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Original Article

Potentially conjugative plasmids harboring Tn*6636*, a multidrug-resistant and composite mobile element, in *Staphylococcus aureus*

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Abstract *Objectives:* This study aimed to provide detailed genetic characterization of Tn*6636*, a multidrug-resistant and composite mobile element, in clinical isolates of *Staphylococcus aureus*.
Methods: A total of 112 *ermB*-positive methicillin-susceptible *S. aureus* (MSSA) and 224 *ermB*-positive methicillin-resistant *S. aureus* (MRSA) isolates collected from 2000 to 2015 were tested for the presence of Tn*6636*. Detection of the plasmids harboring Tn*6636* was performed by S1 nuclease digestion pulsed-field gel electrophoresis (PFGE) analysis, conjugation test, and whole genome sequencing (WGS).
Results: Prevalence of Tn*6636* in MSSA is higher than that in MRSA. Ten MSSA isolates and 10

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MRSA isolates carried Tn*6636*. The 10 MSSA isolates belonged to three sequence types (ST), including ST7 (n = 6), ST5 (n = 3), and ST59 (n = 1). The 10 MRSA isolates belonged to ST188 (n = 8) and ST965 (n = 2). Analysis of plasmid sequences revealed that Tn*6636* was harbored by six different mosaic plasmids. In addition to resistance genes, some plasmids also harbored toxin genes.

Conclusion: The presence of multi-resistant Tn*6636* in plasmids of both MSSA and MRSA with various STs suggests its broad dissemination. Results indicate that Tn6636 has existed for at least 16 years in Taiwan. The mosaic plasmids harboring Tn*6636* can be transferred by conjugation. Ongoing surveillance of Tn*6636* is essential to avoid continued spreading of resistant plasmids.

Introduction

Antimicrobial resistance of *Staphylococcus aureus* is a major problem in effective prevention and treatment due to its high capacity to acquire drug resistance genes by mobile DNA elements, including insertion sequences (ISs), transposons (Tns), plasmids, phages, and *S. aureus* pathogenicity islands by horizontal transfer.[1,2] Our previous study reported that the most prevalent resistance gene in erythromycin-resistant blood isolates of methicillin-susceptible *S. aureus* (MSSA) collected from 2000 to 2012 in Taiwan was *ermB*.[3] Although the majority (92%) of *ermB*-positive MSSA isolates carried structures resembling the mobile element structure (MES) that has been reported in sequence type 59 (ST59) methicillin-resistant *S. aureus* (MRSA),[4,5] we identified the unique structure of the *Enterococcus faecium*-originated *ermB*-positive Tn*1546*-like element, which we redesignated more precisely as Tn*6636*.[3]

Tn*6636* encodes erythromycin/gentamicin/kanamycin resistance. Tn*6636* belonged to Tn*3* family transposons, and generates a 5-bp duplication (direct repeat) of target sequences upon insertion. The broad Tn*3* family transposons are 38-bp terminal imperfect inverted repeats.[2] Tn*3* family members demonstrate transposition immunity, but homologous and/or res-mediated recombination between related elements can occur, creating hybrid elements.[6]

In the present study, the presence of Tn*6636* in MSSA and MRSA collected in a 16-year period from 2000 to 2015 was tracked. Four clonal complexes have been found to carry Tn*6636*, either on plasmids or into the chromosome. Sequence analysis revealed high conservation of Tn*6636* in *S. aureus* over the 14 years. Filter mating demonstrated low transfer efficiency of the Tn*6636*-carrying plasmids, which may limit the dissemination of the multidrug resistance elements.

Materials and methods

Bacterial isolates

A total of 340 erythromycin-resistant MSSA recovered from the Bacteriology Laboratory, National Taiwan University Hospital between 2000 and 2015 were collected. For MRSA, a total of 640 erythromycin-resistant MRSA were collected between 2006 and 2015. The criteria of selected MRSA according (i) resistance to erythromycin and gentamicin, but susceptible to trimethoprim/sulfamethoxazole (SXT), were collected between 2006 and 2010, to include strains harboring Tn*6636* and to exclude ST239 MRSA (usually SXT-resistant)[7] that harbored *ermA* as the major erythromycin resistant genes; (ii) resistant to erythromycin and gentamicin were collected between 2011 and 2012; (iii) erythromycin-resistant isolates were collected between 2013 and 2015. All of the isolates were recovered from blood cultures, and only one isolate per patient was collected in this study. Identification of *S. aureus* and confirmed by *nuc* gene detection.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing were preliminarily determined by commercial identification systems (containing erythromycin, clindamycin, gentamicin, oxacillin, and SXT). Minimum inhibitory concentrations (MICs) were determined using the agar dilution method. The MICs of erythromycin, clindamycin, gentamicin, kanamycin, and SXT (Sigma—Aldrich) were determined using the agar dilution method with Mueller-Hinton agar (MHA) as described by the Clinical and Laboratory Standards Institute.[8] *S. aureus* ATCC 29213 was used as the reference strain. Erythromycin resistance was confirmed by the disc diffusion method according to the 2020 guidelines of CLSI.[8] Resistance to methicillin was determined by *mecA* PCR.

Detection of Tn*6636* element structure in erythromycin-resistant isolates

Tn*6636* was initially detected by the presence of the *tnp* gene of Tn*6636* and *ermB* gene using PCR.[3] The Tn*6636* structure was mapped by PCR using six primer sets, which are listed in Table 1, and the positions of the primers are indicated in Fig. 1. The Tn*6636* element structures were determined by combining the PCR mapping results and the profiles of resistance determinants. For the 20 Tn*6636*-carrying strains, entire sequences of Tn*6636* were determined using the primer sets described above.

Table 1 Primers used for PCR mapping of Tn*6636*.

Primer name	Sequence (5′ to 3′)	Application
thyX_R	CCTGTACCCTCTTGATGAGAGG	PCR mapping for Tn*6636*
u_tnp_F1	CATAACACTGATTCTATCAGCC	PCR mapping for Tn*6636*
d_tnpR_R	CTACTAGAAAACGGTCAGC	PCR mapping for Tn*6636*
IS1216V_F1	CCGTGGGCTACTATCTTCGTT	PCR mapping for Tn*6636*
aacA-aphD_R3	CATCTTCCCAAGGCTCTG	PCR mapping for Tn*6636*
ermB_F3	GCCAGCGGAATGCTTTCATCCTAAACC	PCR mapping for Tn*6636*
ermB_R1	AGTAACGGTACTTAAATTGTTTAC	PCR mapping for Tn*6636*
tnp_551_F1	CGGTATCCTGGGTGT	PCR mapping for Tn*6636*
tnp_551_R1	ATTTCTGATGCGAGG	PCR mapping for Tn*6636*
tnp_551_F2	ACTAGGTCGCATTGAAAAGAG	PCR mapping for Tn*6636*
tnp_1546_R3	GTGTAGTAGGTTCCTAGCAC	PCR mapping for Tn*6636*
tnp_1546_R1	AGGGATGCTGAAACTTTTCC	PCR mapping for Tn*6636* and detection *tnp* of Tn*6636*
ermB-f	GAAAAAGTACTCAACCAAATA	Detection *ermB*, and *ermB* probe for Southern blot hybridization
ermB-r	AGTAACGGTACTTAAATTGTTTAC	Detection *ermB*, and *ermB* probe for Southern blot hybridization
tnp_1546_F3	GGCGCATGTATGAAGACTC	Detection *tnp* of Tn*6636*

spa typing, multi-locus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE)

spa typing, MLST and PFGE were performed to determine the genetic relatedness of Tn*6636*-carrying isolates. The *spa* typing was performed as described previously.[9] MLST was carried out to determine the sequence types (STs), which were assigned using the *S. aureus* MLST database (https://pubmlst.org/organisms/staphylococcus-aureus/). PFGE was performed as described previously.[10]

S1 nuclease digestion-PFGE

Detection of the presence of plasmids by S1 nuclease digestion-PFGE was performed as described previously.[3,11]

Southern blot hybridization

Southern blot hybridization was performed as described previously.[12] Hybridization with the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Penzberg, Germany) was performed using a Hybridization Incubator Model 1000 (SciGene/Robbins Scientific, Champaign, IL, USA). Detection was performed with the Anti-Digoxigenin-AP and DIG Luminescent Detection Kit (Roche Diagnostics GmbH, Penzberg, Germany) and results were captured with the LAS-4000 Imaging System (FUJI FILM Life Science, Japan).

Sequencing of plasmids

Whole genome sequencing was performed on a PacBio Sequel platform (Pacific Bioscience, Menlo, CA, USA) with a 10–15 kb insert library prepared using the Express Template Preparation Kit 2.0. *De novo* assembly (Microbial Assembly algorithm) was performed using SMRT Analysis version 8.0 (Pacific Bioscience, Menlo, CA, USA). The Microbial Assembly analysis produced a complete *de novo*-assembled genome sequence with at least 300-fold coverage over the entire molecule. The new transposon number was assigned using the Tn Number Registry website (http://transposon.lstmed.ac.uk/).[13] PlasmidFinder (version 2.1, https://cge.cbs.dtu.dk/services/PlasmidFinder/) was used for the detection of plasmid replicons in bacterial whole-genome sequencing data.[14]

Conjugation test

To determine the transfer frequency *in vitro*, strain RN2677 was used as the recipient in the conjugation test, and mating was carried out on Luria Bertani (LB) agar without selection.[15] Bacteria grown overnight on LB agar plates were suspended in 20 ml brain-heart infusion (BHI) broth, and incubated at 37 °C with shaking at 240 rpm for 3 h to an OD_{600} ~1. And then, ~10^{10} colony forming units (CFU) of both the donors and recipient cells were mixed in 50 mL centrifuge tubes. The mixed cells were collected by centrifugation and then transferred to a 3 cm × 3 cm Hybond-Nnylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The membrane was put on LB agar plate at 37 °C. After 24 h, the membrane with mixed cultures was taken from the plate, suspended in 40 ml BHI broth, and incubated at 37 °C with shaking at 240 rpm. After 4 h of incubation, the cells were collected by centrifugation and then plated onto MHA agar containing erythromycin (0.5 μg/mL) and rifampicin (80 μg/mL), at 37 °C for 24 h. Confirmation of transconjugants was carried out by testing for the presence of the *ermB* gene by PCR. The transconjugants were also checked by *spa* typing (the *spa* type of RN2677 is t211).[15] The transfer frequency has counted the transconjugant per donor cell.

Nucleotide sequence accession numbers

The nucleotide sequences of six plasmids: pNTUH_9448 in ST5 MSSA, pNTUH_1027 in ST7 MSSA, pNTUH_3874 and pNTUH_3874-2 in ST59 MSSA, pNTUH_6457 in ST188 MRSA

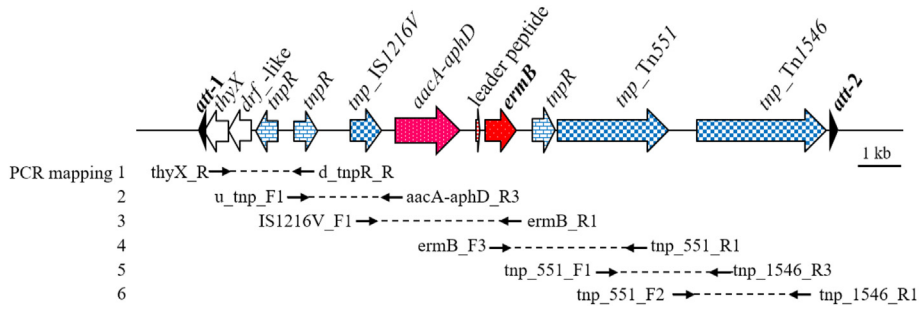

Figure 1. Position of PCR primers for detection of the Tn*6636* structure amplicons for PCR mapping; 1, 2, 3, 4, 5 and 6 are approximately 1.9-kb, 2.8-kb, 3.7-kb, 3.5-kb, 3.1-kb, and 3.2-kb, respectively.

and pNTUH_5066148 in ST965 MRSA were deposited in the DNA Data Bank of Japan (DDBJ) database under accession numbers LC377536 to LC377540 and LC570860.

Results

Distribution of the Tn*6636* in *S. aureus*

Of the 340 erythromycin-resistant MSSA isolates collected between 2000 and 2015, 112 (33%) carried the *ermB* gene. However, of the 640 erythromycin-resistant MRSA isolates collected between 2006 and 2015, only 10 MSSA and 10 MRSA carried Tn*6636*.

Molecular epidemiology of the Tn*6636*-carrying isolates

To determine the clonal relationship among 10 MSSA and 10 MRSA isolates carrying Tn*6636*, *spa* typing, MLST (Table 2) and PFGE (Fig. 2) were performed. Of the 10 MSSA isolates, six belonged to ST7 (*spa* t796), three belonged to ST5 (*spa* t002 and t242) and one isolate belonged to ST59 (*spa* t216). Of the 10 MRSA isolates, eight belonged to ST188 (*spa* t189 and t5529) and two isolates belonged to ST965 (*spa* t575 and t062). The results of PFGE are shown in Fig. 2. Three ST5 MSSA isolates from 2002, 2010 and 2012 belonged to the same pulsotype (with 80% similarity cut-off). Two ST965 (CC5) MRSA isolates obtained from 2013 to 2015 were very closely related to ST5 MSSA. Six ST7 MSSA isolates obtained from 2004 to 2015 belonged to the same pulsotype, and the four isolates from 2012 to 2015 were identical. Eight ST188 MRSA isolates obtained from 2009 to 2014 belonged to the same pulsotype, and six were identical.

Sanger sequencing of the Tn*6636* and homology analysis

Sequences of Tn*6636* from representative strains of each ST (ST5 MSSA NTUH_9448, ST7 MSSA NTUH_1027, ST59 MSSA NTUH_3874, ST188 MRSA NTUH_6457 and ST965 MRSA NTUH_5066148) were compared. The size of Tn*6636* (14,567 bp) was identical in the above strains. Sequences of Tn*6636* in ST7 MSSA NTUH_1027 and ST965 MRSA NTUH_5066148 were 100% identical, while ST5 MSSA NTUH_9448 had a nucleotide difference G1354A in *tnp* gene of Tn*551*, causing an amino acid change to G452R. ST188 MRSA NTUH_6457 had one nucleotide difference downstream of *ermB* gene. ST59 MSSA pNTUH_3874 and pNTUH_3874-2 had four nucleotides difference: (1) point mutation (A299G) of *ermB* gene resulting in an amino acid change to N100S, (2) non-coding region point mutation downstream of *ermB* gene, (3) point mutation (G765A) of *tnp* gene of Tn*551*, (4) premature nonsense mutation in *tnp* gene of Tn*551*, shortening the amino acid length from 972 to 857.

Location of Tn*6636*

To determine the location of the Tn*6636*, the agarose plugs of 10 MSSA and 10 MRSA isolates were digested with S1 nuclease (Fig. 3) and then hybridized with a Dig-labeled *ermB*-specific probe prepared by PCR amplification of *ermB* using primers ermB-f and ermB-r (Table 1). Isolates of

Table 2 Distribution of MLST and *spa* types in Tn*6636* carrying MSSA and MRSA.

Organism	Year	No. of *ermB* carried (%)[a]	No. of Tn*6636* carried (%)[b]	MLST (No. of isolates)	*spa* type (No. of isolates)
MSSA	2000–2015	112 (32.9)	10 (8.9)	ST7 (6)	t796 (6)
				ST5 (3)	t002 (2), t242 (1)
				ST59 (1)	t216 (1)
MRSA	2006–2015	224	10 (4.5)	ST188 (8)	t189 (7), t5529 (1)
				ST965 (2)	t575 (1), t062 (1)

[a] Percentage of *ermB* in erythromycin-resistant isolates.
[b] Rate of Tn*6636* in *ermB*-positive isolates.

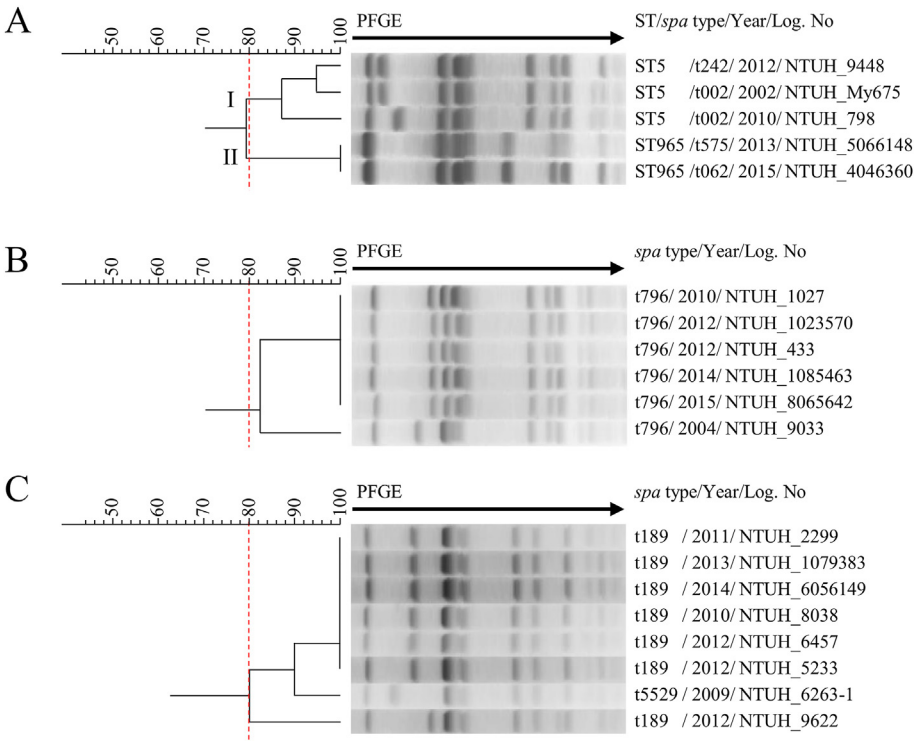

Figure 2. PFGE dendrogram of Tn*6636* carrying isolates. PFGE cluster was assigned to isolates having 80% or greater similarity from the dendrograms: (A) Three ST5 MSSA isolates (pulsotype I) and two ST965 MRSA isolates (pulsotype II); (B) Six ST7 MSSA isolates; (C) Eight ST188 MRSA isolates.

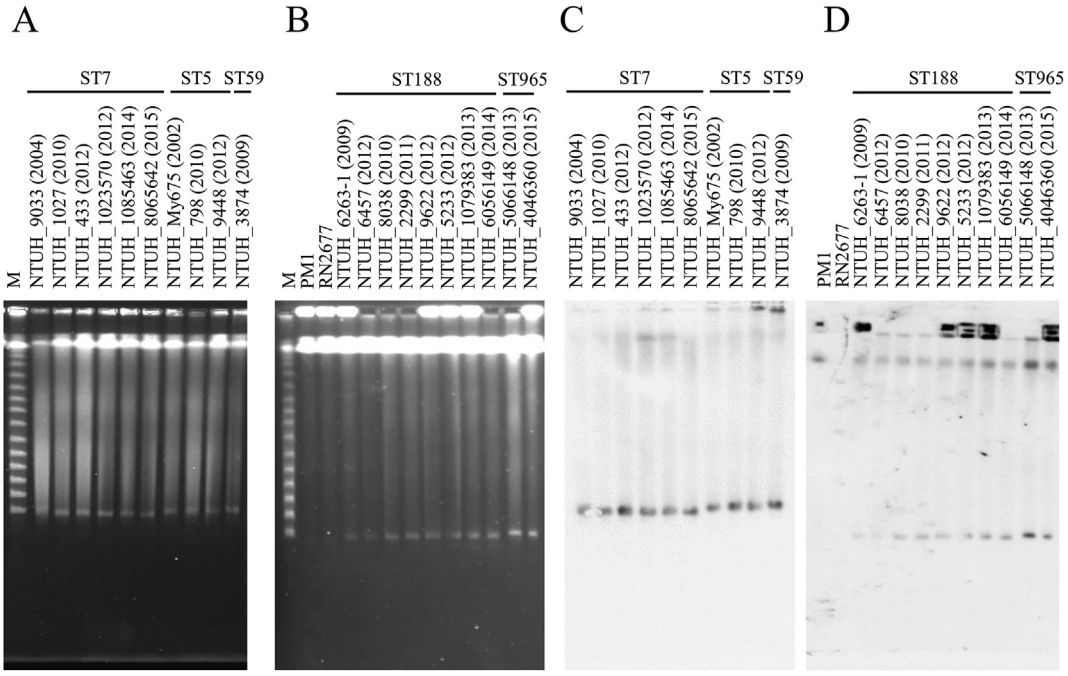

Figure 3. S1 nuclease PFGE and Southern blot hybridization with *ermB*. The S1 nuclease PFGE from three ST5 MSSA, six ST7 MSSA, one ST59 MSSA, eight ST188 MRSA, and two ST965 MRSA were examined. The PM1 strain harboring a 26-kb plasmid and *ermB* gene located on chromosome was used as a positive control. Strain RN2677, lacking the plasmid and *ermB* gene, was used as a negative control. The size of the M marker starts at 48.5-kb and increases 48.5-kb with each successively larger band. (A, B) The bands indicated the plasmids. The size of plasmids is estimated to be between 23.1-kb and 48.5-kb. (C, D) DNA was hybridized with the Dig-labelled *ermB*-specific probe and amplified by PCR using primers ermB-f and ermB-r. Positive signal of *ermB* was detected in respective bands.

the same ST (ST5, ST7, ST188 and ST965) harbored plasmids of similar size containing *ermB* (Fig. 3).

Sequence analysis of Tn*6636*-carrying plasmids

The sequences of plasmids in ST5 MSSA pNTUH_9448, ST7 MSSA pNTUH_1027, ST59 MSSA pNTUH_3874 and pNTUH3874-2, ST188 MRSA pNTUH_6457 and ST965 MRSA pNTUH_5066148 were determined. Fig. 4 presents the genetic structures of the six plasmids. According the results of PlasmidFinder (Table S1), the 14.5-kb Tn*6636* was inserted in four different plasmid-backbones. Among them, the plasmids pNTUH_1027 and pNTUH_6457 (Fig. 4A) showed mosaic structures that included Tn*6636* and a 20.7-kb backbone which is similar to pSaa6159. The *repA* gene and the backbone of the above two plasmids were 100% and 99.9% identical, respectively, to those in pSaa6159. The Tn*6636* was inserted in pNTUH_6457 with the disruption of a *tnp* gene of Tn*552*. Plasmid pNTUH_5066148 and pNTUH_3874-2 (Fig. 4B) were also mosaic plasmids, containing Tn*6636* and a 24.7-kb backbone which is similar to pCA-347 is 99.84% homologous to pN315. The *repA* gene of the pNTUH_5066148 and pNTUH_3874-2 were 100% identical to those in pCA-347. Tn*6636* was inserted in pNTUH_5066148 with the disruption of one of the three *rep* gene, shortening the amino acid length from 286 to 271. Tn*6636* was inserted in pNTUH_3874-2 with disruption of quinone oxidoreductase gene. Additionally, the pNTUH_3874-2 harbored the chloramphenicol resistance gene *fexB*. Plasmid pNTUH_9448 (Fig. 4C) was another mosaic plasmid, in which Tn*6636* was inserted and disrupted an alcohol dehydrogenase gene. The *repA* gene and the backbone of the pNTUH_9448 were 100% and 94.5% identical, respectively, to those in pWBG744. Plasmid pNTUH_3874 (Fig. 4D) showed unique features. Nucleotide–nucleotide BLAST analysis found no significant matches except for the region containing Tn*6636*. Annotation revealed that Tn6636 has 11 ORFs, and the backbone of plasmid pNTUH_3874 has 36 annotated ORFs, including three conjugal transfer genes (*traB*, *traE* and truncated *traK*).

PCR mapping was used to determine the remaining isolates containing Tn*6636* plasmids (Table S2). The results indicated that the plasmids in two ST5 MSSA, six ST7 MSSA, seven ST188 MRSA, and one ST965 MRSA displayed similar plasmid structures corresponding to those in each ST.

Conjugal transfer frequency of plasmids harboring the Tn*6636*

Conjugation tests were performed to determine whether Tn*6636*-carrying plasmids could be transferred *in vitro* from the clinical ST5/7/59/188/965 *S. aureus* to the laboratory strain ST8 RN2677. The Tn*6636*-carrying plasmids could be transferred from ST5 NTUH_9448, ST7 NTUH_1027, ST59 NTUH_3874, and ST965 NTUH_5066148 to RN2677 with a frequency of 3.1×10^{-10}, 10^{-7}, 4.4×10^{-10}, and 1.5×10^{-10} per donor cell, respectively. However, we could not transfer the Tn*6636*-carrying plasmid from ST188 NTUH_6457 to RN2677. Transconjugants were characterized by PCR to test for the presence of *ermB* and *tnp* of Tn*1546*, and by *spa* typing and erythromycin/gentamicin susceptibility testing. The results showed that the four different Tn*6636* plasmids could be transferred *in vitro*, and the

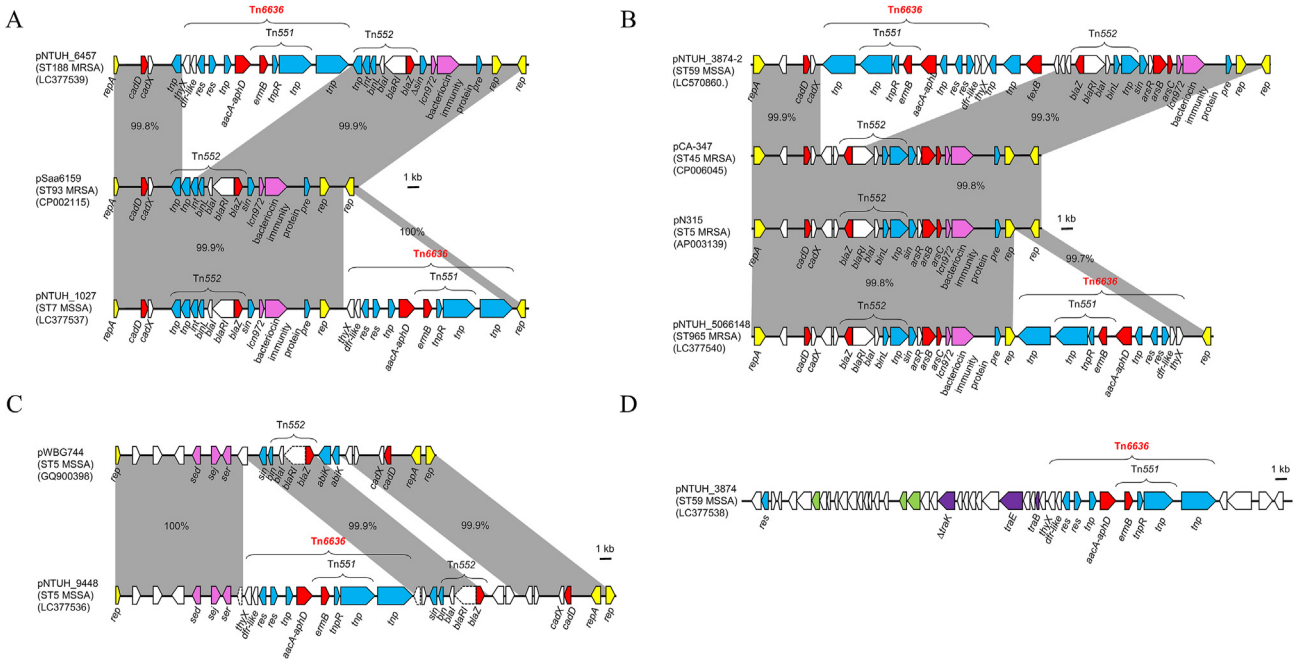

Figure 4. Structure of plasmids. (A) The 35.2-kb ST7 MSSA pNTUH_1027 and ST188 MRSA pNTUH_6457 plasmid included 14.5-kb Tn*6636* and 20.7-kb backbone which is similar to pSaa6159, harboring bacteriocin Lcn972 (*lcn972* gene). (B) The 39.2-kb ST965 MRSA pNTUH_5066148 plasmid and 44.8-kb ST59 pNTUH_3874-2 contained the 14.5-kb Tn*6636* and a 24.7-kb backbone, which is similar to pCA-347, harboring bacteriocin Lcn972 (*lcn972* gene, pink stripe). (C) The 42.5-kb ST5 MSSA pNTUH_9448 plasmid included 14.5-kb Tn*6636* and 27.2-kb pWBG744, harboring three enterotoxin genes *sed*, *sej*, and *ser* (D) The 46.8-kb ST59 MSSA pNTUH_3874 plasmid harbored three conjugal genes *traB*, *traE*, and *traK*.

resulting four transconjugants were resistant to erythromycin and gentamicin (Table 3).

Transposition of Tn*6636*

Two different plasmids were found in ST59 NTUH_3874 (Fig. 4). The Tn*6636* of the two plasmids were identical, showing evidence of transposition of Tn*6636*. Other evidence is provided by the whole genome sequencing of ST7 NTUH_1023570, Tn*6636* located in the genome and plasmid. Only one basic difference exists between the two copies: Tn*6636* inserted in chromosome DNA of ST7 NTUH_1023570 with the disruption of hydrolase gene, which shortened the amino acid length from 383 to 295.

Discussion

This study was a continuation of our previous work in which the novel structure of an *E. faecium*-originated *ermB*-positive Tn*1546*-like element in MSSA was reported.[3] In the present study, we renamed the Tn*1546*-like element to Tn*6636* and examined the presence of Tn*6636* in 980 erythromycin-resistant *S. aureus*. A total of 20 isolates harboring Tn*6636* were identified. They carried Tn*6636* on plasmids with four different backbones, including ST59 NTUH_3874 with two copies of Tn*6636* on two different plasmids, and ST7 NTUH_1023570 with a second Tn*6636* into the chromosome.

The 20 Tn*6636*-carrying isolates were divided into four genotypes: ST188 MRSA (n = 8), ST7 MSSA (n = 6), CC5 *S. aureus* (n = 5) and ST59 MSSA (n = 1). ST188 is one of the most common STs in MSSA in Taiwan.[16] However, ST188 MRSA is rare, and only limited cases have been reported.[17] Recently, Hong Kong,[18] China[19] and Korea[20] reported the occurrence of ST188 MRSA, although they did not report the plasmid information. In the present study, all of the tested ST188 MRSA harboring Tn*6636* plasmid belonged to the same pulsotype (Fig. 2). Simultaneous acquisition of SCC*mec* and Tn*6636* may contribute to emergence of ST188 MRSA, but further study is needed to elucidate the hypothesis. As for CC5 *S. aureus*, three ST5 MSSA and two ST965 MRSA, the single-locus variant (SLV) of ST5, carried the Tn*6636* plasmid. Previous studies have reported that the CC5 *S. aureus* in the United States acquired Tn*1546* to become vancomycin-resistant *S. aureus*. The CC5 isolates may have higher potential for acquisition of the Tn*3* family transposons, such as Tn*1546* and Tn*6636*.[21]

S1 nuclease PFGE analysis showed that each Tn*6636* positive strain harbored only one plasmid except for ST59 NTUH_3874 with two Tn*6636*-plasmids with remarkably similar size. The two plasmids harbored in ST59 NTUH_3874 as demonstrated by NGS, the size of pNTUH_3874 and pNTUH_3874-2 were 46.8-kb and 44.8-kb, respectively. Isolates of the same ST carried a similar size and the same structure of plasmid.

Sequencing of plasmids analyzed by PlasmidFinder revealed that four different plasmid backbones were inserted in the Tn*6636* to form six mosaic plasmids. ST7 pNTUH_1027 and ST188 pNTUH_6457 shared nearly identical plasmid backbones and were the best match to a 20.7-kb pSaa6159 derived from a dominant clone ST93 isolated from community-associated MRSA in Australia.[22] pSaa6159 was also highly similar to the pMW2 from strain MW2 USA400 ST1 MRSA[23] The pMW2-like plasmids are common and have a wide geographical distribution.[24] This is the first report that pMW2-like plasmid obtained Tn*1546*-like element Tn*6636*. The best match of the plasmid backbones from ST965 NTUH_5066148 and ST59 pNTUH_3874-2 was a 24.7-kb pCA-347, derived from a dominant clone USA600 ST45 MRSA from a bacteremia infection in California.[25] pCA-347 was also highly similar to the pN315 plasmid from strain N315 ST5 MRSA, which is prevalent in Japan and the USA.[26] The above two plasmid backbones are popular throughout the world. The drug-resistant element Tn*6636* may be transferred horizontally by those plasmids.

The backbone of the plasmid in ST5 pNTUH_9448 is the 27-kb pWBG744 of ST5 MSSA. pWBG744 belongs to the pIB485-family that has been reported in clinical and colonizing isolates of *S. aureus*.[27] pIB485 is the prototype *sed*/*sej*/*ser*-encoding plasmid of *S. aureus*.[28] The presence of the enterotoxin genes *sed*, *sej* and *ser* in the pIB485-family plasmids have been previously reported.[24,28]

Only one isolate of ST59 in our collection carried the Tn*6636* plasmid. The plasmid backbone of ST59 pNTUH_3874 is a novel plasmid, with no match to its nucleotide sequence in the NCBI database. Since ST59 is the major genotype in both MSSA and MRSA in Taiwan,[29] the occurrence of the Tn*6636* plasmid in ST59 needs more focused attention.

Tn*1546*, which encodes resistance to vancomycin via the *vanA* gene cluster, is the most notable member of the Tn*3*

Table 3 Transfer frequency of plasmids by conjugation.

Donor MSSA			Transfer frequency[a]	MIC of transconjugant (μg/mL)	
Plasmid	Genotype	Plasmid size (kb)		Erythromycin	Gentamicin
pNTUH_9448	ST5/*spa* t242	42.5	3.1×10^{-10}	>256	8
pNTUH_1027	ST7/*spa* t796	35.2	10^{-7}	>256	8
pNTUH_3874	ST59/*spa* t216	46.8	4.4×10^{-10}	>256	8
pNTUH_6457	ST188/*spa* t189	35.2	$<9.2 \times 10^{-12}$	-[b]	-[b]
pNTUH_8038	ST188/*spa* t189	~35.2	$<6.0 \times 10^{-12}$	-[b]	-[b]
pNTUH_6263-1	ST188/*spa* t5529	~35.2	$<5.1 \times 10^{-12}$	-[b]	-[b]
pNTUH_5066148	ST965/*spa* t575	39.2	1.5×10^{-10}	>256	64

[a] Transconjugant per donor cell.
[b] No transconjugant was obtained.

family in Gram-positive bacterial species.[30] Our previous study demonstrated high homology of the *tnpA*, *tnpR*, *res* sites, and terminal IRs between Tn*6636* and Tn*1546*.[3] In enterococci, high transfer of Tn*1546* is largely facilitated by its associated plasmids, such as RepA_N (pRUM/pLG1) or Inc18 plasmid families.[31] In *S. aureus*, although Tn1546 has been delivered into MRSA by pLW1043[32] and pBRZ01,[33] limited transferability has been noted. In the present study, we demonstrated that the low prevalence of Tn*6636* may be attributed to low conjugation frequency of the Tn*6636*-carrying plasmids in *S. aureus* (Table 3), similar to what has been observed in Tn*1546*.

The earliest strain from this study was ST5 MSSA NTUH_My675 isolated from 2002, which indicates that although acquisition of the Tn*6636* in *S. aureus* happened more than 18 years ago, Tn*6636* still revealed high conservation. However, the Tn*1546* in enterococci displayed heterogeneity by insertion of various insertion sequences, such as IS*19*, IS*1216V*, IS*1251*, and IS*1542*.[34–36] Considering that the 14.5-kb Tn*6636* is more similar to that in pMCCL2 of *Macrococcus caseolyticus*,[3] studies of other species in which Tn*6636* originates may provide more information.

In addition, transposon Tn*1546* is the prototype of *vanA*-carrying transposons; if *S. aureus* was able to acquire the Tn*6636*, it is likely that it could obtain the Tn*1546-vanA* element. The transfer of vancomycin resistance to *S. aureus* has occurred by interspecies transfer of Tn*1546* from a co-isolate of *Enterococcus faecalis*.[32] Recently, Rossi et al. reported that a conjugative plasmid carrying the Tn*1546-vanA* element could be transferred to other staphylococci.[37] Thus, the presence of Tn*6636* in *S. aureus* or other species should continue to be monitored.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.03.003.