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Analysis of genomic diversity within the *Xr*-region of the protein A gene in clinical isolates of *Staphylococcus aureus*

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SUMMARY

Protein A of *Staphylococcus aureus* contains a polymorphic Xr-region characterized by a tandem repeat of eight amino acid units. In this study, the diversity of genes encoding the repeat regions and their relatedness among *S. aureus* strains was analyzed. Ten different protein-A types characterized by repeat numbers 4–13 were identified in a total of 293 clinical isolates. The protein-A type with 10 repeat units (10 repeats) in the Xr-region was most frequently detected in methicillin-resistant *S. aureus*, whereas the majority of methicillin-susceptible strains were distributed almost evenly into protein-A types with 7–11 repeats. Strains that belonged to a single coagulase type were classified into multiple protein-A types, e.g. strains with the common coagulase types II and VII were differentiated into 7 and 8 protein-A types, respectively.

Nucleotide sequence analysis of the *Xr*-region of 42 representative strains revealed the presence of 37 different genotypes (*spa* types), which were constituted by a combination of several of 24 different repeat unit genotypes. Based on the similarity in arrangement of repeat unit genotypes, 34 strains with different repeat numbers were classified into 5 genetic clusters (C1–C5). The clusters C1, C2 and C3 consisted exclusively of strains with identical coagulase types II, III, and IV, respectively. These findings suggested that the protein-A gene of *S. aureus* has evolved from a common ancestral clone in individual clusters independently.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a major cause of nosocomial infection around the world, and is potentially a great threat to medical therapy [1]. Typing of *S. aureus* strains is recognized to be important in the study of transmission routes of this organism in both hospitals and the community, and to determine the mode of dissemination of methicillin-resistance gene (*mecA*) among *S. aureus* strains. Hence a number of typing methods based on biological properties and genomic polymorphisms have been designed and applied for epidemiologic studies [2, 3]. Protein-A typing is a genetic method which employs diversity of the gene encoding the Xr-region of protein A [4]. Whereas the N-terminal part of protein A is involved in binding with IgG Fc portion, the C-terminal domain associated with cell-wall attachment contains the Xr-region constituted by a tandem repeat of 8-amino acid units [5, 6]. The number of repeat units in the Xr-region is diverse among *S. aureus* strains and can be determined by polymerase chain reaction (PCR) amplification of DNA containing the *Xr*-region. To date variable numbers of repeats between 3 and 15 have been identified [4]. Since protein-A type is a clearly defined genetic marker, this typing system was introduced

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Table 1. S. aureus *strains of which the* Xr-*regions of protein A were sequenced*

Protein-A type (no. of repeat)	*S. aureus* strain (coagulase type)	
	MRSA	MSSA
4		SH64(II), SH182(VII)
5	SH147(VII)	SH456(VII)
6		SH63(III), SH409(V), SH484(VII)
7	SH432(II), SH73(IV), SH408(VII)	SH492(VII)
8	SH58(IV)	SH60(I), SH461(II), SH345(IV), SH445(VII), SH401(VIII)
9	SH489(II), SH380(IV)	SH487(II), SH416(IV), SH434(V), SH427(VII)
10	SH149(II), SH220(II), SH320(II), SH494(II), SH475(III)	SH198(II), SH479(II), SH20115(II), SH495(III), SH454(IV), SH478(VII)
11	SH203(II), SH497(III)	SH87(II), SH325(III), SH323(VII)
12	SH463(IV)	SH472(III)
13	SH423(II)	
Total no. of strains	16	26

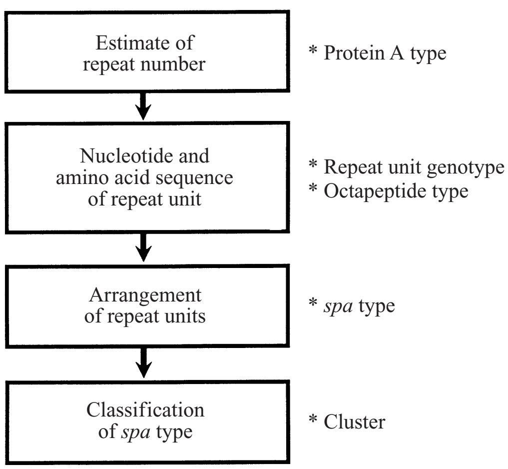

Fig. 1. Flow diagram of analysis of the *Xr*-region of the protein-A gene. Individual 'type' (or classification) shown with an asterisk was derived from the study approach within the box.

into some epidemiologic analysis of MRSA [7–9]. Furthermore, epidemiologic efficacy of protein-A gene type (*spa* type) based on nucleotide sequence of the *Xr*-region in a number of clinical isolates was also described [10]. In these reports, *spa* typing was found to be sensitive enough to allow further differentiation of strains within a specific phage type, although these studies were carried out for strains isolated exclusively in European countries. However, the following points which have epidemiological significance remain unclarified: difference in protein-A or *spa* type between MRSA and methicillin-susceptible *S. aureus* (MSSA), and association of protein-A or *spa* type with coagulase type, a representative biological type of *S. aureus*. In addition, although nucleotide sequences of the *Xr*-region with repeat numbers 6, 7, 10, 11, and 12 have been published [10], sequences with other repeat numbers and genomic relatedness among strains with various repeat numbers in the *Xr*-region have not been investigated. In order to elucidate these points, we analysed a number of MRSA and MSSA clinical isolates in this study.

MATERIALS AND METHODS

Bacterial strains

A total of 293 *S. aureus* strains that comprised 223 MRSA and 70 MSSA were analysed. They were obtained from Sapporo Medical University Hospital in 1993 (Jan. to June), 1994 (Jan. to June), 1995 (Feb. to June) and 1997 (Jan. to June). A single isolate from individual patients was subjected to this study. Identification of bacterial species and antimicrobial susceptibility tests were performed by the use of MicroScan WalkAway 96 (Baxter Diagnostics Inc., West Sacramento, USA). Presence of *mecA* gene which defines methicillin-resistance was examined for all *S. aureus* strains by PCR as described previously [11]. A total of 42 representative MRSA and MSSA strains with various protein-A and coagulase types (Table 1) were selected for DNA sequencing of the *Xr*-region of protein-A gene.

Coagulase typing

Coagulase type was determined by a neutralization test using coagulase type (I–VIII)-specific antisera

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Table 2. *Sequence of oligonucleotide primers and their locations in protein-A gene*

Primer name	Nucleotide sequence (5′–3′)	Location* (nucleotide nos.)
spa-1	+CAAGCACCAAAAGAGGAA	1153–1170
spa-2	−CACCAGGTTTAACGACAT	1475–1492
spa-3	+GCTAAAAAGCTAAACGAT	1132–1149
spa-4	+CCTTCGGTGAGCAAAGAA	1102–1119
spa-5	+GACGATCCTTCGGTGAGC	1096–1113
spa-6	−TCAGCAGTAGTGCCGTTTGC	1516–1535

* Nucleotide number is described according to the protein-A gene sequence of *S. aureus* strain 8325-4 [5].

(Denka Seiken Inc., Japan), as described previously [12].

Coagulase gene typing

In order to discriminate the bacterial isolates genetically, coagulase gene typing was also performed based on the method described previously [13]. Briefly, a PCR product derived from a hypervariable region of coagulase gene was digested with restriction enzyme *Alu*I, and the size of the resultant fragments (restriction fragment length polymorphism; RFLP) was examined by electrophoresis in agarose gel. Since the coagulase gene typing is based on the diversity of the gene encoding C-terminal region, this typing is independent of coagulase type (serological type of coagulase) which is determined by the antigenicity of N-terminal region of this protein [14].

Analysis on diversity of the protein-A Xr-region

Nucleotide and amino acid sequence diversity of the protein-A Xr-region was investigated through direct DNA sequencing of the *Xr*-region amplified by PCR. The process of the genetic analysis is shown in the flow diagram (Fig. 1). First, protein-A type indicating a repeat number was determined by assay of the PCR product size in agarose gel electrophoresis. Secondly, using sequence data, diversity of repeat units (repeat unit genotype and octapeptide type) was analysed. Furthermore, genetic type of the whole *Xr*-region (*spa* type), expressed by arrangement of repeat unit genotypes, was assigned to each bacterial isolate and the *spa* types were classified into clusters based on homology.

Amplification of protein-A gene Xr-region

Bacterial DNA was extracted using achromopeptidase as described previously [11]. DNA sequence including that of the *Xr*-region in the *S. aureus* gene was amplified by PCR using a pair of primers spa-2 and spa-5 (Table 2). The size of the PCR product was determined by electrophoresis in 2% NuSieve 3:1 agarose (FMC BioProducts) at 100 V for 1·5 h, followed by staining with ethidium bromide. The PCR product contained additional 72 and 37 nucleotides at the 5′- and 3′-end of the repeat region, respectively. Consequently the relation of the repeat number of 24-base units to the size of the PCR product is expressed by the following formula:

$$\text{size (bp) of PCR product} = (\text{repeat no.}) \times 24 + 109.$$

Direct DNA sequencing

Direct DNA sequencing of the PCR product from representative strains was performed by dideoxynucleotide chain termination method using Sequenase PCR product sequencing kit (United States Biochemical, Cleveland, Ohio), employing the primers listed in Table 2. In addition to the DNA amplified with primers spa-2 and spa-5, PCR products generated with primers spa-5 and spa-6 were also used as templates for sequencing of all the strains examined except for strains SH64, SH147, SH182, and SH456.

RESULTS

Coagulase typing and coagulase gene typing

Table 3 shows the distribution of coagulase types and coagulase gene types of *S. aureus* isolates employed in

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Table 3. *Coagulase types and coagulase gene type* (coa-*RFLP patterns*) *of* S. aureus *employed in this study*

S. aureus (total no. of isolates)	Coagulase type	Coagulase gene type (*coa*-RFLP pattern*)																Total (%)
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	UT†	
MRSA (223)	II	192				2												194 (87·0)
	III						2											2 (0·9)
	IV			6														6 (2·7)
	VII		17						1					1				19 (8·5)
	UT†	1														1		2 (0·9)
MSSA (70)	I											1						1 (1·4)
	II	7								1	2							10 (14·2)
	III						6											6 (8·6)
	IV			4														4 (5·7)
	V				4									1				5 (7·1)
	VII		11					3	6		2			10		1		33 (47·1)
	VIII												2					2 (2·9)
	UT†			1	1				1		3				1		2	9 (12·9)

* The *coa*-RFLP patterns A to M were described previously [13]. Patterns N and M were identified in the present study and defined by generation of DNA fragments of 243 and 405 bases, and 81 and 567 bases, respectively.
† Untypable.

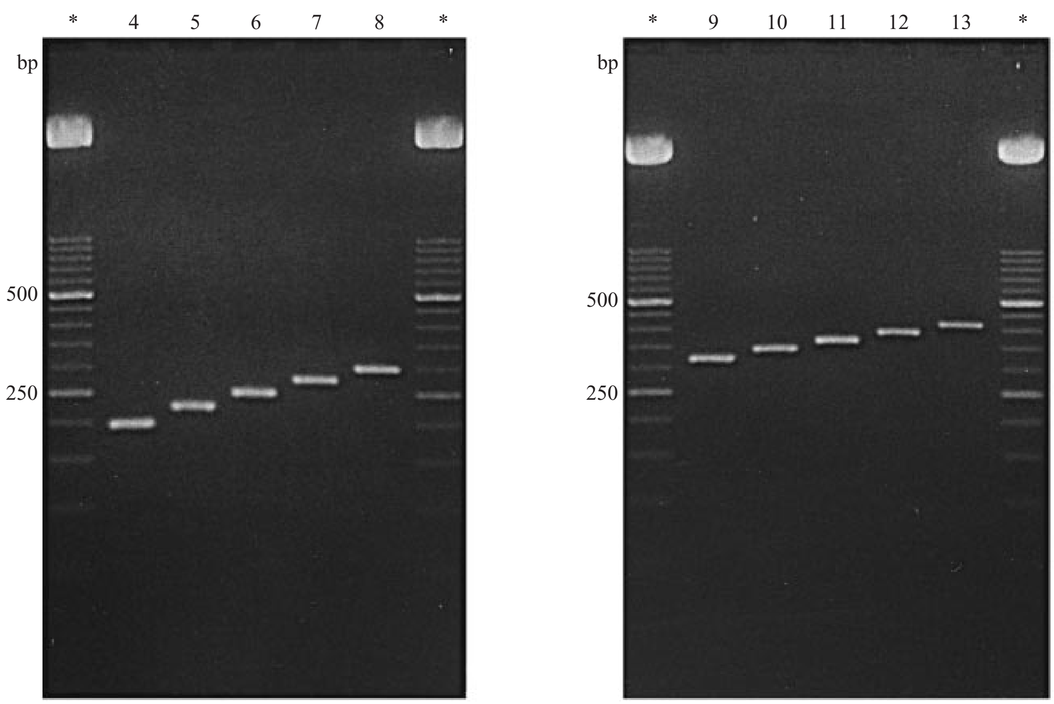

Fig. 2. PCR products containing the whole Xr-region of representative *S. aureus* strains. Estimated number of repeats is indicated above each lane. Repeat number (size of PCR product) and ID of strains are as follows: 4 repeats (205 bp), SH450; 5 repeats (229 bp), SH456; 6 repeats (253 bp), SH484; 7 repeats (277 bp), SH432; 8 repeats (301 bp), SH461; 9 repeats (325 bp), SH427; 10 repeats (349 bp), SH428; 11 repeats (373 bp), SH315; 12 repeats (397 bp), SH349; 13 repeats (421 bp), SH423. Lanes for molecular weight standard (50-bp ladder) are shown by an asterisk.

this study. Although four coagulase types were detected in MRSA, most of the isolates (87%) belonged to coagulase type II. In contrast, coagulase type VII was the most frequently detected (47·1%) in MSSA. In coagulase gene typing, a total of 15 distinct RFLP patterns (A to O) were identified. The result of coagulase gene typing indicated that *S. aureus* isolates belonging to a single coagulase type II, V, or VII, contained multiple clones. For example, coagulase type VII MSSA consisted of six genetically different

Table 4. *Frequency of protein-A types in* S. aureus *strains*

	Coagulase type	Protein-A type (number of repeat) 4	5	6	7	8	9	10	11	12	13	UD*	Total no. of isolates
MRSA	II				1		2	188	2		1		194
	III							1	1				2
	IV				2	2	1			1			6
	VII		1		18								19
	UT*							2					2
	Total		1		21	2	3	191	3	1	1		223
	(%)		(0·4)		(9·4)	(0·9)	(1·3)	(85·7)	(1·3)	(0·4)	(0·4)		(100)
MSSA	I					1							1
	II	1				1	1	6	1				10
	III			1				1	2	2			6
	IV					1	1	2					4
	V			3			1					1	5
	VII	3	1	2	11	5	4	4	2			1	33
	VIII					2							2
	UT*	1					3		5				9
	Total	5	1	6	11	10	10	13	10	2		2	70
	(%)	(7·1)	(1·4)	(8·6)	(15·7)	(14·3)	(14·3)	(18·6)	(14·3)	(2·9)		(2·9)	(100)
Total		5	2	6	32	12	13	204	13	3	1	2	293
(%)		(1·7)	(0·7)	(2·0)	(10·9)	(4·1)	(4·4)	(69·6)	(4·4)	(1·0)	(0·3)	(0·7)	(100)

* Undetermined.

Table 5. *Nucleotide and amino acid sequences of repeat units in the Xr-region of protein A*

Nucleotide sequence*	Repeat unit genotype	Amino acid sequence	Octapeptide type
GAAGACAACAACAAGCCTGGTAAA	A_1	Glu-Asp-Asn-Asn-Lys-Pro-Gly-Lys	(a)
........T...............	A_2		
..............A.........	A_3		
....................C...	A_4		
........T...........C...	A_5		
..............A.....C...	A_6		
GAAGATGGCAACAAGCCTGGTAAA	B_1	Glu-Asp-Gly-Asn-Lys-Pro-Gly-Lys	(b)
.....C..................	B_2		
..............A.........	B_3		
....................C...	B_4		
.....C........A.........	B_5		
.....C..............C...	B_6		
..............A.....C...	B_9		
.....C........A.....C...	B_8		
GAAGACAACAAAAAACCTGGCAAA	C_1	Glu-Asp-Asn-Lys-Lys-Pro-Gly-Lys	(c)
....................T...	C_2		
..............G.........	C_3		
GAAGATGGCAACAATCCTGGTAAA	D	Glu-Asp-Gly-Asn-Asn-Pro-Gly-Lys	(d)
GAAGACGGCAAAAAACCTGGCAAA	E	Glu-Asp-Gly-Lys-Lys-Pro-Gly-Lys	(e)
GAAGACAACAAAAAAACTGGTAAA	F	Glu-Asp-Asn-Lys-Lys-Thr-Gly-Lys	(f)
GAAGATGGCAACAAGCCTAGTAAA	G	Glu-Asp-Gly-Asn-Lys-Pro-Ser-Lys	(g)
GAAGACAACAACAAGCCTGGTCAA	H	Glu-Asp-Asn-Asn-Lys-Pro-Gly-Gln	(h)
GAAGACAACAAACCTGGCAAA	I	Glu-Asp-Asn-Lys-Pro-Gly-Lys	(i)
GAAGACGGCAACAAAAAACCTGGTAAA	J	Glu-Asp-Gly-Asn-Lys-Lys-Pro-Gly-Lys	(j)

* In genotype codes A_{1-6}, B_{1-8} and C_{1-3}, dot indicates identical nucleotide to that of A_1, B_1 and C_1, respectively.

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Table 6. *Arrangement of repeat units in Xr-region of protein A*

Cluster	Strain*	No. of eight amino acid-repeat	Coagulase type	Arrangement of 24 base-unit†	(*spa* type)
C1	SH423	13	II	$C_2B_8B_2C_2B_2A_6B_2A_1A_6B_1A_1B_2B_5$	(C1-a)
	SH87, SH203	11	II	. . . . $A_1A_1B_5$	(C1-b)
	SH149, SH320, SH494	10	II	. . . . B_1 . . . B_2B_5	(C1-c)
	SH479, SH20115	10	II	. . . . A_1B_5	(C1-d)
	SH220	10	II	. J . . . . . . B_2B_5	(C1-e)
	SH487	9	II	. . . . . C_1 . . B_5	(C1-f)
	SH489	9	II	. . . . − . . . B_2B_5	(C1-g)
	SH461	8	II	. . . . . $A_3A_1B_5$	(C1-h)
	SH432	7	II	. . . A_1 . B_2B_5	(C1-i)
	SH198	10	II	A_2A_4H . . . . $A_1A_1B_2$	(C1-j)
	SH64	4	II	$A_2A_1A_1B_2$	(C1-k)
C2	SH472	12	III	$A_5A_5A_5A_3A_1A_4B_2C_2B_1$ − $C_2B_6B_3$	(C2-a)
	SH325	11	III	. . − − . . . . . B_2 . . .	(C2-b)
	SH497	11	III	. . . − . . . . . . − . . .	(C2-c)
	SH475	10	III	. . − − . A_3 . . . . − . . .	(C2-d)
	SH63	6	III	. . − −	(C2-e)
C3	SH463	12	IV	$A_4A_1B_5B_5C_1B_5C_1B_3B_5B_2D\ B_1$	(C3-a)
	SH454	10	IV	. − . . . . B_2 . .	(C3-b)
	SH380, SH416	9	IV	. . . − . . . . B_2B_1	(C3-c)
	SH345	8	IV	. . . − − − . . $B_2B_1A_1$	(C3-d)
	SH58	8	IV	. . . . − − . . B_2B_1	(C3-e)
	SH73	7	IV	. . − − − − . . . B_2B_1	(C3-f)
C4	SH478	10	VII	$A_1B_5C_1B_5C_2A_3B_2C_2B_5C_2$	(C4-a)
	SH434	9	V	. . . . . . −	(C4-b)
	SH427	9	VII	−	(C4-c)
	SH147	5	VII	. . . . .	(C4-d)
C5	SH492	7	VII	$A_3B_8A_4B_5C_2B_4A_3$	(C5-a)
	SH408	7	VII	C_2B_3	(C5-b)
	SH484	6	VII	. . −	(C5-c)
	SH456	5	VII	. − −	(C5-d)
	SH60	8	I	I . . C_3B_2 . .	(C5-e)
Not grouped	SH182	4	VII	$A_3C_2B_1C_2$	(N-a)
	SH409	6	V	$A_3A_1B_2B_8G\ B_2$	(N-b)
	SH445	8	VII	$A_2A_6B_2A_6B_2B_7B_5C_2$	(N-c)
	SH401	8	VIII	$A_3E\ B_2A_4F\ C_2B_6C_2$	(N-d)
	SH323	11	VII	$A_2B_8A_1C_2C_2A_3A_1B_8C_1A_1B_8$	(N-e)
	SH495	10	III	$A_5A_1A_4A_1C_2B_1B_1C_1B_6B_5$	(N-f)

* MRSA strain is shown by an underline.

† Nucleotide sequence of the *Xr*-region of *spa* gene is represented by arrangement of repeat unit genotype codes shown in Table 5. In each cluster, dots indicate identical genotypes to those of strains listed on top (SH423, C1; SH472, C2; SH463, C3; SH478, C4; SH492, C5), while dashes denote gaps.

clones which showed *coa*-RFLP patterns B, G, H, J, M, and O.

Protein-A type

The protein-A type of all the *S. aureus* strains examined was expressed as the repeat number of a 24-nucleotide unit estimated by the size of the PCR product amplified with primers spa-2 and spa-5. As shown in Figure 2, PCR products having 10 different repeat numbers, 4–13 repeats, were detected. No PCR product was obtained from two MSSA strains (SH151 and SH152). The distribution of protein-A types was considerably different between MSSA and MRSA, as shown in Table 4. In MRSA, the 10-repeat type was predominant (85·7%), followed by 7 repeats (9·4%), while other strains showed six different repeat numbers. In contrast, in MSSA, no predominant

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protein-A type was found among the nine different types identified. The majority of MSSA strains were grouped into 7–11 repeat types almost evenly, although the 10-repeat type was the one most frequently detected (18·6%).

Comparison of protein-A type and coagulase type indicated that these markers seemed to have no definite correlation (Table 4). In general, *S. aureus* belonging to a single coagulase type was differentiated into multiple protein-A types. Seven and eight protein-A types were detected in strains with coagulase type II and VII, respectively, although the majority of coagulase type II strains showed the 10-repeat type, and the 7-repeat type was most frequently found in coagulase type VII strains (Table 4).

Octapeptide types and repeat unit genotypes

As shown in Table 5, ten octapeptide types [(a) to (j)] and 24 different nucleotide sequences (repeat unit genotypes) were identified. Octapeptide types (a), (b), and (c) were encoded by 6 (A_{1-6}), 8 (B_{1-8}), and 3 (C_{1-3}) different genotypes, respectively, each having a few synonymous nucleotide substitutions. Only peptide units (i) and (j) comprised 7 and 9 amino acids, respectively. Glu-Asp were conserved as the first two amino acids in all the repeat units. Although Pro-Gly-Lys were the C-terminal residues in most repeat units, it was of note that proline was substituted by threonine in octapeptide type (f), glycine by serine in type (g), and lysine by glutamine in type (h).

spa types and their classification into cluster

The genotype of the whole *Xr*-region (*spa* type) was expressed as a series of repeat unit genotypes (Table 6). Consequently, 37 *spa* types were identified among the 42 strains. We classified these *spa* types on the basis of partial similarity in arrangement of genotypes; that is, different *spa* types were classified into a single cluster when at least three consecutive genotypes were commonly shared by those *spa* types. The *spa* types of all the strains were aligned in individual clusters as shown in Table 6. Except for 6 strains, all *S. aureus* strains were classified into one of the 5 clusters (C1–C5) each consisting of 4–15 strains with different repeat numbers. Even among strains with identical protein-A types (same repeat numbers), *spa* types of some strains were not identical and classified into different clusters; e.g. *spa* types C1-c, C2-d, C3-b, and C4-a were found in strains with a 10-repeat type. It was of note that the strains assigned to C1, C2, and C3 belonged to coagulase types II, III, and IV, respectively. While 4 out of the 5 strains in C5 showed coagulase type VII, C4 consisted of coagulase type V and VII strains. In the *spa* types of most clusters, repeat unit genotypes located at the 5′- and 3′-ends of the *Xr*-region were common. For example, in C2, genotypes A_5A_5 at the 5′-end and $C_2B_6B_3$ at the 3′-end were conserved in 4 strains. In contrast, gaps of repeat units in *spa* types with smaller repeat numbers were mostly found in the internal portion of those with larger repeat numbers, as typically seen in the cluster C3.

DISCUSSION

The efficacy of protein-A typing (or *spa* typing) of MRSA compared with phage typing has been reported previously [7, 10]. In the present study, protein-A typing proved useful in differentiating strains with identical coagulase type in MRSA as well as in MSSA. Although definite agreement between coagulase type and protein-A type was not observed, it was notable that most MRSA with coagulase type II showed the 10-repeat type. A similar tendency in genetic homogeneity of MRSA was also found in coagulase gene typing (Table 3); 99% (192 out of 194 isolates) of coagulase type II MRSA exhibited *coa*-RFLP pattern A. Therefore, the majority of the coagulase type II MRSA is suggested to be a hospital strain of clonal derivation. On the other hand, in the study on MRSA isolated from The Netherlands, 11-repeat and 7-repeat protein-A types were detected with high frequency [4, 10], indicating that predominant MRSA strains in The Netherlands are different from those detected in our hospital.

Among the 10 different protein-A types identified, octapeptide types (a), (b), and (c) appeared most frequently in the Xr-region and are encoded by a total of 17 different genotypes. Although nucleotide sequences encoding these common repeat units were mostly identical to those reported by Frénay and coworkers [10], octapeptide types (d), (e), (f) and (h), and a repeat unit genotype C_3 are newly identified in the present study. Common to most of the repeat units, amino acids at both N- and C-terminals are conserved, whereas amino acid diversity was seen at the 3rd to 5th amino acids of repeat units; asparagine or glycine was located at the 3rd position, and lysine or asparagine was present as 4th and 5th residues. Proline and glycine, which are nonpolar amino acids found in repeat units, are considered to be responsible for formation of protein structure. These findings on amino acid composition of repeat units are consistent with those reported previously for *S. aureus* prototype strains [15] and clinical isolates [10]. However, we detected unusual repeat units (f) and (h) lacking proline or C-terminal residue lysine, respectively. These repeats were found only in *spa* types N-d and C1-j, and located between common repeat units (a), (b) or (c). Furthermore, we found repeat unit (i) with 7 amino acids and (j) with 9 amino acids, which were also detected previously [10], although the repeat unit genotypes differed by a few nucleotides.

According to our coding system of repeat unit genotypes, *spa* type of prototype *S. aureus* strains 8325-4 and Cowan 1 (NCTC8530) [15] are expressed as $A_5A_5A_5A_4A_1A_4B_2C_2B_1C_2B_6B_3$ and $A_1B_5B_5C_1B_5C_1B_3B_2B_1B_1$, which are similar to *spa* types C2-a and C3-b, respectively. Furthermore, *spa* type code 01 assigned to the predominant MRSA with phage type III-29 in a previous study [10] is indicated as $A_5A_5A_4A_1A_4B_2C_2B_1C_2B_6B_3$ in our notation, which is identical to C2-c except for a single nucleotide, while *spa* code 35 assigned to strains with phage type N corresponded to *spa* type C3-f. The agreement or similarity in *spa* types between our study and previous studies in European countries suggest that variations in the *Xr*-region may be stably conserved among *S. aureus* distributed ubiquitously.

It was of interest that most nucleotide sequences of the *Xr*-region with different repeat numbers were classified into five genetic clusters based on their similarity. Moreover, it was of note that the three clusters C1, C2 and C3 comprised strains with coagulase types II, III or IV, respectively, and that most strains in C4 and C5 belonged to coagulase type VII. From these findings, it is suggested that each cluster consists of strains that evolved from a common ancestral clone. Further, it is conceivable that *spa* type was differentiated after establishment of coagulase types II, III, and IV. In contrast, coagulase type VII *S. aureus* were found in clusters C4 and C5 together with coagulase types V and I strains, respectively. Some coagulase type VII strains were not grouped into the five clusters mentioned above. This observation suggested that coagulase type VII strains may have originated from several different clones or have been generated by an evolutionary mechanism other than coagulase types II, III and IV strains.

It has been suggested that the repeat region of the protein-A gene has evolved through multiple duplications [5, 15]. In the present study, comparison of *spa* types within each cluster showed that the gaps of the repeat unit reside in the internal portion of *spa* types with larger repeat numbers, and that repeat unit genotype(s) at the 5′- and 3′-termini of the *Xr*-region were generally conserved. These results, together with the finding that two unusual repeat units (f) and (h) are also located inside the Xr-region, suggest that multiple duplication may have readily occurred in the internal part of a series of repeat units. These findings of our study suggest that dynamic evolution of protein-A *Xr*-region might have occurred in nature, although the *spa* type is stable through multiple passages in an experimental condition [10]. The evolutionary process of protein-A gene divergence may be further elucidated by the same analysis as that employed in the present study for more *S. aureus* strains derived from a variety of sources.

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