *mecR1* and *mecI* as well as *mecA* are phenotypically methicillin susceptible because of the repression of PBP-2a production by *mec* regulator elements. However, deletion or mutation in the *mec* regulators was detected in clinical MRSA isolates and methicillin-resistant coagulase negative staphylococci (MR-CNS) highly resistant to methicillin [18]. Such genomic changes in *mec* regulator genes are considered to alter or remove their repressor function on *mecA* gene transcription, which may lead to constitutive production of PBP-2a. Therefore, it is important to analyse the genomic diversity found in *mec* regulator genes of staphylococci in order to understand the molecular basis for methicillin resistance. Several studies reported for the past three decades have shown genomic variation in *mec* regulator genes among MRSA and MR-CNS strains isolated from different countries and at different times [17–19]. However, the extent of diversity in *mec* regulator genes has not been studied among staphylococcal isolates from a limited area or from a single hospital.

In the present study, we investigated the distribution of *mec* regulator genes and the presence of the previously reported mutations in *mecI* among *S. aureus* and *S. epidermidis* strains derived from a single hospital, using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. Results obtained revealed the present situation of *mec* regulator gene distribution in staphylococcal clinical isolates and suggested the occurrence of greater genomic variation in these genes than has been reported previously.

MATERIALS AND METHODS

Bacterial strains

A total of 176 *S. aureus* and 33 *S. epidermis* strains were employed in this study. The *S. aureus* strains consisted of 122 and 28 clinical isolates of MRSA and methicillin-susceptible *S. aureus* (MSSA), respectively, and of 26 environmental isolates of MRSA. All *S. epidermidis* isolates were derived from clinical specimens and 31 strains were identified as MRSE. Clinical isolates of *S. aureus* and *S. epidermidis* were isolated at Sapporo Medical University Hospital, Sapporo, Japan, in 1993 (between January and March), 1994 (between January and April), and 1995 (between February and May). The *S. epidermidis* isolates were derived from 33 different patients, whereas 122 MRSA were isolated from 97 patients. Multiple isolates of MRSA were obtained from different sites of 22 patients over a period of 2 weeks from a half of these patients, while almost simultaneously from the other patients. Environmental MRSA were isolated from swabs from floors and sinks of wards in three clinical departments of the hospital in January, March and April in 1994; an overall detection rate throughout the study period was 59·4%. Identification of bacterial species and antimicrobial susceptibility tests were performed with MicroScan WalkAway™-96 (Baxter Diagnostics, Inc., West Sacramento, USA). Differentiation of *S. aureus* used in this study was made by coagulase typing with coagulase type-specific antisera. Coagulase types of MRSA clinical isolates consisted of type II (91·8%), VII (5·7%), and IV (2·5%); all the environmental MRSA isolates belonged to type II. In contrast MSSA clinical isolates consisted of type VII (46·4%), II (14·3%), V (14·3%), III (7·1%), IV (7·1%), and untypable strains (10·7%).

Preparation of bacterial DNA and PCR

DNA samples for PCR were prepared as described previously [20]. Three sets of primers indicated in Table 1 were used for PCR to detect *mec* regulator genes. As shown in Figure 1, primers mecI1 and mecI2 amplified the whole open reading frame (ORF) of *mecI* gene, whereas primer pairs mecRA1 and mecRA2, and mecRB1 and mecRB2 amplified the regions located on the half portion of 5′ end (*mecR1A*) and 3′ end (*mecR1B*) of *mecR1* gene, respectively. In addition, *mecA* gene fragment was also amplified by PCR as described in our previous study [20], in which detection of each *mecA* gene was performed with isolates obtained in 1993. Methicillin resistance of staphylococci was essentially defined by the presence of *mecA* gene encoding PBP-2a irrespective of the degree of phenotypic methicillin susceptibility [21, 22]. Therefore, in the present study, *S. aureus* and *S. epidermidis* possessing *mecA* gene were designated MRSA and MRSE, respectively.

DNA amplification was performed on a thermal cycler in 100 μl of reaction mixture containing 200 μM each of dATP, dCTP, dGTP, and dTTP; 100 pM each of a pair of primers; 2·5 U of AmpliTaq DNA polyermase (Perkin Elmer); 10 mM Tris–HCl (pH 8·3); 50 mM KCl; and 1·5 mM $MgCl_2$. DNA fragments were amplified for 25 cycles, with each cycle consisting of 1 min at 97 °C for denaturation, 1 min at 57 °C for

Table 1. *Primers used for PCR*

Amplified gene*	Oligodeoxynucleotide primer			
	Pair	Sequence (5′ to 3′)†	Position‡	Size of PCR product (bp)
mecR1 (*mecR1A*)	mecRA1	+GTCTCCACGTTAATTCCATT	133–152	310
	mecRA2	−GTCGTTCATTAAGATATGACG	422–442	
mecR1 (*mecR1B*)	mecRB1	+AAGCACCGTTACTATCTGCACA	986–1007	236
	mecRB2	−GAGTAAATTTTGGTCGAATGCC	1200–1221	
mecI	mecI1	+AATGGCGAAAAAGCACAACA	1699–1718	481
	mecI2	−GACTTGATTGTTTCCTCTGTT	2159–2179	

* *mecR1A* and *mecR1B* represent half portion of the 5′ and 3′ end of *mecR1* gene, respectively. Amplified regions of *mecR1* and *mecI* genes are shown in Fig. 1.

† +, sense primer; −, antisense primer.

‡ Positions are expressed as the nucleotide number from the first ORF of *mec* regulator gene (*mecR1*) based on the sequence data reported previously [14].

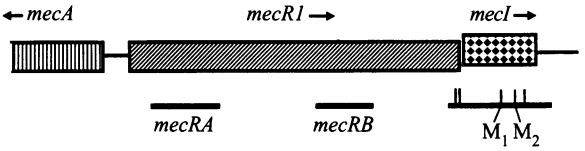

Fig. 1. Schematic representation of *mecA* and *mec* regulator genes, and amplified regions by PCR (*mecR1A*, *mecR1B*, and *mecI*), which are shown as horizontal bars. Vertical lines on the amplified region of *mecI* denote cleavage sites of restriction enzyme *Mse* I. Two *Mse* I sites indicated as M_1 and M_2 are not present in normal *mecI* gene but appear as a result of point mutations previously reported, a nucleotide substitution at positions 202 and 260, of *mecI* gene, respectively [18]. Arrows indicate directions of transcription.

annealing, and 2 min at 72 °C for primer extension. The amplified PCR product (10 μl) was analysed by electrophoresis on 1 % agarose gel and visualized with u.v. light after staining with ethidium bromide.

RFLP analysis of *mecI* gene

RFLP analysis was carried out for *mecI* gene in order to detect point mutations which had been described previously by Suzuki and colleagues [18]. They identified three point mutations in *mecI* gene of the 6 clinical MRSA strains derived from 5 different countries; nucleotide substitution (C to T) at position 202 (mutation 1), nucleotide substitution (T to A) at position 260 (mutation 2), and deletion of nucleotide (T) at position 143 (mutation 3). Mutations 1 and 2 were detected in 5 of the 6 MRSA strains examined, suggesting these to be dominant types of mutation. The mutations 1 and 2 result in a new nucleotide sequence (5′-TTAA-3′) which is recognized by restriction endonuclease *Mse* I (5′-T↓TAA-3′). Hence RFLP analysis was done in this study using the PCR product of *mecI* gene and *Mse* I. Whereas the PCR

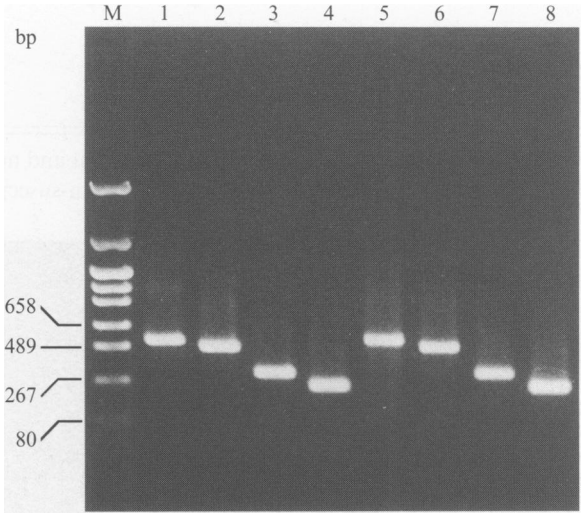

Fig. 2. PCR products obtained from clinical MRSA isolates (strain SH162 for lanes 1–4, strain SH163 for lanes 5–8). Amplified genes are as follows: lanes 1 and 5, *mecA* (533 bp); lanes 2 and 6, *mecI* (481 bp); lanes 3 and 7, *mecR1A* (310 bp); lanes 4 and 8, *mecR1B* (236 bp). M, molecular weight marker.

product for *mecI* gene originally contained 3 *Mse* I sites as shown in Figure 1, generation of fragmentation patterns distinct from that of normal *mecI* gene implied the emergence of novel *Mse* I sites due to mutation 1 or 2. After PCR product was digested with *Mse* I (New England Biolabs) according to the manufacturer's instructions, DNA fragments were separated by electrophoresis on a 1 % agarose gel.

RESULTS

DNA fragments with the size of 310 bp, 236 bp, and 481 bp were amplified by PCR from *mecR1A*, *mecR1B*, and *mecI* gene of MRSA, respectively, as shown in Figure 2. On the basis of detection pattern

Table 2. *Detection of* mecR1 *and* mecI *genes in* S. aureus *and* S. epidermidis

Bacterial species*	Number of isolates	PCR product				Detection patterns of PCR product		Number of oxacillin-susceptible strain
		mecA	*mecR1A*	*mecR1B*	*mecI*	Type	Number of isolates (%)	
MRSA (clinical isolates)	122	+	+	+	+	I	115 (94·3)	0
		+	+	+	−	II	3 (2·5)	0
		+	+	−	−	III	1 (0·8)	1
		+	−	−	−	IV	3 (2·5)	2
MRSA (environmental strains)	26	+	+	+	+	I	23 (88·5)	0
		+	−	−	−	IV	3 (11·5)	0
MSSA	28	−	−	−	−	V	28 (100)	28
MRSE	31	+	+	+	+	I	26 (83·9)	1
		+	+	+	−	II	1 (3·2)	0
		+	+	−	−	III	3 (9·7)	1
		+	−	−	−	IV	1 (3·2)	0
MSSE	2	−	−	−	−	V	2 (100)	2

* MRSA and MSSA represent methicillin-resistant and methicillin-susceptible *S. aureus*, respectively. Similarly, MRSE and MSSE indicate methicillin-resistant and methicillin-susceptible *S. epidermidis*, respectively.

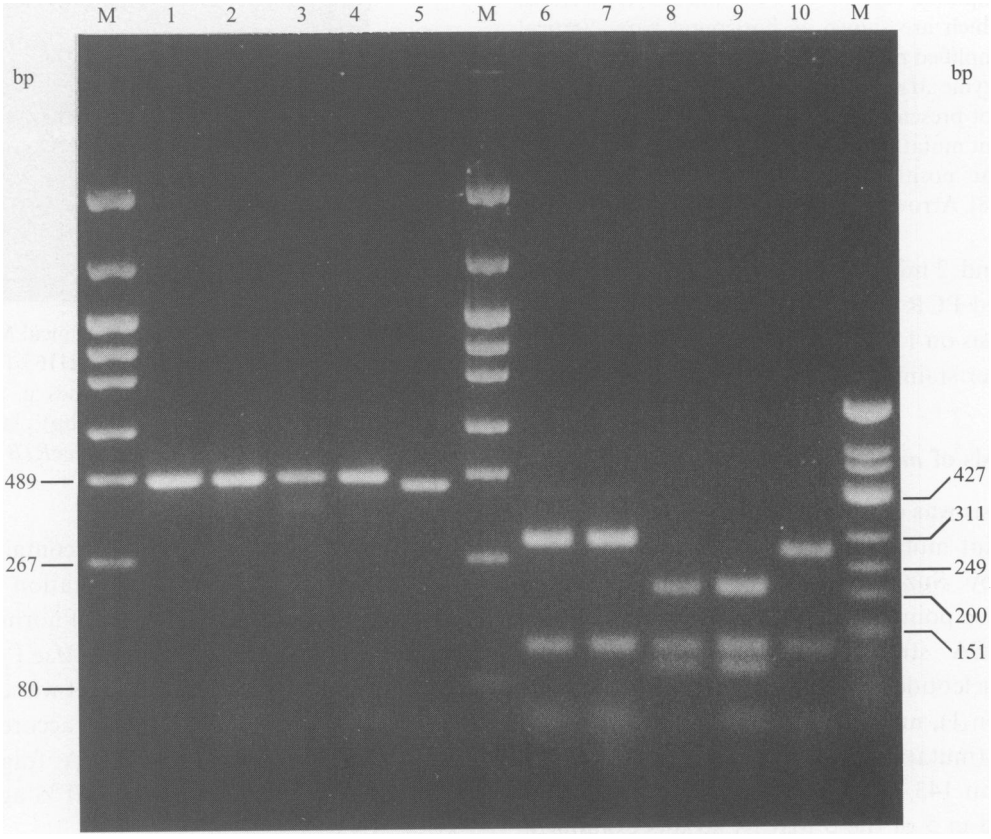

Fig. 3. RFLP analysis of *mecI* gene with *Mse* I restriction endonuclease. Lanes 6–10 represent RFLP patterns derived from *mecI*-PCR product arranged in the same order in lanes 1–5. Lanes 6 and 7, RFLP pattern 1; lanes 8 and 9, pattern 2; lane 10, pattern 3. *mecI*-PCR products in lanes 1 to 5 are obtained from MRSA strains SH21795, SH22058, SH19651, SH212, and SH13, respectively. M, molecular weight markers.

of *mecA* gene and *mec* regulator genes, *S. aureus* and *S. epidermidis* strains were classified into five types (I–V), as shown in Table 2. In 28 MSSA and 2 methicillin-susceptible *S. epidermidis* (MSSE), neither *mecA* nor *mec* regulator genes was detected (type V). The *mecR1* (*mecR1A* and *mecR1B*) and *mecI*-positive pattern (type I) were detected in 94·3% of MRSA clinical isolates, 88·5% of MRSA environmental isolates, and 83·9% of MRSE. A few strains were assigned to type II lacking *mecI*, type III possessing only *mecR1A*, or type IV in which no *mec* regulator genes were detectable. In clinical isolates of MRSA and MRSE, all the four types were observed, whereas environmental MRSA strains were classified as either type I or IV. Two MRSA clinical strains that belonged to type II were isolated from different specimens (pus and sputum) derived from the same patient, and two environmental MRSA strains that belonged to type IV were isolated from different sites in the same department. Besides these, all MRSA and MRSE strains grouped into types II, III and IV were isolated from different patients.

None of MSSA and MSSE strains possessed *mecA* and they were all susceptible to methicillin. However, a few MRSA and MRSE strains also showed phenotypic oxacillin-susceptibility and they belonged to type III or IV in *S. aureus*, and type I or III in *S. epidermidis* (Table 2).

RFLP analysis with *Mse* I was performed for 164 strains in which PCR product of *mecI* was obtained. Most of the staphylococcal strains showed RFLP pattern 1 (160 strains, 97·6%) which represented the absence of novel *Mse* I site, and yielded fragments of 303 bp and 122 bp, and smaller fragments (37 bp and 13 bp) (pattern 1) (Fig. 3, lanes 6 and 7). Three MRSA clinical strains isolated from different patients in 1993 and 1994 showed RFLP pattern 2 which suggested mutation 1, consisted of fragments of 206 bp, 122 bp an 95 bp, and smaller fragments (Fig. 3, lanes 8 and 9). From a single MRSA (a clinical isolate in 1993), a slightly smaller sized *mecI* gene-PCR product was obtained (Fig. 3, lane 5). Restriction fragments of the PCR product digested by *Mse* I contained a DNA fragment with the approximate size of 280 bp (pattern 3) (Fig. 3, lane 10) instead of 303 bp-fragment which was seen in pattern 1. All the strains that showed RLFP patterns 2 and 3 possessed both *mecR1A* and *mecR1B*.

DISCUSSION

It has been accepted that intact *mec* regulator genes strongly repress the transcription of *mecA* gene, whereas the repression activity is lost by mutation or deletion which occurred in *mec* regulator genes, resulting in constitutive production of PBP-2a [15, 17, 18]. Suzuki and colleagues [18] studied in detail the genomic diversity in the *mec* regulator gene and distinguished four different groups; group A, strains carried the whole *mec* regulator region including *mecI* which contained a point mutation; group B, the strain in which *mecI* only was deleted; group C, the strain in which *mecI* and nearly half of the 3′ end of *mecR1* were deleted; group D, strains lacked almost all *mecI* and *mecR1* genes. In 26 epidemic MRSA strains isolated from various countries, groups A (59·5%) and C (40·5%) were predominant, whereas groups B and D were not detected. In contrast, all the four groups were found in 27 MR-CNS isolated in Japan, although no *S. epidermidis* was assigned for group D. Our present study using recent isolates from a single hospital in Japan indicated that the major type of *mec* regulator gene variation was type I (corresponding to group A by Suzuki and colleagues [18]). Moreover, our results demonstrated that greater genomic variation existed in MRSA and MRSE than reported previously; types II and IV (groups B and D, respectively) were found in MRSA and type IV (group D) was detected in MRSE in our study.

Results in RFLP analysis of *mecI* revealed that most *mecI* gene tested in our study did not harbour the same point mutations as those identified previously [18]. This result suggested the presence of mutations in other sites of *mecI* which cannot be detected by the *Mse* I digestion. However, it was of note that the *mecI*-mutation 1 (RFLP pattern 2) found in 3 MRSA strains in our study had been identified in 3 MRSA isolates derived from different European countries. This might imply either that the mutation 1 readily occurs in MRSA strains worldwide, or that an MRSA clone with this mutation has disseminated worldwide.

The *Mse* I-RFLP pattern 3 which was observed in only one MRSA strain appears to represent a novel type of genomic diversity in *mecI*. The size of *mecI*-PCR product showing pattern 3 was slightly smaller than those of all other strains, and the smaller size of DNA fragment was generated instead of 303 bp-fragment which was observed in RFLP pattern 1. This finding suggested that the *mecI* gene that showed pattern 3 had an internal deletion with approximate

length of 20 bp, which may be confirmed by further sequence analysis.

Since the emergence of MRSA in England in 1961 [23], distribution of MRSA has been expanding globally. Several speculations have been presented for origin and evolution of *mec* locus including *mecA* and *mec* regulator genes. Kreiswirth and colleagues [24] hypothesized that all MRSA evolved vertically from a single progenitor clone which had acquired *mecA*. Other studies also supported the idea of a clonal origin of European MRSA strains [25–27]. However, occurrence of horizontal transfer of *mecA* has also been suggested by studies on diversity of *mecA* and chromosomal DNA [28]. Musser and Kapur [29], using multilocus enzyme electrophoresis, found that while MRSA derived from 1960s showed a single genotype, a fact favouring clonal dissemination, more recent isolates showed extensive diversity of chromosome, suggesting polyclonality. Hence they hypothesized that multiple episodes of horizontal transfer of *mec* locus and recombination have occurred recently.

Similar change in distribution of *mec* regulator genes has also been observed; most of the MRSA isolated from various countries in 1960s lacked the *mec* regulator genes, whereas these genes existed in most strains isolated since 1980 [17–19]. Consistent with these observations, the presence of *mecI* and *mecR1* was demonstrated in most clinical and environmental MRSA in our present study. These findings may indicate that old strains had only *mecA* gene and acquired the *mec* regulator genes later. Alternatively, MRSA with *mec* regulator genes surpassed those with *mecA* only after the period when both types of MRSA existed. Archer and colleagues [19] recently presented a hypothesis that *mec* regulator genes including those with deletion or mutation have been transferred from CNS to *S. aureus*. However, it is still unclear why MRSA with dysfunctional *mec* regulator genes became predominant recently in place of old type MRSA that possessed only *mecA*. It was noted in our study that 3 MRSA and 1 MRSE strains lacking *mecI* (type III and IV) were phenotypically oxacillin-susceptible. Thus, the involvement of other unknown mechanism for regulation of PBP-2a production is suggested. Further investigation of the distribution, genetic divergence and function of *mec* regulator genes will be required to understand the evolution of MRSA and the basis for its global spread.

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