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Novel Characteristics of Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Strains Belonging to Multilocus Sequence Type 59 in Taiwan▽

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Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains, which often produce Panton-Valentine leucocidin (PVL), are increasingly noted worldwide. In this study, we examined 42 MRSA strains (25 PVL-positive [PVL$^+$] strains and 17 PVL-negative [PVL$^-$] strains) isolated in Taiwan for their molecular characteristics. The PVL$^+$ MRSA strains included CA-MRSA strains with multilocus sequence type (ST) 59 (major PVL$^+$ MRSA in Taiwan), its variants, and worldwide CA-MRSA ST30 strains. The PVL$^-$ MRSA strains included the pandemic Hungarian MRSA ST239 strain, the Hungarian MRSA ST239 variant, MRSA ST59 (largely hospital-acquired MRSA strains) and its variants, the pandemic New York/Japan MRSA ST5 strain (Japanese type), and the MRSA ST8 strain. The major PVL$^+$ CA-MRSA ST59 strain possessed a tetracycline resistance-conferring (*tetK* positive) penicillinase plasmid and a drug resistance gene cluster (a possible composite transposon) for multidrug resistance. Moreover, it carried a novel staphylococcal cassette chromosome *mec* (SCC*mec*) with two distinct *ccrC* genes (*ccrC2-C8*). This SCC*mec* (previously named SCC*mec* type V_T) was tentatively designated SCC*mec* type VII. Sequencing of the PVL genes revealed the polymorphisms, and the PVL$^+$ CA-MRSA ST59 strain possessed the ST59-specific PVL gene sequence. The data suggest that a significant amount of clonal spread is occurring in Taiwan and that the major PVL$^+$ CA-MRSA ST59$_{Taiwan}$ strain exhibits unique genetic characteristics, such as a novel SCC*mec* type and an ST59-specific PVL gene sequence.

Methicillin-resistant *Staphylococcus aureus* (MRSA), alternatively called hospital-acquired MRSA (HA-MRSA), has been a major cause of nosocomial infections since the 1960s (6). MRSA is considered to have emerged from *S. aureus* through the acquisition of staphylococcal cassette chromosome *mec* (SCC*mec*), which carries the *mecA* gene for methicillin resistance (10, 41, 69). Several such pandemic clones have spread worldwide (1), including a Hungarian clone (belonging to multilocus sequence type [ST] 239) that has spread to, e.g., Taiwan, and a New York/Japan (ST5) clone that has spread to the United States and Japan.

In addition, since the period from 1997 to 1999, community-acquired MRSA (CA-MRSA) has also become a major concern worldwide (8, 59, 68). CA-MRSA is associated with skin and soft tissue infections in young, otherwise healthy individuals in the community (68) and also with severe diseases such as necrotizing pneumonia and sepsis (8, 68). CA-MRSA often carries genes for Panton-Valentine leucocidin (PVL) (31, 59, 68), a harmful toxin which destroys bacterium-engulfing immune cells and also respiratory tissue (19, 33). The rates of carriage of the PVL toxin gene are, e.g., >75% for CA-MRSA strains (21) and 67% and 80% for CA-MRSA strains with ST8 and ST1, respectively, in the United States (37).

Some PVL-positive (PVL$^+$) CA-MRSA clones are rather continent specific; e.g., clones with ST1 and ST8 are mostly found in the United States and Canada (20, 38, 59), and clones with ST80 are mostly found in Europe (59). In contrast, PVL$^+$ CA-MRSA strains with ST30 are found worldwide, e.g., in the United States, Europe, Oceania, Japan, and Russia (3, 16, 46, 52, 59). PVL$^+$ CA-MRSA strains with ST59 have been reported from Taiwan and the United States (5, 7, 16). Although in many cases PVL$^+$ CA-MRSA strains are susceptible to non-β-lactam antimicrobial agents, the PVL$^+$ CA-MRSA ST59$_{Taiwan}$ strain is multidrug resistant (5). In this study, we investigated the genetic, virulence, and drug resistance characteristics of PVL$^+$ CA-MRSA ST59$_{Taiwan}$ and compared with them with those of other PVL$^+$ and PVL-negative (PVL$^-$) MRSA clones.

MATERIALS AND METHODS

Bacterial strains. Forty-two MRSA strains (25 PVL$^+$ strains and 17 PVL$^-$ strains) were isolated in National Taiwan University Hospital from 2000 to June 2006; of those, 32 strains were isolated from patients in the pediatric department and the emergency surgery room and from patients with skin and soft tissue

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infections in other departments and 10 strains were randomly chosen from among isolates recovered in all departments. All PVL$^-$ strains examined in this study were isolated from inpatients (48 h after hospitalization), indicating that they were HA-MRSA strains. An additional 10 MRSA strains belonging to the pandemic New York/Japan ST5 clone were also obtained from inpatients at the same hospital in 2006. CA-MRSA ST59 strains TSGH17 from Taiwan (5) and USA1000 from the United States (38) were kindly provided by C. C. Wang and by L. K. McDougal and L. C. McDonald, respectively. The following strains were also employed for PVL gene sequence analysis: PVL$^+$ methicillin-susceptible *S. aureus* (MSSA) strains GN1, GN3, GN4, and GN5 (isolated in Japan in 2005 [this study]); CA-MRSA ST30 strain EB00449 (isolated in Japan in 2002 [67]); CA-MRSA ST30 strain RS08 (isolated in Russia in 2006 [3]); CA-MRSA ST30 strain ER7 (isolated in Egypt in 2007 [this study]); CA-MRSA ST80 strain HT20030345 (isolated in The Netherlands [67]); and CA-MRSA ST80 strain HT20030442 (isolated in France [67]). The pandemic New York/Japan ST5 clone (strain BK2464) originated in the United States (1) and was kindly provided by H. de Lencastre.

Media and bacterial growth. For bacterial growth, we used LB broth (Difco, Sparks, MD) as the liquid medium, which was inoculated and incubated at 37°C with agitation until the isolates reached log phase. Nutrient agar (Eiken Chemical, Tokyo, Japan) was used as the solid medium. Mueller-Hinton agar (Difco) was used for susceptibility testing.

Molecular typing. Multilocus sequence typing was performed by using seven housekeeping genes, as described previously (17). An allelic profile (allele number) was obtained from the multilocus sequence typing website (http://www.mlst.net/), and the ST data were further analyzed by using eBURST software (18) to determine the clonal complex to which each ST belonged. *spa* (staphylococcal protein A gene) typing was performed as described previously (49). The *spa* type was determined by using a public *spa* type database (http://tools.egenomics.com/). Detection of the accessory gene regulator (*agr*) allele group was done by PCR with the primers described previously (50). The strains were analyzed for their SCC*mec* types (types I to V) by PCR, as described previously (10, 27, 41, 69), by using reference strains. In the case of SCC*mec* type IV, four subtypes (subtypes IVa, IVb, IVc, and IVd) were further analyzed by PCR with the primers described previously (26). PCR primers for the detection of SCC*mec* type V_T were designed on the basis of the *ccrC2* gene sequence of strain TSGH17 (GenBank accession no. AY894416). They were ccrC2-F2 (5′-ATAAGTTAAAAGCACGACTCA) and ccrC2-R2 (5′-TTCAATCCTATTTTTCTTTGTG), which generate a 257-bp product.

Coagulase typing. The MRSA and MSSA strains were examined for their coagulase types by using a staphylococcal coagulase antiserum kit (Denka Seiken, Tokyo, Japan), in accordance with the manufacturer's instructions.

Virulence gene analysis. Forty-one staphylococcal virulence genes (except the *lukE-lukD* genes) were detected by PCR by using the primers reported previously. The targeted genes were 3 leucocidin genes (29, 40), 5 hemolysin genes (29), 17 staphylococcal enterotoxin (SE) genes (4, 14, 23, 28, 43, 70), 1 putative staphylococcal enterotoxin gene (34), 3 exfoliative toxin genes (4, 66), an exotoxin-like gene cluster (63), the epidermal cell differentiation inhibitor gene (29), and 14 adhesin genes (39, 44, 55, 58, 60). The *lukE-lukD* genes were assayed with primers LUKED-F1 (5′-CAGATGTGAAGGGTAGTGGA) and LUKED-R5 (5′-TCATTATCAGATGTTGCTGTTG), designed in this study.

PVL gene diversity analysis. The PVL genes were sequenced as described previously (40), and the phylogenetic diversity of the PVL genes was analyzed by use of the CLUSTAL W program (http://clustalw.ddbj.nig.ac.jp/top-j.html).

Drug resistance gene analysis. Resistance genes were detected by PCR. They included genes for penicillin resistance (53), tetracycline resistance (56), aminoglycoside resistance (9, 11), macrolide and lincosamide resistance (51), chloramphenicol resistance (32), and cadmium resistance (*cadDX* [36]); for the detection of the other cadmium resistance genes (*cadA* and *cadC*), primer set cadA-F1 (5′-GTTCGATTGTAATTGGCGG) and cadA-R1 (5′-TTTCCTGACCATTCCGC) and primer set cadC-F1 (5′-GAAGATAAGGTAAACAGGGCT) and cadC-R1 (5′-CAAGCTGTTTAACATGCTC), respectively, were designed in this study on the basis of the gene sequences of MRSA strain 252 (GenBank accession no. BX571856). Stock strains in our laboratory were used as positive controls for PCR analysis for resistance genes. For fluoroquinolone resistance, mutations in *gyrA* (for DNA gyrase) and *grlA* (for topoisomerase IV) were determined as described previously (24, 61). In order to detect Tn*551*, which hopped onto a plasmid, PCR primer sets were designed on the basis of the sequence of Tn*551* (GenBank accession no. Y13600): primer set tnpR-F (5′-ATGATTTTTGGCTATGCTCG) and tnpR-R (5′-TAAGACCAGAGTTAGTTCGTTC), which generated a 382-bp product for the resolvase gene (*tnpR*), and primer set tnpA-F (5′-ACAACTTCTTTCTGTAGACCAC) and tnpA-R (5′-GTCTTTTAGCCAAGCGAG), which generated a 604-bp product for the transposase gene (*tnpA*). PCR was conducted for the detection of three genes of Tn*551* (*ermB*, *tnpR*, and *tnpA*).

Plasmid analysis. Plasmid DNA was isolated by using a plasmid midi kit (Qiagen, Hilden, Germany) and lysostaphin (Wako Pure Chemicals, Osaka, Japan), according to the manufacturers' instructions.

PCase plasmid transfer. Penicillinase (PCase) plasmid DNA was introduced into *S. aureus* RN2677 (52, 67) by electroporation by using a Gene Pulser II electroporator (Bio-Rad, Tokyo, Japan), according to the manufacturer's instructions, or by the filter mating method. In the latter method, transconjugants were selected for both the donor resistance (PCase plasmid) marker (with cadmium acetate at 10 μg/ml) and the recipient marker (with novobiocin at 5 μg/ml or rifampin at 1 μg/ml).

Susceptibility testing. Susceptibility testing of the bacterial strains was done by the agar dilution method with Mueller-Hinton agar according to previously described procedures (12). The antimicrobial agents were gifts from their manufacturers.

Nucleotide sequence accession numbers. The GenBank accession no. for the *rgc* region is AB300568. The GenBank accession no. for the novel SCC*mec* sequence is AB353125. The GenBank accession numbers for the PVL gene are AB303648 for strain EB00449, AB256036 for strain GN1, AB256038 for strain GN3, AB256039 for strain GN4, AB256037 for strain GN5, AB303647 for strain PM7, AB303649 for strain RS08, AB353126 for strain ER7, AB295470 for strain PM1, AB295471 for strain PM4, AB295472 for strain PM35, AB303645 for strain PM13, AB303646 for strain PM17, AB295473 for strain USA1000, AB331244 for strain HT20030345, and AB331245 for strain HT20030442.

RESULTS

Characterization and identification of MRSA strains. Forty-two MRSA strains (25 PVL$^+$ strains and 17 PVL$^-$ strains) from Taiwan were examined for their genetic types, toxin and adhesin gene patterns, and oxacillin resistance. The data for these strains and their identities are summarized in Table 1.

The major PVL$^+$ ST59 clone (group 1; which accounted for 96% of the PVL$^+$ MRSA strains) exhibited SCC*mec* type V_T, *spa143*, *agr-1*, and coagulase type VII (CoaVII) and was positive for all hemolysin genes examined; and most of the strains of this clone (80%) were positive for the SEB gene (*seb*). It was positive for 10 (common) adhesin genes, and most of the strains of this clone (85%) were positive for the fibrinogen adhesin gene (*adrE*) but negative for the collagen adhesin gene (*cna*) and the bone sialoprotein adhesin gene (*bbp*). The oxacillin resistance levels of the strains of the major PVL$^+$ ST59 clone were relatively low (MICs, ≤32 μg/ml), and most strains of this clone (85%) were isolated from abscesses, in good agreement with the findings for CA-MRSA strains. There were several other related strains with minor differences (groups 1-v1 to 1-v4). The remaining PVL$^+$ MRSA clone (group 2; which accounted for 4% of the PVL$^+$ MRSA strains) was CA-MRSA ST30, which has a worldwide distribution. The oxacillin resistance levels of these strains were also relatively low (MICs, 32 μg/ml).

One major PVL$^-$ clone (group 3; which accounted for 41.2% of the PVL$^-$ MRSA strains) was the pandemic Hungarian MRSA ST239 clone, which is the predominant HA-MRSA clone in Taiwan. Strains of this clone exhibited high-level oxacillin resistance (MICs, ≥256 μg/ml) and were mostly (85.7%) isolated from blood. A variant (group 3-v1) of the Hungarian MRSA ST239 clone exhibited a novel ST type, ST900, and is a single-locus variant of ST239.

Another major PVL$^-$ clone (group 4; which accounted for 23.5% of the PVL$^-$ MRSA strains) was MRSA ST59, which closely resembled the major PVL$^+$ ST59 clone (Table 1), except that it exhibited SCC*mec* type IVx and had high levels of

TABLE 1. Characteristics and identification of MRSA strains from Taiwan

Group	No. of strains	Type			Presence of toxin gene		Adhesin gene[c]	Oxacillin MIC(s) (μg/ml [% of strains])	Isolation site (% of strains)	Identification of MRSA
		ST (clonal complex)	*SCCmec* type[a]	Other	*luk*$_{PV}$*SF* (PVL)	Other[b] (% of strains)				
1	20	59 (59)	V_T	*spa143*, *agr1*, CoaVII	+	*hlb*, *hlg-v*, *seb* (80)	*sdrE* (85%)	≤32 (60); ≥64 (40)	Abscess (85), bacteremia (5), RTI[d] (5), surgical wound (5)	Major PVL$^+$ MRSA clone in Taiwan, ST59
1-v1	1	59 (59)	V	*spa143*, *agr1*, CoaVII	+	*hlb*, *hlg-v*, *seb*	*sdrE*	64	Surgical wound	PVL$^+$ ST59 variant
1-v2	1	59 (59)	X	*spa143*, *agr1*, CoaVII	+	*hlb*, *hlg-v*, *seb*	*sdrE*	64	RTI	PVL$^+$ ST59 variant
1-v3	1	59 (59)	V	*spa775*,[e] *agr1*, CoaVII	+	*hlb*, *hlg-v*, *seb*	*sdrE*	4	UTI[f]	PVL$^+$ ST59 variant
1-v4	1	59 (59)	V_T	*spa776*,[e] *agr1*, CoaVII	+	*hlb*, *hlg-v*, *seb*	*sdrE*	4	Abscess	PVL$^+$ ST59 variant
2	1	30 (30)	IVc	*spa19*, *agr3*, CoaIV	+	*egc*,[g] *seu*	*cna*, *bbp*	32	UTI	PVL$^+$ MRSA clone, worldwide ST
3	7	239 (8)	III	*spa3*, *agr1*, CoaIV	−	*lukE-lukD*, *hlb* (14.3), *hlg-v*, *sea*	*cna*, *sdrD*, *sdrE*	≥128	Abscess (14.3), bacteremia (85.7)	Major PVL$^-$ MRSA clone in Taiwan, Hungarian clone
3-v1	1	900[h] (8)	III	*spa3*, *agr1*, CoaIV	−	*lukE-lukD*, *hlg-v*, *sea*	*cna*, *sdrD*, *sdrE*	≥128	Bacteremia	Hungarian clone variant
4	4	59 (59)	IVx	*spa143, agr1,* CoaVII	−	*hlb*, *hlg-v*, *seb*	*sdrE*	≥128	Bacteremia (75), RTI (25)	Major PVL$^-$ MRSA clone in Taiwan, ST59
4-v1	1	59 (59)	X	*spa143*, *agr1*, CoaVII	−	*hlb*, *hlg-v*, *seb*	*sdrE*	32	Bacteremia	PVL$^-$ ST59 variant
4-v2	1	59 (59)	IVx	*spa777*,[e] *agr1*, CoaVII	−	*hlb*, *hlg-v*, *seb*	*sdrE*	64	Abscess	PVL$^-$ ST59 variant
4-v3	1	59 (59)	IVc	*spa778*,[e] *agr1*, CoaVII	−	*hlb*, *hlg-v*, *seb*	*sdrE*	32	Abscess	PVL$^-$ ST59 variant
5	1	5 (5)	II	*spa2*, *agr2*, CoaII	−	*lukE-lukD*, *hlb*, *hlg-v*, *tst*, *sec*, *egc*	*sdrD*, *sdrE*	≥128	Bacteremia	New York/Japan clone (Japanese type)
6	1	8 (8)	IVx	*spa779*,[e] *agr1*, CoaIII	−	*lukE-lukD, hlb, hlg-v*	*sdrD, sdrE*	32	Bacteremia	MRSA clone, worldwide ST

[a] SCC*mec* type V_T was detected by PCR with primers ccrC2-F2 and ccrC2-R2, designed in this study. SCC*mec* type V was negative by PCR with the SCC*mec* type V_T-specific primers. SCC*mec* type X is an unknown type (i.e., it is a type other than type I, II, III, IV, or V). SCC*mec* type IVx is type IV with unknown subtypes (a subtype other than subtype IVa, IVb, IVc, or IVd).
[b] Other than the common four genes, which consist of three hemolysin genes (*hla*, *hlg*, *hld*) and the SE gene (*set*).
[c] Other than the 10 genes *icaA*, *icaD*, *eno*, *fnbA*, *fnbB*, *ebpS*, *clfA*, *clfB*, *fib*, and *sdrC*.
[d] RTI, respiratory tract infection.
[e] Novel *spa* types.
[f] UTI, urinary tract infection.
[g] *egc,* enterotoxin gene cluster, which carries *seg, sei, sem, sen*, and *seo* (30).
[h] Single-locus variant of ST239.

TABLE 2. Drug resistance of MRSA strains from Taiwan

Drug, resistance gene	Presence of gene or gene mutation in the following MRSA strains (% of strains)[a]:					
	PVL$^+$ ST59 (n = 24)	PVL$^+$ ST30 (n = 1)	PVL$^-$ ST239/ST900 (n = 8)	PVL$^-$ ST59 (n = 7)	PVL$^-$ ST5 (n = 1)	PVL$^-$ ST8 (n = 1)
Tetracycline						
tetK	+ (75)[b]	−	+ (63)	+ (71)[c]	−	−
tetM	−	−	+	+ (14)	−	−
Gentamicin-kanamycin, *aac(6′)-aph(2″)*	−	−	+	+ (71)	+	−
Kanamycin						
aph(3′)-IIIa	+ (92)[d]	−	+	+ (71)	−	−
ant(4′)-Ia	−	−	−	−	+	−
Streptomycin						
aadE	+ (92)[d]	−	+ (25)	+ (14)	−	−
spc	−	−	+	−	+	−
Erythromycin-clindamycin						
ermA	−	−	+	−	+	+
ermB	+ (92)[d]	−	−	+	−	−
ermC	−	−	−	+ (14)	−	−
Erythromycin, *msrA*/*msrB*	−	−	−	−	−	−
Chloramphenicol, *cat*	+ (75)	−	−	+ (57)	−	−
Fluoroquinolones[e]						
gyrA mutation	−	−	S84L (88), E88K (12)	−	S84L	−
grlA mutation	−	−	S80F	−	S80Y	−
Ampicillin, *blaZ*	+ (96)[b]	+	+	+ (86)[c]	+[f]	+
Cadmium						
cadDX	+ (96)[b]	+	+ (63)	+ (86)[c]	+[f]	−
cadA	−	−	+ (50)	−	−	−
cadC	−	−	+ (50)	−	−	−

[a] Strains are from Table 1. PVL$^+$ ST59 includes groups 1 and groups 1-v1 to 1-v4. PVL$^+$ ST30 corresponds to group 2. PVL$^-$ ST239/ST900 includes group 3 and group 3-v1. PVL$^-$ ST59 includes group 4 and groups 4-v1 to 4-v3. PVL$^-$ ST5 and PVL$^-$ ST8 correspond to groups 5 and 6, respectively.
[b] The PCase plasmids of strains PM1 and PM9 code for tetracycline resistance (*tetK*), ampicillin resistance (*blaZ*), and cadmium resistance (*cadDX*).
[c] The PCase plasmids of strains PM26 and PM34 code for tetracycline resistance (*tetK*), ampicillin resistance (*blaZ*), and cadmium resistance (*cadDX*).
[d] The resistance to kanamycin, streptomycin, and erythromycin-clindamycin of strain PM1 was encoded by *rgc* (drug resistance gene cluster), shown in Fig. 1.
[e] +, fluoroquinolone resistance with *gyrA* and *grlA* mutations; −, fluoroquinolone susceptible.
[f] The PCase plasmid of strain PM29 codes only for ampicillin resistance (*blaZ*) and cadmium resistance (*cadDX*).

oxacillin resistance (MICs, ≥256 μg/ml), and most of the strains of this clone (75%) were isolated from blood. Since these strains were isolated from inpatients, they were classified as HA-MRSA. There were several other related strains with minor differences (groups 4-v1 to 4-v3).

Minor PVL$^-$ MRSA clones (which were isolated infrequently) included the pandemic New York/Japan ST5 clone (group 5; which accounted for 5.9% of the PVL$^-$ MRSA strains). These strains were positive for *hlb* (which codes for β-hemolysin) and the mobile pathogenicity island (SaPI1 [48]), which carries *tst* (which codes for toxic shock syndrome toxin 1), *sec* (which codes for SEC), and *sel* (which codes for SEL), like Japanese strains and unlike strain BK2464 from the United States (data not shown). This indicates that the Taiwanese New York/Japan clone is of the Japanese type. PVL$^-$ clone ST8 (group 6) was also isolated infrequently.

Drug resistance of MRSA strains. The drug resistance and the resistance genes of the MRSA strains belonging to each ST type are shown in Table 2. Strains of the major PVL$^+$ MRSA ST59 clone were mostly (75%) positive for *tetK*, which codes for tetracycline resistance. The *tetK* gene was carried on a PCase (*blaZ*) plasmid (designated pTPC1 [42 kb] in strain PM1); strain PM1 carried only this plasmid. pTPC1 also carried *cadDX*, which codes for cadmium resistance. Moreover, most strains of this clone (90%) were positive for *aph(3′)-IIIa*, which codes for kanamycin resistance; *aadE*, which codes for streptomycin resistance; and *ermB*, which codes for erythromycin and clindamycin resistance. The three genes constituted a resistance gene cluster (*rgc*) and were parts of Tn*5405* and Tn*551* (Fig. 1), indicating the possibility of a novel composite transposon. Of those structures, Tn*551* occasionally translocated onto pTPC1; the *ermB* gene on the Tn*551*-containing plasmid (pTPC1-EC1) encoded (and expressed constitutively) resistance to both erythromycin and clindamycin (data not shown). Strains of the major PVL$^+$ MRSA ST59 clone were mostly (75%) positive for *cat*, which codes for chloramphenicol resistance; the *cat* gene was located independently of *tetK* and, probably, *rgc*. The characteristics of PVL$^+$ MRSA ST59 strain

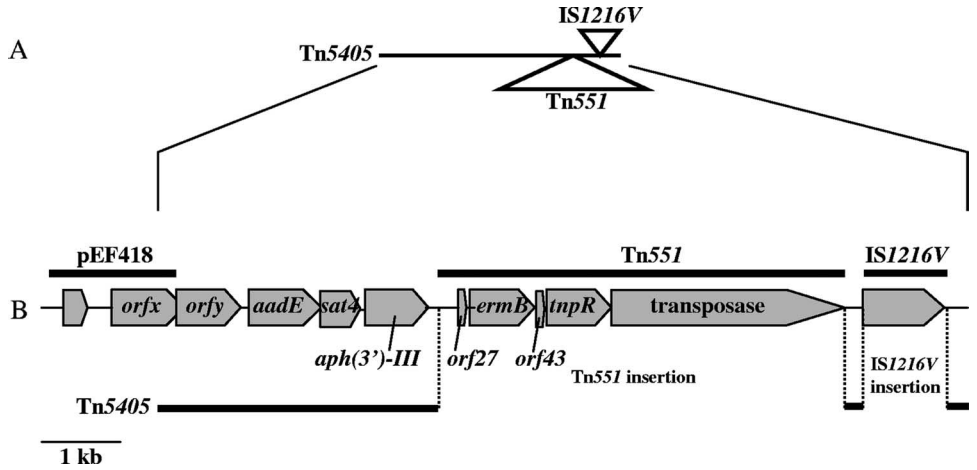

FIG. 1. Schematic structure of the drug resistance gene cluster (*rgc*) of PVL$^+$ CA-MRSA ST59$_{Taiwan}$ strain PM1. Two mobile genetic elements, transposon Tn*551* (which carries *ermB*, which encodes constitutive resistance to erythromycin and clindamycin [65]) and insertion sequence IS*1216V*, were inserted into transposon Tn*5405*, which carries *aadE* (which encodes streptomycin resistance) and *aph(3′)-III* (which encodes kanamycin resistance). Occasionally, Tn*551* transposed onto pTPC1 (PCase plasmid) in strain PM1.

TSGH17, described previously, were the same as those of strain PM1 (data not shown).

PVL$^-$ MRSA ST59 strain PM34 also carried a *tetK*-positive PCase plasmid (designated pTPC34 [46 kb]), in addition to a 4.5-kb plasmid. All strains (including the MRSA ST8 strains) listed in Tables 1 and 2 were negative for macrolide resistance genes (*msrA*/*msrB*), which is characteristic of many CA-MRSA strains, such as USA300 (ST8).

Novel structure of SCC*mec* type V$_T$. Analysis of the SCC*mec* type V$_T$ sequence of CA-MRSA ST59$_{Taiwan}$ (strain PM1) revealed two distinct *ccrC* genes within the SCC*mec* region, one of which, *ccrC2*, has been reported previously and the other one of which was a novel *ccrC* gene (designated *ccrC8*) (Fig. 2). The sequences of the *ccrC2* region and the *mec* complex C2 (*mecA*-Δ*mecR1*-ΔIS*431*) region were the same as those of previously described strain TSGH17. The *ccrC2* gene was 1,680 bp (560 amino acids) long, and the *ccrC8* gene was 1,677 bp (559 amino acids) long, and they showed 92.5% and 93.7% homologies at the nucleotide and amino acid levels, respectively. SCC*mec* type V$_T$, which contains a novel *ccr* arrangement (*ccrC-C*) and *mec* complex (*mec* complex C2), is a novel class of SCC*mec* rather than a variant of SCC*mec* type V. This novel SCC*mec* type was tentatively designated SCC*mec* type VII.

Polymorphisms and ST type dependency of the PVL gene sequences. Sequencing of the PVL genes revealed polymorphisms. The phylogenetic diversity of the PVL genes of strains of various STs (31 strains) is summarized in Fig. 3. The PVL gene sequences were ST dependent (except for those of ST30 and ST50). Thus, the PVL gene sequence of CA-MRSA ST59 had a unique nucleotide substitution which was distinct from those of strains of the other STs (Fig. 3B). Nonsynonymous substitutions (which cause amino acid changes) were found in three cases (CA-MRSA ST30$_{Egypt}$ strain ER7, CA-MRSA ST1 [USA400, MW2], and strain YSSA15) and were found only in the *luk$_{PV}$S* gene region (Fig. 3A and B).

DISCUSSION

In Taiwan, the macrolide-resistant (*ermB*-positive), PVL$^+$ CA-MRSA ST59 clone was reported by Wang et al. (62) in 2004. The SCC*mec* type of most PVL$^+$ CA-MRSA ST59 strains was reported to be untypeable (62); and in 2005, it was classified as SCC*mec* type V$_T$, which contains a *ccrC* recombinase gene variant (*ccrC2*) and *mec* complex C2, by Boyle-Vavra et al. (5). Boyle-Vavra et al. (5) also designed a particular primer to detect SCC*mec* type V$_T$ and proposed strain TGSH17 as a prototype of the PVL$^+$ CA-MRSA ST59 clone from Taiwan, which shows resistance to four non-β-lactam antimicrobial agents (erythromycin, clindamycin, tetracycline, and chloramphenicol). They also speculated that the PVL$^+$ CA-MRSA ST59 clone in the United States (the San Francisco clone) might have originated from Taipei. Lo et al. described in 2006 that PVL$^+$ CA-MRSA, isolated from patients who lived in Taipei City or Taipei County, Taiwan, was SEB positive, and the majority of the strains exhibited SCC*mec* type V$_T$, while some were SCC*mec* type IV (35).

Aires de Sousa et al. reported in 2003 that the majority of strains of the PVL$^-$ MRSA clone in Taiwan were the pandemic Hungarian clone (ST239, *spa3*, SCC*mec* type III) or an ST241 clone (a single-locus variant of ST239) (2). Huang et al. (25) described in 2006 that a new epidemic clone (ST5, SCC*mec* type II) had increased in prevalence and also that the PVL$^+$ CA-MRSA ST59 clone had a link to health care settings and had not previously been detected.

In this study, we found that SCC*mec* type V$_T$ possessed two distinct *ccrC* genes (*ccrC2* and a novel *ccrC* gene designated *ccrC8*), demonstrating the first case of *ccr* arrangement (*ccrC2-C8*). *ccrC8* was found in a region that Boyle-Vavra et al. (5) had not examined for the SCC*mec* type V$_T$ sequence.

The previously described *ccr* complexes include *ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4*, and *ccrC* (22); and *ccrC* is carried only by SCC*mec* type III (which has a *ccr* arrangement with a *ccrA3B3* complex and *ccrC3*) and by SCC*mec* type V (which

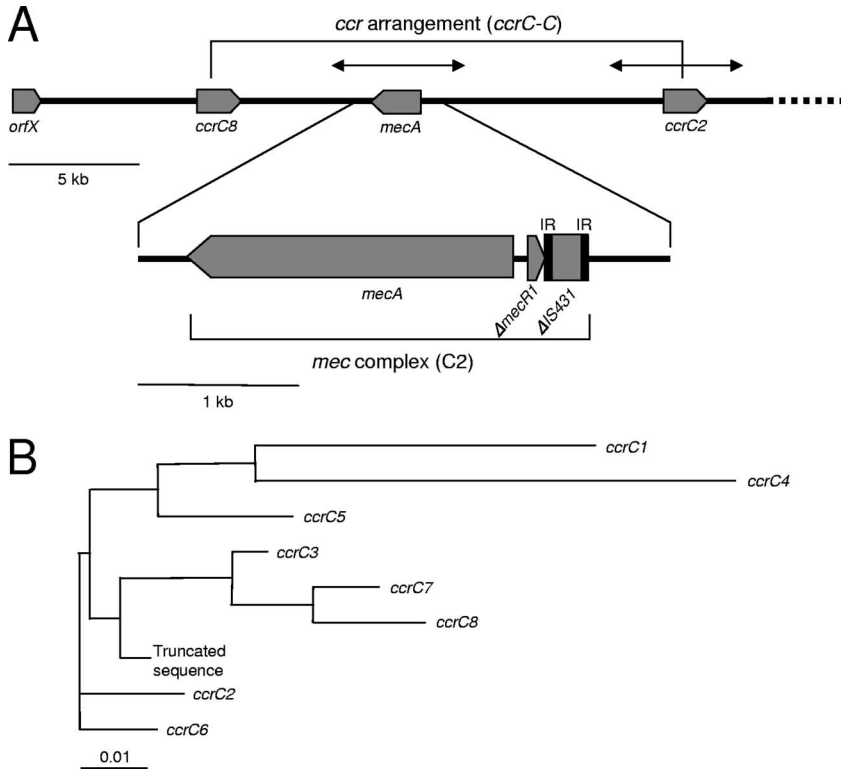

FIG. 2. Structure of the novel SCC*mec* (previously named SCC*mec* type V_T) of PVL^+ CA-MRSA $ST59_{Taiwan}$ strain PM1 (A) and the phylogenetic diversity of the *ccrC* genes (B). (A) Arrows above the novel SCC*mec* structure indicate the sequence region of strain TSGH17, determined by Boyle-Vavra et al. (5). IR, inverted repeats. (B) Phylogenetic diversity was analyzed by use of the CLUSTAL W program (http://clustalw.ddbj.nig.ac.jp/top-j.html), and the graph was constructed by using the Tree View X program. GenBank accession nos. for the previously described *ccrC* gene sequences are AB121219 for *ccrC1*, AY894416 for *ccrC2*, AB037671 for ccr*C3*, U10927 for *ccrC4*, AP006716 for *ccrC5*, EF190467 for *ccrC6*, EF190468 for *ccrC7*, and AB047089 for the truncated sequence. *ccrC8* (GenBank accession no. AB353125) is a novel *ccrC* found in the SCC*mec* of strain PM1 (A) and showed 97.5% homology to *ccrC7*. The scale bar represents nucleotide differences.

has a *ccr* arrangement with *ccrC1*) (10, 22). SCC*mec* type V_T (of CA-MRSA $ST59_{Taiwan}$ strain PM1), which has a *ccr* arrangement (*ccrC2-C8*), should be a new class of SCC*mec*. This class was tentatively designated SCC*mec* type VII, following SCC*mec* type VI, which possesses a *ccrAB* complex (42).

We also demonstrated the polymorphisms of the PVL gene sequences by the exhaustive sequencing of the PVL genes. The PVL genes were divergent in many cases (as we have described previously [40]) and were ST dependent, except in two cases. PVL consists of S and F proteins (31, 45), and it is considered that the modification of the S protein to the phosphorylated version by the protein kinase of human polymorphonuclear leukocytes (PMNs) after its initial binding to PMNs (probably GM1), followed by the subsequent binding of the F protein, is essential for the induction of cell lysis (31). Interestingly, amino acid changes were observed only in the S-protein region, indicating the possibility that PVL variants (with amino acid changes) show a different host (or tissue) specificity. Further studies are necessary to elucidate this possibility. It has been considered that PVL could be a serious toxin (33, 68). However, since the description of the PVL destruction of white blood cells and lung tissue is based only on in vitro and animal studies, its role in human infections remains to be defined.

The *tetK* gene is usually carried on small plasmids (4.3 to 4.4 kb) in CA-MRSA strains (15, 54). In contrast, PVL^+ CA-MRSA $ST59_{Taiwan}$ (strain PM1) as well as PVL^- MRSA $ST59_{Taiwan}$ (strain PM34) carried the *tetK* gene on a large PCase plasmid (42 or 46 kb). Such multidrug-resistant PCase plasmids have been found in PVL^+ MRSA strains derived from MRSA outbreaks in the 1980s to 1990s in Japan (53). Although strain PM34 carried an additional small (4.5-kb) plasmid, the role of this plasmid remains to be defined.

PVL^+ CA-MRSA ST30 is the worldwide type, being *egc* positive and highly adhesive (*cna* and *bbp* positive). *egc* is frequently found in *S. aureus* strains from carriers, suggesting the possibility for a role of *egc* in bacterial colonization (57).

PVL^- MRSA $ST5_{Taiwan}$ was classified as the Japanese type of the New York/Japan clone (52) and not the USA type (14). Strains from Japanese patients with toxic shock syndrome were *sea* positive, while those from patients with neonatal toxic shock syndrome-like exanthematous disease (NTED) were *sea* negative (52). Thus, the New York/Japan clone from Taiwan resembled that from patients with NTED. The New York/Japan clone, which has the ability to replace preexisting MRSA clones in hospital settings (13), may be expanding to the western-most areas in Pacific regions.

MRSA $ST8_{Taiwan}$ seems to be different from MRSA ST8, which is found in both Europe and the United States. In the United States, the majority of such strains carry the *msrA* gene (which codes for macrolide resistance) and not the *erm* gene

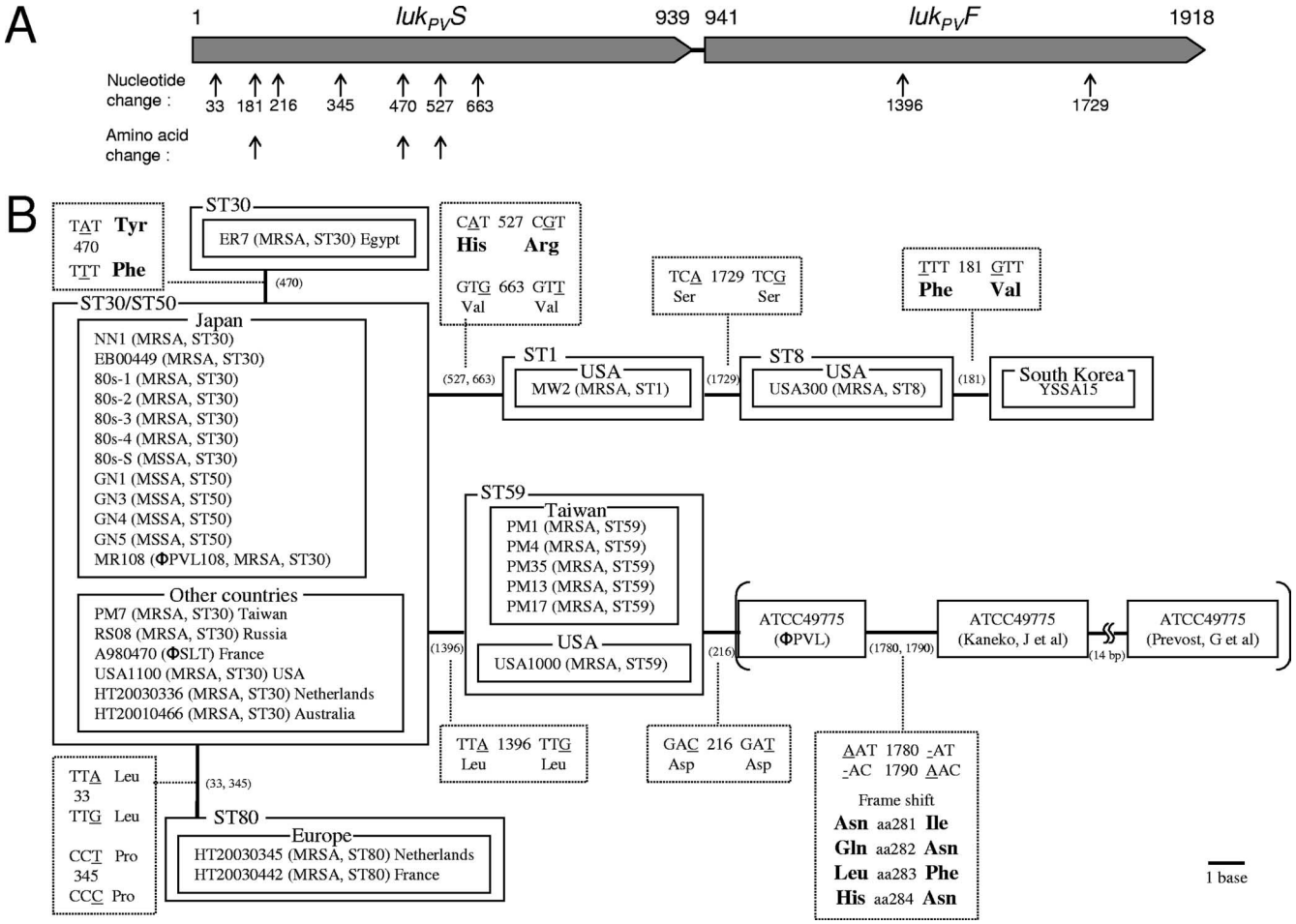

FIG. 3. Phlogenetic diversity of the PVL genes. (A) The structure of the PVL gene for *luk$_{PV}$S* and *luk$_{PV}$F* is from PVL$^+$ CA-MRSA ST30$_{Japan}$ strain NN1 (GenBank accession no. AB186917). The numbering starts at the 5′ end of *luk$_{PV}$S* (1). Arrows below the PVL gene structure indicate the positions of nucleotide changes or the amino acid changes observed with the PVL variants shown in panel B; the data for strain ATCC 49775 are not shown. (B) A bar represents 1 base substitution (distance), and the position (nucleotide number) of substitutions corresponds to that in panel A. An amino acid for a codon in a nonsynonymous substitution is shown in boldface. The PVL genes of PVL$^+$ MSSA$_{Japan}$ strains (GN1, GN3, GN4, and GN5), PVL$^+$ CA-MRSA ST30$_{Taiwan}$ strain (PM7; C6 in Table 1), PVL$^+$ CA-MRSA ST30$_{Japan}$ strain (EB00449; [67]), PVL$^+$ CA-MRSA ST30$_{Russia}$ strain (RS08 [3]), PVL$^+$ CA-MRSA ST30$_{Egypt}$ strain (ER7), PVL$^+$ CA-MRSA ST59$_{Taiwan}$ strains (PM1, PM4, PM13, PM17, and PM35; C1, C4, C3, C2, and C5 in Table 1, respectively), PVL$^+$ CA-MRSA ST59$_{USA}$ strain (USA1000 [54]), the PVL$^+$ CA-MRSA ST80$_{Netherlands}$ strain (HT20030345), and the PVL$^+$ CA-MRSA ST80$_{France}$ strain (HT20030442) were determined in this study. The GenBank accession nos. are AB186917 for strain NN1; AB303648 for strain EB00449; AB245449 for strain 80s-1; AB245450 for strain 80s-2; AB245451 for strain 80s-3; AB245452 for strain 80s-4; AB245448 for strain 80s-S; AB256036 for strain GN1; AB256038 for strain GN3; AB256039 for strain GN4; AB256037 for strain GN5; AB243556 for strain MR108 (φ108); AB303647 for strain PM7; AB303649 for strain RS08; AB045978 for strain A980470 (φSLT); AB245453 for strain USA1100; AB245454 for strain HT20030336; AB245455 for strain HT20010466; AB353126 for strain ER7; BA000033 for strain MW2; CP000255 for strain USA300; DQ993352 for strain YSSA15; AB295470 for strain PM1; AB295471 for strain PM4; AB295472 for strain PM35; AB303645 for strain PM13; AB303646 for strain PM17; AB295473 for strain USA1000; AB009866, AB006796, and X72700 for strain ATCC49775; AB331244 for strain HT20030345; and AB331245 for strain HT20030442.

(which codes for macrolide-lincosamide-streptogramin B resistance), and some strains carry both the *msrA* and the *erm* genes (64). In this study, all MRSA strains, including PVL$^-$ MRSA ST8$_{Taiwan}$, were negative for the *msrA* gene.

Although the PVL$^+$ CA-MRSA ST59$_{Taiwan}$ and PVL$^-$ MRSA ST59$_{Taiwan}$ (identified as HA-MRSA in this study) clones shared many common characteristics, the evolutionary relationship between the two clones remains to be clarified. A previous study demonstrated that descendants of early pandemic PVL$^+$ *S. aureus* strains have acquired SCC*mec* and have reemerged as CA-MRSA (47).

As for oxacillin resistance, HA-MRSA strains manifest high levels of resistance (MICs, ≥256 μg/ml), in contrast to those for CA-MRSA strains (MICs, ≤32 μg/ml), in many cases in Japan (52, 53). This seems to be the case even in some instances in Taiwan.

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