Comparative Genomics of Community-Acquired ST59 Methicillin-Resistant *Staphylococcus aureus* in Taiwan: Novel Mobile Resistance Structures with IS*1216V*

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) with ST59/SCC*mec*V and Panton-Valentine leukocidin gene is a major community-acquired MRSA (CA-MRSA) lineage in Taiwan and has been multidrug-resistant since its initial isolation. In this study, we studied the acquisition mechanism of multidrug resistance in an ST59 CA-MRSA strain (PM1) by comparative genomics. PM1's non-β-lactam resistance was encoded by two unique genetic traits. One was a 21,832-bp composite mobile element structure (MES$_{PM1}$), which was flanked by direct repeats of enterococcal IS*1216V* and was inserted into the chromosomal *sasK* gene; the target sequence (*att*) was 8 bp long and was duplicated at both ends of MES$_{PM1}$. MES$_{PM1}$ consisted of two regions: the 5'-end side 12.4-kb region carrying Tn*551* (with *ermB*) and Tn*5405*-like (with *aph[3']-IIIa* and *aadE*), similar to an *Enterococcus faecalis* plasmid, and the 3'-end side 6,587-bp region (MES$_{cat}$) that carries *cat* and is flanked by inverted repeats of IS*1216V*. MES$_{cat}$ possessed *att* duplication at both ends and additional two copies of IS*1216V* inside. MES$_{PM1}$ represents the first enterococcal IS*1216V*-mediated composite transposon emerged in MRSA. IS*1216V*-mediated deletion likely occurred in IS*1216V*-rich MES$_{PM1}$, resulting in distinct resistance patterns in PM1-derivative strains. Another structure was a 6,025-bp *tet*-carrying element (MES$_{tet}$) on a 25,961-bp novel mosaic penicillinase plasmid (pPM1); MES$_{tet}$ was flanked by direct repeats of IS*431*, but with no target sequence repeats. Moreover, the PM1 genome was deficient in a copy of the restriction and modification genes (*hsdM* and *hsdS*), which might have contributed to the acquisition of enterococcal multidrug resistance.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major public health problem worldwide. It was first isolated in 1961 as a nosocomial pathogen [1,2], now known as hospital-acquired MRSA (HA-MRSA). Another class of MRSA, designated community-acquired MRSA (CA-MRSA), emerged in the community from 1997 to 1999, posing a novel threat worldwide [1–4]. CA-MRSA infection usually occurs in children and young adults, or even the elderly, without exposure to hospital environments, and includes common skin and soft tissue infections (SSTIs) and occasionally life-threatening invasive infections, such as sepsis, necrotizing pneumonia and osteomyelitis [1–5].

CA-MRSA is characterized by the presence of staphylococcal cassette chromosome *mec* type IV (SCC*mec*IV) or SCC*mec*V [1,2,4,6]. SCC*mec*IV likely contributes to the spread in the community because it is smaller in size compared to other SCC*mec* elements [4,6,7] and has no effect on MRSA fitness (engendering no biological cost) [4,8]. CA-MRSA strains often produce Panton-Valentine leukocidin (PVL), which acts against neutrophils [4,9–11]. Moreover, CA-MRSA strains are only resistant to β-lactam antimicrobial agents or to some agents in

restricted classes, although some recently successful CA-MRSA strains, such as multilocus sequence type 8 (ST8) CA-MRSA USA300, became multidrug-resistant [12,13]; USA300 has even spread into hospitals as a nosocomial pathogen [14,15].

A major CA-MRSA lineage in Taiwan is ST59 with SCC*mec*V and *luk$_{PV}$SF* genes encoding PVL. Wang et al. [16] and Boyle-Vavra et al. [17] demonstrated that ST59 CA-MRSA in Taiwan was already resistant to multidrugs (including erythromycin, clindamycin, and chloramphenicol) when it began emerging in 1997, in contrast to the general understanding of CA-MRSA. Boyle-Vavra et al. [17] also characterized the SCC*mec* of Taiwanese ST59 CA-MRSA (representative strain, TSGH17) as SCC*mec* V$_T$. The ST59 CA-MRSA lineage (with SCC*mec* type V$_T$ and *luk$_{PV}$SF*) has also been reported in Singapore [18], Hong Kong [19], Japan [20,21], and Western Australia [22].

We further characterized the Taiwanese ST59 CA-MRSA lineage and found that it possessed a novel SCC*mec* with two distinct *ccrC* genes (*ccrC1* allele 2 and *ccrC1* allele 8) [23–25]. We determined the entire SCC*mec* sequence of the ST59 CA-MRSA lineage (strain PM1) and tentatively designated it as SCC*mec*VII [24]; later, the SCC*mec* of the Taiwanese ST59 CA-MRSA lineage (strains TSGH17 and PM1) was reclassified as SCC*mec*V by the

International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements [6]. As for non-β-lactam resistance, three resistance determinants, *ermB* (encoding erythromycin/clindamycin resistance), *aph(3′)-IIIa* (encoding kanamycin resistance), and *aadE* (encoding streptomycin resistance), were clustered in a region, previously named the drug resistance gene cluster (*rgc*) [23].

IS*1216V* is an enterococcal insertion sequence [26] and is rarely found in *S. aureus*. IS*1216V*-positive *S. aureus* cases include vancomycin-resistant MRSA (VRSA), in which IS*1216V* is inserted in a vancomycin resistance transposon (Tn*1546*) [27]. The *rgc* region of strain PM1 also carried IS*1216V* [23], raising the question of whether the *rgc* region of the Taiwanese ST59 CA-MRSA lineage (strain PM1) originated in enterococci through an IS*1216V* function.

In order to further understand the mechanism of multidrug resistance acquisition by PVL-positive ST59 CA-MRSA in Taiwan, we performed comparative genomics of strain PM1 and found that strain PM1 became multidrug resistant by acquiring two mobile genetic elements, an enterococcal IS*1216V*-mediated composite mobile element structure (MES$_{PM1}$) on the chromosome and an IS*431*-mediated element (MES$_{tet}$) on a plasmid, besides β-lactam resistance by SCC*mec*V. We also searched for the genetic background of the ST59 CA-MRSA lineage, which allowed (or stimulated) the acquisition of multidrug resistance.

Results

Susceptibility to Non-β-lactam Agents of PVL-positive ST59/SCCmecV CA-MRSA Strains in Taiwan

Drug resistance patterns of ST59/SCC*mec*V CA-MRSA strains isolated in Taiwan, compared to an ST59 MRSA-type strain USA1000, are summarized in Table 1. Half of the strains (including PM1) were resistant to erythromycin/clindamycin, kanamycin, streptomycin, chloramphenicol, and tetracycline, in addition to β-lactam agents, showing marked multidrug resistance (in contrast to USA1000). The rest showed five distinct patterns in terms of resistance to non-β-lactam agents: resistance to erythromycin/clindamycin, kanamycin, streptomycin, and chloramphenicol; resistance to erythromycin/clindamycin, kanamycin, streptomycin, and tetracycline; resistance to erythromycin/clindamycin, kanamycin, and streptomycin; resistance to chloramphenicol and tetracycline; and non-resistance (to any non-β-lactam agent).

Comparative Genomics of PVL-positive Multidrug-resistant ST59/SCCmecV CA-MRSA (Strain PM1)

The PM1 genome was analyzed by pyrosequencing (GenBank Accession number BAFA01000000) and compared with the genomes of ST398 MRSA human strain S0385 and PVL-positive ST8 CA-MRSA USA300, since ST398 MRSA carried SCC*mec*V [28], similar to PM1, and showed the highest similarity to ST59 MRSA [29] in terms of the seven housekeeping gene sequences used for multilocus sequence typing, and USA300 was one of the best characterized CA-MRSA [30]. The data are summarized in Figure 1. The alignment of PM1 contigs on the reference MRSA genome was the same in both cases (Figure 1A); the similarities of the PM1 genome to the ST398 and ST8 genomes were both approximately 98%, albeit with divergence in, for example, the carriage of mobile elements (Figure 1A). The PM1 genome was estimated to be at least 2.8 Mb. Strain PM1 carried a 25,961-bp plasmid (pPM1), encoding tetracycline resistance; plasmid DNA analysis of strain PM1 also demonstrated a single species of plasmid of 26.0 kb (Table 1).

When PM1 was compared with S0385 and USA300, the four regions were unique to PM1: i) SCC*mec*V with two *ccrC* genes (*ccrC1* allele 2 and *ccrC1* allele 8); ii) φSA1$_{PM1}$, which was 70.2% homologous to φETA3, a member of the φSA1 family (φETA3 is *eta*-positive, while φSA1$_{PM1}$ was negative); iii) MES$_{PM1}$, a novel mobile element structure on the chromosome, encoding multidrug resistance; and iv) a tetracycline resistance plasmid pPM1. Moreover, νSAβ on the PM1 genome had a large deletion.

Structure of MES$_{PM1}$

The entire sequence of MES$_{PM1}$ and its surrounding region was determined (GenBank Accession number AB699882); the structure is summarized in Figure 2. MES$_{PM1}$ was 21,832 bp long and flanked by direct repeats of IS*1216V* at both ends. It was inserted into the *att* site (8-bp target sequence) within the *sasK* gene; the 8-bp *att* sequence was duplicated at both ends of MES$_{PM1}$, indicating that MES$_{PM1}$ is a large transposon. The target gene (*sasK*) with the 8-bp *att* sequence was present on the genome of ST59 MRSA USA1000, but not in strains S0385 and USA300 (Figure 1A). Interestingly, MES$_{PM1}$ was IS*1216V*-rich and contained three additional copies of IS*1216V* (a total of five copies of IS*1216V*), as shown Figure 2.

Table 1. Resistance patterns of ST59 CA-MRSA strains isolated in Taiwan, compared to ST59 MRSA USA1000.

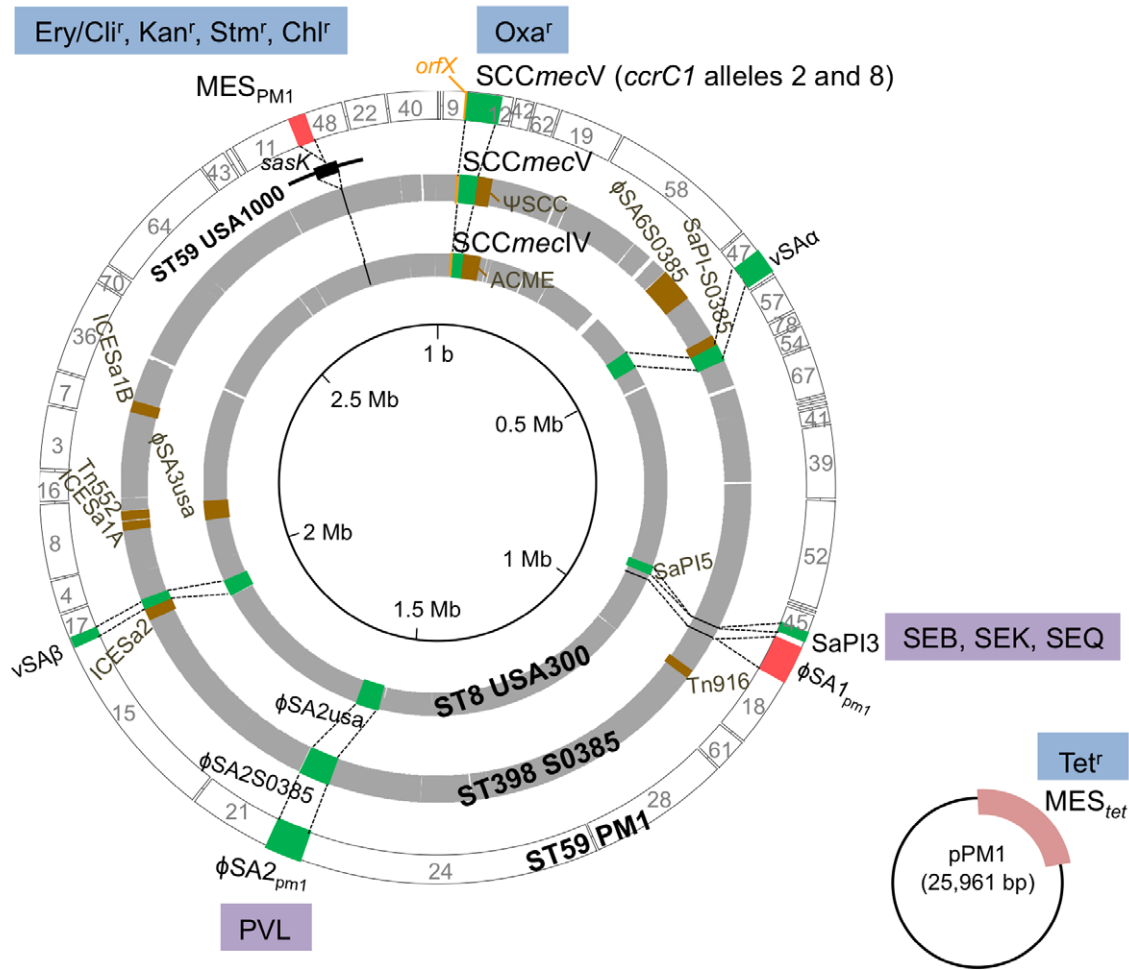| Resistance patterns[a]					Percentage (no./total)	Example (strain)	Plasmid[b] (size)
Ery/Cli	Kan	Stm	Chl	Tet			
R	R	R	R	R	50% (12/24)	PM1, PM22	26 kb
R	R	R	R	S	16.7% (4/24)	PM11	21 kb
R	R	R	S	R	16.7% (4/24)	PM9	26 kb
R	R	R	S	S	8.3% (2/24)	PM12	None
S	S	S	R	R	4.2% (1/24)	PM8	26 kb
S	S	S	S	S	4.2% (1/24)	PM18	21 kb
R(Ery)	S	S	S	S	-	USA1000	ND[c]

[a]The number of strains tested was 24. Non-β-lactam agents: Ery, erythromycin; Cli, clindamycin; Kan, kanamycin; Stm, streptomycin; Chl, chloramphenicol; Tet, tetracycline. USA1000 is a type strain of PVL-positive ST59 MRSA.
[b]Plasmid analysis was carried out for example strains.
[c]Not determined.
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A

B

Relevant genotype of strain PM1

Genotype	ST59/*spa*143(t437)/*agr*1a/SCC*mec*V/CoaVII
Drug resistance	*ermB* (Ery/Cli), *aph(3')-IIIa* (Kan), *aadE* (Stm), *cat* (Chl), *tetK* (Tet)
Virulence factor	
Leukocidin	$luk_{PV}SF$ (PVL)
Hemolysin	*hla*, *hlb*, *hlg*, *hlg-v*, *hld*
Enterotoxin	*seb*, *sek*, *seq*
Adhesin	*icaA*, *icaD* (for biofilm formation); *eno* (for laminin-adhesin); *fnbA*, *fnbB* (for fibronectin-adhesin); *ebpS* (for elastin-adhesin); *clfA*, *clfB*, *fib*, *sdrC*, *sdrE* (for fibrinogen-adhesin)
Phage	$\phi SA1_{pm1,}$ $\phi SA2_{pm1}$ ($luk_{PV}SF$)
Pathogenicity island	SaPI3 (*seb*, *sek*, *seq*)
Mobile element	MES$_{PM1}$ (*ermB*, *aph[3']-IIIa*, *aadE*, *cat*), MES$_{tet}$ (*tetK*)
Transposon	Tn*551* (*ermB*), Tn*5405*-like (*aph[3']-IIIa*, *aadE*), Tn*552*-like (*bla*)
Insertion sequence	IS*232*-like, IS*431*, IS*1216V*, IS*1272*, IS*200*-like family

The left side of MES$_{PM1}$ was a composite transposon region consisting of Tn*551* carrying *ermB* and Tn*5405*-like carrying *aph(3′)-IIIa* and *aadE*. The composite transposon region was identical to the corresponding region of a plasmid (pLG2) of *Enterococcus faecalis* (Figure 2); the entire sequence of pLG2 has not been reported.

Analysis of the right-side region of MES$_{PM1}$ revealed an independent transposon (MES$_{cat}$) carrying *cat* (encoding chloramphenicol resistance). MES$_{cat}$ was 6,587 bp long and flanked by the inverted repeats of IS*1216V*; moreover, the 8-bp *att* sequence was duplicated at both ends of MES$_{cat}$ (Figure 2). MES$_{cat}$ contained two additional copies of IS*1216V*. The central *cat* region in MES$_{cat}$ was highly homologous (99.5%) to the *cat* region of SAP084A plasmid in *S. aureus*.

Based on the data, we conclude that MES$_{PM1}$ is a highly composite transposon, which originated in enterococci, but acquired an additional drug resistance gene (*cat*) in *S. aureus*. Another multidrug-resistant strain PM22 (Table 1) also possessed an MES structure (MES$_{PM22}$) very similar to MES$_{PM1}$, although there was a small deletion within the structure (data not shown).

Structure of MES$_{PM1}$ Segregants with Different Resistance Patterns

The MES$_{PM1}$-corresponding region of ST59 CA-MRSA strains with different resistance patterns (Table 1) was determined by sequencing (Figure 3). MES$_{PM1}$ seems genetically unstable, and recombination likely takes place between two direct repeats of IS*1216V*, resulting in deletion, which could explain the distinct resistance patterns of ST59 CA-MRSA.

For instance, strain PM9, which was resistant to erythromycin/clindamycin, kanamycin, and streptomycin, but susceptible to chloramphenicol, seemed to be a segregant, generated through deletion (1) in Figure 3 (A, B). Likewise, strain PM8, which was resistant to chloramphenicol only, seemed to be a segregant, generated through deletion (2) in Figure 3 (A, C). Strain PM18, which was susceptible to all the non-β-lactam agents, seemed to be a segregant, generated through deletion (3) in Figure 3 (A, D).

MES$_{PM1}$ segregants, MES$_{PM9}$ (Figure 3B) and MES$_{PM8}$ (Figure 3C), may also behave as transposons, since they were flanked by direct repeats of IS*1216V* at both ends with *att* sequence duplication and inserted into the *sasK* gene, just like MES$_{PM1}$. MES$_{PM18}$ (corresponding to IS*1216V*), which was inserted into the
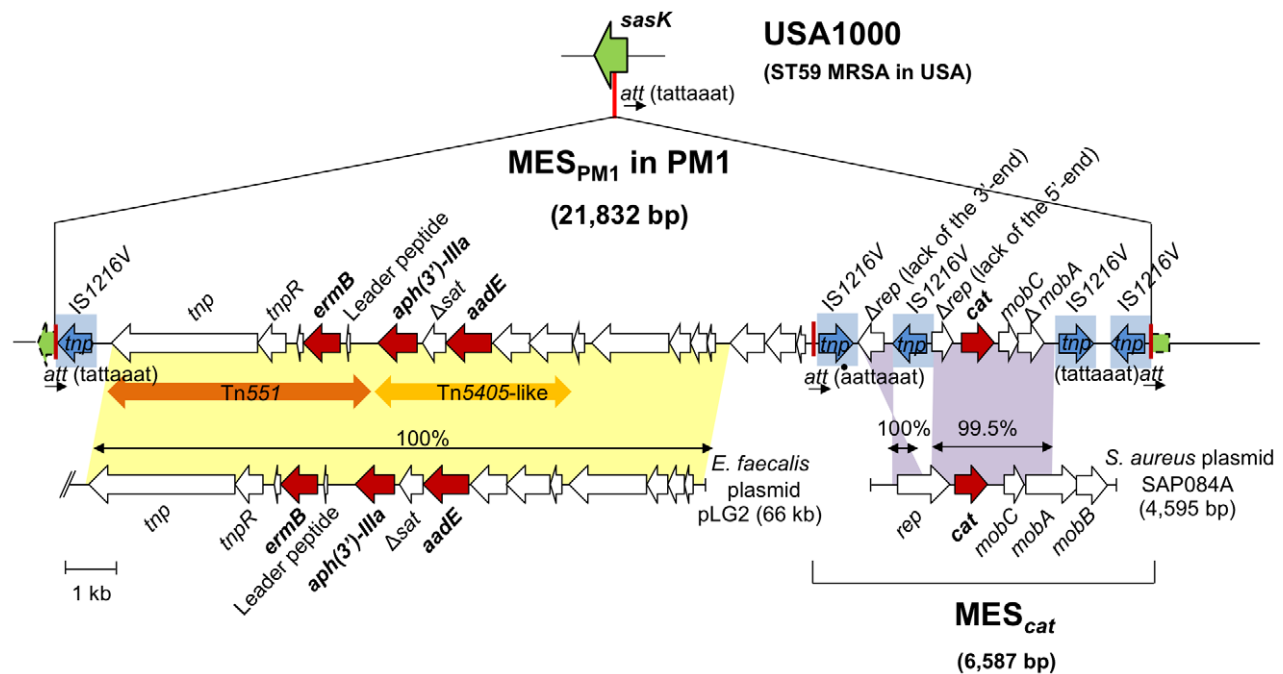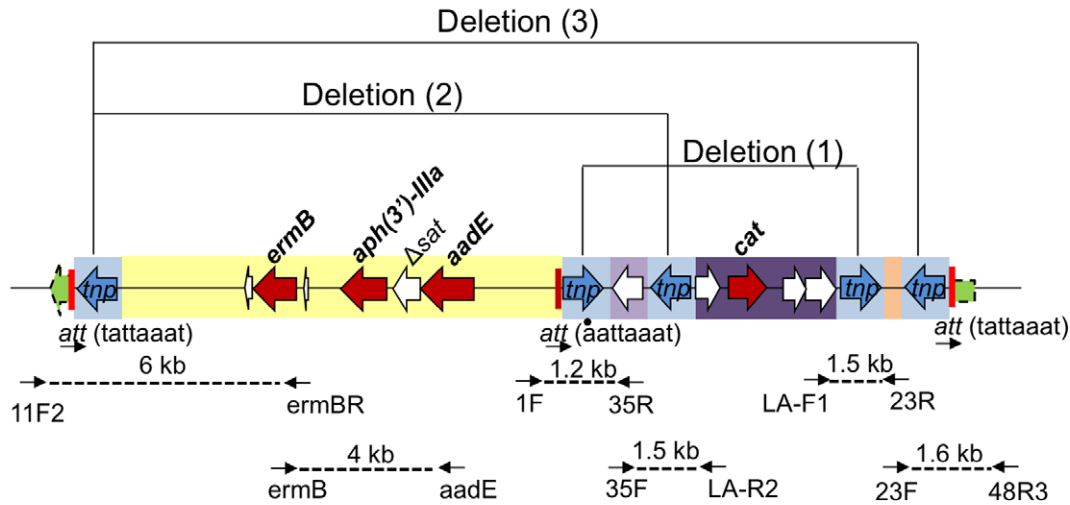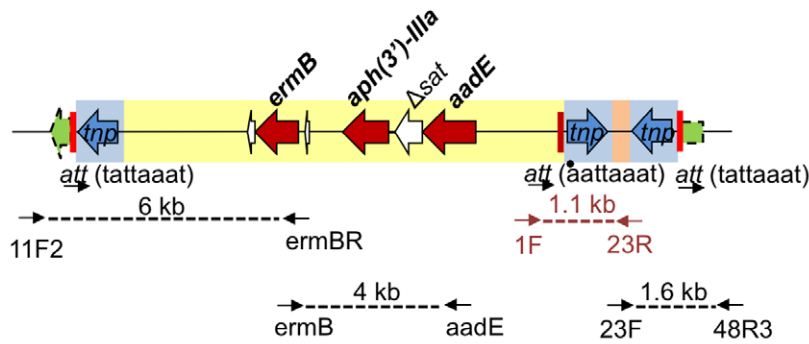

Figure 2. Genetic structure of MES$_{PM1}$ carrying four resistance genes, *ermB*, *aph(3′)-IIIa*, *aadE*, and *cat*. MES$_{PM1}$ was integrated into and disrupted the *sasK* gene, which encoded a putative cell-wall anchored surface protein with the LPXTG-motif. The intact *sasK* gene was present in PVL-positive ST59 CA-MRSA type strain USA1000. Three *att* sites are in a vertical red line. The region of IS*1216V* with the transposase gene (*tnp*) is shaded in blue. Homology regions with *E. faecalis* pLG2 plasmid (GenBank accession number HQ426665) and *S. aureus* SAP084A (GenBank accession number GQ900436) are shaded in yellow and purple, respectively. A Greek delta symbol indicates truncated coding sequence.
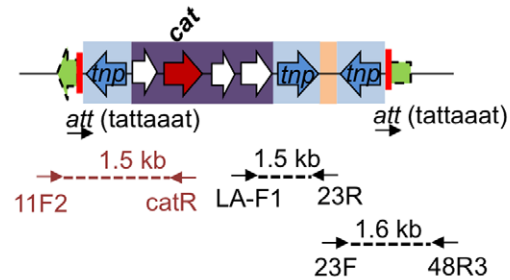doi:10.1371/journal.pone.0046987.g002

A MES$_{PM1}$ in PM1 (Ery/Cli^r, Kan^r, Stm^r, Chl^r)

B MES$_{PM9}$ in PM9 (Ery/Cli^r, Kan^r, Stm^r)

C MES$_{PM8}$ in PM8 (Chl^r)

D MES$_{PM18}$ (IS$1216V$) in PM18

sasK gene and possessed *att* sequence duplication at both ends (Figure 3D), may behave as an IS.

Structure of pPM1

The entire sequence of pPM1 was determined (GenBank Accession number AB699881) and its structure is summarized in Figure 4A. pPM1 was a novel composite plasmid, consisting of a replication (*rep*) region (homologous to pSK156 plasmid), cadmium resistance (*cad*) region (homologous to SAP063A plasmid), ampicillin resistance (*bla*) region (homologous to SAP063A plasmid), and tetracycline resistance (*tetK*) region (homologous to SAP014A plasmid). The *tetK*-carrying region was 6,025 bp long and was flanked by direct repeats of IS431 at both ends; this structure was designated MES_tet. There was no *att* sequence duplication at either end of MES_tet.

Analysis of Tetracycline-susceptible Strains

Of three tetracycline-susceptible strains (Table 1) examined, two strains, PM11 and PM18, carried a pPM1-related plasmid (pPM11 and pPM18, respectively). Entire sequence analysis revealed that the two plasmids carried one copy of IS431 only at the MES_tet insertion site on pPM1, as shown in Figure 4B; therefore, pPM11 and pPM18 could be a segregant of pPM1 generated through a recombination/deletion event between two

direct repeats of IS431. The remaining strain (PM12) lacked pPM1 plasmid.

Conjugative Transfer of Drug Resistance

Conjugative transfer of drug resistance was performed between PM1 (donor strain) and *S. aureus* RN2677 (recipient strain) by filter mating. As shown in Table 2, resistance to ampicillin, tetracycline, and cadmium was transferred to *S. aureus* RN2677 with equal frequency; all transconjugants obtained carried a 26-kp penicillinase plasmid (pPM1), as expected. However, no MES_PM1-associated transconjugants (exhibiting resistance to erythromycin, clindamycin, chloramphenicol or streptomycin) were obtained.

Restriction and Modification Genes (*hsdS* and *hsdM*) in Strain PM1

Genomic island vSAβ of MRSA strains, e.g. USA300, carried intact *hsdS* and *hsdM*, while vSAβ of PM1 carried truncated *hsdS* and lacked *hsdM* (Figure 5), although genomic island vSAα of PM1 carried an intact set of *hsdS* and *hsdM*, similar to USA300 (Figure 1). Lack of a set of *hsdS* and *hsdM* in PM1 may have contributed, in part, to PM1's potential to acquire foreign DNA.
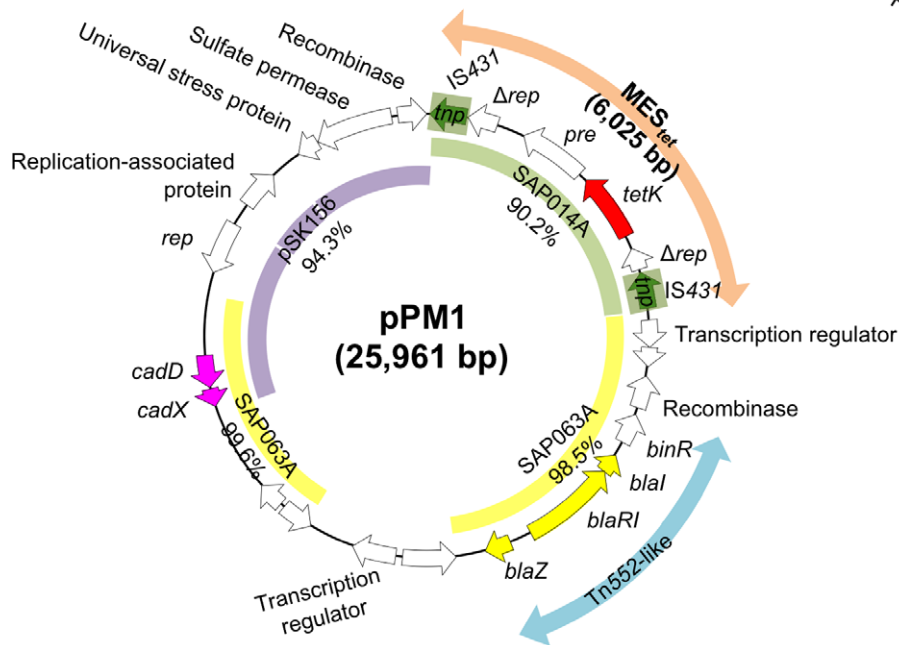
Figure 4. Genetic structure of penicillinase plasmid pPM1 and its segregant. In A, pPM1 structure is shown. Inner circle indicates homologous regions to *S. aureus* plasmid SAP014A (GenBank accession number GQ900379), SAP063A (GenBank accession number GQ900418), and pSK156 (GenBank accession number GQ900448). The IS431 region with the transposase gene (*tnp*) is shaded in light green. A Greek delta symbol indicates truncated coding sequence. In B, a segregant of pPM1 in MES_tet locus is shown. Tet^r, tetracycline-resistant; Tet^s, tetracycline-susceptible.
doi:10.1371/journal.pone.0046987.g004

Table 2. Transfer of drug resistance in mating between ST59 CA-MRSA strain PM1and *S. aureus* RN2677.

Selection of transconjugant[a]	Transfer frequency: drug-resistant recipient/donor	Minimal inhibitory concentration (µg/ml)		
		Transconjugants	Recipient (RN2677)	Donor (PM1)
Ampicillin	1.6×10^{-7}	1[b]	0.13	32
Tetracycline	1.6×10^{-7}	32	0.25	32
Cadmium	1.6×10^{-7}	32	4	32
Erythromycin	$<1.4 \times 10^{-9}$	–[c]		
Clindamycin	$<1.4 \times 10^{-9}$	–[c]		
Chloramphenicol	$<1.0 \times 10^{-8}$	–[c]		
Streptomycin	$<1.0 \times 10^{-8}$	–[c]		

[a]Novobiocin was used for selection of RN2677.
[b]MIC of ampicillin for transconjugants was lower than that for PM1 (1 µg/ml vs. 32 µg/ml).
[c]No transconjugants were obtained.
doi:10.1371/journal.pone.0046987.t002

Discussion

In this study, we performed comparative genomic analysis of a multidrug-resistant, PVL-positive ST59/SCC*mec*V CA-MRSA strain (PM1) and found MES$_{PM1}$ on the PM1 chromosome, encoding resistance to erythromycin, clindamycin, kanamycin, streptomycin, and chloramphenicol. As shown in Figure 6A, MES$_{PM1}$ exhibited typical features of a transposon, such as the presence of terminal repeats with 8-bp att duplications at both ends. The unique features of MES$_{PM1}$ include the presence of IS*1216V* as a terminal direct repeat and a composite transposon region containing Tn*551* and Tn*5405*-like, both of which
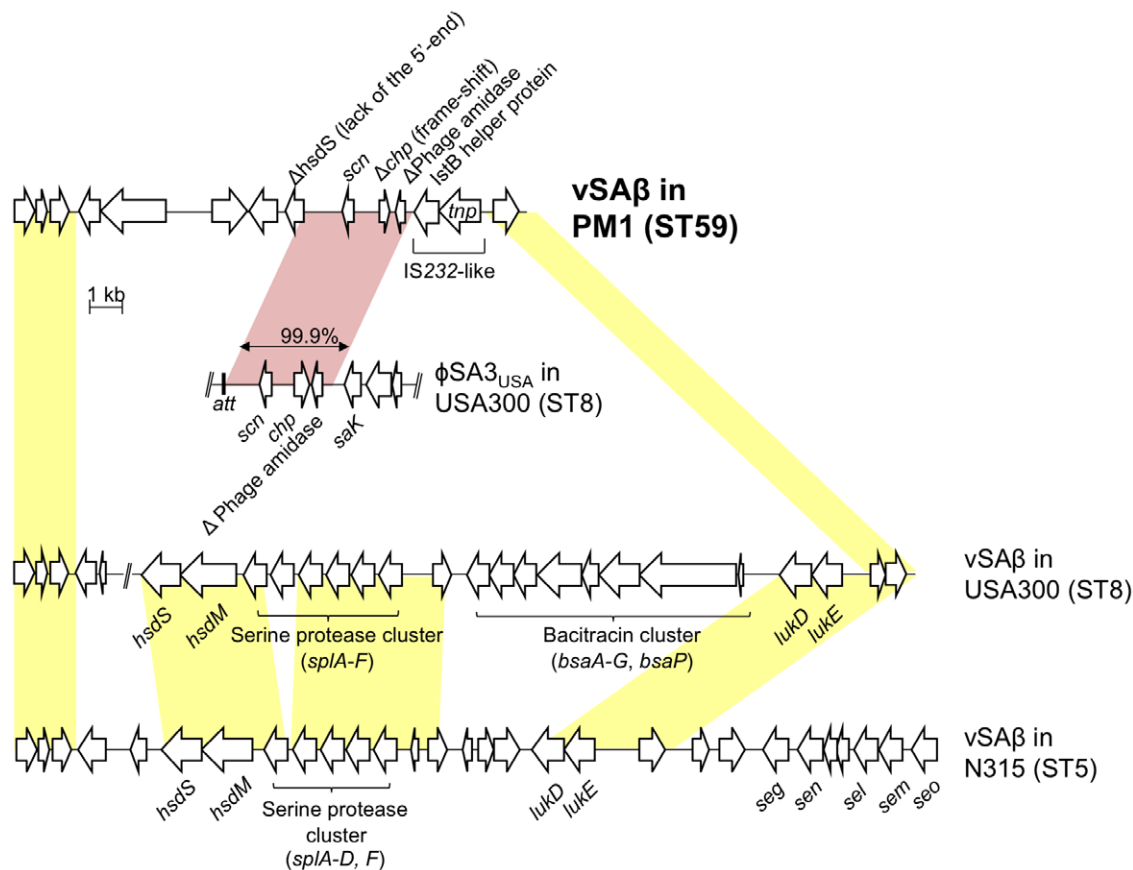
Figure 5. Structure of νSAβ in PM1. The 15,551-bp νSAβ structure in PM1 is shown in the upper part. This sequence was compared to the data of ST8 CA-MRSA strain USA300 FPR3757 (GenBank accession number NC_007793) and ST5 HA-MRSA strain N315 (GenBank accession number BA000018). PM1 regions homologous to φSA3$_{USA}$ (of USA300) and νSAβs (of USA300 and N315) are shaded in pink and yellow, respectively. A Greek delta symbol indicates truncated coding sequence.
doi:10.1371/journal.pone.0046987.g005
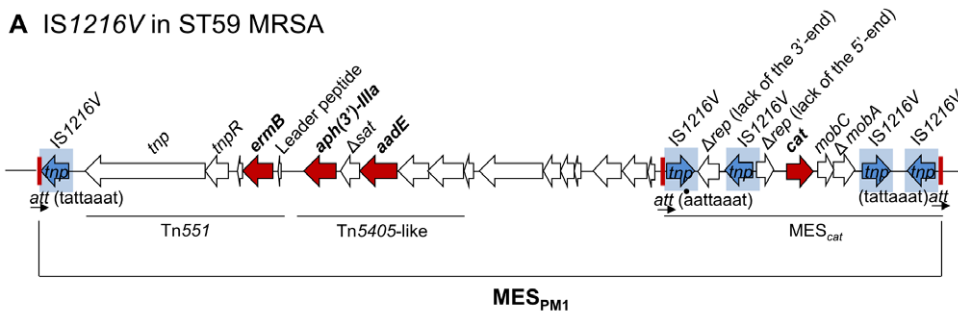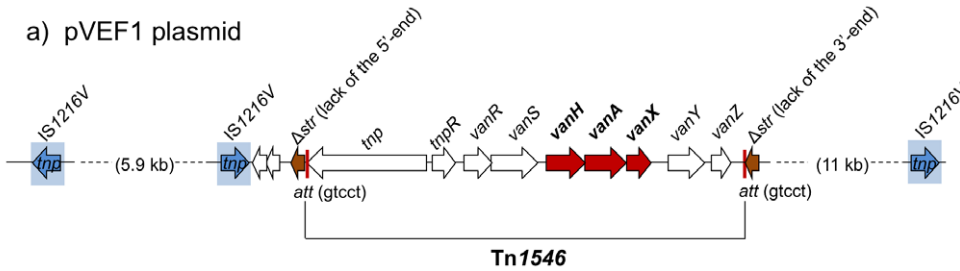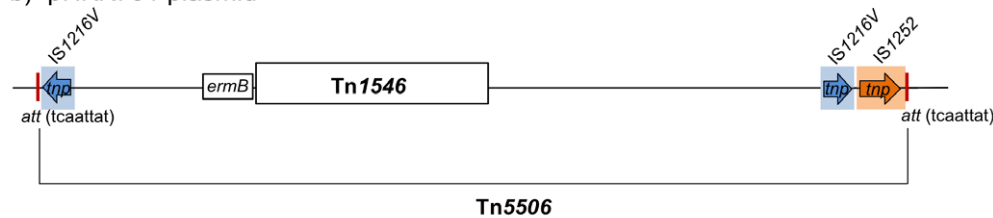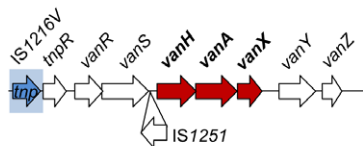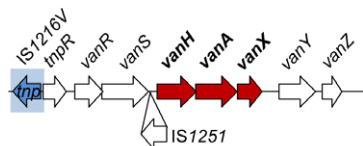
Figure 6. Comparison of IS*1216V*-containig mobile elements in MRSA and *E. faecium*. The structures of IS*1216V*-containing mobile elements: A, MES$_{PM1}$ determined in this study; B, Tn*1546* and Tn*5506* in *E. faecium*; C, vancomycin resistance transposon Tn*1546* in VRSA. The IS*1216V* region with the transposase gene (*tnp*) is shaded in blue. The *att* site is indicated by a vertical red line. The data for pVEF1 are from GenBank (GenBank accession number AM296544). The data for pHKK701 and Tn*1546* in VRSA-2 and VRSA-3 were obtained from previously described maps [31,32]. doi:10.1371/journal.pone.0046987.g006

originate in enterococci (such as *E. faecium* and *E. faecalis*) [26,33,34], strongly suggesting the origin of MES$_{PM1}$ in enterococci.

In *E. faecium*, IS*1216V* is considered to be part of a large mobile element containing Tn*1546* with *vanA* (encoding vancomycin resistance) [35], as shown in Figure 6B (a); however, a possible mobile structure with *att* duplication has not been reported. Moreover, in *E. faecium*, a large mobile element (Tn*5506*), which

contains Tn*1546* and is flanked by hetero terminal sequences, IS*1216V* and IS*1252*, with 8-bp *att* duplications at both ends, has been described [32], as shown in Figure 6B (b); however, the *att* of Tn*5506* was different from the *att* of MES$_{PM1}$ (Figure 6A and B [b]), and in the case of Tn*5506*, another copy of IS*1216V* was located adjacent to IS*1252* (Figure 6B [b]).

Transfer of the *van* genes (such as *vanA*) from enterococci to MRSA would be a great public health concern and threat, because

vancomycin is the first-line agent for MRSA infections [3,36,37]. Unfortunately, Tn*1546* has already emerged in MRSA [27], as shown in Figure 6C; in this case, IS*1216V* is present near the *van* genes (although possible IS*1216V* repeats and *att* duplication have not been reported).

Therefore, this study demonstrates the first IS*1216V*-mediated mobile element structure with terminal direct repeats of IS*1216V* with *att* duplication at both ends. Interestingly, MES$_{PM1}$ also contained another mobile element structure (MES$_{cat}$) within the structure, which was flanked by inverted repeats of IS*1216V* with *att* duplication at both ends. Chloramphenicol resistance in MRSA is usually encoded by a plasmid [31,38]; however, in this case, it was encoded by MES$_{cat}$ (as a part of MES$_{PM1}$) on the chromosome. The chloramphenicol resistance region seemed to originate in *S. aureus*; therefore, MES$_{PM1}$ is a highly composite structure with mobile elements originating in *Enterococcus* and *Staphylococcus* species.

This study clearly concludes that the acquisition mechanism of multidrug resistance in ST59 CA-MRSA in Taiwan is quite different from many other MRSA strains, but shows some similarity to VRSA cases, and that the MES$_{PM1}$ acquisition event occurred most probably around 2007 or earlier in Taiwan, and then ST59 CA-MRSA carrying MES$_{PM1}$ spread in Taiwan, followed by segregation events generating different resistance patterns.

The reason why ST59 CA-MRSA in Taiwan acquired MES$_{PM1}$ is not known. The type I restriction-modification system, composed of HsdR (restriction), HsdM (modification), and HsdS (sequence specificity), is known to block horizontal gene transfer from other species into *S. aureus*, as well as to limit DNA exchange between the different lineages of *S. aureus* [39,40]. Deficiency in the type I restriction-modification system would allow foreign DNA acquisition with high frequency. For example, a bovine pathogenic *S. aureus* strain, which is naturally mutated in both *hsdS* genes, has been reported displaying a "hyper-recipient" phenotype to acquire DNA from *Enterococcus* species [41]. In the case of ST59 CA-MRSA (strain PM1) in Taiwan, a copy of *hsdM* and *hsdS* in vSAβ was truncated, which might contribute to the acquisition of foreign DNA from enterococci such as IS*1216V* and MES$_{PM1}$; further study is needed for elucidation.

In this study, a conjugative penicillinase plasmid (pPM1) in strain PM1 was successfully transferred by filter mating, while MES$_{PM1}$ was not, suggesting that MES$_{PM1}$ may not be a conjugative transposon.

In strain PM1, we also identified a novel mosaic penicillinase plasmid encoding tetracycline resistance (pPM1). The tetracycline resistance region (MES$_{tet}$) could be a novel transposon flanked by IS*431*, which most probably originated in *S. aureus*. This structure also generated segregants (tetracycline-susceptible penicillinase plasmid), resulting in strains PM11 and PM18.

Regarding other genetic structures in ST59 CA-MRSA in Taiwan, we confirmed the sequence of SCC*mec*V of strain PM1 (formerly SCC*mec*VII) [24] and the sequence of PVL phage (ϕ5967PVL; ϕSA2$_{PM1}$ in this study), which was originally reported with another strain (JCSC5967) by Zhang et al. [42]; they were 99.9% homologous.

During the course of this study, Huang et al. described the genome sequence of ST59 CA-MRSA strain M013 in Taiwan [43], although they did not describe the plasmid(s). According to their data, their strain (M013) is not multidrug-resistant; most probably it corresponds to strain PM18 in this study, a minor derivative of PM1 carrying only IS*1216V* at the MES$_{PM1}$ insertion site and showing no resistance to non-β-lactam agents. M013 also does not carry ϕSA1$_{PM1}$. In addition, three genes (*sasA*, *fnbA*, and

sdrC) in M013 have more than 24-bp deletions or insertions. The comparison between the PM1 and M013 genomes is summarized in Table S1.

In conclusion, an IS*1216V*-mediated mobile element structure (MES$_{PM1}$), encoding multidrug resistance and originating in enterococci, has emerged in PVL-positive ST59 CA-MRSA (e.g. strain PM1) in Taiwan. Although IS*1216V*, related to vancomycin resistance transposon Tn*1546*, has been reported in MRSA, MES$_{PM1}$ represents the first mobile element flanked by IS*1216V* direct repeats with *att* duplication at both ends. MES$_{PM1}$ also includes additional MES$_{cat}$ flanked by IS*1216V* inverted repeats with *att* duplication at both ends, although the *cat* region originated in *S. aureus*, indicating a highly composite formation in its evolution. The deficiency of a copy of *hsdM* and *hsdS* (in vSAβ) in strain PM1 might contribute to the acquisition of foreign DNA, such as IS*1216V* and MES$_{PM1}$, from enterococci.

Materials and Methods

Bacterial Strains

Twenty-four PVL-positive ST59/SCC*mec*V CA-MRSA strains were collected from 2000 to 2006 in the Bacteriology Laboratory, National Taiwan University Hospital. These strains were isolated from patients with SSTI, bacteremia, respiratory tract infection, and surgical wounds [23]. USA1000 is an ST59 MRSA-type strain (of the SmaI PFGE type) isolated in the United States, and was kindly provided by L. K. McDougal and L.L. McDonald.

Pyrosequence Analysis of the Genome

The PM1 genome was analyzed by pyrosequencing using a genome sequencer FLX system (Roche Diagnostics, Branford, CT, USA). It yielded 93 Mb raw sequences by 236,943 reads, corresponding to approximately 33.2-fold of the genome size. Contigs were then assembled according to the two genomes of ST8 MRSA USA300 FPR3757 (GenBank accession number NC_007793) and ST398 MRSA human strain S0385 (GenBank accession number AM990992). Open reading frames were searched for using the software in silico MolecularCloning (version 4.2) (In Silico Biology, Yokohama, Japan) or DNAman software package (Version 6) (Lynnon Biosoft, Quebec, Canada).

Entire Sequencing of Mobile Elements and a Plasmid

The gaps between contigs, related to mobile elements, were filled by PCR and sequencing. We also assembled contigs using an LA PCR in vitro cloning kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. In brief, after digestion with suitable restriction enzymes and ligation with the corresponding cassette adapters, amplification was performed with cassette primers and target-specific primers.

PCR Analysis of the MES$_{PM1}$ Structure in Other PVL-positive ST59 CA-MRSA Strains

Based on the determined entire sequence of MES$_{PM1}$, a series of primer sets was designed (Table 3) and used to screen the genetic constitution of MES$_{PM1}$. In PCR reactions, 10% glycerol or 4% DMSO was added in order to dissolve the secondary structure of IS*1216V*.

Susceptibility Testing

Antimicrobial susceptibility tests were performed by the agar dilution method with Hueller-Hinton agar (Difco, Sparks, MD, USA) according to the guidelines of the Clinical and Laboratory Standards Institute [44].

Table 3. Primers used for PCR mapping of the MES$_{PM1}$ locus.

Primer name	Sequence (5′ to 3′)
11F2	AGGCGAAACATTGGAAGT
ermBR	GCAATGAAACACGCC
ermB	AGTAACGGTACTTAAATTGTTTAC
aadE	ACTGGCTTAATCAATTTGGG
1F	AGTAGCCTTTCCCTCACTT
35R	GCTTTGACGCTATGACGA
35F	CCTTACCAGTTGTTCCGAA
LA-R2	CCCATGCAGGTTTCAAAATGTGTAAGTCA
LA-F1	ACACCAGAACAATGTAATCAACTAGGGT
23R	TGTTCCAAAGAGCCATCA
23F	CATCAGGAGGGCAAGAA
48R3	TCAGGCGTGAGAATAAATGA
catR	GCATTTGATTCTGTCCA

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Conjugative Transfer

Donor strains were mated with *S. aureus* RN2677 (recipient strain, which is restriction-negative and resistant to rifampicin and novobiocin) on membrane filters on tryptic soy agar (Difco) (filter mating), as previously described [45]; RN2677 was used as a recipient because it carried no plasmids and had non-transmissible drug resistance (recipient) markers. The resistance genes of transconjugants were examined by PCR as previously described [23].

Plasmid Analysis

Plasmid DNA was isolated using a plasmid midi kit (Qiagen, Hilden, Germany) and lysostaphin (Wako Pure Chemicals, Osaka, Japan), following the manufacturer's instructions.

Supporting Information

Table S1 Comparative genomic analysis between PM1 and M013.
(XLS)

Author Contributions

Conceived and designed the experiments: TY WCH TT. Performed the experiments: TY HCH TT WH YI. Analyzed the data: TY HCH TT WH YI. Contributed reagents/materials/analysis tools: WCH TT WH YI OK LJT TY. Wrote the paper: TY WCH.

References

1. Grundmann H, Aires-de-Sousa M, Boyce J, Tiemersma E (2006) Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet 368: 874–885.
2. Yamamoto T, Nishiyama A, Takano T, Yabe S, Higuchi W, et al. (2010) Community-acquired methicillin-resistant *Staphylococcus aureus*: community transmission, pathogenesis, and drug resistance. J Infect Chemother 16: 225–254.
3. Zetola N, Francis JS, Nuermberger EL, Bishai WR (2005) Community-acquired meticillin-resistant *Staphylococcus aureus*: an emerging threat. Lancet Infect Dis 5: 275–286.
4. Diep BA, Otto M (2008) The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol 16: 361–369.
5. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, et al. (2003) Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA 290: 2976–2984.
6. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) (2009) Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother 53: 4961–4967.
7. Daum RS, Ito T, Hiramatsu K, Hussain F, Mongkolrattanothai K, Jamklang M, et al. (2002) A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. J Infect Dis 186: 1344–1347.
8. Diep BA, Stone GG, Basuino L, Graber CJ, Miller A, et al. (2008) The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. J Infect Dis 197: 1523–1530.
9. Löffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, et al. (2010) *Staphylococcus aureus* Panton-Valentine leukocidin is a very potent cytotoxic factor for human neutrophils. PLoS Pathog 6: e1000715.
10. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, et al. (2003) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 9: 978–984.
11. Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, et al. (2007) *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. Science 315: 1130–1133.
12. Chua K, Laurent F, Coombs G, Grayson ML, Howden BP (2011) Antimicrobial resistance: Not community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA)! A clinician's guide to community MRSA - its evolving antimicrobial resistance and implications for therapy. Clin Infect Dis 52: 99–114.
13. Diep BA, Chambers HF, Graber CJ, Szumowski JD, Miller LG, et al. (2008) Emergence of multidrug-resistant, community-associated, methicillin-resistant *Staphylococcus aureus* clone USA300 in men who have sex with men. Ann Intern Med 148: 249–257.
14. Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, et al. (2006) Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. Clin Infect Dis 42: 647–656.
15. Gonzalez BE, Rueda AM, Shelburne SA, 3rd, Musher DM, Hamill RJ, et al. (2006) Community-associated strains of methicillin-resistant *Staphylococcus aureus* as the cause of healthcare-associated infection. Infect Control Hosp Epidemiol 27: 1051–1056.
16. Wang CC, Lo WT, Chu ML Siu LK (2004) Epidemiological typing of community-acquired methicillin-resistant *Staphylococcus aureus* isolates from children in Taiwan. Clin Infect Dis 39: 481–487.
17. Boyle-Vavra S, Ereshefsky B, Wang CC, Daum RS (2005) Successful multiresistant community-associated methicillin-resistant *Staphylococcus aureus* lineage from Taipei, Taiwan, that carries either the novel staphylococcal chromosome *mec* (SCC*mec*) type V$_T$ or SCC*mec* type IV. J Clin Microbiol 43: 4719–4730.
18. Hsu LY, Tristan A, Koh TH, Bes M, Etienne J, et al. (2005) Community associated methicillin-resistant *Staphylococcus aureus*, Singapore. Emerg Infect Dis 11: 341–342.
19. Ho PL, Cheung C, Mak GC, Tse CW, Ng TK, et al. (2007) Molecular epidemiology and household transmission of community-associated methicillin-resistant *Staphylococcus aureus* in Hong Kong. Diagn Microbiol Infect Dis 57: 145–151.
20. Higuchi W, Hung WC, Takano T, Iwao Y, Ozaki K, et al. (2010) Molecular characteristics of the Taiwanese multiple drug-resistant ST59 clone of Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* from pediatric cellulitis. J Infect Chemother 16: 144–149.
21. Kawaguchiya M, Urushibara N, Kuwahara O, Ito M, Mise K, et al. (2011) Molecular characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* in Hokkaido, northern main island of Japan: identification of sequence types 6 and 59 Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus*. Microb Drug Resist 17: 241–250.
22. Coombs GW, Monecke S, Ehricht R, Slickers P, Pearson JC, et al. (2010) Differentiation of clonal complex 59 community-associated methicillin-resistant *Staphylococcus aureus* in Western Australia. Antimicrob Agents Chemother 54: 1914–1921.
23. Takano T, Higuchi W, Otsuka T, Baranovich T, Enany S, et al. (2008) Novel characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* strains belonging to multilocus sequence type 59 in Taiwan. Antimicrob Agents Chemother 52: 837–845.
24. Higuchi W, Takano T, Teng LJ, Yamamoto T (2008) Structure and specific detection of staphylococcal cassette chromosome *mec* type VII. Biochem Biophys Res Commun 377: 752–756.

25. Yamamoto T, Takano T, Higuchi W, Iwao Y, Singur O, et al. (2012) Comparative genomics and drug resistance of a geographic variant of ST239 methicillin-resistant *Staphylococcus aureus* emerged in Russia. PLoS One 7: e29187.
26. Mahillon J, Chandler M (1998) Insertion sequences. Microbiol Mol Biol Rev 62: 725–774.
27. Perichon B, Courvalin P (2009) VanA-type vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53: 4580–4587.
28. Schijffelen MJ, Boel CH, van Strijp JA, Fluit AC (2010) Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. BMC Genomics 11: 376.
29. Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, et al. (2011) A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One 6: e17936.
30. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, et al. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet 367: 731–739.
31. Lyon BR, Skurray R (1987) Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. Microbiol Rev 51: 88–134.
32. Heaton MP, Discotto LF, Pucci MJ, Handwerger S (1996) Mobilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an *Enterococcus faecium* sex pheromone-response plasmid. Gene 171: 9–17.
33. Werner G, Hildebrandt B, Witte W (2003) Linkage of *erm(B)* and *aadE-sat4-aphA-3* in multiple-resistant *Enterococcus faecium* isolates of different ecological origins. Microb Drug Resist 9 Suppl 1: S9–16.
34. Laverde Gomez JA, Hendrickx AP, Willems RJ, Top J, Sava I, et al. (2011) Intra- and interspecies genomic transfer of the *Enterococcus faecalis* pathogenicity island. PLoS One 6: e16720.
35. Sletvold H, Johnsen PJ, Wikmark OG, Simonsen GS, Sundsfjord A, et al. (2010) Tn*1546* is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal *Enterococcus faecium* lineages. J Antimicrob Chemother 65: 1894–1906.
36. Levine DP (2006) Vancomycin: a history. Clin Infect Dis 42 Suppl 1: S5–12.
37. Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, et al. (2011) Clinical practice guidelines by the infectious diseases society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children: executive summary. Clin Infect Dis 52: 285–292.
38. Schwarz S, Cardoso M (1991) Nucleotide sequence and phylogeny of a chloramphenicol acetyltransferase encoded by the plasmid pSCS7 from *Staphylococcus aureus*. Antimicrob Agents Chemother 35: 1551–1556.
39. Corvaglia AR, Francois P, Hernandez D, Perron K, Linder P, et al. (2010) A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical *Staphylococcus aureus* strains. Proc Natl Acad Sci U S A 107: 11954–11958.
40. Waldron DE, Lindsay JA (2006) Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. J Bacteriol 188: 5578–5585.
41. Sung JM, Lindsay JA (2007) *Staphylococcus aureus* strains that are hypersusceptible to resistance gene transfer from enterococci. Antimicrob Agents Chemother 51: 2189–2191.
42. Zhang M, Ito T, Li S, Jin J, Takeuchi F, et al. (2011) Identification of the third type of PVL phage in ST59 methicillin-resistant *Staphylococcus aureus* (MRSA) strains. FEMS Microbiol Lett 323: 20–28.
43. Huang TW, Chen FJ, Miu WC, Liao TL, Lin AC, et al. (2012) Complete genome sequence of *Staphylococcus aureus* M013, a *pvl*-positive, ST59-SCC*mec* type V strain isolated in Taiwan. J Bacteriol 194: 1256–1257.
44. Clinical and Laboratory Standards Institute (2011) Performance standards for antimicrobial susceptibility testing: 21st informational supplement M100-S21. Wayne, PA, USA: Clinical and Laboratory Standards Institute.
45. Takizawa Y, Taneike I, Nakagawa S, Oishi T, Nitahara Y, et al. (2005) A Panton-Valentine leucocidin (PVL)-positive community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) strain, another such strain carrying a multiple-drug resistance plasmid, and other more-typical PVL-negative MRSA strains found in Japan. J Clin Microbiol 43: 3356–3363.