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1 Emergence of mosaic plasmids harboring Tn*1546-ermB* element in *Staphylococcus*
2 *aureus* isolates
3
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20 Running Head: Tn*1546-ermB* plasmids in *Staphylococcus aureus*

21 Keywords: *Staphylococcus aureus*, Tn*1546-ermB*, plasmid

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23 **ABSTRACT** Antimicrobial resistance in *Staphylococcus aureus* is a major problem
24 and the acquisition of resistance genes may occur by horizontal gene transfer (HGT).
25 The transposon, an important means of HGT, is recognized as a mobile genetic
26 element that can integrate in plasmids, replicate and transfer to other strains. We have
27 previously reported a novel structure of the *Enterococcus faecium*-originated Tn*1546*-
28 *ermB* element in *S. aureus*. The emergence of the Tn*1546*-like element is an emerging
29 problem that requires continuous monitoring. In the present study, we expand the
30 examination of Tn*1546*-*ermB* element to *ermB*-positive methicillin-susceptible *S.*
31 *aureus* (MSSA) (n = 116) and *ermB*-positive methicillin-resistant *S. aureus* (MRSA)
32 (n = 253) during a 16-year period, from 2000 to 2015. PCR mapping showed that 10
33 MSSA and 10 MRSA carried the Tn*1546*-*ermB* element. The 10 MSSA belonged to
34 three sequence types (ST), ST7 (n = 6), ST5 (n = 3), and ST59 (n = 1), and the 10
35 MRSA belonged to two STs, ST188 (n = 8) and ST965 (n = 2). Since only clonal
36 complex 5 (including ST5, ST85, ST231, and ST371) MRSA, ST8 MRSA and ST5
37 MSSA have been previously reported to carry Tn*1546* plasmids, this is the first report
38 describing the presence of the Tn*1546*-*ermB* element in ST7/5/59 MSSA and
39 ST188/965 MRSA. Plasmid sequencing revealed that the Tn*1546*-*ermB* element was
40 harbored by five different mosaic plasmids. In addition to resistance genes, some
41 plasmids also harbored toxin genes.

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43 **INTRODUCTION**

44 Antimicrobial resistance in *Staphylococcus aureus* is a major problem due to its
45 high capacity to acquire drug resistance genes by horizontal transfer (1). The problem
46 of multidrug-resistant *S. aureus* is becoming more serious, and the available drugs to
47 combat it are decreasing (2).

48 Antibiotic resistance is highly prevalent among common pathogenic bacteria in
49 Taiwan. For example, the rate of oxacillin resistance in 33,305 *S. aureus* isolates from
50 eight medical centers in Taiwan is approximately 60%, and the rates of resistance to
51 erythromycin, clindamycin, gentamicin, and tetracycline are also very high (3).
52 Erythromycin, a macrolide class antibiotic, is an old and well-established
53 antimicrobial agent that was approved in Taiwan in 1968 (4). Due to its long history
54 of usage in Taiwan, *S. aureus* isolates are highly resistant to erythromycin (3).

55 There are two major mechanisms for resistance to erythromycin. One uses
56 methylase (encoded by *erm*) which methylates 23S rRNA, thereby altering the drug
57 binding site, thus conferring resistance not only to macrolides (erythromycin) but also
58 to lincosamides (clindamycin) and streptogramin B (MLS phenotype) antibiotics. The
59 common *erm* gene in *S. aureus* is in Tn*554*-*ermA* (5, 6), Tn*551*-*ermB* (7, 8), and *ermC*
60 (5). The second mechanism is through an efflux pump encoded by the *msrA* or *msrB*,
61 which confers resistance to the macrolides and streptogramin B only (MS phenotype)

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62 (9).

63 We previously reported that the most prevalent resistance gene in erythromycin-
64 resistant blood isolates of methicillin-susceptible *S. aureus* (MSSA) collected from
65 2000 to 2012 in Taiwan was *ermB* (10). Although the majority (92%) of *ermB*-
66 positive MSSA isolates carried structures resembling the mobile element structure
67 (MES) that has been reported in sequence type 59 (ST59) MRSA (8, 11), we found a
68 unique structure, the Tn*1546*-*ermB* element (10). The significance of the Tn*1546*-
69 *ermB* element is that Tn*1546* is also responsible for vancomycin resistance in
70 *Enterococcus* spp. (12, 13). The Tn*1546*-*vanA* was mainly located on the RepA_N
71 (pRUM/pLG1) and Inc18 plasmid families in vancomycin-resistant *E. faecium*
72 (VREfm) (14). The plasmids RepA_N (pRUM/pLG1) were able to yield plasmid
73 mosaics and acquire Tn*1546*-*vanA*, which was the main reason that *vanA* spread in
74 VREfm (14).

75 Tn*1546* could acquire different insertion sequences (ISs), including the most
76 frequently acquired IS*1216V*, as well as IS*1542*, IS*1251*, and IS*19* (15-18). A 92-kb
77 plasmid of *E. faecium*, pHKK701, contains a 39-kb Tn*5506*, which carries *ermB* and
78 IS*1216V*, and contains Tn*1546* with *vanA* (8, 19). The novel Tn*1546*-*ermB* element,
79 Tn*1546*-carrying Tn*551*-*ermB* and IS*1216V* was recently reported in *S. aureus* by our
80 group (10).

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81 In this study, we examined the prevalence of the Tn*1546-ermB* element in MSSA
82 and MRSA during a 16-year period, from 2000 to 2015. The presence of a Tn*1546*-
83 like element in different plasmids and lineages of *S. aureus* is an indication that *S.*
84 *aureus* may have the chance to acquire Tn*1546-vanA* by horizontal gene transfer
85 (HGT).

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86 **RESULTS**

87 **Prevalence of the Tn*1546-ermB* element.** Of 340 erythromycin-resistant MSSA
88 isolates collected between 2000 and 2015, 112 (33%) carried the *ermB* gene. Of 1429
89 erythromycin-resistant MRSA isolates collected between 2006 and 2015, 224 (16%)
90 carried the *ermB* gene. However, only 10 MSSA and 10 MRSA carried the Tn*1546-*
91 *ermB* element.

92 **Molecular epidemiology of the Tn*1546-ermB* element-carrying isolates.** To
93 determine the clonal relation between 10 MSSA and 10 MRSA isolates carrying the
94 Tn*1546-ermB* element, we performed *spa* typing, multi-locus sequence typing
95 (MLST) (Table 1) and pulsed-field gel electrophoresis (PFGE) (Fig. 1). Of the 10
96 MSSA isolates, six belonged to ST7 (*spa* type t796), three belonged to ST5 (*spa* type
97 t002 and t242) and one isolate belonged to ST59 (*spa* type t216). Of the 10 MRSA
98 isolates, eight belonged to ST188 (*spa* type t189 and t5529) and two isolates belonged
99 to ST965 (*spa* type t575 and t062). The results of PFGE are shown in Fig. 1. Three
100 ST5 MSSA isolates from 2002, 2010 and 2012 samples belonged to the same
101 pulsotype (with 80% similarity cut-off). Two ST965 (CC5) MRSA isolates obtained
102 from 2012 and 2014 samples were very closely related to ST5 MSSA. Six ST7 MSSA
103 isolates obtained from 2004 to 2016 samples belonged to the same pulsotype, and the
104 four isolates from 2012 to 2015 samples were identical. Eight ST188 MRSA isolates

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105 obtained from 2009 to 2014 samples belonged to the same pulsotype, and six of them

106 were identical.

107 **Sanger sequencing of the Tn*1546-ermB* element and homology analysis.**

108 Representative strains of each STs (ST5 MSSA NTUH_9448, ST7 MSSA

109 NTUH_1027, ST59 MSSA NTUH_3874, ST188 MRSA NTUH_6457 and ST965

110 MRSA NTUH_5066148) were chosen for sequencing to determine the sequence of

111 Tn*1546*-*ermB* element. The size of the Tn*1546*-*ermB* element in all of the above

112 strains is 14,567 bp. The sequences of Tn*1546*-*ermB* element in ST7 MSSA

113 NTUH_1027 and ST965 MRSA NTUH_5066148 were 100% identical. ST5 MSSA

114 NTUH_9448 had a nucleotide difference G1354A in *tnp* gene of Tn*551* causing

115 G452R. ST188 MRSA NTUH_6457 had a nucleotide difference downstream of *ermB*

116 gene. ST59 MSSA NTUH_3874 had four nucleotides difference: (1) point mutation

117 (A299G) of *ermB* gene resulting in N100S, (2) non-coding region point mutation

118 downstream of *ermB* gene, (3) point mutation (G765A) of *tnp* gene of Tn*551*, (4)

119 premature nonsense mutation in *tnp* gene of Tn*551*, shortening the amino acid length

120 from 972 to 857.

121 **Location of Tn*1546-ermB* element.** To determine the location of the Tn*1546*-

122 *ermB* element, the agarose plugs of 10 MSSA and 10 MRSA isolates were digested

123 with S1 nuclease (Fig. 2) and then hybridized with a Dig-labeled *ermB*-specific probe

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124 prepared by PCR amplification of *ermB* using primers ermB-f and ermB-r (Table 2).

125 Isolates of the same ST (ST5, ST7, ST188 and ST965) harbored similar size of

126 plasmids containing *ermB* (Fig. 2).

127 **Sequence analysis of five plasmids harboring the Tn*1546-ermB* element.** The

128 sequences of plasmids in ST5 MSSA NTUH_9448, ST7 MSSA NTUH_1027, ST59

129 MSSA NTUH_3874, ST188 MRSA NTUH_6457 and ST965 MRSA NTUH_5066148

130 were determined. Fig. 3 presents the genetic structures of the five plasmids. The 14.5-

131 kb Tn*1546*-*ermB* element was inserted in four different backbones. Among them, the

132 plasmids pNTUH_1027 and pNTUH_6457 (Fig. 3A and 3B) showed mosaic

133 structures that included Tn*1546*-*ermB* and a 20.7-kb backbone which is similar to

134 pSaa6159 (NCBI accession no. CP002115) of ST93 MRSA. The *repA* gene and the

135 backbone of the above two plasmids were 100% and 99.9% identical, respectively, to

136 those in pSaa6159. The Tn*1546*-*ermB* element was inserted at the eleven o'clock

137 position in pNTUH_1027. The Tn*1546*-*ermB* element was inserted at three o'clock

138 position in pNTUH_6457 with the disruption of a *tnp* gene of Tn*552*. Plasmid

139 pNTUH_5066148 (Fig. 3C) was also a mosaic plasmid and contained the Tn*1546*-

140 *ermB* and a 24.7-kb backbone which is similar to pCA-347 (NCBI accession no.

141 CP006045) of ST45 MRSA is essentially identical to pN315 (NCBI accession no.

142 AP003139) of ST5 MRSA. The *repA* gene and the backbone of the pNTUH_5066148

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143 was 100% and 99.8% identical, respectively, to those in pCA-347. The Tn*1546-ermB*
144 element was inserted at the eleven o'clock position in pNTUH_5066148 with the
145 disruption of one of the three *rep* gene, shortening the amino acid length from 286 to
146 271. Plasmid pNTUH_9448 (Fig. 3D) was another mosaic plasmid, in which the 14.5-
147 kb Tn*1546-ermB* element was inserted at the five o'clock position and disrupted an
148 alcohol dehydrogenase gene. The *repA* gene and the backbone of the pNTUH_9448
149 was 100% and 94.5% identical, respectively, to those in pWBG744.

150 Plasmid pNTUH_3874 (Fig. 3E) showed unique features. Nucleotide-nucleotide
151 BLAST analysis found no significant matches except for the region containing the
152 Tn*1546-ermB* element. Annotation revealed that Tn*1546-ermB* element has 11 ORFs,
153 and the backbone of plasmid pNTUH_3874 has 35 annotated ORFs, including three
154 conjugal transfer genes (*traB*, *traE* and truncated *traK*), one resolvase gene, one DNA
155 topoisomerase I gene and two DNA topoisomerase III genes.

156 **Tn*1546-ermB* plasmids in other isolates.** PCR mapping was used to determine
157 whether the other Tn*1546-ermB*-carrying plasmids contain similar structures in each
158 ST (Fig. S1, Table S1). Our results indicated that the plasmids in two remaining ST5
159 MSSA, six ST7 MSSA, seven ST188 MRSA, and one ST965 MRSA display similar
160 plasmid structures corresponding to each ST.

161 **Conjugal transfer frequency of plasmids harboring the Tn*1546-ermB* element.**

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162 To determine whether Tn*1546-ermB* element-carrying plasmids could be transferred
163 *in vitro* from the clinical ST5/7/59/188/965 *S. aureus* to the laboratory strain ST8
164 RN2677, we performed conjugation tests. The Tn*1546-ermB* element-carrying
165 plasmids could be transferred from ST5 NTUH_9448, ST7 NTUH_1027, ST59
166 NTUH_3874, and ST965 NTUH_5066148 to RN2677 with a frequency of $3.1x10^{-10}$,
167 10^{-7}, $4.4x10^{-10}$, and $1.5x10^{-10}$ per recipient cell, respectively. Transconjugants were
168 characterized by PCR to test for the presence of *ermB* and *tnp* of Tn*1546*, and by *spa*
169 typing, and by erythromycin/gentamicin susceptibility testing. The results showed that
170 the four different Tn*1546-ermB* plasmids could be transferred *in vitro*, and the
171 resulting four transconjugants were resistant to erythromycin and gentamicin (Table
172 3).

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173 **DISCUSSION**

174 This study continued the work of our previous study in which the novel structure of
175 an *E. faecium*-originated Tn*1546*-*ermB* element in MSSA was reported (10). In the
176 present study, we examined the presence of Tn*1546*-*ermB* plasmids in more isolates
177 of MSSA and MRSA. The overall prevalence of Tn*1546*-*ermB* plasmid in
178 erythromycin-resistant *S. aureus* was low, and was slightly higher in MSSA than
179 MRSA. This result is expected since most of our MRSA isolates were resistant to
180 erythromycin and belonged to ST5/SCC*mec*II or ST239/SCC*mec*III harboring *ermA*
181 located on Tn*554* (20).

182 The most frequent ST of 20 Tn*1546*-*ermB* positive isolates is ST188 SCC*mec*IVa
183 MRSA (n = 8), followed by ST7 MSSA (n = 6). The ST188 was the common ST of
184 MSSA in Taiwan (21). However, the ST188 in MRSA is rare in Taiwan; only one
185 nasal carriage and one bloodstream report have referred to the ST188 MRSA (22, 23).
186 ST188 MRSA is also rare in other countries. Recently, Hong Kong (24, 25), China
187 (26) and Korea (27) showed the occurrence of ST188 MRSA. We examined the
188 ST188 MRSA isolates recovered from blood from 2006 to 2012 in NTUH, and found
189 that all the tested ST188 MRSA isolates harbored the Tn*1546*-*ermB* plasmid. The
190 reason for this finding is unclear. PFGE analysis shows that ST188 MSSA and ST188
191 MRSA belong to different pulsotypes (data not shown), suggesting some level of

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192 genetic diversity.

193 The ST7 MSSA is one of the major sequence types of MSSA bacteremia in Taiwan
194 (21) and is a prevalent clonotype (13/92, 14.1%) for invasive community-acquired
195 MSSA infection (28). ST7 MSSA was the dominant MSSA carrying Tn*1546-ermB*
196 element in this study.

197 Three ST5 MSSA and two ST965 MRSA carried the Tn*1546-ermB* plasmid. ST965
198 is a single-locus variant (SLV) of ST5. Our data shows that ST5 MSSA and ST965
199 harbored different plasmids. However, according to PFGE data, the pulsotypes of ST5
200 MSSA and ST965 MRSA were very close. Twelve cases of vancomycin-resistant *S.*
201 *aureus* (VRSA) infection have been reported in the United States—all CC5 strains,
202 each have Tn*1546-vanA*. The CC5 isolates appear to be very well adapted for
203 acquiring Tn*1546* (29). In the present study ST5-CC5 MSSA and ST965-CC5 MRSA
204 acquired Tn*1546*.

205 The S1 nuclease PFGE analysis showed that each Tn*1546-ermB* positive strain
206 harbored only one plasmid. Isolates of the same ST carried a similar size and the same
207 structure of plasmid. The earliest strain of this study was ST5 MSSA NTUH_My675
208 isolated from 2002, which indicates that the acquisition of the Tn*1546-ermB* element
209 in *S. aureus* did not occur recently.

210 Sequencing of plasmids revealed that there were four different plasmid backbones

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211 inserted with the Tn*1546-ermB* element to form five mosaic plasmids. The ST7
212 pNTUH_1027 and ST188 pNTUH_6457 shared nearly identical plasmid backbones
213 and best match to a 20.7-kb pSaa6159 (NCBI accession no. CP002115) derived from
214 a dominant clone ST93 isolated in 2004 from community-associated MRSA (CA-
215 MRSA) in Australia (30, 31). Plasmid pSaa6159 was also highly similar to the pMW2
216 plasmid (NCBI accession no. AP004832) from strain MW2 USA400 ST1 MRSA,
217 which caused fatal septicemia and septic arthritis in a 16-month-old girl in North
218 Dakota, USA, in 1998 (32). The pMW2-like plasmids are common with a wide
219 geographical distribution (33). This is the first report that pMW2-like plasmid
220 obtained Tn*1546*.
221 The best match of the plasmid backbones from ST965 NTUH_5066148 was a 24.7-
222 kb pCA-347 (NCBI accession no. CP006045), derived from a dominant clone
223 USA600 ST45 MRSA from a bacteremia infection in 2005 in California (34). Plasmid
224 pCA-347 was also highly similar to the pN315 plasmid (NCBI accession no.
225 AP003139) from strain N315 ST5 MRSA, was isolated in 1982 from the pharyngeal
226 smear of a Japanese patient, which is prevalent in Japan and the USA (35).
227 The plasmids from ST7 pNTUH_1027, ST188 pNTUH_6457 and ST965
228 NTUH_5066148 contained the identical origin-of-transfer gene *oriT* mimic sequence
229 of the pWBG749-family. Since the *oriT* may facilitate horizontal transmission (36-

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230 38), if this kind of plasmid contains the Tn*1546*-*ermB* element, then it may also
231 acquire the Tn*1546*-*vanA* element. This is alarming since it raises the possibility of
232 the occurrence of the Tn*1546*-*vanA* element in *S. aureus*.

233 The backbone of the plasmid in ST5 pNTUH_9448 is the 27-kb pWBG744 of ST5
234 MSSA. The mobilized plasmid pWBG744 belongs to the pIB485-family that has been
235 reported in clinical and colonizing isolates of *S. aureus* (39). Plasmid pIB485 is the
236 prototype *sed*/*sej*/*ser*-encoding plasmid of *S. aureus*. (40-42). The presence of the
237 enterotoxin genes *sed*, *sej* and *ser* in the pIB485-family plasmids have been
238 previously reported (33, 41).

239 Only one isolate of ST59 in our collection carried the Tn*1546*-*ermB* plasmid. The
240 plasmid backbone of ST59 pNTUH_3874 is a novel plasmid, since there was no
241 match to its nucleotide sequence in the NCBI database. This plasmid harbored three
242 conjugal transfer genes *traB*, *traE* and truncated *traK*. Since the ST59 is the major
243 genotype in both MSSA and MRSA in Taiwan (43), the occurrence of the Tn*1546*-
244 *ermB* plasmid in ST59 needs more attention.

245 Previously the Tn*1546* element has only been found in pLW1043 (44) and pBRZ01
246 (45) in *S. aureus*. The *repA* gene of the four plasmids in the present study was
247 different from that in pLW1043 (44) or pBRZ01 (45). The size of the *repA* gene is
248 960 bp in pLW1043, 984 bp in pBRZ01, 861 bp in pSaa6159 (backbone of

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249 pNTUH_1027 and pNTUH_6457945), 984 bp in pCA-347 (backbone of

250 pNTUH_5066148), and 945 bp in pWBG744 (backbone of pNTUH_9948). This is

251 the first report of the occurrence of the Tn*1546* element in new plasmids.

252 Although the overall prevalence of Tn*1546-ermB* or its plasmids in *S. aureus*

253 isolates was low, the Tn*1546-ermB* element has existed from at least 2002 to the

254 present; perhaps there are reservoirs in other species. It is known that the Tn*1546*

255 element originated in *E. faecium*. However, the sequence of the 14.5-kb Tn*1546-ermB*

256 element is more similar to that in pMCCL2 of *Macrococcus caseolyticus* (6, 10).

257 Studies on other species may provide more information. In addition, transposon

258 Tn*1546* is the prototype of *vanA*-carrying transposons; if *S. aureus* was able to

259 acquire the Tn*1546-ermB* element, it is likely that it could obtain the Tn*1546-vanA*

260 element. The transfer of vancomycin resistance to *S. aureus* has occurred *in vivo* by

261 interspecies transfer of Tn*1546* from a co-isolate of *E. faecalis* (44). Recently, Rossi

262 et al. reported that a conjugative plasmid carrying the Tn*1546-vanA* element could be

263 transferred to other staphylococci (46). Thus, the occurrence of Tn*1546* in *S. aureus*

264 should be monitored.

265

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266 **MATERIALS AND METHODS**

267 **Bacterial isolates.** All isolates were recovered from blood. The MSSA were
268 collected during the period between 2000 and 2015 and the MRSA were between
269 2006 and 2015 at the Bacteriology Laboratory, National Taiwan University Hospital,
270 a 2,500-bed teaching hospital in northern Taiwan. Only one isolate per patient was
271 collected in this study. *S. aureus* was identified by the Vitek2 system and *nuc* gene
272 detection (47). Resistance to methicillin was confirmed by *mecA* PCR.

273 **Detection of Tn*1546-ermB* element structure in erythromycin-resistant**
274 **isolates.** The Tn*1546-ermB* element was initially detected by the presence of the *tnp*
275 gene of Tn*1546* and *ermB* gene by PCR (10). The Tn*1546-ermB* element structure
276 was mapped by PCR using six primer sets which are listed in Table 2, and the
277 positions of the primers are indicated in Fig. 4. The Tn*1546-ermB* element structures
278 were determined by combining the PCR mapping results and the profiles of resistance
279 determinants.

280 ***spa* typing, multi-locus sequence typing (MLST), and pulsed-field gel**
281 **electrophoresis (PFGE).** To determine the genetic relatedness of Tn*1546-ermB*-
282 carrying isolates, *spa* typing, MLST, and PFGE were performed. The *spa* typing was
283 performed as described previously (48). MLST was carried out to determine the
284 sequence types (STs), which were assigned using the *S. aureus* MLST database

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285 (www.mlst.net) (49). PFGE was performed as described previously (50). The DNA in
286 gel plugs were digested with SmaI (New England BioLabs, Ipswich, MA, USA) and
287 then separated in a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA,
288 USA). Plugs were applied to wells in 0.8% (w/v) agarose gels (Bio-Rad). PFGE was
289 carried out at 200 V and 12°C for 20 h, with a pulse angle of 120° and pulse times
290 ranging from 5 to 60 s. The pulsotypes were analyzed by BioNumerics software
291 version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).
292 **S1 nuclease digestion-PFGE.** Detection of the presence of plasmids by S1
293 nuclease digestion-PFGE was performed as described previously (50). Plug slices
294 were incubated at 37°C for 45 min with 1-10 unit of *Aspergillus oryzae* S1 nuclease
295 (Invitrogen) in 150 μl of 50 mM NaCl, 30 mM sodium acetate (pH 4.6) and 1 mM
296 zinc acetate. The plugs were applied to wells of 1.2% (w/v) agarose gels (Bio-Rad),
297 and run in a CHEF-DR III apparatus (Bio-Rad Laboratories) with a pulse angle of
298 120° and pulse times of 45 s for 14 h and 25 s for 6 h, at 200 V in 0.5X Tris-Borate-
299 EDTA (TBE) buffer (51). The linear form of the plasmids separated from the
300 chromosome DNA and the size of plasmids were estimated.
301 **Conjugation test.** To determine the transfer frequency *in vitro*, strain RN2677 was
302 used as the recipient in the conjugation test, and mating was carried out on LB agar
303 medium without selection (52). After 24 h, the mixed cultures were taken from the

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304 plates, suspended in brain-heart infusion (BHI) broth medium, and then plated onto
305 MHA agar medium containing erythromycin (0.5 μg/ml) and rifampicin (80 μg/ml), at
306 37°C and 24 h. Confirmation of transconjugants was carried out by testing for the
307 presence of the *ermB* gene by PCR. The transconjugants were also checked by *spa*
308 typing (the *spa* type of RN2677 is t211) (52).

309 **Southern blot hybridization.** DNA was electrophoresed, depurinated, denaturated,
310 neutralized, and transferred to a Hybond-N^+ nylon membrane (Amersham Pharmacia
311 Biotech, Buckinghamshire, UK) using the Vacuum Blotting System (VacuGene^{TM}
312 XL, Amersham Pharmacia Biotech, Buckinghamshire, UK). Hybridization with the
313 PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Penzberg, Germany) was
314 performed using a Hybridization Incubator Model 1000 (Robbins Scientific).
315 Detection was performed with the Anti-Digoxigenin-AP and DIG Luminescent
316 Detection Kit (Roche Diagnostics GmbH, Penzberg, Germany) and results were
317 captured with the LAS-4000 Imaging System (FUJI FILM Life Science, Japan).

318 **Sequencing of plasmids.** We used "Long accurate PCR *in vitro* cloning kit"
319 (Takara Shuzo Co. Ltd., Japan) to amplify and clone the fragments of plasmids in ST5
320 NTUH_9448 MSSA, ST7 NTUH_1027 MSSA, ST59 NTUH_3874 MSSA, ST188
321 NTUH_6457 MRSA and ST965 NTUH_5066148 MRSA. The PCR products were
322 analyzed on the ABI 3730xl DNA Analyzer (Applied Biosystems).

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323 **Nucleotide sequence accession numbers.** The nucleotide sequences of five
324 plasmids: pNTUH_9448 in ST5 MSSA, pNTUH_1027 in ST7 MSSA, pNTUH_3874
325 in ST59 MSSA, pNTUH_6457 in ST188 MRSA and pNTUH_5066148 in ST965
326 MRSA have been deposited in the DNA Data Bank of Japan (DDBJ) database under
327 accession numbers LC377536 to LC377540.

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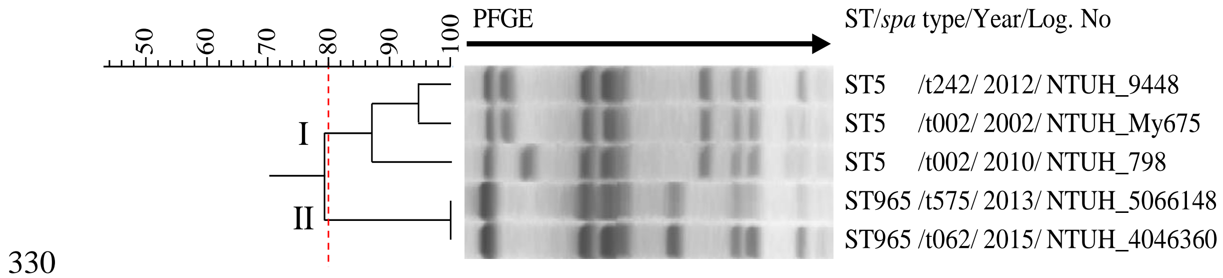
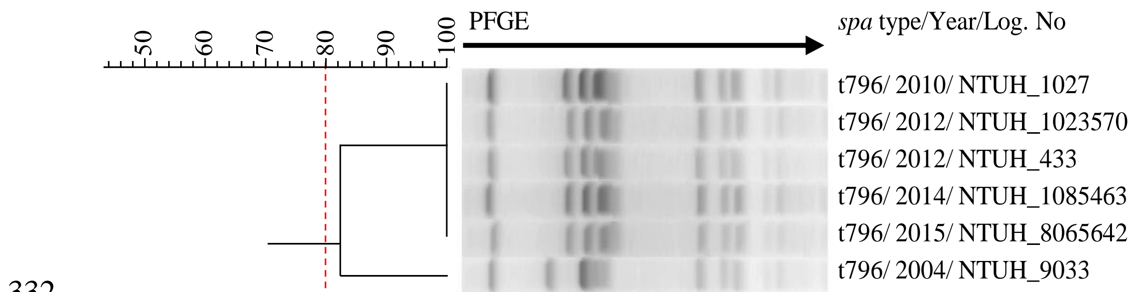
328 **FIGURE**

329 A



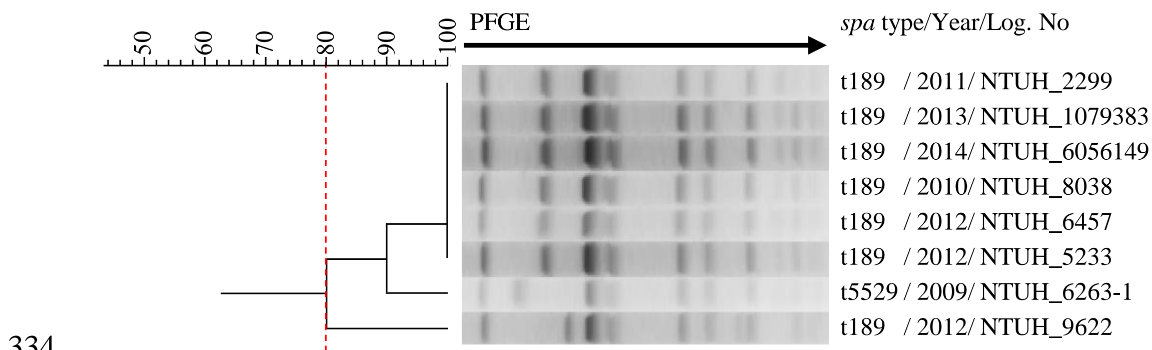
330

331 B



332

333 C



334

335 **FIG 1** PFGE dendrogram of Tn*1546-ermB* carrying isolates. PFGE cluster was

336 assigned to isolates having 80% or greater similarity from the dendrograms (A) Three

337 ST5 MSSA isolates (pulsotype I) and two ST965 MRSA isolates (pulsotype II) (B)

338 Six ST7 MSSA isolates (C) Eight ST188 MRSA isolates.

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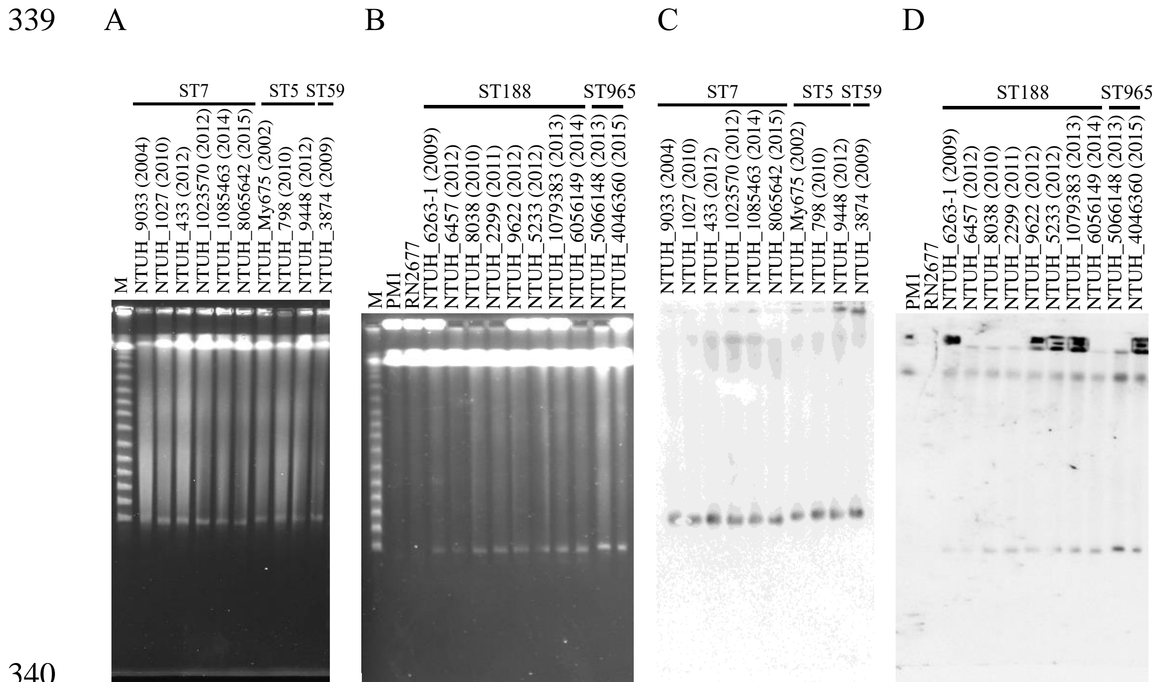
339 A B C D



340

341 **FIG 2** S1 nuclease PFGE and Southern blot hybridization with *ermB*. The S1

342 nuclease PFGE from three ST5 MSSA, six ST7 MSSA, one ST59 MSSA, eight

343 ST188 MRSA, and two ST965 MRSA was examined. The PM1 strain harboring a 26-

344 kb plasmid and *ermB* gene located on chromosome was used as a positive control.

345 Strain RN2677, lacking the plasmid and *ermB* gene, was used as a negative control.

346 The size of the M marker starts at 48.5 kb and increases 48.5 kb with each

347 successively larger band. (A, B) The bands indicated the plasmids. The size of

348 plasmids is estimated between 23.1 kb and 48.5 kb. (C, D) DNA was hybridized with

349 the Dig-labelled *ermB*-specific probe and amplified by PCR using primers ermB-f and

350 ermB-r. Positive signal of *ermB* was detected in respective bands.

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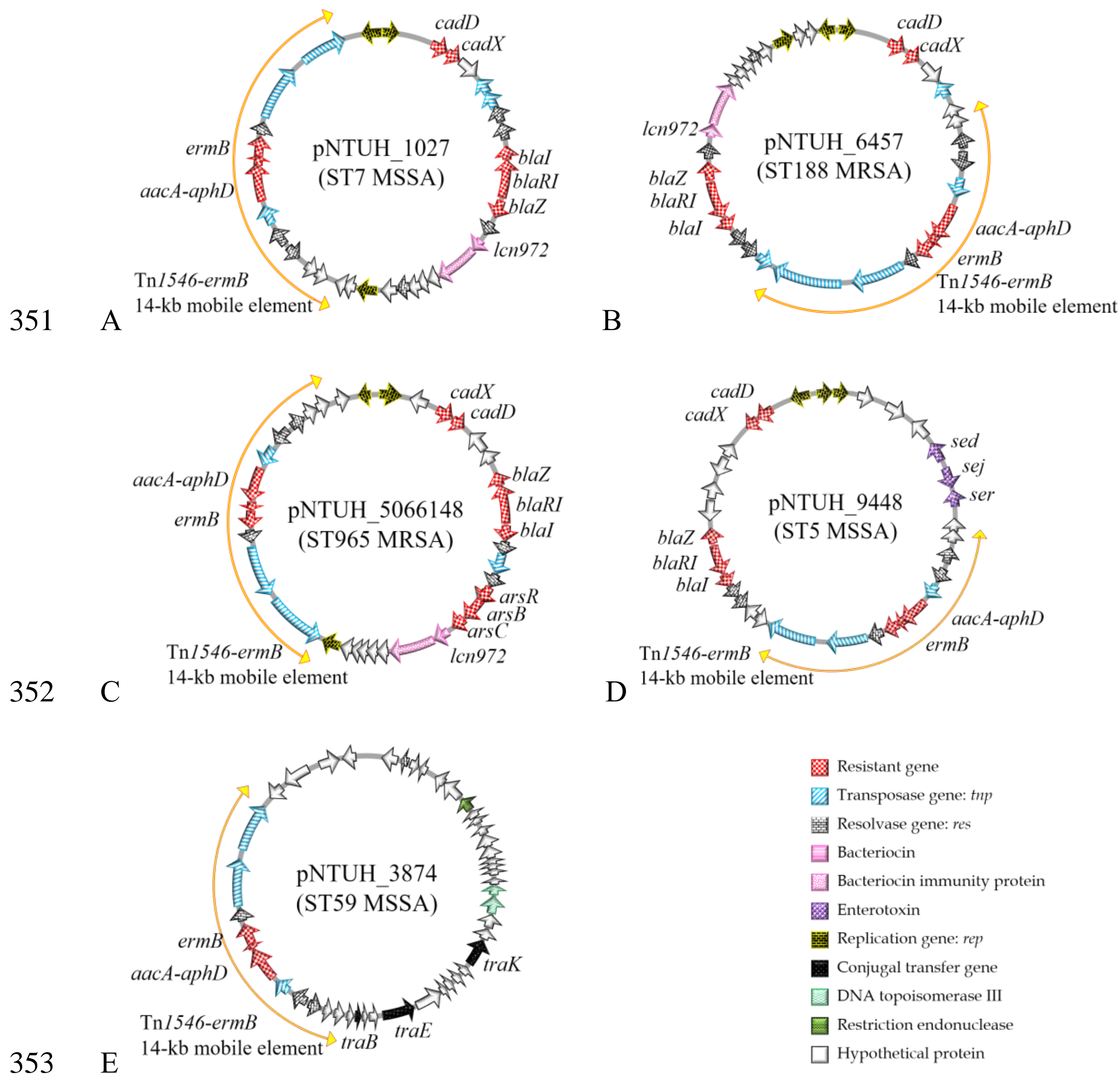

351
352
353

354 **FIG 3** Structure of plasmids. (A) The 35.2-kb ST7 MSSA NTUH_1027 plasmid
355 included 14.5-kb Tn*1546-ermB* element and 20.7-kb backbone which is similar to
356 pSaa6159, harboring bacteriocin Lcn972 (*lcn972* gene, pink stripe). The Tn*1546-*
357 *ermB* element was inserted at the eleven o'clock position in pNTUH_1027. (B) The
358 35.2-kb ST188 MRSA NTUH_6457 plasmid included 14.5-kb Tn*1546-ermB* element
359 and 20.7-kb backbone which is similar to pSaa6159, harboring bacteriocin Lcn972
360 (*lcn972* gene, pink stripe). The Tn*1546-ermB* element was inserted at the three o'clock
361 position in pNTUH_6457. (C) The 39.2-kb ST965 MRSA NTUH_5066148 plasmid
362 contained the 14.5-kb Tn*1546-ermB* and a 24.7-kb backbone which is similar to pCA-

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363 347, harboring bacteriocin Lcn972 (*lcn972* gene, pink stripe). (D) The 42.5-kb ST5
364 MSSA NTUH_9448 plasmid included 14.5-kb Tn*1546*-*ermB* element and 27.2-kb
365 pWBG744, harboring three enterotoxin genes *sed*, *sej*, and *ser* (purple mesh). (E) The
366 46.8-kb ST59 MSSA NTUH_3874 plasmid harbored three conjugal genes *traB*, *traE*,
367 and *traK*.
368

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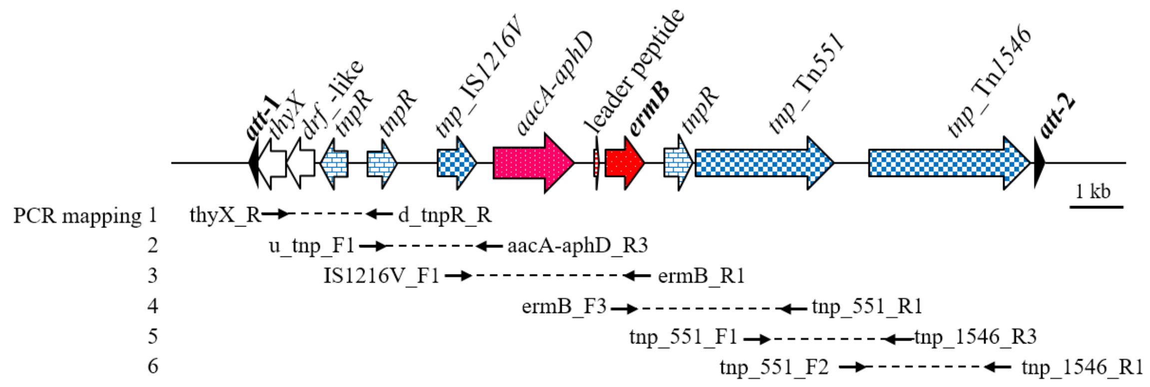

369

370 **FIG 4** Position of PCR primers for detection of the Tn*1546*-*ermB* element structure

371 amplicons for PCR mapping 1, 2, 3, 4, 5 and 6 are approximately 1.9 kb, 2.8 kb, 3.7

372 kb, 3.5 kb, 3.1 kb, and 3.2 kb, respectively.

373 **TABLE**

374 **TABLE 1** Distribution of MLST and *spa* types in Tn*1546*-*ermB* carrying MSSA and MRSA

Organism	Year	No. of *ermB* carried (%)[a]	No. of Tn*1546* 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 (15.7)	10 (4.5)	ST188 (8)	t189 (7), t5529(1)
				ST965 (2)	t575 (1), t062 (1)

375

376 a: Percentage of *ermB* in erythromycin-resistant isolates.

377 b: Rate of Tn*1546* in *ermB*-positive isolates.

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378 **TABLE 2** Primers used for PCR mapping of Tn*1546*-*ermB* element

Primer name	Sequence (5' to 3')	Application
thyX_R	CCTGTACCCTCTTGATGAGAGG	PCR mapping for Tn*1546*-*ermB* element
u_tnp_F1	CATAACACTGATTCTATCAGCC	PCR mapping for Tn*1546*-*ermB* element
d_tnpR_R	CTACTAGAAAACGGTCAGC	PCR mapping for Tn*1546*-*ermB* element
IS1216V_F1	CCGTGGGCTACTATCTTCGTT	PCR mapping for Tn*1546*-*ermB* element
aacA-aphD_R3	CATCTTCCCAAGGCTCTG	PCR mapping for Tn*1546*-*ermB* element
ermB_F3	GCCAGCGGAATGCTTTCATCCTAAACC	PCR mapping for Tn*1546*-*ermB* element
ermB_R1	AGTAACGGTACTTAAATTGTTTAC	PCR mapping for Tn*1546*-*ermB* element
tnp_551_F1	CGGTATCCTGGGTGT	PCR mapping for Tn*1546*-*ermB* element
tnp_551_R1	ATTTCTGATGCGAGG	PCR mapping for Tn*1546*-*ermB* element
tnp_551_F2	ACTAGGTCGCATTGAAAAGAG	PCR mapping for Tn*1546*-*ermB* element
tnp_1546_R3	GTGTAGTAGGTTCCTAGCAC	PCR mapping for Tn*1546*-*ermB* element
tnp_1546_R1	AGGGATGCTGAAACTTTTCC	PCR mapping for Tn*1546*-*ermB* element and detection *tnp* of Tn*1546*
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*1546*

379

380 **TABLE 3** Transfer frequency of plasmids by conjugation

Donor MSSA			Transfer frequency [a]	MIC of transconjugant (μg/ml)	
Strain	Genotype	Plasmid size (kb)		Erythromycin	Gentamicin
NTUH_9448	ST5/*spa* t242	42.5	3.1 X 10^{-10}	>256	8
NTUH_1027	ST7/*spa* t796	35.2	10^{-7}	>256	8
NTUH_3874	ST59/*spa* t216	46.8	4.4 X 10^{-10}	>256	8
NTUH_5066148	ST965/*spa* t575	35.2	1.5 X 10^{-10}	>256	64

381 [a] Transconjugant per donor cell.

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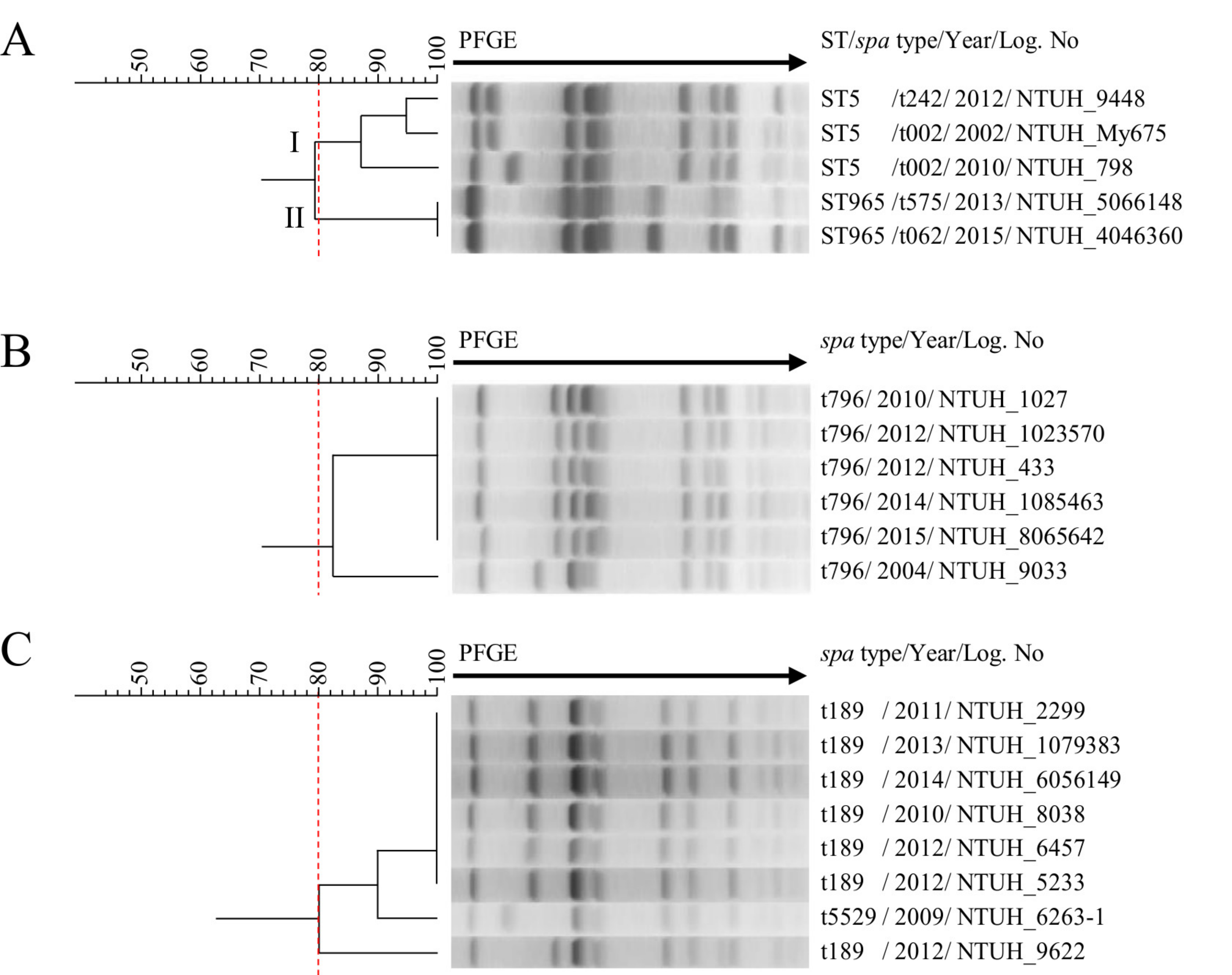

FIG 1 PFGE dendrogram of Tn*1546*-*ermB* carrying isolates. PFGE cluster was assigned to isolates having 80% or greater similarity from the dendrograms (A) Three ST5 MSSA isolates (pulsotype I) and two ST965 MRSA isolates (pulsotype II) (B) Six ST7 MSSA isolates (C) Eight ST188 MRSA isolates.

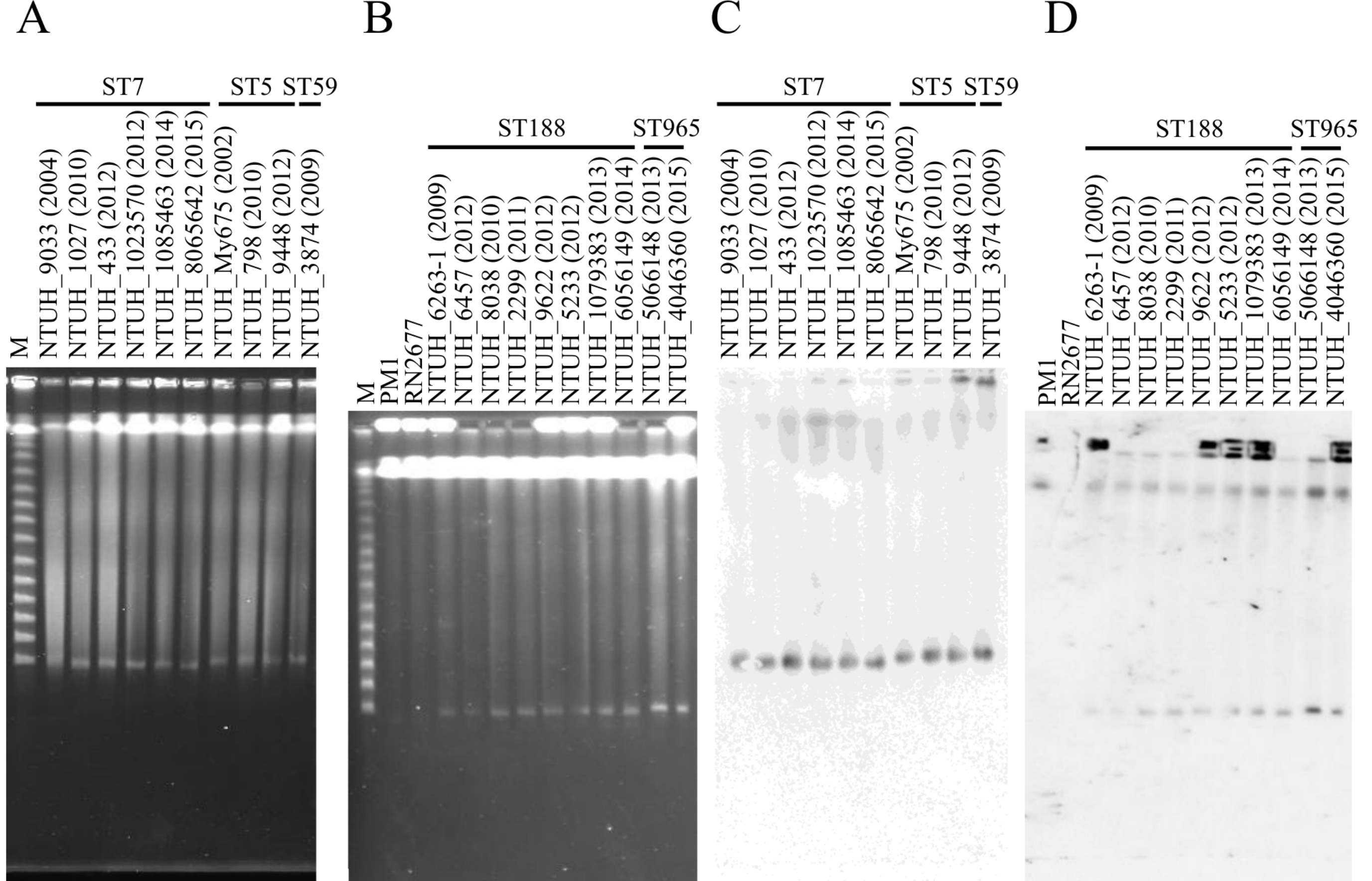

FIG 2 S1 nuclease PFGE and Southern blot hybridization with *ermB*. The S1 nuclease PFGE from three ST5 MSSA, six ST7 MSSA, one ST59 MSSA, eight ST188 MRSA, and two ST965 MRSA was examined. The PM1 strain harboring a 26-kb plasmid and *ermB* gene located on chromosome was used as a positive control. 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 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.

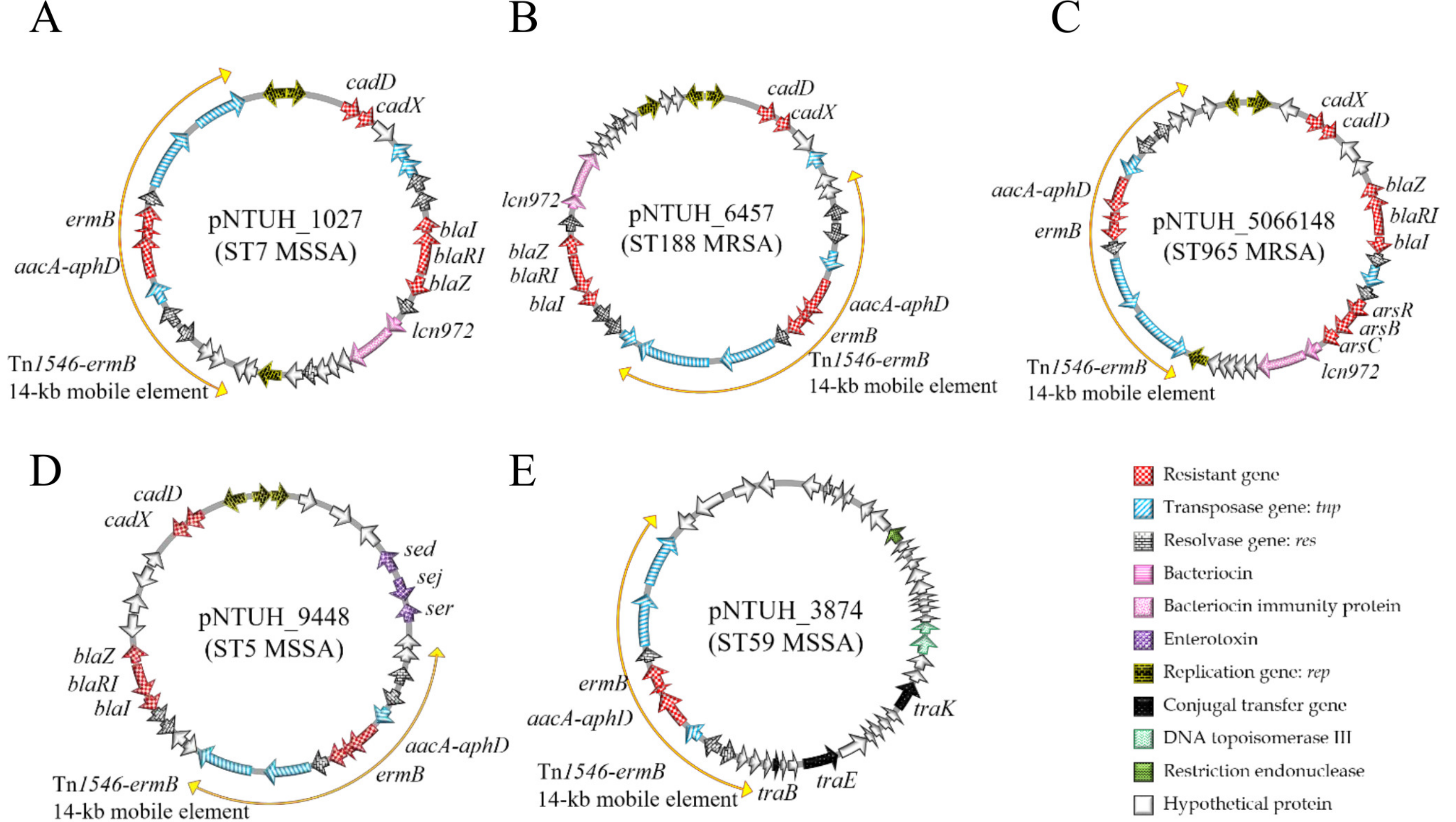

FIG 3 Structure of plasmids. (A) The 35.2-kb ST7 MSSA NTUH_1027 plasmid included 14.5-kb Tn*1546*-*ermB* element and 20.7-kb backbone which is similar to pSaa6159, harboring bacteriocin Lcn972 (*lcn972* gene, pink stripe). The Tn*1546*-*ermB* element was inserted at the eleven o'clock position in pNTUH_1027. (B) The 35.2-kb ST188 MRSA NTUH_6457 plasmid included 14.5-kb Tn*1546*-*ermB* element and 20.7-kb backbone which is similar to pSaa6159, harboring bacteriocin Lcn972 (*lcn972* gene, pink stripe). The Tn*1546*-*ermB* element was inserted at the three o'clock position in pNTUH_6457. (C) The 39.2-kb ST965 MRSA NTUH_5066148 plasmid contained the 14.5-kb Tn*1546*-*ermB* and a 24.7-kb backbone which is similar to pCA-347, harboring bacteriocin Lcn972 (*lcn972* gene, pink stripe). (D) The 42.5-kb ST5 MSSA NTUH_9448 plasmid included 14.5-kb Tn*1546*-*ermB* element and 27.2-kb pWBG744, harboring three enterotoxin genes *sed*, *sej*, and *ser* (purple mesh). (E) The 46.8-kb ST59 MSSA NTUH_3874 plasmid harbored three conjugal genes *traB*, *traE*, and *traK*.

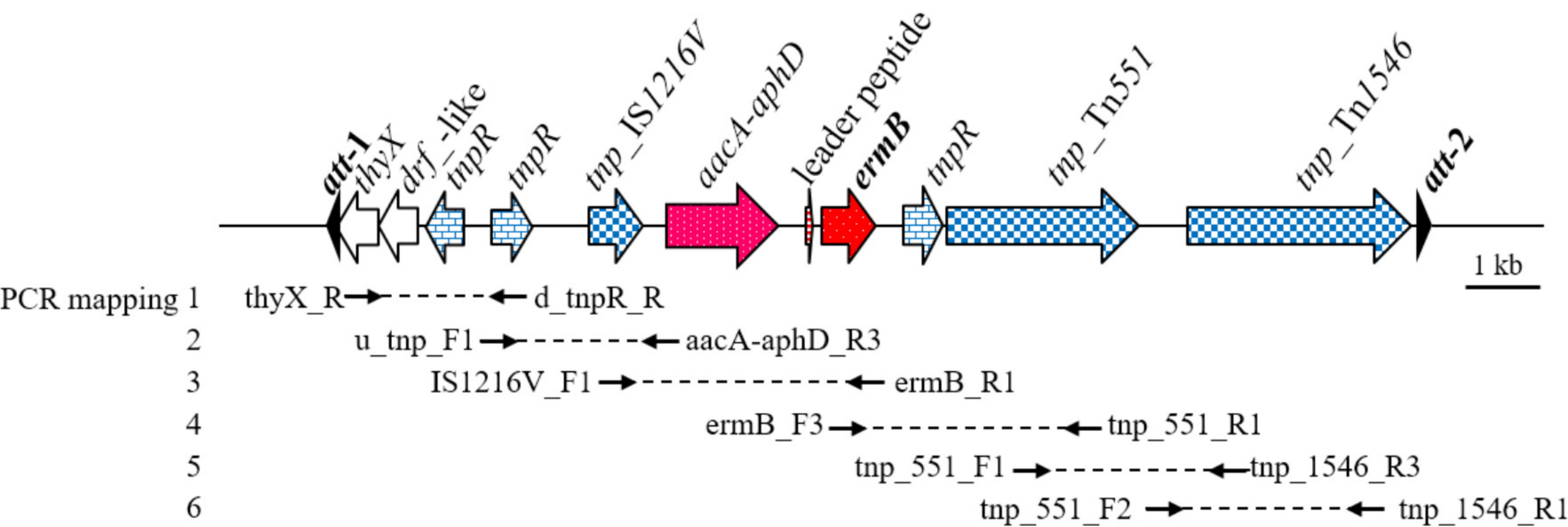

FIG 4 Position of PCR primers for detection of the Tn*1546*-*ermB* element structure amplicons for PCR mapping 1, 2, 3, 4, 5 and 6 are approximately 1.9 kb, 2.8 kb, 3.7 kb, 3.5 kb, 3.1 kb, and 3.2 kb, respectively.