JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2006, p. 4515–4527 Vol. 44, No. 12
0095-1137/06/$08.00+0 doi:10.1128/JCM.00985-06

Predominance of Clones Carrying Panton-Valentine Leukocidin Genes among Methicillin-Resistant *Staphylococcus aureus* Strains Isolated in Japanese Hospitals from 1979 to 1985▽

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Received 11 May 2006/Returned for modification 3 July 2006/Accepted 5 October 2006

We examined 97 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated between 1979 and 1985, the period of time when the appearance of MRSA strains increased, and we determined that these strains are distinct from the MRSA clones predominating in today's Japanese hospitals. Type IV staphylococcal cassette chromosome *mec* (SCC*mec*) strains were the most frequent, comprising 53.6% of all strains, followed by type I (22.7%) and type II (21.6%) SCC*mec* strains. Among the type IV SCC*mec* strains, the frequencies of two new subtypes, type IV.3 (IVc) and type IV.4 (IVd), were very high, comprising 38.1 and 10.3% of all strains, respectively. Forty-four of the 97 strains (45.3%) were Panton-Valentine leukocidin (PVL) positive. Among the PVL-positive strains, sequence type 30 (ST30)-SCC*mec* type IV strains producing type 4 coagulase were the most frequent. This is in striking contrast to the MRSA strains isolated in the 1990s, most of which were ST5-SCC*mec* type II strains producing type 2 coagulase and positive for the toxic shock syndrome toxin 1 gene. We also identified a new PVL-carrying prophage lysogenized in a type IV.3 SCC*mec* strain, 81/108. ϕ108PVL was distinct from the three extant PVL-carrying phages and was presumed to be carried by ST30-type IV.3 SCC*mec* strains isolated in Japan. These results provide genetic bases for the transition of MRSA clones in Japan that is commonly considered the transition from coagulase type 4 MRSA strains to coagulase type 2 MRSA strains. The results also suggested that MRSA strains that predominated between 1979 and 1985 were generated from PVL-positive methicillin-susceptible *S. aureus* strains through the integration of SCC*mec* elements.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the major human pathogens and causes a wide range of infections in health care settings and community environments (5, 11, 36). The first MRSA strain was reported in England in 1961, after which it appeared worldwide (25). In Japan, *S. aureus* strains exhibiting low-level resistance to methicillin were reported in the early 1960s, albeit at very low frequencies of less than 3% (20, 35). However, from the late 1970s to the early 1980s, a period when third-generation cephems with a wide spectrum of antimicrobial activity were introduced in the clinical field, MRSA strains began to dominate in Japanese hospitals (8). Since some of the third-generation cephems had weak antibacterial activities toward *S. aureus*, it was suspected that the excessive use of these antibiotics was responsible for the expansion of MRSA strains in hospitals throughout Japan during this time. The frequency of isolation of MRSA strains increased drastically in the early 1980s from 34% (from 1982 to 1983) to 43% (from 1986 to 1987) and from 18.6% (from July to December 1981) to 35.9% (from January to June 1982) (34, 38, 43). From the mid-1980s to the early 1990s, β-lactam antibiotics with improved antibacterial activities towards *S. aureus* were introduced, but by 1990, these antibiotics had become less effective against clinical isolates.

MRSA strains have been characterized by susceptibility testing and coagulase isotyping. Coagulase typing is a method developed in Japan which can classify *S. aureus* strains based on the antigenic difference in coagulase, the protein causing coagulation of plasma (47). In the early 1980s, coagulase type 4 MRSA strains, which showed a heterogeneous profile of oxacillin resistance, disseminated in Japanese hospitals. But from the mid 1980s to 1990s, the majority of hospital MRSA isolates were coagulase type 2 MRSA strains, most of which exhibit high resistance to oxacillin as well as to other many antibiotics. It was suggested that MRSA strains disseminated in Japanese hospitals had undergone a transition (9, 29, 31, 46). We have investigated the genotypes by conducting ribotyping and the carriage of toxin genes and reported that a shift of MRSA strains from coagulase type 4 MRSA strains to coagulase type 2 MRSA strains should be regarded as a shift of MRSA clones. The coagulase type 2 MRSA strains, which were characterized by the production of enterotoxin A and by their specific ribotyping patterns, were prevalent in the early 1980s but declined drastically in the 1990s. On the other hand, coagulase type 4 MRSA strains were characterized by the production of enterotoxin C and toxic shock syndrome toxin 1 (TSST-1) and by having their specific ribotyping patterns (16, 46).

We now know that MRSA strains have evolved from methicillin-susceptible *Staphylococcus aureus* (MSSA) by the acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) carrying the *mecA* gene (24, 30). There are several types of SCC*mec* elements that differ in their genomic organi-

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▽ Published ahead of print on 18 October 2006.

TABLE 1. Primers used in this study

Primer purpose	Primer name	Nucleotide sequence (5′–3′)	Reference or location on ϕ108PVL
Subtyping of type IV SCC*mec* elements			
IV.1 (IVa)	4a1	TTTGAATGCCCTCCATGAATAAAAT	Okuma et al. (39)
	4a2	AGAAAAGATAGAAGTTCGAAAGA	Okuma et al. (39)
IV.2 (IVb)	4b1	AGTACATTTTATCTTTGCGTA	Okuma et al. (39)
	4b2	AGTCATCTTCAATATCGAGAAAGTA	Okuma et al. (39)
IV.3 (IVc)	4c1	TCTATTCAATCGTTCTCGTATTT	Ma et al. (32)
	4c2	TCGTTGTCATTTAATTCTGAACT	Ma et al. (32)
IV.4 (IVd)	4d1	TTTGAGAGTCCGTCATTATTTCTT	Ma et al. (32)
	4d2	AGAATGTGGTTATAAGATAGCTA	Ma et al. (32)
Identification of virulence factor			
LukS and LukF	PVL-F	ATGTCTGGACATGATCCAA	Ma et al. (32)
	PVL-R	AACTATCTCTGCCATATGGT	Ma et al. (32)
TSST-1	TSST-1A	TGATATGTGGATCCGTCAT	Ma et al. (32)
	TSST-1B	AAACACAGATGGCAGCAT	Ma et al. (32)
CNA	cna-1	ACACCAGACGGTGCAACAATTA	Ma et al. (32)
	cna-2	AGCAATACCGTTTGCATCTGTTA	Ma et al. (32)
SEH	entH-F	ATTCACATCATATGCGAAAGCAG	Ma et al. (32)
	entH-R	ATGTCGAATGAGTAATCTCTAG	Ma et al. (32)
Amplification of the whole genome of ϕ108PVL			
From chromosome to integrase	phiMW-DN	GCAGAAAAAGATGCGATTGAA	
	int-R	CGGATACAAAGACACTACAAA	800–780
From integrase to antirepressor	int-F	TTTGTAGTGTCTTTGTATCCG	780–800
	anti-R	CCGAATGTGTGATTGATGTTT	5229–5209
From antirepressor to terminase large subunit	anti-F	ATTGTATTTGCAGATGCAGTAG	5001–5022
	termi-R	TTAATACATCTTCAATGTCTGTC	19338–19316
From terminase large subunit to portal protein	termi-F	AAACAAGGTAAGTCTCTAATCG	19116–19137
	portal-R	TCTAAATTAGCATCCGTGATAC	21616–21637
From portal protein to tail protein	portal-F	ACACGTGATAAAACAGGAGAA	21069–21089
	tail-R	TTCGACTTTTATCTTCGACTTTTC	27382–27359
From tail protein to LukS-PV	tail-F	TTAAAAGACATGCAAAGAGAGC	27566–27587
	LukS-R	CAGCTTTAGATTCATGAAACGAA	42344–42322
From LukS-PV to chromosome	LukS-F	TGGTCAACTATATCGTGGTTTT	42038–42059
	phiMW-UP	TCGCCACGTTTAGCAATTTTAT	
Amplification of the leftmost region specific to three extant phages in combination with a primer, int-c			
	int-c	TTTGTAGTGTCTTTGTATCCG	
ϕPVL	recϕPVL	TCGAAACCTTATCAAAAGAAACT	
ϕSLT	recϕSLT	AAGCTACTGTACAGCGTTATG	
ϕSa2mw	recϕSa2mw	AATCTACCTTATCCCGATACGA	
Amplification of the rightmost region specific to three extant phages in combination with LukSR listed above			
ϕPVL	ϕPVL-RE	TCTTAACTTACCTATTAGAACG	
ϕSLT, ϕSa2mw	ϕSLT-MW-RE	ATTACAAGCGTATGACTTATCGA	
Amplification of the regions located on ϕ108PVL			
A	int-F2	ATGTTTTCGAGTTTTTGAGTTAG	393–415
	int-R2	CATTTTAATTGCCAGCATCTTA	929–950
B	termi-2	GTAGAGGTCAATACAGAATGTT	19066–19087
	portal-R2	AATCTTATGATACGATGTCCC	19743–19763
C	portal-F2	ACACGTGATAAAACAGGAGAA	21069–21089
	portal-R	TCTAAATTAGCATCCGTGATAC	21616–21637
D	tail-F2	CGTTTGTGGGATAAACTACGTAAT	29952–29975
	tail-R2	TAGTAGTACCGAAATGAAATCTC	30386–30408
E	tail-F3	CTTGATTAGACTCAACCAAACT	31232–31257
	tail-R3	ATAATTGGGATAGCAACGCAA	31704–31725
F	108RE-F	AGGCTTCACCCTTACTTATTGA	42038–42059
	108RE-R	GATACCTGATAGCGAATTCG	44363–44832

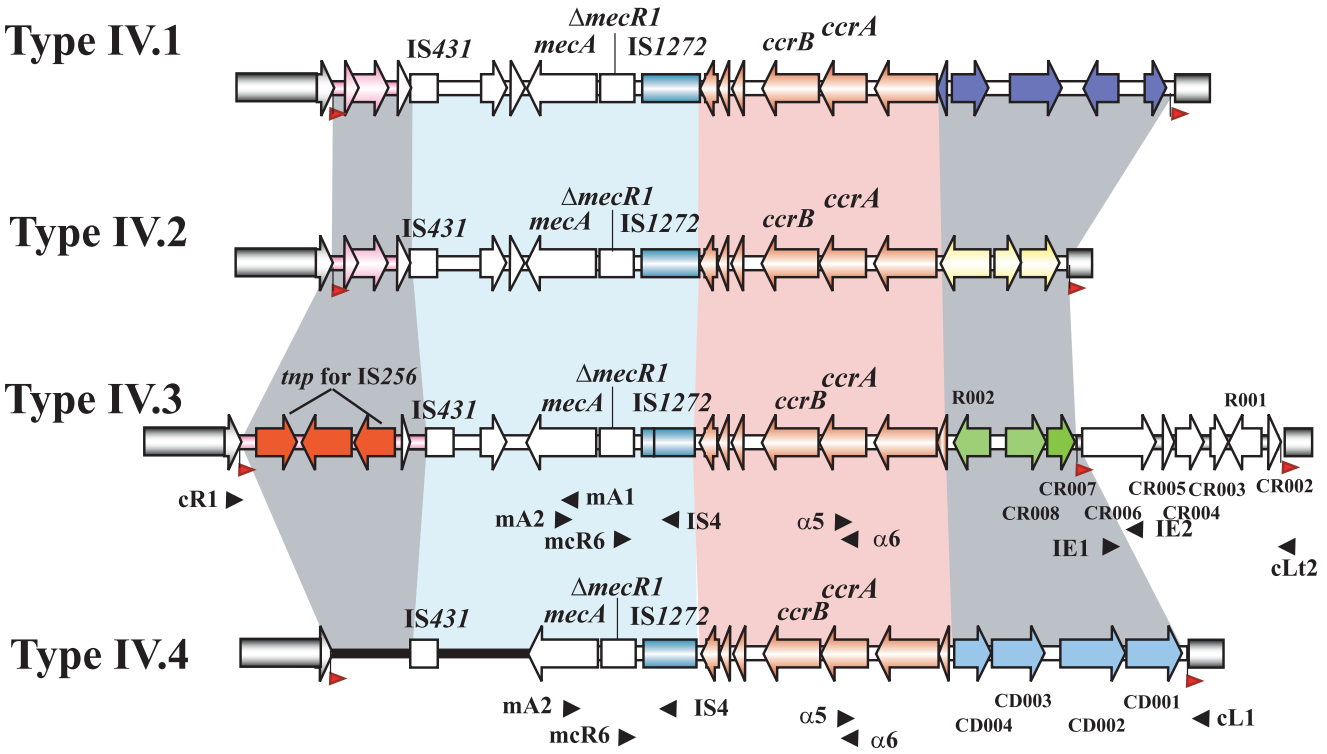

FIG. 1. Structural comparison of different subtypes of type IV SCC*mec* elements. The structures of four subtypes of type IV SCC*mec* elements are illustrated based on the nucleotide sequences deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB063172 (type IV.1 SCC*mec*), AB063173 (type IV.2 SCC*mec*), AB096217 (type IV.3 SCC*mec*), and AB097677 (IV.4 SCC*mec*). The SCC*mec* element is composed of two essential gene complexes, the *ccr* gene complex (pink) and the *mec* gene complex (light blue). The *ccr* gene complex consists of *ccr* genes which are responsible for the mobility of the SCC*mec* element and surrounding ORFs. The *mec* gene complex is responsible for methicillin-cephem resistance. Other areas (light gray) of SCC*mec* are nonessential and are divided into three regions, J1 to J3. Direct repeats containing integration site sequences for SCC*mec* elements are indicated by red arrowheads. The locations of the primer sets used for PCR amplification of an entire SCC*mec* element of strain 81/108 and a part of SCC*mec* element of strain JCSC4469 are indicated by arrows.

zations and structures (22, 23, 33, 40, 44), and it is generally accepted that MRSA clones could be defined by SCC*mec* type and genotype. The classification of MRSA clones is important for epidemiological study to distinguish MRSA clones that have predominated through a hospital setting and a community setting.

In the current study, we conducted a retrospective analysis of MRSA strains isolated from Japanese hospitals between 1979 and 1985 and during the early 1990s using several different molecular typing methods: SCC*mec* element typing, virulence gene repertoire, and multilocus sequence typing (MLST) (13). Our results provided genetic proof for the transition of MRSA clones predominating in Japanese hospitals. Furthermore, we found that approximately one-half of MRSA strains disseminated in the early 1980s in Japan were Panton-Valentine leukocidin (PVL)-positive clones, possibly harboring a novel PVL-carrying phage.

MATERIALS AND METHODS

MRSA strains used in this study. MRSA strains isolated during three different time periods were tested: 97 MRSA strains isolated from 1979 to 1985 in Japanese hospitals (24 strains isolated at Tokyo University Hospital in 1982, 49 strains isolated at Gunma University Hospital from 1981 to 1985, 22 strains isolated at Jikei University Hospital from 1979 to 1981, and two strains isolated at other hospitals in 1981), 22 strains isolated in 1992 at Tokyo University Hospital, and 138 strains isolated in 1999 in 14 Japanese hospitals (Teikyo University, Fukushima Prefectural Medical College, Iwate Medical College, Kobe University, Akita University, Saga Medical College, Tokyo University, Hiroshima University, Fukuoka University, Mie University, Kurume Medical College, Shiga University, Jikei Medical College, and National Defense Medical College).

Susceptibility testing. MICs were determined using the agar dilution method according to the procedure recommended by the Clinical and Laboratory Standards Institute. The antibiotics tested were oxacillin, tetracycline, erythromycin, and gentamicin (Sigma Chemical Co., St. Louis, Mo.), ceftizoxime (Fujisawa Pharmacy Co., Osaka, Japan), imipenem (Banyu Pharmaceutical Co., Tokyo, Japan), and levofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan).

***SCCmec* typing and identification of virulence genes.** SCC*mec* typing was performed using PCR as described previously (21, 22, 39). The presence of four virulence-related genes, *lukS-PV–lukF-PV*, *tsst-1*, *seh*, and *cna*, was investigated by PCR using the primers listed in Table 1.

Nucleotide sequencing of type IV.3 and type IV.4 SCC*mec* elements. Several DNA fragments spanning the entire nucleotide sequence of the SCC*mec* element and an SCC-like element of strain 81/108 were amplified by long-range PCR with the five primer sets (Fig. 1): the region spanning *orfX* (an open reading frame [ORF] that works as a port of the gene cassettes SCC; all SCC elements are integrated into the 3′ end of *orfX* or into the regions having the specific nucleotide sequence that is very similar to the nucleotide sequence of the 3′ end of *orfX*) to *mecA* was amplified using primers cR1 and mA1; the region spanning *mecA* to IS*1272* was amplified using primers mA2 and IS*4*; the region spanning *mecR1* to the *ccr* complex was amplified using primers mcR6 and α6; the region spanning the *ccr* complex to IE25923 was amplified using primers α5 and IE2 (5′-TCCACAAAATTTACATATACTCTCT-3′); the region spanning IE25923 to left-flanking chromosomal region flanked to left end of SCC*mec* was amplified by PCR with primers IE1(5′-AGAAATTTTGTAGCGAATGATGA-3′) and cLt2. The DNA fragments encompassing the region from the J1 region to the *mec* gene complex of the SCC*mec* element of strain JCSC4469 were amplified by

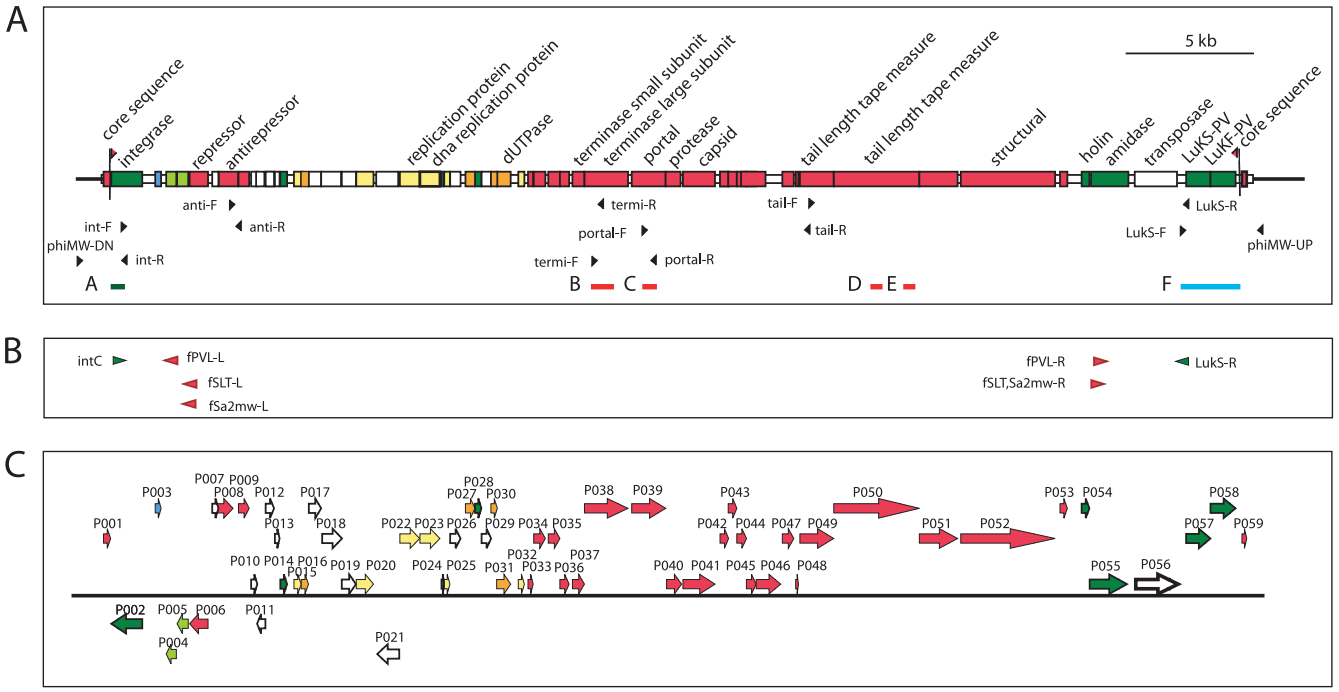

FIG. 2. (A) Essential structural and functional components of ϕ108PVL element are illustrated. Black arrowheads indicate the locations of primers used to amplify the entire ϕ108PVL genome. The two red arrowheads flanking the core sequence indicate the *att* sites on the phage element. (B) The locations of the primers used to amplify regions specific to ϕPVL, ϕSLT, and ϕSa2MW. (C) The ORFs in and around the ϕ108PVL element are illustrated as arrows in six possible reading frames. The direction of the arrows indicates the transcriptional direction for each ORF. Color codes are as follows: dark green, ORFs (or the parts of ORFs) that are well conserved among all other three PVL-carrying phages, ϕPVL, ϕSLT, and ϕSa2MW; red, ORFs that are highly homologous to ϕPVL, which is lysogenized in the *S. aureus* ATCC 49775 strain; yellow, ORFs that are highly homologous to ϕSLT; blue, ORFs that are highly homologous to ϕSa2mw; orange, ORFs that are highly homologous to both ϕPVL and ϕSLT; green, ORFs that are highly homologous to both ϕSLT and ϕSa2mw; white, ORFs that are unique in ϕ108PVL.

long-range PCR using the following three sets of primers, mA2 and IS*4*, mcR6 and α6, and α5 and cL1, as indicated in Fig. 1. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany), and their nucleotide sequencing was carried out by fluorescence dideoxy chain termination chemistry using the BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, CA) and an ABI Prism 3100 genetic analyzer (Applied Biosystems, CA). PCR and long-range PCR were performed as described previously (24).

PCR amplification and nucleotide sequencing of PVL-carrying prophage ϕ108PVL. The DNA fragments encompassing the entire phage-specific region in the bacterial chromosome, along with the primers used to amplify them by long-range PCR, were as follows: the phage-flanking chromosomal region to integrase, primers phiMW-DN and intR; integrase to antirepressor, primers int-F and anti-R; anti-repressor to terminase large subunit, primers anti-F and termi-R; terminase large subunit to portal gene, primers termi-F and portal-R; portal gene to tail gene, primers, portal-F and tail-R; tail gene to the *lukS-PV* gene, primers tail-F and LukS-R; and *lukS-PV* gene to flanking chromosomal region, primers, LukS-F and phiMW-UP.

The nucleotide sequences of the primers used in these studies are listed in Table 1, and their locations in the phage region of the genome are illustrated in Fig. 2. PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany), and their nucleotide sequences were determined by primer walking.

Coagulase isotyping. Coagulase type was determined by using an inhibition test for the coagulation of plasma with eight specific antisera (Denka Seiken, Niigata, Japan) according to the method of Ushioda et al. (47). Briefly, bacterial strains were grown overnight in brain heart infusion broth and culture supernatants were collected by centrifugation. An appropriately diluted 0.1-ml aliquot of the supernatant was mixed with 0.1 ml of a solution containing each antiserum and incubated at 37°C for 1 h. Diluted rabbit plasma (0.2 ml) was added to each tube, followed by incubation at 37°C for 1 h or more until coagulation of the plasma was observed by visual inspection. The serotype of cogulase produced by a given strain was determined by the specificity of the antiserum which inhibited coagulation.

MLST. Genotypes of representative strains were determined by MLST according to the procedure of Enright et al. (13). Alleles of the seven loci were assigned by a comparison of their sequences to the corresponding loci in the *S. aureus* MLST database (www.mlst.net). Sequence types were determined according to the combined pattern of the seven alleles, and clonal complexes were defined by the BURST (based upon related sequence types) program available on the MLST website.

Nucleotide sequence accession numbers. The entire nucleotide sequence of ϕ108PVL has been deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB243556. The sequences of the type IV.3 (VIc) SCC*mec* of strain 81/108 and the type IV.4 (IVd) SCC*mec* of strain JCSC4469 have been deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB096217 and AB097677, respectively.

RESULTS

SCC*mec* typing of MRSA strains isolated between 1979 and 1985 and the 1990s. We characterized the SCC*mec* elements carried by 97 MRSA strains isolated between 1979 and 1985 (1979-1985 strains) and 22 MRSA strains isolated in 1992 and compared them to the types of SCC*mec* elements of MRSA strains isolated in 1999, which have previously been reported (6). As shown in Table 2, 95 of the 1979-1985 strains (97.9%) could be classified into one of three types of SCC*mec* elements, judging from the combinations of *mec* gene complex and *ccr* gene complex identified by PCR. Type IV SCC*mec* strains (53.6%) were the most frequent overall (53.6%), followed by

TABLE 2. Presence of virulence determinants in MRSA strains and genotypes of the strains representing each combination[a]

Group (yr of isolation)[b]	Characteristics of MRSA strains isolated at each period							Genotypes of chosen MRSA strains as indicated by:				No. of tested strains
	SCC*mec* type	Ratio (%)	Presence of indicated virulent determinant				No. of strains with indicated results	Coagulase isotype	MLST			
			PVL	CNA	TSST-1	SEH			CC	ST	Allelic profile	
A (1979-1985)	I (n = 22)	22.7	+	+	−	−	4	4	30	30	2, 2, 2, 2, 6, 3, 2	2
			+	−	−	−	7	4	30	30	2, 2, 2, 2, 6, 3, 2	1
			−	+	−	−	5	4	30	30	2, 2, 2, 2, 6, 3, 2	1
			−	−	−	−	6	4	30	30	2, 2, 2, 2, 6, 3, 2	1
	II.1 (IIa) (n = 21)	21.6	+	−	+	−	1	2	5	5	1, 4, 1, 4, 12, 1, 10	1
			−	−	+	−	20	2	5	5	1, 4, 1, 4, 12, 1, 10	6
	IV.1 (IVa) (n = 1)	1.0	+	+	−	−	1	4	N[c]	N	2, 2, N, 2, 6, 3, 2	1
	IV.3 (IVc) (n = 37)	38.1	+	+	−	−	30	4	30	30	2, 2, 2, 2, 6, 3, 2	7
			−	−	+	−	1	3	8	8	3, 3, 1, 1, 4, 4, 3	1
			−	−	−	−	6					
	IV.4 (IVd) (n = 10)	10.3	−	−	−	−	10	2	5	5	1, 4, 1, 4, 12, 1, 10	1
	IV.n (IVn) (n = 4)	4.1	+	+	−	−	1	4	30	30	2, 2, 2, 2, 6, 3, 2	1
			−	+	−	−	3	4	30	30	2, 2, 2, 2, 6, 3, 2	1
	Nontypeable (n = 2)	1.0	−	−	−	−	1					
			−	−	+	−	1	2	5	5	1, 4, 1, 4, 12, 1, 10	1
B (1992)	I (n = 1)	4.5	−	−	−	−	1					
	II.1 (IIa) (n = 20)	91.0	−	−	+	−	19	2	5	5	1, 4, 1, 4, 12, 1, 10	3
			−	−	−	−	1					
	IV.3 (IVc) (n = 1)	4.5	−	−	−	−	1					
C (1999)	I (n = 1)	0.7	−	−	−	−	1					
	II.1 (IIa) (n = 120)	91.3	−	−	+	−	114	2	5	5	1, 4, 1, 4, 12, 1, 10	6
			−	−	−	−	6					
	II.n (n = 6)	4.3	−	+	−	−	2					
			−	−	+	−	4					
	IV.3 (IVc) (n = 6)	4.3	−	+	−	+	4	7	1	81	1, 1, 1, 9, 1, 1, 1	2
			−	−	−	−	2					
	Nontypeable (n = 5)	3.6	−	−	+	−	5					

[a] CNA, collagen adhesion protein; CC, clonal complex; N, nontypeable; SEH, staphylococcal enterotoxin H; ST, sequence type. +, present; −, absent.
[b] For group A (n = 97), the numbers of PVL-positive and TSST-1-positive strains were 44 (45.3) and 23 (23.7%), respectively, for group B (n = 22), the numbers of PVL-positive and TSST-1-positive strains were 0 and 19 (86.3%), respectively, and for group C (n = 138), the numbers of PVL-positive and TSST-1-positive strains were 0 and 123 (89.1%), respectively.
[c] The sequence type of the strains could not be assigned since the *glpF* gene could not be amplified by PCR.

type I SCC*mec* strains (22.7%) and type II SCC*mec* strains (21.6%). The frequencies of each SCC*mec* strain, grouped according to hospital and ranked in order from highest to lowest, were as follows: Tokyo University Hospital, type IV, 17/24 (70.8%), type I, 6/24 (25.0%), and type II, 1/24 (4.2%); Gunma University Hospital, type IV, 32/49 (65.3%), type II, 8/49 (16.3%), type I, 7/49 (14.3%), and nontypeable, 2/49 (4.1%); and Jikei University Hospital, type II, 12/22 (54.5%), type I, 9/22 (4.1%), and type IV, 1/22 (4.5%). Although the frequency of each SCC*mec* strain at the three hospitals was different, it was clear that three types of SCC*mec* strains were disseminated and over three-quarters of the 1979-1985 strains (76%) carried either a type IV or a type I SCC*mec* element. In contrast, 126 out of 138 (93%) MRSA strains isolated in 1999 in 14 hospitals carried a type II SCC*mec* element. When we looked at the MRSA strains isolated in Tokyo University Hospital in particular, 91% of the strains isolated in 1992 and 100% of the strains isolated in 1999 carried a type II SCC*mec* element, indicating that type II SCC*mec* strains predominated in the early 1990s, at least at Tokyo University Hospital.

New subtypes of type IV MRSA strains, IV.3 (IVc) and IV.4 (IVd), predominated in the early 1980s in Japan. We further classified the SCC*mec* type II and type IV elements based on the nucleotide sequences of their J1 regions. The majority of SCC*mec* type II strains were classified as subtype type II.1 (type IIa), while six strains isolated in 1999 could not be classified into subtype 1 (type IIa) or 2 (type IIb). When we examined the type IV SCC*mec* elements by PCR experiments using primers that would amplify type IV.1- and type IV.2-specific J1 sequences, we identified one SCC*mec* type IV.1 strain among 58 SCC*mec* type IV strains examined. This result suggested that most type IV strains carried SCC*mec* type IV of unknown subtype. Therefore, we amplified and sequenced several large DNA fragments spanning the entire SCC*mec* element from MRSA strain 81/108. The nucleotide sequence of the J1 region of the SCC*mec* element carried by 81/108 was not homologous to any previously reported type IV.1 or type IV.2 SCC*mec* elements, so we have designated this element type IV.3 SCC*mec* (Fig. 1). We then designed specific primers to amplify type IV.3-specific J1 region sequences and conducted PCR experiments using chromosomal DNA from strains whose subtypes, based on the sequence of the J1 region, were unclassifiable. According to this type of analysis, 40 of 55 SCC*mec* type IV strains were type IV.3 SCC*mec* strains and 15

TABLE 3. Comparison among MICs to 10 antibiotics of MRSA strains isolated in each period

Antibiotic	MIC (μg/ml) of MRSA isolated in:			
	1979 to 1985		1999	
	50%	90%	50%	90%
Oxacillin	32	128	256	>512
Ceftizoxime	>512	>512	>512	>512
Imipenem	0.5	64	32	128
Ampicillin	64	256	32	64
Gentamicin	128	512	32	128
Tobramycin	128	512	256	512
Erythromycin	>512	>512	>512	>512
Tetracycline	2	4	64	64
Levofloxacin	0.25	0.5	8	64
Ciprofloxacin	1	2	NT[a]	NT

[a] NT, not tested.

strains still remained unclassifiable. We then amplified and sequenced DNA fragments from MRSA strain JCSC4469 that spanned the region of the SCC*mec* element from *mecA* to J1. The J1 region of the SCC*mec* element of strain JCSC4469 was not homologous to that of a type IV.1, IV.2, or IV.3 SCC*mec* element, so we have designated it type IV.4 (Fig. 1). We then carried out PCR experiments using primers that amplified type IV.4-specific J1 sequences and found that 10 of 15 previously unclassified strains belonged to this type, leaving 5 strains still nontypeable. These results indicated that type IV.3 SCC*mec* strains, which are infrequent among recent isolates, predominated in Japan between 1979 and 1985 (Table 2).

Antibiotic susceptibilities. We determined the MICs of eight antibiotics (oxacillin, imipenem, ampicillin, gentamicin, tobramycin, erythromycin, tetracycline, and levofloxacin) for MRSA strains isolated between 1979 and 1985 and compared them to MICs of MRSA strains isolated in 1992 and 1999 (Table 3). Most of the strains isolated between 1979 and 1985 were susceptible to tetracycline, levofloxacin, and imipenem and showed low-level resistance to oxacillin. In contrast, the majority of MRSA strains isolated in 1999 were highly resistant to all of the antibiotics tested. The MICs of 1992 isolates for nine antibiotics reported by Tanaka et al. (46) are very similar to those of the 1999 isolates, indicating that MRSA strains became highly resistant to many antibiotics in the early 1990s.

Distribution of virulence-related genes. The prevalence of virulence-related genes in all 257 MRSA strains was examined by PCR amplification of four gene loci: *lukS-PV–lukF-PV*, encoding Panton-Valentine leukocidin; *cna*, encoding collagen adhesion protein; *seh*, encoding staphylococcal enterotoxin H; and *tsst-1*, encoding toxic shock syndrome toxin-1. Three of these genes, *lukS-PV–lukF-PV*, *cna*, and *seh*, were identified in the highly virulent community-acquired MRSA (C-MRSA) strain MW2. The results are summarized in Table 2. Forty-four of 97 MRSA strains (45.3%) isolated between 1979 and 1985 carried *lukS-PV–lukF-PV* genes. In contrast, none of the MRSA strains isolated in 1992 and 1999 carried these genes. We detected *cna* in 44 of 97 MRSA strains isolated between 1979 and 1985, whereas only a few MRSA strains isolated in 1992 and 1999 carried this gene. The prevalence of the *seh* gene was very low, being identified in only four strains isolated in 1999, indicating that this gene was rarely carried by hospital-associated MRSA (H-MRSA) strains in Japan. There was a low prevalence of TSST-1-positive strains among those isolated between 1979 and 1985; however, the majority of the MRSA strains isolated in both the early and late 1990s carried *tsst-1* (86.4% [19 of 22] and 92.0% [127 of 138], respectively).

Comparison of genotypes of strains chosen from different periods. To identify the genotypes of MRSA strains isolated at different periods of time, MLST and coagulase typing were conducted on strains representing each combination of SCC*mec* type and *lukS-PV–lukF-PV*- or *tsst-1*-positive gene profiles (Table 2). Among MRSA strains isolated between 1979 and 1985, ST30-coagulase type 4 strains were the most dominant, followed by ST5-coagulase type 2 strains and ST8-coagulase type 3 strains. All PVL-positive and TSST-1-negative strains were ST30-type 4 coagulase producers and carried a type I or type IV SCC*mec* element. Curiously, we found a PVL-positive/TSST-1-positive strain that carried a type II SCC*mec* and was a ST5-coagulase type 2 producer. Among the MRSA strains isolated in the 1990s, ST5-coagulase-type 2 strains were the most dominant, followed by ST81-coagulase type 7 strains. All

TABLE 4. PCR identification for four integrated PVL-carrying genes[a]

Name of tested strain	Coagulase isotype	MLST ST	SCC*mec*	Regions common to ϕ108PVL						Leftmost regions specific to:			Rightmost regions specific to:	
				A	B	C	D	E	F	ϕPVL	ϕSLT	ϕSa2mw	ϕPVL	ϕSLT and ϕSa2mw
JCSC2973	4	30	1B (I)	+	+	+	−	+	−	−	−	−	−	−
JCSC4467	4	30	1B (I)	+	+	+	−	+	−	−	−	−	−	−
JCSC4483	4	30	1B (I)	+	+	+	−	+	−	−	(+)	−	−	−
JCSC2958	2	5	2A.1	+	−	−	−	−	(+)	−	−	−	−	(+)
JCSC4451	4	30	2A.n (II)	+	+	+	+	+	+	−	−	−	−	−
JCSC2913	4	N	2B.1 (IVa)	+	+	+	+	+	+	−	−	−	−	−
81/108	4	30	2B.3 (IVc)	+	+	+	+	+	+	−	−	−	−	−
JCSC4458	4	30	2B.3 (IVc)	+	+	+	+	+	+	−	−	−	−	−
JCSC4461	4	30	2B.3 (IVc)	+	+	+	+	+	+	−	(+)	−	−	(+)
JCSC4485	4	30	2B.3 (IVc)	+	+	+	+	+	+	−	−	−	−	−
155-2	4	30	2B.3 (IVc)	+	+	+	+	+	−	−	−	−	+	−
174-2	4	30	2B.3 (IVc)	+	+	+	(+)	+	(+)	−	(+)	−	−	−
JCSC4465	4	30	2B.n	+	+	+	−	+	−	−	+	−	−	−

[a] N, nontypeable; ST, sequence type. +, present; (+), might be present (the amount of amplified DNA fragment is not much); −, absent.

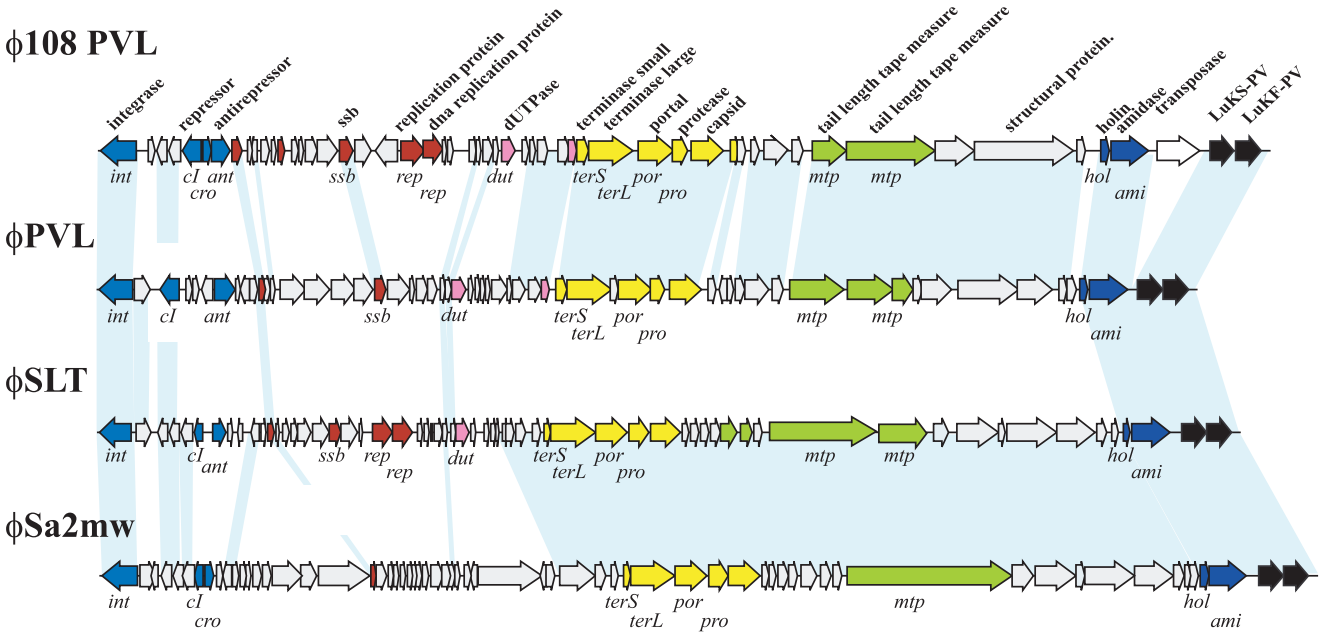

FIG. 3. Alignment of the four PVL-carrying phages. Structures of ϕ108PVL, ϕPVL, ϕSLT, and ϕSa2mw are indicated based on the following nucleotide sequences: ϕ108PVL (DDBJ, EMBL, and GenBank databases under accession no. AB243556), ϕPVL (DDBJ, EMBL, and GenBank accession no. AB009866), ϕSLT (DDBJ, EMBL, and GenBank accession no. NC_002661), and ϕSa2mw (DDBJ, EMBL, and GenBank accession no. BA000033). Genes having sequence identities of more than 90% are linked by light blue shading. Known functions of ORFs are colored as follows: lysogeny, blue; DNA replication, red; recombination, pink; DNA packaging and head, yellow; tail, green; lysis, dark blue; *lukS-PV–lukF-PV*, black.

tested type II.1 SCC*mec* strains, which were PVL negative/TSST-1 positive, were ST5-coagulase type 2 producers.

Identification of a novel PVL-carrying phage ϕ108PVL. It has been well established that *lukS-PV* and *lukF-PV* are encoded by a prophage that integrated into the *S. aureus* chromosome. To date, the nucleotide sequences of three temperate phages carrying the *lukS-PV–lukF-PV* genes, ϕPVL, ϕSLT, and ϕSa2mw, have been reported (2, 28, 37). To determine whether MRSA strains isolated in the early 1980s harbored one of the three extant PVL-carrying phages, we designed two primer sets to identify three phages specifically: a primer pair composed of a primer specific for integrases that are common to three phages and three primers specific for the repressor gene of each PVL-carrying phage, ϕPVL, ϕSLT, and ϕSa2mw; a primer pair composed of a primer specific for the *lukS* gene that is common to three phages and another two primers, one is specific for an ORF, P052, in ϕPVL and the other is specific for ORFs of unknown function that are conserved in two phages, ϕSLT and ϕSa2mw. PCR was carried out using chromosomal DNAs from 34 of the 44 PVL-positive MRSA strains. The results from selected strains, representing different combinations of SCC*mec* type and exotoxin repertoire, are presented in Table 4. There were no strains that carry either one of three phages. A strain showed a positive result by PCR for identifying the gene lineage *int* to *rep*, and a strain showed a positive result by PCR for identifying the gene lineage *lukS* to an ORF of unknown function, However, no strain showed a positive result with both primer sets, indicating that these strains did not carry one of three extant PVL-carrying phages.

To determine whether these strains carried a prophage different from those of the three extant phages, DNA fragments spanning the entire phage genome were amplified by long PCR and sequenced using the chromosomal DNA of MRSA strain 81/108, a ST30-type IV.3 SCC*mec* strain. The characteristic 25-bp sequences *attP* left and *attP* right, which are located at both ends of the phage genome, were present. Judging from the locations of *attP* left and *attP* right, we estimated that the size of the prophage carried by MRSA strain 81/108, which we termed ϕ108PVL, was 44,107 bp in length. This was comparable to the sizes of the three extant PVL phages, ϕPVL, ϕSLT, and ϕSa2mw, which are 41,421, 42,942, and 45,924 bp in size, respectively. Two 29-bp conserved core sequences were identified adjacent to the *attP* left and *attP* right sequences. The core sequences in ϕ108PVL were identical to the corresponding sequences in ϕPVL but differed by 2 bp from the conserved core sequences in ϕSLT and ϕSa2mw.

Figure 2A illustrates the genomic organization of ϕ108PVL. Using BLAST to search for homologies to the three PVL-carrying phages (ϕPVL, DDBJ/EMBL/GenBank accession no. AB009866; ϕSLT, DDBJ/EMBL/GenBank accession no. NC_002661; and ϕSa2mw, DDBJ/EMBL/GenBank accession no. BA000033) (Fig. 2C), a total of 59 predicted ORFs larger than 99 bp were identified in and around ϕ108PVL. The organization of the genome of ϕ108PVL was very similar to that of the extant PVL-carrying phages and contained regions related to lysogeny, DNA replication/transcriptional regulation, packaging/head, tail, and lysis as well as *lukS-PV–lukF-PV* (Fig. 3; Table 5). Although all four phages were similar in their genomic organizations, not all of the ORFs encoded by the four phages were homologous. There were complexes of ORFs that were highly homologous, and those that appeared to be distantly related.

TABLE 5. ORFs in and around 108PVL and their similarities to three extant PVL-carrying phages

ORF or special structure[a]	Starting position	Ending position	Size (bp)	aa[b]	Gene	Function	Data for phage								
							ϕPVL			ϕSLT			ϕSa2mw		
							% Identity[c] to genome	Corresponding ORF (bp)	% aa identity	% Identity to genome	Corresponding ORF (bp)	% aa identity	% Identity to genome	Corresponding ORF (bp)	% aa identity
(P001)	80	340	261	87		Hypothetical protein									
attB1	246	270	25			Attachment site on chromosome									
Core	271	299	29			Core sequence									
attP left	300	324	25			Attachment site on phage									
P002	1588	383	1,206	402	*int*	Phage integrase	98.6	Integrase (1,206)	98.6	98.4	Integrase (1,206)	98.4	99.9	Integrase (1,206)	99.9
P003	2094	2321	228	76		Putative membrane protein	54.6			51.1			100	Putative membrane protein MW1441 (624)	100
P004	2916	2521	396	132		Hypothetical protein	49.1			99.7	phiSLTp03 (396)	99.7	99.5	MW1439 (396)	99.5
P005	3379	2945	435	145		Putative lipoprotein	50.3			99.5	phiSLTp04 (435)	99.5	99.5	Putative lipoprotein MW1438 (435)	99.5
P006	4150	3434	717	239	*cl*	cl-like repressor	81.4	Repressor (771)	81.4	46.8	Repressor (315)	48.7	49.5	Repressor (324)	45.2
P007	4314	4556	243	81	*cro*	Putative cro-like repressor	49.8			53.3			51.2	Putative cro-like repressor (249)	54.5
P008	4572	5324	753	251	*ant*	Putative antirepressor	84.3	Antirepressor (750)	84.3	45.7	Antirepressor (549)	47.6	45	No corresponding gene	
P009	5337	5753	417	139		Transcriptional regulator	97.9	orf32 (276)	98.4	48			47.8		
P010	5830	6069	240	80		Hypothetical protein	48.7			50.4			49.8		
P011	6346	6023	324	108		Hypothetical protein	48.4			49.4			46.9		
P012	6396	6722	327	109		Hypothetical protein	52.9			47.5			52.2		
P013	6755	6937	183	61		Hypothetical protein	49			52.6			55.9		
P014	6967	7230	264	88		Putative DNA binding protein	99.6	orf37 (264)	99.6	95.1	phiSLTp12 (264)	95.1	82.3	MW1431 (264)	81.7
P015	7498	7779	282	94		Hypothetical protein	50.7			75.6	phiSLTp14 (303)	75.6	48		
P016	7780	8064	285	95		Hypothetical protein	95.7	orf39 (261)	98.1	99.3	phiSLTp15 (261)	99.2	48.8		
P017	8079	8558	480	160		phiPV83	48.1			51.3			48.3		
P018	8558	9331	774	258		Hypothetical protein	46.8			47			46.7		
P019	9361	9912	552	184	*ssb*	Single-strand binding protein	47.9	Ssb (471)	49.9	48.8	Ssb (444)	50.1	47.6		
P020	9925	10596	672	224		Hypothetical protein	47.4			70.1	phiSLTp19 (675)	70.1	46.2		
P021	11559	10702	858	286		Hypothetical protein	46.4			47.4			49.5		
P022	11624	12394	771	257		Phage replication protein	48.1			80	phiSLTp21 (771)	80	45.3		
P023	12404	13183	780	260		DNA replication protein	48.3			97.4	phiSLTp22 (780)	97.4	52.1		
P024	13240	13338	99	33		Hypothetical protein	55.2			61.6			58.2		
P025	13351	13572	222	74		Hypothetical protein	50.7			96.8	phiSLTp23 (222)	96.8	47.8		
P026	13583	13987	405	135		Hypothetical protein	50.4			68.3			51		
P027	14178	14549	372	124		Hypothetical protein	85.7	orf50 (369)	85.9	86.5	phiSLTp26 (369)	86.7	57.2		
P028	14550	14798	249	83		Hypothetical protein	92.3	orf51 (243)	93.8	93.8	phiSLTp27 (243)	93.8	90.4	MW1420 (243)	92.1
P029	14807	15178	372	124		Hypothetical protein	50			51.2			50.5		
P030	15171	15419	249	83		Hypothetical protein	97.2	orf52 (249)	97.2	94.4	phiSLTp28 (249)	94.4	51.2		
P031	15394	15939	546	182	*dut*	dUTPasc	87.2	dUTPase (528)	87.2	100	dUTPase (528)	100	47.4		
P032	16237	16473	237	79		Hypothetical protein	48.7			100	phiSLTp32 (237)	100	48.1		
P033	16615	16815	201	67		Hypothetical protein	99.5	orf60 (204)	99.5	62.6			48.1		
P034	16838	17290	453	151		Hypothetical protein	100	orf61 (471)	100	51.8			47		
P035	17405	17857	453	151		Hypothetical protein	99.6	orf62 (453)	99.6	48.3			49.4		
P036	17857	18216	360	120		Phage endonuclease	100	orf63 (342)	100	49.7			50.6		
P037	18346	18813	468	156	*terS*	Phage terminase, small subunit	100	Terminase (468)	100	49.1	Terminase (306)	47.9	48.9	Terminase (306)	47.6

P038	18816	20510	1,695	565	*terL*	Putative terminase, large subunit	99.7	Terminase (1,695)	99.7	47.1	Terminase (1,692)	50.6	46.5	Terminase (1,692)	50.7
P039	20655	21980	1,326	442	*por*	Portal protein	99.5	Portal protein (1,251)	99.6	47.4	Portal protein (1,239)	48.9	45.2	Portal protein (1,239)	49
P040	21973	22557	585	195	*pro*	Prohead protease	99.5	orf5-a (585)	99.5	48.8	Protease (774)	45.6	47	Protease (774)	46.3
P041	22645	23892	1,248	416	*capsid*	Capsid protein	99.9	Capsid protein (1,248)	99.9	49	Capsid protein (1,164)	49.6	47.8	Capsid protein (1,164)	49.5
P042	24095	24427	333	111	*mhp*	DNA packaging protein	99.1	orf9 (333)	99.1	50.8			46.3		
P043	24414	24749	336	112		Hypothetical protein	100	orf10 (336)	100	50.4			48.9		
P044	24749	25126	378	126		Hypothetical protein	99.5			46.6			49.6		
P045	25123	25503	381	127		Hypothetical protein	99.7	orf12 (381)	99.7	47			50.4		
P046	25504	26457	954	318		Hypothetical protein	99.8	orf13 (954)	99.8	47			44.3		
P047	26522	26968	447	149		Hypothetical protein	99.1	orf14 (447)	99.1	47.9			44.8		
P048	27043	27150	108	36		Hypothetical protein	100			52.1			55.4		
P049	27206	28522	1,317	439	*mtp*	Tail-length tape measure protein	99.9	orf15 (2,085)	99.9	49	Tape measure protein (6,204)	48.9	45.4	Tail tape measure protein (6,204)	45.5
P050	28527	31859	3,333	1111	*mtp*	Tail-length tape measure protein	97.9	orf16 (1,620), orf17 (672)	96.3, 99.7	47.7	Tape measure protein (6,204)	47.6	45.2	Tail tape measure protein (6,204)	45.4
P051	31859	33349	1,491	497		Hypothetical protein	99.6	orf18 (360), orf19 (1,134)	98.6, 99.9	46.9			44.9		
P052	33473	37147	3,675	1225		Structural protein	89.1	orf20 (2,280)	96.9	46.8			45.3		
P053	37338	37625	288	96		Hypothetical protein	83.3	orf22 (288)	83.3	52.9			48.5		
P054	38181	38483	303	101	*hol*	Holin	100	Holin (303)	100	97.1	Holin (303)	96	100	Holin (303)	100
P055	38494	39948	1,455	485	*ami*	Amidase (peptidoglycan hydrolase)	98.3	Amidase (1,455)	98.3	91.5	Amidase (1,455)	94.6	99.9	Amidase (1,455)	99.9
P056	40249	41895	1,647	549	*tra*	Transposase									
P057	42251	43198	948	316	*lukS-PV*	LukS-PV	99.8	LukS-PV (939)	99.9	100	LukS-PV (939)	100	99.8	LukS-PV (939)	99.8
P058	43200	44177	978	326	*lukF-PV*	LukF-PV	99.9	LukF-PV (978)	99.9	100	LukF-PV (978)	100	100	LukF-PV (978)	100
attP right	44382	44406	25			Attachment site on phage									
Core	44407	44435	29			Core sequence									
attB2	44436	44460	25			Attachment site on chromosome									
(P059)	44426	44611	186	62		Hypothetical protein									

[a] ORFs in parentheses were located outside of the ϕ108PVL. Two sets of core sequences and attachment site sequences were also identified flanking the ϕ108PVL.
[b] aa, amino acid.
[c] The percentages of identity for each ORF against three phages were determined by homology hit in nucleotide sequence.

Among the four phages, *lukS-PV* and *lukF-PV* genes were conserved with a predicted amino acid identity of greater than 99.8%. In addition, two genes encoding holin (*hol*) and amidase (*ami*), which are located upstream of *lukS-PV–lukF-PV*, were also well conserved, with predicted amino acid identities of more than 94.6%. We also identified 49 nucleotides located upstream of *hol* that were conserved among all four phages, with 98.0% nucleotide identity, while 555 nucleotides upstream of *hol* were highly homologous between ϕPVL and ϕ108PVL. The 204 nucleotides located between *lukS-PV–lukF-PV* and *attP* right were also highly conserved among the four phages, with nucleotide identities of more than 99.5%.

Integrases from four phages were identical with a ratio of more than 98% amino acid identity, suggesting that all four phages had integrated at the same position on the staphylococcal chromosome. In addition, 58 nucleotides between *attP* left and *int* were highly conserved with 98.3 to 100% nucleotide identities. A region located upstream of *int* and an additional sequence of 886 nucleotides in ϕ108PVL were identical to the corresponding regions in ϕSa2mw, and a 489-nucleotide region in ϕ108PVL had very high nucleotide identity to ϕSLT and ϕPVL (90.9 to 91.1%).

Other highly conserved ORFs included a putative DNA binding protein and a hypothetical protein, P028.

The ORFs that were conserved among at least three of the phages were as follows: P004 and P005, conserved among ϕ108PVL, ϕSLT, and ϕSa2mw with predicted amino acid identities of more than 99.5% (Fig. 2C and 3), and P027, P030, and P031, conserved among ϕ108PVL, ϕPVL and ϕSLT with predicted amino acid identities of more than 85.7% (Fig. 2C).

Twenty-four ORFs in ϕ108PVL were highly homologous to only ϕPVL (Fig. 2C and 3): P006 (cI-like repressor), P008 (antirepressor), P009 (transcriptional regulator), and P033 to P053 (DNA packaging and head and tail formation). The high identities between ϕ108PVL and ϕPVL among genes involved in morphogenesis suggested that ϕ108PVL belonged to *Siphoviridae*, the same group as ϕPVL, based on the taxonomy criteria based on the genetic organization of the structural gene cluster (4).

Six ORFs in ϕ108PVL related to DNA replication, P015, P020, P022, P023, P025, and P032, were homologous to only the corresponding ORFs in ϕSLT (Fig. 2). It is notable that only a small ORF, P003, located downstream of *int*, was homologous to the corresponding ORF in ϕSa2mw (Fig. 2). The remaining ORFs were unique to ϕ108PVL (Fig. 2). Among them, ORF P056, which encodes a putative transposase, was located just upstream of *lukS-PV*. BLAST searches of this ORF revealed that it was identical to transposase encoded by IS*1272*-SA, which was identified in a strain, MRSA 252, and a phage, PV83.

Dissemination of ϕ108PVL among Japanese MRSA strains. To determine whether PVL-positive MRSA strains isolated between 1979 and 1985 carried ϕ108PVL, we performed PCR experiments using chromosomal DNA from selected strains and six primer sets designed to amplify the following six genes or gene alleles in ϕ108PVL: set A, integrase; set B, terminase large subunit; set C, portal protein; set D, tail-length tape measure protein; set E, tail-length tape measure protein; and set F, transposase and the region at the rightmost end of ϕ108PVL (Fig. 2A and Table 1). All strains were positive in PCR with primer set A, indicating that they shared the same integrase and that the integration site was located at the same position on the bacterial chromosome. We found a strain (ST5-SCC*mec* type II.1) negative by PCR with the remaining five primer sets, indicating that it might carry a PVL-carrying phage which was distantly related to ϕ108PVL. Three ST30-SCC*mec* type I strains and an ST30-type IV.n SCC*mec* (a type IV SCC*mec* strain of which the J1 region was not classified into extant J1 regions of type IV SCC*mec* elements) were positive by PCR with primer sets B, C, and E and negative by PCR with primer sets D and F. A SCC*mec* type IV.1 strain and five SCC*mec* type IV.3 strains, all of which belonged to ST30, gave positive results with all other five sets of primers, indicating that they carried ϕ108PVL. A SCC*mec* type IV.3 strain was positive using four sets of primers, indicating that it carried a phage similar to ϕ108PVL, differing only in the presence of transposase encoded by IS*1272*-SA, which is found in MRSA strain 252 in its intact form.

DISCUSSION

The changing epidemiology of MRSA strains disseminated in Japanese hospitals. We conducted a retrospective study of MRSA strains disseminated in Japanese hospitals between 1979 and 1980 using molecular epidemiological methods. We showed that MRSA clones predominating in Japanese hospitals have changed drastically in 20 years. In contrast to MRSA clones predominated in 1999, most of which were ST5-SCC*mec* type II strains, the majority of MRSA strains isolated between 1979 and 1980 belonged to ST30 and carried either a type IV or a type I SCC*mec* element. In contrast to MRSA strains isolated in 1999, which were highly resistant against all tested antibiotics, MRSA strains isolated between 1979 and 1980 showed heterogeneous resistance to oxacillin and were susceptible to carbapenems, new quinolones, and tetracycline. They were different in toxin repertoire, too. The *lukS-PV–lukF-PV* genes, which are identified in the majority of C-MRSA strains, were identified in only MRSA strains isolated between 1979 and 1985. Since 22 MRSA strains isolated in Tokyo University Hospital in 1992 showed characteristics similar to those of MRSA strains isolated in 1999, we suppose that the shift of MRSA clones might have occurred in the early 1990s, at least in the case of Tokyo University Hospital. The type of coagulase correlated very well to the MLST genotype.

The change of MRSA clinical isolates, noticed in many facilities as the change of antibiotic susceptibility patterns and coagulase types, can be regarded as the change of MRSA clones from ST30-type I SCC*mec* or ST30-type IV SCC*mec* to ST5-type II SCC*mec*. In Japan, carbapenems, new quinolones, and minocyclines have been used for the treatment of MRSA infections since the mid-1980s to the late 1980s. Extensive use of antibiotics might exert selective pressures on bacteria, and only those strains that carry or acquire resistance genes or that acquire resistance through mutation are able to adapt and survive. Under the selective pressure caused by the extensive use of antibiotics, MRSA clones carrying type II SCC*mec*, which carries several resistance genes, might have replaced to the MRSA strains predominating in early 1980s, which could to adapt to the environmental change.

The historical shift of MRSA clones is not limited to Japanese hospitals. In 1960s, MRSA clones represented by strain COL or NCTC10442 (ST250-type 1 SCC*mec*) predominated in the United Kingdom. But, other clones, e.g., epidemic MRSA 16, represented by MRSA 252 (ST36-type II SCC*mec*), began to predominate in the United Kingdom in 1990s (15, 17, 26). In a Greece hospital, a change from ST30-type IV SCC*mec* strains to ST239-type III or type IIIA SCC*mec* IV had occurred (1). The ancient MRSA clones might have been replaced by the highly resistant MRSA clone, which has a strong capacity to spread or to survive under the selective pressure of antibiotics.

Characteristics of MRSA strains isolated between 1979 and 1985. It is interesting that approximately three-fourths of MRSA strains isolated between 1979 and 1985 carried either a type IV SCC*mec* element, which was identified primarily in C-MRSA strains, or a type I SCC*mec* element, which was identified in the first reported MRSA strains in England. Since we used strains that were stocked 20 years ago, it was very difficult to classify them into hospital-associated MRSA strains or community-associated MRSA strains due to the lack of detailed information that is required for the definition, e.g., the date of hospitalization or previous association to medical facilities. Using the records from two hospitals, we could classify the MRSA strains into two groups, strains obtained from outpatients and strains obtained from inpatients. They were as follows (the type is followed by the number obtained from inpatients and outpatients, respectively): isolates from Gunma Hospital, type I, 2 and 5; type II.1, 3 and 5; type IV.1, 0 and 1; type IV.3, 14 and 5; type IV.4, 8 and 2; type IV.n, 2 and 0; and nontypeable, 1 and 1; isolates from Tokyo University Hospital, type I, 2 and 4; type II.1, 0 and 1; type IV.3, 11 and 4; and type IV.n, 2 and 0. This analysis indicated that there was no significant difference among SCC*mec* types carried by outpatients and those carried by inpatients. It could be presumed that MRSA strains, which were similar to hospital-associated MRSA strains, predominated in the community as well. In the case of a large outbreak of *S. aureus* in Uruguay, which was caused by a highly virulent Uruguay clone represented by UR6 (ST30-type IV.3 SCC*mec*), the Uruguay clones were identified not only from community isolates but also from hospital isolates (32). These data suggested that both C-MRSA strains and H-MRSA strains could not be defined by their genotypes and SCC*mec* types, although representative C-MRSA clones and H-MRSA clones have been identified.

MRSA strains isolated between 1979 and 1985 carried *lukS-PV–lukF-PV* genes in a ratio of 45.3%. Since none of the tested MRSA strains isolated in 1992 and 1999 carried *lukS-PV–lukF-PV* genes, it is a remarkable characteristic of MRSA strains isolated between 1979 and 1985. Oka et al. reported that mortality associated with bacteremia was very high at the ratio of 47.8% (93 cases total) at Tokyo Metropolitan Geriatric Hospital from 1973 through 1984 (38). Although *lukS-PV–lukF-PV* genes were identified in highly virulent C-MRSA strains, e.g., MW2 that caused the death of healthy infants, we do not have the data to conclude whether the cause of high mortality is due to the presence of PVL-positive MRSA strains. Further retrospective studies of MRSA strains isolated from patients with well-documented clinical histories will help answer that question.

Evolution of PVL-carrying phages. It is well known that PVL-positive *S. aureus* strains harbor a bacteriophage carrying *lukS-PV–lukF-PV*. All extant PVL-carrying phages belonged to the class of Shi21-like *Siphoviridae*. Canchaya et al. classified the *Staphylococcus* prophages into five groups based on the similarities in genomic structures, predominantly of the genes related to morphogenesis, such as those specifying the phage head and tail (4). ϕSLT and ϕSa2mw were determined to be part of one group based on nucleotide sequence identity in the region encoding the head and tail genes. ϕSLT has an elongated shape, and ϕSa2mw is presumed to have a similar morphology. Similarly, ϕPV83 and ϕPVL were determined to belong to a second group and exhibit an icosaheadral head morphology (27). The novel phage that we identified in the current study, which we designated ϕ108PVL, shared basic structural components with all three extant PVL-carrying phages and showed the highest similarity to ϕPVL.

The four PVL-carrying phages had two highly homologous regions, the regions in and around the gene encoding integrase and the regions in and around the lysis-related genes and *lukS-PV–lukF-PV*. These observations strongly suggested that PVL-carrying phages evolved from non-PVL-carrying phage by acquiring the region containing *lukS-PV–lukF-PV*, although it is not certain whether the acquisition of the region containing *lukS-PV–lukF-PV* was earlier than the acquisition of the region carrying the gene for integrase. Discrepancies in the lengths of the homologous regions among these phages suggested that the acquisition of these two regions might have occurred independently by one or more illegitimate recombination events.

Characteristics of ϕ108PVL. The ϕ108PVL carried a transposase which is not present in the three extant PVL phages. The noncoding region between the genes for amidase and transposase had very low similarity to other phages, but some noncoding regions between transposase and *lukS–lukF* showed rather high similarity, although the length of this region differed for each, i.e., the lengths were 339 bp (ϕPVL, 98.2%), 340 bp (ϕSLT, 97.4%), and 362 bp (ϕSa2mw, 99.5%). These results suggested that ϕ108PVL acquired transposase, along with the noncoding region between the genes for amidase and transposase, as encoded by IS*1272*-SA. It seems that IS*1272* integrated into the phage, as is the case for ϕPV83.

Kaneko and Kamio found no evidence for a tail structure on the ϕPVL phage particle using electron microscopy, although the nucleotide sequence of this phage suggests that it carries tail-related genes and that ϕPVL was defective in its ability to infect any *S. aureus* strains experimentally (27). Since most of the ST30 MRSA strains examined to date carry ϕ108PVL by PCR, it can be presumed that the phage was responsible for disseminating the PVL gene among MRSA strains isolated in the early 1980s in Japan. However, when we examined the genes encoding tail-length tape measure protein in ϕ108PVL, we found that the coding region was split into two ORFs, P049 and P050, similar to what is observed in ϕPVL. In contrast, ϕSLT, which is able to infect *S. aureus* strains experimentally, encodes a large tail-length tape measure protein of 2,067 amino acids. It therefore seemed that ϕ108PVL and ϕPVL are both inactive and that the spread of phages among the *S. aureus* strains occurred in an ancient time, and since which time, lysogenized phages have become inactive. Our data re-

vealed that the genome of ϕ108PVL is a mosaic, suggesting that ϕ108PVL was produced as a result of lateral gene transfer and illegitimate recombination events. Thus, it seemed reasonable to assume that ϕ108PVL and ϕPVL originated from a relatively close ancestor during their revolution.

The origin of PVL-positive ST30 MRSA strains isolated in Japan. Infections caused by C-MRSA have been reported worldwide, and their genetic background and the carriage of virulence factors have been examined. Vandenesch et al. reported that all tested C-MRSA strains shared type IV SCC*mec* and *lukS-PV–lukF-PV* genes (48). Although it has been pointed out that the presence of *lukS-PV–lukF-PV* genes could not be used for defining C-MRSA strains, *lukS-PV–lukF-PV* genes have been identified in many C-MRSA strains. They reported that the genotypes of PVL-carrying C-MRSA strains were classified into six strains, ST1, -8, -30, -59, -80, and -93. Enright et al. reported eight major international epidemic MRSA strains, ST5, -8, -22, -36, -45, -289, -247, and -250 (12). Pandemic clones were also reported as New York/Japan clone (ST5-type II SCC*mec*), pediatric clone (ST5-type IV SCC*mec*), archaic clone (ST250-type I SCC*mec*), Iberian clone (ST247-SCC*mec* type 1A), Hungarian clone (ST239-type III SCC*mec*), and Brazilian clone (ST239-SCC*mec* type IIIA) by Oliveira et al. (40). The genotypes of PVL-carrying C-MRSA strains were different from those of pandemic clones other than ST8, which was identified mostly in MSSA strains (12). Subsequent study revealed that most PVL-carrying MRSA strains isolated worldwide belonged to one of six genotypes reported by Vandenesch et al. (48): ST1, from the United States; ST30, from the United States, Australia, England, Singapore, Belgium, Uruguay, and Japan; ST80, from England, The Netherlands, Denmark, Greece, Belgium, France, Switzerland, and Germany; ST8, from Belgium and the United States; and ST59, from the United States and Taiwan (3, 7, 10, 14, 18, 19, 32, 41, 45, 49, 50).

It is noteworthy that Japanese PVL-positive MRSA strains are mostly ST30, known as the southwest Pacific clone after the region where it was first described (48).

But when we investigated the subtypes of type IV SCC*mec* elements carried by ST30 strains, we found that different subtypes of SCC*mec* elements were carried by these strains. Australian strains carried type IV.1 SCC*mec* elements, whereas the Uruguay and Japanese PVL-positive ST30 MRSA clones carried a type IV.3 SCC*mec* similar to those of Japanese MRSA strains. However, their banding patterns under pulsed-field gel electrophoresis were closely related (32).

Robinson et al. reported that phage type 80/81 MSSA strains, which have been spread throughout the world, were PVL positive and belonged to ST30 and suggested that these MSSA strains might have changed to MRSA strains through the acquisition of different types of SCC*mec* elements independently (42). It seems likely that ST30 MSSA strains carrying *lukS-PV–lukF-PV* existed in the Japanese community as well and evolved into MRSA strains through the acquisition of a type of SCC*mec* element. This is partly supported by the observation that 38% (15 of 39) of MSSA strains isolated in the 1960s carried *lukS-PV–lukF-PV* (X. X. Ma et al., unpublished data). The diversity in the types of SCC*mec* elements identified in these isolates indicates that integration of SCC*mec* elements occurred independently in several different time periods.

In this paper, we introduced a method to distinguish PVL-carrying phage by PCR. By adopting the method, we found that most Japanese MRSA strains disseminated in 1979 to 1985 carried ϕ108PVL and that a ST5-type II SCC*mec* MRSA strain carried a phage which was different from three extant PVL phages as well as ϕ108PVL (X. X. Ma et al., unpublished). By using our methods, we will be able to classify PVL phages to know which phage was integrated in MSSA strains and whether ST30 MRSA strains isolated in Australia and Uruguay carried a phage homologous to ϕ108PVL. Further studies are awaited to learn the origin or spread of PVL-positive MRSA strains.

ACKNOWLEDGMENTS

We thank Toyoji Okubo and Shizuko Iyobe of Gunma University for providing us with MRSA strains.

This work was supported by a grant-in-aid for 21st Century COE Research and a grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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