A Possible Role of Insertion Sequence IS1216V in Dissemination of Multidrug-Resistant Elements MES\textsubscript{PM1} and MES\textsubscript{6272-2} between \textit{Enterococcus} and ST59 \textit{Staphylococcus aureus}

Yu-Tzu Lin 1, Sung-Pin Tseng 2, Wei-Wen Hung 3, Chen-Chia Chang 4, You-Han Chen 4, Ya-Ting Jao 5, Yen-Hsu Chen 6,7,8,9, Lee-Jene Teng 10 and Wei-Chun Hung 4,11,*

1 Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung 404333, Taiwan; yutzulin@mail.cmu.edu.tw
2 Department of Medical Laboratory Science and Biotechnology, College of Health Sciences, Kaohsiung Medical University, Kaohsiung 807378, Taiwan; tsengsp@kmu.edu.tw
3 Division of Endocrinology and Metabolism, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 807377, Taiwan; hung4488@ms57.hinet.net
4 Department of Microbiology and Immunology, College of Medicine, Kaohsiung Medical University, Kaohsiung 807378, Taiwan; wacsun@gmail.com (C.-C.C.); hady85427@gmail.com (Y.-H.C.)
5 Infection Control Center, Kaohsiung Medical University Hospital, Kaohsiung 807377, Taiwan; ya-tine@yahoo.com.tw
6 Division of Infectious Diseases, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 807377, Taiwan; infchen@gmail.com
7 School of Medicine, Graduate Institute of Medicine, Sepsis Research Center, Center of Tropical Medicine and Infectious Diseases, Kaohsiung Medical University, Kaohsiung 807378, Taiwan
8 Department of Biological Science and Technology, College of Biological Science and Technology, National Chiao Tung University, HsinChu 300093, Taiwan
9 Institute of Medical Science and Technology, National Sun Yat-sen University, Kaohsiung 804201, Taiwan
10 Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, Taipei 100229, Taiwan; lj teng@ntu.edu.tw
11 Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung 807377, Taiwan

* Correspondence: wchung@kmu.edu.tw; Tel.: +886-7-3121101 (ext. 2150-16)

Received: 4 November 2020; Accepted: 27 November 2020; Published: 30 November 2020

Abstract: Sequence type 59 (ST59) is the dominant type of community-associated methicillin-resistant \textit{Staphylococcus aureus} (MRSA) in Taiwan. Previously, we reported that ST59 MRSA harbors enterococcal IS1216V-mediated multidrug-resistant composite transposons MES\textsubscript{PM1} or MES\textsubscript{6272-2}. The MES were found to have a mosaic structure, largely originating in enterococci and partly native to \textit{S. aureus}. The current study aimed to track the origin of the MES and how they disseminated from enterococci to ST59 \textit{S. aureus}. A total of 270 enterococcal isolates were analyzed, showing that two ST64 \textit{Enterococcus faecalis} isolated in 1992 and 11 clonal complex 17 \textit{Enterococcus faecium} harbored MES\textsubscript{PM1}-like and MES\textsubscript{6272-2}-like structures, respectively. Sequence analysis revealed that ST64 \textit{E. faecalis} strain N48 acquired the MES\textsubscript{PM1}-like structure on the plasmid pEffis48. The pEffis48 harbored the enterococci-originated region (erythromycin, kanamycin, and streptomycin resistances) and the \textit{S. aureus}-originated region (chloramphenicol resistance) of MES\textsubscript{PM1} but was separated by the replication region of the plasmid. Homologous recombination between the two direct repeats of IS1216V resulted in excision of the replication region of the plasmid to regenerate MES\textsubscript{PM1}. The p4780-1 and pV19 of \textit{E. faecium} carried MES\textsubscript{6272-2}-like structures with IS1216V, albeit with multiple
insertions by other insertion sequences. The findings show that IS1216V plays important roles in bidirectional gene transfer of multidrug resistance between enterococci and S. aureus.

**Keywords:** Staphylococcus aureus; enterococci; IS1216V; gene transfer

1. Introduction

Sequence type 59 (ST59) methicillin-resistant Staphylococcus aureus (MRSA) is the dominant type of community-associated MRSA (CA-MRSA) in Taiwan and can be divided into two clones: Panton–Valentine leukocidin (PVL)-positive/staphylococcal cassette chromosome mec (SCCmec) V and PVL-negative/SCCmec IV [1-3]. Previously, we reported that ST59 S. aureus acquires IS1216V-mediated enterococcal composite transposons MES, which are responsible for multidrug resistance [4,5]. ST59 PVL-positive/SCCmec V MRSA acquires MES\textsubscript{pm}, which confers resistances to erythromycin, kanamycin, streptomycin, and chloramphenicol; ST59 PVL-negative/SCCmec IV MRSA usually acquires MES\textsubscript{S272-1}, which confers resistances to erythromycin, kanamycin, gentamicin, and chloramphenicol. Both the MES\textsubscript{pm} and MES\textsubscript{S272-1} are inserted into the chromosomal \textit{sasK} gene with a 8 bp \textit{att} sequence and are flanked by direct repeats of IS1216V at both ends, indicating that MES\textsubscript{pm} and MES\textsubscript{S272-1} are composite transposons mediated by IS1216V [4,5]. IS1216V, belonging to the \textit{Is6}/\textit{Is26} family, is 809 bp in length with 18 inverted repeats [6,7]. Although IS1216V is an enterococcal insertion sequence rarely found in \textit{S. aureus}, up to five copies of IS1216V are located in MES\textsubscript{pm} and MES\textsubscript{S272-1} of ST59 \textit{S. aureus} [6].

Regarding multidrug resistance in ST59 \textit{S. aureus}, four resistance determinants originate in enterococci, including \textit{ermB} (erythromycin resistance), \textit{aph(3')-IIa} (kanamycin resistance), \textit{aadE} (streptomycin resistance), and \textit{aacA-aphD} (gentamicin resistance), while the \textit{cat} gene (chloramphenicol resistance) and its surrounding genetic environment is native to \textit{S. aureus}. MES\textsubscript{pm} includes \textit{ermB}, \textit{aph(3')-IIa}, \textit{aadE}, and \textit{cat}, while MES\textsubscript{S272-1} includes \textit{ermB}, \textit{aph(3')-IIa}, truncated \textit{aadE}, \textit{aacA-aphD}, and \textit{cat}. On the left side of MES\textsubscript{pm}, the IS1216V-\textit{ermB}-[\textit{aph(3')-IIa}-\textit{aadE}] cluster displays 100% DNA sequence similarity to the corresponding region in pLG2 of \textit{Enterococcus faecalis} [5]. On the right side of MES\textsubscript{pm}, the \textit{cat} gene and its surrounding environment show high DNA sequence similarity (>99.5%) to the corresponding region of the SAP084A plasmid in \textit{S. aureus}, albeit with four copies of IS1216V nearby [5]. The genetic organization of MES\textsubscript{S272-1} is similar to that of MES\textsubscript{pm} except with disruption of the \textit{aadE} gene by \textit{aacA-aphD} and replacement of the region downstream of the \textit{aadE} gene by group II introns [4].

\textit{E. faecalis} and \textit{Enterococcus faecium} are frequently encountered multidrug-resistant microorganisms and have rapidly emerged as troublesome pathogens causing nosocomial infections [8-11]. Mobile genetic elements (MGEs), including plasmids and transposons, have been shown to play a major role in dissemination of antibiotic resistance among the \textit{Enterococcus} species [12,13]. Some enterococcal MGEs have even been shown to be transferrable to other pathogenic species, such as \textit{Staphylococcus aureus}. Tn1546, which encodes vancomycin resistance via \textit{vanA}, originates in enterococci and can transfer to \textit{S. aureus}, leading to emergence of vancomycin-resistant \textit{S. aureus} (VRSA) [12,14]. The structure of Tn1546 displays heterogeneity, such as an enterococcal insertion sequence IS1216V inserted into the backbone [15]. IS1216V is also considered to be part of a large mobile element containing Tn1546 and may cotransfer with Tn1546 to other species [16].

MES\textsubscript{pm} and MES\textsubscript{S272-1} in ST59 MRSA, the composite transposons mediated by IS1216V, are examples of insertion sequences playing a pivotal role in the dissemination of antibiotic resistance. DNA sequence analysis revealed that more than half of the structures originate in enterococci, not in staphylococci. In the current study, we characterized enterococcal clinical isolates with MES-like structures and found evidence of IS1216V-mediated interspecies transfer of multidrug resistance genes between staphylococci and enterococci.
2. Materials and Methods

2.1. Bacterial Isolates

A total of 270 enterococcal isolates collected from clinical specimens were included in this study: (i) 95 randomly selected isolates collected between 1991 and 1993 at the National Taiwan University Hospital (NTUH), (ii) 26 randomly selected isolates from blood cultures collected between 2002 and 2003 at NTUH, and (iii) 149 isolates collected from January 2013 to October 2014 at the Kaohsiung Medical University Hospital (KMUH). Among the 149 isolates, E1–E51 ($n = 50$) were randomly selected vancomycin-susceptible enterococci collected from blood cultures, while V1–V99 ($n = 99$) were first identified as vancomycin-resistant enterococci and collected within the period. Enterococcal multiplex PCR or groEL sequencing was used for species identification [17]. Detailed information about the bacterial strains is shown in Table S1.

2.2. PCR Mapping of MES Structures

The resistance determinants $ermB$, $aph(3')-IIIa$, $aadE$, $aacA-aphD$, and $cat$ were detected as previously described [4]. For $ermB$, $aph(3')-IIIa$, and $aadE$-positive isolates, six primer sets (I–VI) were used to screen the MES structures. The entire region of MES$\beta$-like structures in the two E. faecalis strains was further mapped using two PCR primer sets (A and B) with the methods we had previously described [5]. Information about the primers is indicated in Table 1, and the locations of the primers are indicated in Figure S1.

| Primer Set | Primer Name | Sequence (5' to 3') | PCR Product Size (bp) |
|------------|-------------|---------------------|-----------------------|
| I          | IS1216VF    | AGTTTACGCCACTGCCTCT | 2170                  |
|            | tnF         | CGGTATCCTGGGTGT     |                       |
| II         | IS1216V-fo  | CTTCGCGTCATCAAACCTGC| 1384                  |
|            | tnp-rev     | TCAAATCACCTCCTACTACCC|                      |
| III        | ermB-rev    | TTGAACAGGTAAGGCC    | 2727                  |
|            | ermB-fo     | ATCTGTGATATGCGGGTA  |                       |
| IV         | aphIIIa-rev | ATGACATTGCGCTTCTCCG | 1432                  |
|            | aphIIIa-fo | TGTCAATACCTTGCCGCG  |                       |
| V          | aadE-rev    | GCTGCCTGAGATACACATA | 1345                  |
|            | aadE-R      | (2331 if ISfem1 insertion) |                |
| VI         | aacA-aphD-F | ATACAGAGCTTGGAAG     | 2254                  |
|            | 1F          | ATAGCTTTACTGAGCG     | 1301                  |
| A          | 35R         | GCTTTGACGCTATGACGA   |                       |
|            | 35F         | CTTTACCAGTTCCCGAAA   | 1619                  |

2.3. Antimicrobial Susceptibility Testing

Susceptibility testing of vancomycin, erythromycin, kanamycin, streptomycin, gentamicin, and chloramphenicol was performed by the agar dilution method according to the 2020 guidelines of the Clinical and Laboratory Standards Institute [18]. E. faecalis ATCC 29,212 was used as the reference strain. High-level resistance of streptomycin and gentamicin was defined as minimal inhibitory concentrations of $>2000$ and $>500$ mg/L, respectively.

2.4. Multilocus Sequence Typing (MLST)

MLST of the 11 MES-carrying enterococcal isolates (2 E. faecalis and 11 E. faecium) was performed as previously described [19,20]. Sequence types (STs) were assigned according to the program on the MLST website (https://pubmlst.org). Clonal complex (CC) was defined by geoBURST [21].
2.5. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed as previously described [22]. In brief, agarose-embedded bacterial DNA was digested with Smal (New England BioLabs, Ipswich, MA, USA) and then was separated using a CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, CA, USA). PFGE was carried out at 200 V and 14 °C for 22.5 h, with the pulse times ranging from 5 to 35 s. The pulsortypes were analyzed by BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The dendrogram of pulsortype relationships was produced by the unweighted pair group method using arithmetic averages (UPGMA) based on Dice similarity indices.

2.6. S1 Nuclease-Digested PFGE

S1 nuclease-digested PFGE analysis was performed as previously described to determine the sizes of the plasmids [23]. The agarose-embedded bacterial DNA was incubated at 37 °C for 45 min with 10 units of Aspergillus oryzae S1 nuclease (Invitrogen). The reaction was stopped by transferring the agarose-embedded bacterial DNA to ES buffer (0.5 mM EDTA, 1% (w/v) Sarkosyl, pH 9.0) at 4 °C for 10 min. The plugs were applied to wells of 1.2% (w/v) agarose gels (Bio-Rad) and run in a CHEF-DRII apparatus (Bio-Rad) with a pulse angle of 120° and pulse times of 45 s for 14 h and 25 s for 6 h at 200 V in 0.5× Tris-borate-EDTA (TBE). Each band was considered a unit length linear plasmid.

2.7. Southern Blot

The linear plasmids in the gel separated by S1-PFGE were depurinated, denatured, neutralized, and transferred to a Hybond-N+ nylon membrane (GE Healthcare, Chicago, IL, USA) by vacuum-blotting. Hybridization was achieved using the digoxigenin-labeled DNA probe specific for the aadE gene generated by PCR. The detection of hybridization was performed using an alkaline-phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) and the substrate CSPD (Roche Diagnostics GmbH) according to the manufacturer’s instructions.

2.8. Sequencing and Analysis of the MES Structures and Their Adjacent Environments

The sequence of the MES\textsubscript{6272} like structure in \textit{E. faecium} 4780-1 was determined using the primer sets for PCR mapping described above, and extension of the IS12126\textsubscript{V} upstream sequence was achieved by inverse PCR. For \textit{E. faecalis} strain N48 and \textit{E. faecium} V19, Illumina MiSeq 300 bp paired-end sequencing was used to determine the MES\textsubscript{48} and MES\textsubscript{6272} like structures. Total Illumina sequencing output corresponded to approximately 146- and 301-folds of the N48 and V19 genome size, respectively. Contigs were obtained using de novo assembly with SPAdes v3.10.1 [24]. A total of 92 and 261 contigs were yielded for the N48 and V19 genomes, respectively. The gaps between the contigs were filled up by Sanger sequencing. The open reading frame (ORF) was determined using BLAST. The plasmid family was determined using PlasmidFinder 2.0 [25].

2.9. Filter Mating

Filter mating was carried out on BHI agar using \textit{E. faecalis} N48 or \textit{E. faecium} V19 as donors, and \textit{E. faecalis} JH2-2 or \textit{S. aureus} RN2677 as recipients. The donor–recipient mix cultures were placed on a filter with incubation at 37 °C for 24 h, followed by resuspension and shaking in BHI broth with incubation at 37 °C for 1 h. The cells were collected and plated on BHI agar containing 250 mg/L erythromycin, 100 mg/L rifampin, 100 mg/L streptomycin (if donor was \textit{E. faecalis} N48), 100 mg/L gentamicin (if donor was \textit{E. faecium} V19), or 25 mg/L fusidic acid (if recipient was \textit{E. faecalis} JH2-2).

2.10. Nucleotide Sequence Accession Numbers

The nucleotide sequences (Supplementary Data) of pEflis48, p4780-1, and pV8919 characterized in this study have been deposited in GenBank under accession numbers MT877066–MT877068.
3. Results

3.1. Molecular Characteristics of MESPM1- or MES6272-2-Carrying Enterococcal Strains

The 270 enterococcal isolates were first screened by PCR for five resistance determinants (ermB, aph(3′)-IIIa, aadE, aacA-aphD, and cat) and antimicrobial susceptibility testing (Table S1). A total of 128 isolates that were positive for ermB, aph(3′)-IIIa, and aadE by PCR were selected for further analysis because MESPM1 carried the ermB, aph(3′)-IIIa, and aadE, while MES6272-2 carried ermB, aph(3′)-IIIa, and the nearly complete aadE gene with 5′-end disruption by aacA-aphD. The PCR mapping of MES structures revealed that two E. faecalis strains isolated in 1992 harbored a MESPM1-like structure; one E. faecium strain isolated in 2003 and 10 E. faecium strains isolated from 2013 to 2014 harbored MES6272-2-like structures (Table 2). The phylogenetic relatedness of the 13 strains was analyzed by PFGE and MLST (Figure 1). The two E. faecalis strains belonged to ST64 and had identical SmaI PFGE patterns. For the 11 E. faecium strains, all of them belonged to clonal complex 17 (CC17) with 5 different STs (ST18, ST262, ST612, ST787, and ST1693). The E. faecium strains were all resistant to vancomycin except strain 4780-1 (Table S1). The eight strains were clustered together, distant from V18, V76, and 4780-1 (the only strain isolated in 2003).

Table 2. Summary of the bacterial strains collected in this study.

| Isolation Year | Enterococcus species | No. of Isolates | Total |
|---------------|----------------------|----------------|-------|
| 1991–1993     | E. faecalis          | 82             | 126   |
| 2002–2003     | E. faecalis          | 14             | 30    |
| 2013–2014     | E. faecalis          | 30             | 63    |
|               | Others (E. hirae)    | 10             | 117   |
|               | Positive for ermB, aph(3′)-IIIa, aadE | 54 | 128 |
|               | MESPM1-like          | 2 (E. faecalis)| 0     |
|               | MES6272-2-like       | 0 (E. faecium) | 10    |

Figure 1. PFGE analysis of the 13 MES-carrying enterococcal strains. The dendrogram was produced by BioNumerics software, showing distance calculated by the Dice similarity index of SmaI-digested DNA fragments. The degree of similarity is shown in the scale. The strain number, ST, species, isolation year, isolation hospital, and MES types are indicated in the figure.

3.2. Localization of the MESPM1- or MES6272-2-Like Elements in Enterococcal Strains

To determine the location of the MESPM1- or MES6272-2-like elements in enterococcal strains, E. faecalis strain N48 isolated in 1992, E. faecium 4780-1 isolated in 2003, and E. faecium V19 isolated in 2013 were chosen for S1 nuclease-digested PFGE analysis. The S1 nuclease-digested DNAs were...
separated by PFGE, followed by Southern blot hybridization with the *aadE* probe (Figure 2). Southern blot hybridization after S1 nuclease-digested PFGE revealed that the MES-like elements of *E. faecalis* strain N48 and *E. faecium* 4780-1 were located on the plasmids with estimated sizes ranging from 48.5 to 97 kb, while the MES$_{522}$-like elements of *E. faecium* V19 were located on a relatively large plasmid with an estimated size ranging from 194 to 242.5 kb. The size of the MES$_{6272}$-like elements of *E. faecium* strains V32, V35, V47, and V76 corresponded to *E. faecalis* N48 and *E. faecium* V19, respectively (data not shown).

![Figure 2](image)

**Figure 2.** S1 nuclease PFGE analysis followed by Southern blot hybridization with the *aadE* probe of *E. faecalis* N48, *E. faecium* V19, and *E. faecium* 4780-1: (a) S1 nuclease-digested DNAs separated by PFGE; (b) Southern blot hybridization of the *aadE* probe of the S1 nuclease-digested DNAs.

### 3.3. Sequencing of the MES$_{PM1}$-Like Elements and the Adjacent Genetic Environments in Enterococcal Strains

*E. faecalis* N48 was chosen for DNA sequencing to determine the MES-like structure and the surrounding genetic environment. An incomplete plasmid sequence of 50,564 bp in length was determined, and sequence analysis revealed that the MES$_{PM1}$-like structure was located on a mosaic plasmid, pEflis48 (Figure 3a). Positions 1–9944 nt showed 99.7% DNA sequence identity to the corresponding region of the *E. faecalis* plasmid pTEF3. Positions 9136–14,615 and 34,166–50,564 showed 98.4% DNA sequence identity to MES$_{PM1}$ in ST59 MRSA. Positions 14,616–16,723 and 16,724–34,144 showed 99.3% and 99.0% DNA sequence identity to the corresponding regions of the *E. faecalis* plasmid pEF10748 and *E. faecalis* ATCC 29,212 plasmid 2, respectively. Detailed sequence comparison of the MES$_{PM1}$-like structure in *E. faecalis* N48 is shown in Figure 3b, and the results are highly identical to those for ST59/SCCmec V MRSA PM1, except (i) an additional 344 bp sequence near the IS1216Vφ; (ii) an additional region corresponding to *E. faecalis* plasmid pTEF3, including a rep gene belonging to the Inc18 family of broad-host-range conjugative plasmid, a truncated gene-encoding pheromone-binding protein, and a copy of IS1216V; and (iii) lack of a 298 bp noncoding sequence and an IS1216V on the right side of MES$_{PM1}$. Interestingly, the cat gene and its surrounding environment that originated in *S. aureus* were also found in pEflis48 and showed 99.9% sequence identity to MES$_{PM1}$. Moreover, PCR and DNA sequencing using the primer set A located in MES$_{PM1}$ (one was located upstream of IS1216Vφ and the other was located downstream of IS1216Vφ, as indicated in Figures 3 and S1) outside the pTEF3 region yielded a 1301 bp amplicon corresponding to a copy of IS1216V, indicating that homologous recombination occurred between the two direct repeats of IS1216V to excise the pTEF3 region. PCR of *E. faecalis* N46 generated the identical size to that of *E. faecalis* N48 (Figure S1b).
Figure 3. Structure of pEfliis48 in *E. faecalis* N48. (a) Schematic map of pEfliis48 is shown. Blue, pink, green, and purple colors in the circle indicate homologous regions to *E. faecalis* plasmid pTEF3 (GenBank accession number NC_004670), ST59 MRSA MES$_{6272}$ (GenBank accession number AB699882), *E. faecalis* plasmid pEF10748 (GenBank accession number MK993385), and *E. faecalis* ATCC 29,212 plasmid 2 (GenBank accession number CP008814). The black triangle indicates the position of IS$_{1216V}$. The dashed line in the circle indicates an unresolved sequence. (b) Genetic organization of the fan-shaped region indicated in (a) is shown. The arrow indicates an open reading frame. The rectangle indicates a truncated gene. Black, grey, red, and blue arrows indicate tnp (transposase) of IS$_{1216V}$, tnp of other insertion sequences, resistance determinates, and rep (replication initiator) gene, respectively. Homologous regions to *E. faecalis* plasmid pTEF3, *S. aureus* plasmid SAP084A, and MES$_{6272}$ are shaded in blue, yellow, and red, respectively.

3.4. Sequencing of the MES6272-2-Like Elements and the Adjacent Genetic Environments in Enterococcal Strains

*E. faecium* 4780-1 and V19 both carried MES$_{6272}$-2-like structures. Sequence analysis revealed that the IS$_{1216V}$-ermB-[aph(3’)-IIIa]-sat-$\Delta$aadE-[aacA-aphD] cluster of the MES$_{6272}$-2-like structure in *E. faecium* 4780-1 showed >99.7% identity to the corresponding region in ST59/SCC$_{mec}$ IV MRSA 6272-2, except an ISE$_{Efm1}$ insertion into the sat gene (Figure 4).

Figure 4. Genetic organization of MES$_{6272}$-2-like structures in p4780-1 and pV19. The arrow indicates an open reading frame. The rectangle indicates a truncated gene. Black, grey, and red colors indicate tnp (transposase) of IS$_{1216V}$, tnp of other insertion sequences, and resistance determinates, respectively. Homologous regions are shaded.

For the MES$_{6272}$-2-like structures of *E. faecium* V19, Illumina sequencing revealed that the MES$_{6272}$-2-like structure was located on plasmid, and 24,402 bp in length was determined. The ermA-[aph(3’)-IIIa]-sat-$\Delta$aadE-[aacA-aphD] cluster of MES$_{6272}$-2-like structures in *E. faecium* V19 showed 99.9% identity to the corresponding region in ST59/SCC$_{mec}$ IV MRSA 6272-2. However, the 3’-end of transposase of
Microorganisms 2020, 8, 1005

Tn551 was absent in *E. faecium* V19, and the direction of IS1216V in front of *ermB* was different between *E. faecium* V19 and ST59/SCCmec IV MRSA 6272-2. The region between the aacA-aphD gene and the second IS1216V of *E. faecium* V19 displayed different structures compared with MESPM1, harboring multiple resistance determinants of In1(B) (lincomamide resistance), Isa(E) (pleuromutilin, lincomamide, and streptogramin A resistance), spw (pectinomycin resistance), and an *aadE* gene.

3.5. Horizontal Transfer of MESPM1 or MES6272-2 Encoding Drug Resistance

Conjugative transfer of MES-like structures between enterococci and *S. aureus* was performed by filter mating. *E. faecalis* N48 or *E. faecium* V19 were used as donors, while *E. faecalis* JH2-2 or *S. aureus* RN2677 were used as recipients. The MES-related resistances were successfully transferred from *E. faecalis* N48 or *E. faecium* V19 to *E. faecalis* JH2-2, with transfer frequencies of $3.7 \times 10^{-2}$ and $2.5 \times 10^{-2}$, respectively. Resistances were transferred to transconjugants (Table 3). All of the transconjugants tested acquired plasmids with sizes similar to their donors detected by S1 nuclease PFGE with Southern blot hybridization. However, no transconjugants were obtained in the cases of transfer from *E. faecalis* N48 or *E. faecium* V19 to *S. aureus* RN2677 (efficiency < $6.5 \times 10^{-10}$).

Table 3. Transfer of drug resistance in filter mating.

| Recipient: *E. faecalis* JH2-2 | MIC (mg/L) |
|-------------------------------|------------|
| Donor: *E. faecalis* N48      | 0.25       |
|                               | 64         |
|                               | 128        |
|                               | 16         |
|                               | 8          |
| Donor: *E. faecium* V19       | 3.7 $\times$ 10^{-2} |
| Transconjugants (*E. faecalis* N48) | >256 $>$1024 |
|                               | >1024      |
|                               | >1024      |
|                               | >1024      |
|                               | 64         |
| Transconjugants (*E. faecium* V19) | >256 $>$1024 |
|                               | >1024      |
|                               | 32         |
|                               | >1024      |
|                               | 8          |
|                               | 8          |
|                               | -          |
|                               | -          |
|                               | -          |

Abbreviation: *E*, erythromycin; *KM*, kanamycin; *SM*, streptomycin; *GM*, gentamicin; *C*, chloramphenicol. “−”, not detected.

4. Discussion

Previously, we described MESPM1 and MES6272-2 in ST59 MRSA, the novel multidrug-resistant composite transposons mediated by enterococcal IS1216V and mingled with *S. aureus*- and enterococci-originated sequences [4,5]. This raised a question about how the mosaic composite transposons emerged and disseminated into ST59 MRSA, the dominant CA-MRSA in Taiwan identified in 1997 [26]. In the current study, we collected enterococcal clinical isolates from as early as 1991 and analyzed their resistance elements. As expected, MESPM1-like and MES6272-2-like structures, both surrounded by IS1216V, were found in *E. faecalis* and *E. faecium* isolates, respectively. Furthermore, the MESPM1-like structure of *E. faecalis* isolated in 1992 had already acquired the *cat* gene and its surrounding genetic environment that was native to *S. aureus*, suggesting that horizontal gene transfer from *S. aureus* to enterococci might have occurred before the emergence of multidrug-resistant ST59 MRSA.

The IS1216 isoform groups, including IS1216, IS1216E, and IS1216V, are one of the most multiply represented insertion sequences in enterococci and play important roles in the dissemination of resistance determinants [7,12]. They form composite transposons encoding antibiotic resistance in enterococci, such as Tn5385 (encoding gentamicin, streptomycin, penicillin, erythromycin, and tetracycline/minocycline resistances), Tn5482, and Tn5506 (both encoding vancomycin resistance) [27–29]. MESPM1 and MES6272-2 are rare cases of composite transposons mediated by IS1216V found in *S. aureus* [4,5]. We previously reported that MESPM1 would be excised, resulting in a single IS1216V remaining in MESPM1 [4]. The same phenomenon has been demonstrated in pE37BA of *E. faecium* with the php5 gene surrounded by two direct repeats of IS1216V [30]. In the current study, the pEflis48 of ST64 *E. faecalis* carried a MESPM1-like structure, within which a region included the *rep* gene and a truncated *prgZ* gene encoding pheromone-binding proteins corresponding to that in pTEF3 of vancomycin-resistant *E. faecalis* (Figure 3). PCR with primer set A located upstream of IS1216V© and downstream of IS1216V© generated a 1031 bp amplicon, indicating that RecA-mediated homologous
recombination would occur to delete the replication region of pTEF3 and leave a copy of IS1216V (Figures 3 and S1). As a result, MES\textsuperscript{SM1}, a composite transposon mediated by IS1216V highly similar to that in ST59/SCC\textit{mec} V MRSA with erythromycin, kanamycin, streptomycin, and chloramphenicol resistances, would be regenerated. This indicates the important role of IS1216V in the dissemination of multidrug resistance between enterococci and ST59 \textit{S. aureus}, the most dominant CA-MRSA in Taiwan.

The \textit{cat} genes found in staphylococci and enterococci are frequently located on small multicopy plasmids and are seldom associated with large multiresistant plasmids [31,32]. In pEfli\textsuperscript{s}48 of \textit{E. faecalis} and MES\textsuperscript{SM1} of ST59/SCC\textit{mec} V MRSA, the \textit{cat} gene and its surrounding environments were both highly related to a small \textit{S. aureus} plasmid SAP084A (>99.5% DNA sequence similarity, Figure 3), which indicates that this region originated in \textit{S. aureus}. Furthermore, the \textit{S. aureus}-originated \textit{cat} region in pEfli\textsuperscript{s}48 and MES\textsuperscript{SM1} was flanked by two direct repeats of enterococcal IS1216V in which another copy embedded, resulting in disruption of the \textit{rep} gene encoding replication initiator of the \textit{S. aureus} plasmid. This implies that the region transferred from \textit{S. aureus} to enterococci, mediated by enterococcal IS1216V. Therefore, we hypothesize that the \textit{S. aureus}-originated \textit{cat} region was initially acquired on a large multiresistant plasmid of enterococci by IS1216V from a small \textit{S. aureus} plasmid, regenerated to MES\textsuperscript{SM1} by RecA-mediated homologous recombination via two direct repeats of IS1216V, and finally disseminated into ST59 \textit{S. aureus}, the dominant CA-MRSA in Taiwan. This would be the first example of IS1216V-mediated bidirectional transfer of resistance in \textit{S. aureus} and enterococci.

The MES\textsuperscript{SM1}-like structure of \textit{E. faecalis} N48 was located on an Inc18 plasmid. The Inc18 plasmids usually possess the ability to confer antibiotic resistance and to transfer to a variety of Gram-positive cocci including enterococci and staphylococci [12,33]. Inc18 plasmids comprising insertion of Tn1546 to facilitate dissemination of vancomycin resistance from enterococci to MRSA have been reported [34]. In the current study, although pEfli\textsuperscript{s}48 displayed high transfer efficiency ($3.7 \times 10^{-3}$/donor) from donor \textit{E. faecalis} N48 to recipient \textit{E. faecalis} JH2-2, it failed to transfer to \textit{S. aureus} RN2677. The reason is unknown and needs to be investigated in the future. A previous study reported that a pSK41-like plasmid is necessary to contribute to successful transfer of the Inc18-like vanA plasmid from \textit{E. faecalis} to MRSA [35]. Further research should be carried out to illustrate the transfer mechanism of MES-like structures between enterococci and \textit{S. aureus}.

ST64 \textit{E. faecalis} belongs to CC8. Although CC8 was not included in the major global clusters associated with healthcare-associated infections such as CC2 [8,9], ST64 \textit{E. faecalis} has been reported to be associated with multidrug resistance to erythromycin, kanamycin, streptomycin, gentamicin, and tetracycline [36,37]. In contrast to \textit{E. faecalis}, all of the \textit{E. faecium} isolates characterized in this study were CC17, the most prevalent cluster worldwide, notably in the nosocomial setting [8,38,39]. It has been proposed that progressive evolution to acquire resistance determinants in CC17 \textit{E. faecium} is associated with its success to adapt to conditions of modern hospitals [38]. In the current study, the MES\textsuperscript{ST59}-like structures of the CC17 \textit{E. faecium} strains isolated in 2003, 2013, and 2014 were inserted by several insertion sequences such as IS\textit{Efm}1, IS\textit{Efl}1, and ISL3, and an additional cluster of resistance determinants \textit{Inut(B)}, \textit{Isut(E)}, \textit{spw}, and \textit{aadE} was acquired (Figure 4). The unpredictable genomic changes of CC17 \textit{E. faecium} would hamper the tracking of the origin of MES-like structures.

In conclusion, the MES\textsuperscript{SM1} structure with the enterococci-originated region (erythromycin, kanamycin, and streptomycin resistances) and the \textit{S. aureus}-originated region (chloramphenicol resistance) found in ST64 \textit{E. faecalis} and ST59 \textit{S. aureus} indicates that bidirectional gene transfer mediated by IS1216V can occur between enterococci and \textit{S. aureus}. Our study is of great importance, as it is the first to demonstrate the role of IS1216V in interspecies transfer of multidrug resistance genes between enterococci and ST59 \textit{S. aureus}.

\textbf{Supplementary Materials}: The following are available online at www.mdpi.com/2076-2607/8/12/1905/s1, Figure S1: MES\textsuperscript{SM1}, pEfli\textsuperscript{s}48, MES\textsuperscript{ST59}, p4780-1, and pV19, Table S1: Information of bacterial strains. Supplementary Data: The nucleotide sequences of pEfli\textsuperscript{s}48, p4780-1, and pV8919 characterized in this study.

\textbf{Author Contributions}: Conceptualization, Y.-T.L. and W.-C.H.; methodology, Y.-T.L., S.-P.T., and W.-C.H.; software, C.-C.C. and W.-C.H.; validation, Y.-T.L., C.-C.C., Y.-H.C., and W.-C.H.; formal analysis, C.-C.C., Y.-
H.C., and W.-C.H.; investigation, C.-C.C., Y.-T.J., and W.-C.H.; resources, S.-P.T., Y.-T.J., Y.-H.C., L.-J.T., and W.-C.H.; data curation, S.-P.T. and W.-C.H.; writing—original draft preparation, Y.-T.L., W.-W.H., and W.-C.H.; writing—review and editing, Y.-T.L., W.-W.H., and W.-C.H.; visualization, W.-C.H.; supervision, S.-P.T., W.-W.H., and W.-C.H.; project administration, W.-C.H.; funding acquisition, Y.-H.C. and W.-C.H. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Ministry of Science and Technology of Taiwan (MOST104-2320-B-037-029 and MOST107-2320-B-037-021-MY3), the Kaohsiung Medical University (KMU-M106003), and partially by a Kaohsiung Medical University Research Center Grant (KMU-Tc109B02).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chen, C.J.; Huang, Y.C. New epidemiology of Staphylococcus aureus infection in Asia. Clin. Microbiol. Infect. 2014, 20, 605–623, doi:10.1111/1469-0691.12705.
2. Huang, Y.C.; Chen, C.J. Community-associated meticillin-resistant Staphylococcus aureus in children in Taiwan, 2000s. Int. J. Antimicrob. Agents 2011, 38, 2–8, doi:10.1016/j.ijantimicag.2011.01.011.
3. Su, Y.C.; Hung, W.W.; Lin, J.M.; Chang, C.C.; Chen, Y.H.; Lai, Y.L.; Tseng, S.F.; Lu, P.L.; Yamamoto, T.; Teng, L.J.; et al. Tracking the evolution of the two successful CC59 meticillin-resistant Staphylococcus aureus clones in Taiwan: The divergence time of the two clades is estimated to be the 1980s. Int. J. Antimicrob. Agents 2020, 106, doi:10.1016/j.ijantimicag.2020.106047.
4. Hung, W.C.; Chan, C.C.; Tsai, J.C.; Lin, Y.T.; Hsu, P.R.; Teng, L.J. Molecular Evolutionary Pathways toward Two Successful Community-Associated but Multidrug-Resistant ST59 Methicillin-Resistant Staphylococcus aureus Lineages in Taiwan: Dynamic Modes of Mobile Genetic Element Salavges. PLoS ONE 2016, 11, e0162526, doi:10.1371/journal.pone.0162526.
5. Hung, W.C.; Takano, T.; Higuchi, W.; Iwao, Y.; Khokhlova, O.; Teng, L.J.; Yamamoto, T. Comparative genomics of community-acquired ST59 meticillin-resistant Staphylococcus aureus in Taiwan: Novel mobile resistance structures with IS1216V. PLoS ONE 2016, 7, e0162526.
6. Mahillon, J.; Chandler, M. Insertion sequences. Microbiol. Mol. Biol. Rev. 1998, 62, 725–774, doi:10.1128/MMBR.62.3.725-774.1998.
7. Harmer, C.J.; Hall, R.M. An analysis of the IS6/IS26 family of insertion sequences: Is it a single family? Microb. Genom. 2019, 5, doi:10.1099/mgen.0.000291.
8. Arias, C.A.; Murray, B.E. The rise of the Enterococcus: Beyond vancomycin resistance. Nat. Rev. Microbiol. 2012, 10, 266–278, doi:10.1038/nrmicro2761.
9. Garcia-Solache, M.; Rice, L.B. The Enterococcus: A model of adaptability to its environment. Clin. Microbiol. Rev. 2019, 32, e00058-00018, doi:10.1128/CMR.00058-18.
10. Ramos, S.; Silva, V.; Dapkevicius, M.L.E.; Igerejas, G.; Poeta, P. Enterococci, from harmless bacteria to a pathogen. Microorganisms 2020, 8, 1118, doi:10.3390/microorganisms8081118.
11. Hollenbeck, B.L.; Rice, L.B. Intrinsic and acquired resistance mechanisms in Enterococcus. Virulence 2012, 3, 421–433, doi:10.4161/viru.21282.
12. Partridge, S.R.; Kwong, S.M.; Firth, N.; Jensen, S.O. Mobile genetic elements associated with antimicrobial resistance. Clin. Microbiol. Rev. 2018, 31, doi:10.1128/CMR.00088-17.
13. Hegstad, K.; Mikalsen, T.; Coque, T.M.; Werner, G.; Sundsfjord, A. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. Clin. Microbiol. Infect. 2010, 16, 541–554, doi:10.1111/j.1469-0691.2010.03226.x.
14. Haaber, J.; Penades, J.R.; Ingmer, H. Transfer of antibiotic resistance in Staphylococcus aureus. Trends Microbiol. 2017, 25, 893–905, doi:10.1016/j.tim.2017.05.011.
15. Clark, N.C.; Weigel, L.M.; Patel, J.B.; Tenover, F.C. Comparison of Tn1546-like elements in vancomycin-resistant Staphylococcus aureus isolates from Michigan and Pennsylvania. Antimicrob. Agents Chemother. 2005, 49, 470–472, doi:10.1128/AAC.49.1.470-472.2005.
16. Sletvold, H.; Johnsen, P.J.; Wikmark, O.G.; Simonsen, G.S.; Sundsfjord, A.; Nielsen, K.M. Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal Enterococcus faecium lineages. J. Antimicrob. Chemother. 2010, 65, 1894–1906, doi:10.1093/jac/dkq219.
17. Hung, W.W.; Chen, Y.H.; Tseng, S.P.; Jao, Y.T.; Teng, L.J.; Hung, W.C. Using groEL as the target for identification of Enterococcus faecium clades and 7 clinically relevant Enterococcus species. J. Microbiol. Immunol. Infect. 2019, 52, 255–264, doi:10.1016/j.jmii.2018.10.008.
Enterococcus faecalis
Zhu, W.; Clark, N.; Patel, J.B. pSK41

McDougal, L.K.; Hageman, J.C.; Olsen, Z.; Schwarz, S.; Kehrenberg, C.; Doublet, B.; Cloeckaert, A.; Roberts, M.C.; Schwarz, S. Tetracycl

Handwerger, S.; Skoble, J. Identification of chromosomal mobile element conferring high

Barton, B.M.; Discotto, L.F.; Pucci, M.J.; Handwerger, S. Mobilization of vancomycin resistance by

Rice, L.B.; Carias, L.L. Transfer of Tn5385, a composite, multiresistance chromosomal element from

Handwerger, S.; Fluit, A.; Coque, T.M.; Schwarz, S. Tetracycline-mediated fusi

Teng, L.J. Skin commensal staphylococci may act as reservoir for fusidic acid resistance genes. PLoS ONE 2015, 10, e0143106, doi:10.1371/journal.pone.0143106.

Carattoli, A.; Zankari, E.; Sverrisdottir, H.; Daum, R.S. Successful multiresistant community

Boyle-Vavra, S.; Ereshefsky, B.; Wang, C.C.; Daum, R.S. Successful multiresistant community-associated

Multilocus sequence typing scheme for

Tribe, D.; Poznanski, S.; Li, M.; Hogg, G.; Spalburg, E.; Van Embden, J.D.; Willems, R.J. Multilocus sequence typing scheme for Enterococcus faecalis. J. Clin. Microbiol. 2002, 40, 1963–1971, doi:10.1128/jcm.40.6.1963-1971.2002.

Ruiz-Garbajosa, P.; Bonten, M.J.; Robinson, D.A.; Top, J.; Nallapareddy, S.R.; Torres, C.; Coque, T.M.; Canton, R.; Baquero, F.; Murray, B.E.; et al. Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination. J. Clin. Microbiol. 2006, 44, 2220–2228, doi:10.1128/JCM.02596-05.

Francisco, A.P.; Bugalho, M.; Ramirez, M.; Carrico, J.A. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics 2009, 10, 152, doi:10.1186/1471-2105-10-152.

Hung, W.C.; Chen, H.J.; Lin, Y.T.; Tsai, J.C.; Chen, C.W.; Lu, H.H.; Tseng, S.P.; Jheng, Y.Y.; Leong, K.H.; Teng, L.J. Skin commensal staphylococci may act as reservoir for fusidic acid resistance genes. PLoS ONE 2015, 10, e0143106, doi:10.1371/journal.pone.0143106.

Barton, B.M.; Harding, G.P.; Zuccarelli, A.J. A general method for detecting and sizing large plasmids. Anal. Biochem. 1995, 226, 235–240, doi:10.1006/abio.1995.1220.

Nurk, S.; Bankevich, A.; Antipov, D.; Gurevich, A.; Korobeynikov, A.; Lapidus, A.; Prijibelsky, A.; Pyshkin, A.; Sirotkin, A.; Sirotkin, Y. Assembling genomes and mini-metagenomes from highly chimeric reads. In Proceedings of Annual International Conference on Research in Computational Molecular Biology; pp. 158-170.

Carattoli, A.; Zankari, E.; Garcia-Fernandez, A.; Voldby Larsen, M.; Lund, O.; Villa, L.; Moller Aarestrup, F.; Hasman, H. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. 2014, 58, 3895–3903, doi:10.1128/AAC.02412-14.

Boyle-Vavra, S.; Ereshefsky, B.; Wang, C.C.; Daum, R.S. Successful multiresistant community-associated methicillin-resistant Staphylococcus aureus lineage from Taipei, Taiwan, that carries either the novel Staphylococcal chromosome cassette mec (SCCmec) type V or SCCmec type IV. J. Clin. Microbiol. 2005, 43, 4719–4730, doi:10.1128/JCM.43.9.4719-4730.2005.

Rice, L.B.; Carias, L.L. Transfer of Tn5385, a composite, multiresistance chromosomal element from Enterococcus faecalis. J. Bacteriol. 1998, 180, 714–721, doi:10.1128/JB.180.3.714-721.1998.

Handwerger, S.; Skoble, J. Identification of chromosomal mobile element conferring high-level vancomycin resistance in Enterococcus faecium. Antimicrob. Agents Chemother. 1995, 39, 2446–2453, doi:10.1128/AAC.39.11.2446.

Heaton, M.P.; Discotto, L.F.; Pucci, M.J.; Handwerger, S. Mobilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an Enterococcus faecium sex pheromone-response plasmid. Gene 1996, 171, 9–17, doi:10.1016/0378-1119(96)00022-4.

Morroni, G.; Brenciani, A.; Litta-Mulondo, A.; Vignaroli, C.; Mangiaterra, G.; Fioriti, S.; Citterio, B.; Cirioni, O.; Giovanetti, E.; Biavasco, F. Characterization of a new transferable MDR plasmid carrying the pbp5 gene from a clade B commensal Enterococcus faecium. J. Antimicrob. Chemother. 2019, 74, 843–850, doi:10.1093/jac/dky549.

Roberts, M.P.; Schwarz, S. Tetracycline and chloramphenicol resistance mechanisms. In Antimicrobial Drug Resistance; Springer: Berlin/Heidelberg, Germany, 2017; pp. 231–243.

Schwarz, S.; Kehrenberg, C.; Doublet, B.; Cloeckaert, A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol. Rev. 2004, 28, 519–542, doi:10.1016/j.femsre.2004.04.001.

Kohler, V.; Vaishampayan, A.; Grohmann, E. Broad-host-range Inc18 plasmids: Occurrence, spread and transfer mechanisms. Plasmid 2018, 99, 11–21, doi:10.1016/j.plasmid.2018.06.001.

Zhu, W.; Murray, P.R.; Huskins, W.C.; Jernigan, J.A.; McDonald, L.C.; Clark, N.C.; Anderson, K.F.; McDougal, L.K.; Hageman, J.C.; Olsen-Rasmussen, M.; et al. Dissemination of an Enterococcus Inc18-like vanA plasmid associated with vancomycin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. 2010, 54, 4314–4320, doi:10.1128/AAC.00185-10.

Zhu, W.; Clark, N.; Patel, J.B. pSK41-like plasmid is necessary for Inc18-like vanA plasmid transfer from Enterococcus faecalis to Staphylococcus aureus in vitro. Antimicrob. Agents Chemother. 2013, 57, 212–219, doi:10.1128/AAC.01587-12.
36. Quinones, D.; Kobayashi, N.; Nagashima, S. Molecular epidemiologic analysis of Enterococcus faecalis isolates in Cuba by multilocus sequence typing. *Microb. Drug Resist.* **2009**, *15*, 287–293, doi:10.1089/mdr.2009.0028.

37. Kudo, M.; Nomura, T.; Yomoda, S.; Tanimoto, K.; Tomita, H. Nosocomial infection caused by vancomycin-susceptible multidrug-resistant Enterococcus faecalis over a long period in a university hospital in Japan. *Microbiol. Immunol.* **2014**, *58*, 607–614, doi:10.1111/1348-0421.12190.

38. Lee, T.; Pang, S.; Abraham, S.; Coombs, G.W. Antimicrobial-resistant CC17 Enterococcus faecium: The past, the present and the future. *J. Glob. Antimicrob. Resist.* **2019**, *16*, 36–47, doi:10.1016/j.jgar.2018.08.016.

39. Leclercq, R. Epidemiological and resistance issues in multidrug-resistant staphylococci and enterococci. *Clin. Microbiol. Infect.* **2009**, *15*, 224–231, doi:10.1111/j.1469-0691.2009.02739.x.

**Publisher’s Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).