





erm(T)-Mediated Macrolide-Lincosamide Resistance in *Streptococcus suis*

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ABSTRACT To investigate the presence and location of *erm(T)* in clinical *Streptococcus suis* isolates and explore the transmission ability and fitness cost of *erm(T)*-carrying mobile genetic elements among *S. suis* isolates, MICs were determined by broth microdilution. The presence of *erm(T)* in *S. suis* was detected by PCR. The genetic environment of *erm(T)* in *S. suis* was explored by whole-genome sequencing (WGS) analysis. Intraspecies and interspecies transmission were examined by electrotransformation. The fitness cost associated with the carriage of an *erm(T)*-harboring plasmid or an integrative and conjugative element (ICE) was examined by competition experiments. Of 237 nonduplicate strains, *erm(T)* was detected in 2 *S. suis* strains (SC262-ST954 and SC117-ST1314), with its location on a 5,125-bp plasmid in *S. suis* SC262 and on a 64,013-bp ICES_{Su}SC117 in *S. suis* SC117, respectively. Both the *erm(T)*-carrying plasmid pSC262 and the ICES_{Su}SC117 were transmissible by transformation. Plasmid pSC262 can replicate and express macrolide-lincosamide resistance in heterologous hosts, including *S. aureus* and *S. pneumoniae*. Both the *erm(T)*-carrying plasmid and the ICE posed a fitness cost to the host *S. suis* isolate. To the best of our knowledge, this is the first report of the macrolide-lincosamide-streptogramin B resistance gene *erm(T)* in *S. suis*. Its location on a plasmid or an ICE will aid in its transmission. The low detection rate of *erm(T)* gene among the *S. suis* population might be due to the fitness cost of the *erm(T)*-carrying plasmid and ICE.

IMPORTANCE Macrolide and lincosamide resistance due to the presence of *erm(T)* have posed a challenge for the treatment of Gram-positive pathogens. Although the low detection rate of *erm(T)* gene among the *S. suis* population due to the fitness cost of the *erm(T)*-carrying plasmid and ICE, the presence of *erm(T)* in *S. suis* and its potential transmission to other Gram-positive pathogens will be of important significance.

KEYWORDS *Streptococcus suis*, *erm(T)*, resistance, macrolide, lincosamide, fitness cost

Streptococcus suis is an important Gram-positive pathogen in the swine industry and also an emerging zoonotic pathogen in humans. Antimicrobial treatment is one of the most important measures to control the *S. suis* infections (1, 2). Penicillins, macrolides, lincosamides, fluoroquinolones, and tetracyclines are often used as first-line treatments. However, antimicrobial resistance has been emerging during the past years (3), which poses a great challenge not only to the swine industry but also to the public health.

Macrolide resistance is commonly mediated by *erm* genes and complemented by *mef* and *msr* genes (4, 5). The *erm(T)* gene was first identified in *Lactobacillus reuteri*; since then, it has been described in various Gram-positive organisms, including the genera *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Enterococcus*, and *Erysipelothrix*, and also in Gram-negative organisms, such as *Glaesserella (Haemophilus) parasuis* (6–11). In most

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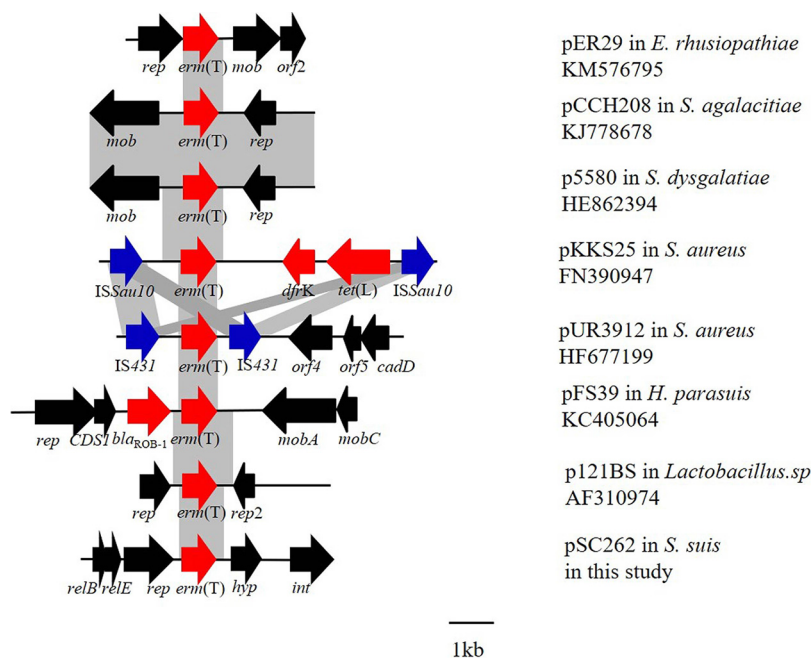


FIG 1 The structural comparison of the *erm(T)* gene in plasmid DNA from *S. suis* in this study with that from *E. rhusiopathiae*, *S. agalactiae*, *S. dysgalactiae*, *S. aureus*, *G. parasuis*, and *Lactobacillus* sp. Resistance genes are shown in red and other genes are shown in black.

cases, the *erm(T)* gene was located on broad host-range plasmids of variable sizes. In *Staphylococcus aureus*, the plasmid-borne *erm(T)* gene was flanked by two copies of IS431 elements (12), or together with other antimicrobial resistance genes, such as *tet(L)* and/or *dfxK*, flanked by two copies of ISSau10 (13). In addition, the *erm(T)* gene was also identified in the chromosomal DNA of *Streptococcus gallolyticus* subsp. *pasteurianus*, where it was flanked by two copies of IS1216V-like elements (14).

To date, the information about *erm(T)* in *S. suis* is still limited. Therefore, this study was initiated to analyze the presence and location of *erm(T)* in clinical *S. suis* isolates. In addition, the transferability and fitness cost of *erm(T)* among *S. suis* isolates were explored.

RESULTS AND DISCUSSION

Plasmid- and ICE-borne *erm(T)* genes were identified in *S. suis*. In our study, 2 *erm(T)*-positive *S. suis* isolates, SC262-ST954 and SC117-ST1314, out of 237 nonduplicate isolates were identified. In *S. suis* SC262, *erm(T)* was located on a small plasmid of 5,125 bp, which is similar to previously described *erm(T)*-carrying plasmids, such as pER29 from *Erysipelothrix rhusiopathiae* (KM576795), pCCH208 from *S. agalactiae* (KJ778678), p5580 from *S. dysgalactiae* (HE862394), pUR2940, pKKS25, and pUR3912 from *S. aureus* (HF583292, FN390947, and HF677199), pFS39 from *Glaesserella parasuis* (KC405064), and p121BS from *Lactobacillus* sp. (AF310974) (Fig. 1). The previous study identified a complete translational attenuator immediately upstream of the *erm(T)* gene on the plasmid pRW35 which consisted of two pairs of inverted-repeat sequences of 12 bp each and a reading frame for a regulatory peptide of 19 amino acids (aa) (15). Comparison of the *erm(T)* regulatory region of pSC262 with that of plasmid pRW35 (EU192194) revealed that the *erm(T)* regulatory region of pSC262 had many point mutations compared with that of pRW35, and only ribosomal binding sites RBS2 and IR2 could match perfectly (Fig. S1). The *rep* gene of plasmid pSC262 was compared with those deposited in NCBI GenBank, and a 58.85% identity with that of plasmid pPTDrAP from *S. aureus* was found, which may point toward the across-genus dissemination potential of this plasmid. As shown in Fig. 2, *erm(T)* is located on an ICE in *S. suis*

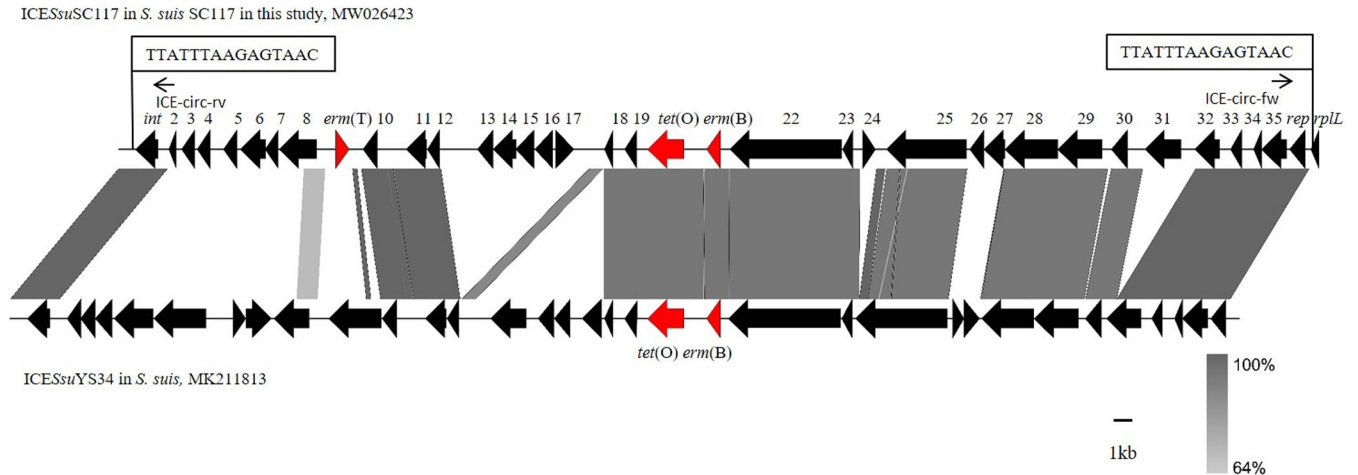


FIG 2 The structural comparison of *erm(T)*-carrying ICESsuSC117 in *S. suis* in this study with ICESsuYS34 in *S. suis* (MK211813) described previously. The locations of the PCR primers for the detection of circularizable forms of the ICE are indicated by arrows. The perfect 15 bp target site duplications at both termini are shown in boxes. Regions with more than 90% nucleotide sequence identity are shaded gray.

SC117, designated ICESsuSC117, which belongs to the ICESa2603 family of ICEs and has a size of 64,013 kb. ICESa2603 is a 54-kb ICE originally found in *S. agalactiae* 2603V/R (16). An ICE that carries an integrase gene closely related to $int_{ICESa2603}$, defined as having >60% gene or protein homology, and has significant sequence alignment (60% nucleic acid identity of core genes) and syntenic core structure was classified as a member of the ICESa2603 family (17). The nucleic acid homology between $int_{ICESsuSC117}$ and $int_{ICESa2603}$ is 94.55%. ICESsuSC117 had 95.90% identity and 55.00% coverage rate with the ICESa2603. In addition, it has a core structure similar to that of ICESa2603. Therefore, ICESsuSC117 was classified into the ICESa2603 family. The DNA sequence of ICESsuSC117 was compared with those deposited in the GenBank, and the BLASTn result indicated that it had 95.42% identity and 60.00% coverage rate with the ICESsuYS34 in *S. suis* (MK211813). The 65.361 kb ICESsuYS34 in the *S. suis* strain carried the resistance genes *erm(B)* and *tet(O)* but not *erm(T)*. ICESsuSC117 was inserted at *rplL* locus, which is one of the common insertion hot spots of mobile genetic elements (MGEs) in *S. suis*, forming perfect 15 bp target site duplications at both termini (5'-TTATTTAAGAGTAAC-3'). The 15 bp sequence (TTATTTAAGAGTAAC) at the 3' end of *rplL*, the insertion hot spot of ICEs, is highly conserved in streptococci (18). To verify the formation of circular ICESsuSC117 structures, specific primers (ICE-circ-fw/ICE-circ-rv) were designed, and then a 2,537 bp amplicon was detected, which confirmed the ability of ICESsuSC117 to excise from the *S. suis* chromosomal DNA and to form a circular intermediate. Similarly, the *erm(T)* upstream regulatory region of ICESsuSC117 was compared with that of plasmid pRW35. The results indicated that the *erm(T)* upstream regulatory region of ICESsuSC117 had 5 bp point mutations and 1 bp insertion compared to pRW35 in the regulatory peptide open reading frame (ORF). This 1 bp insertion resulted in a frameshift mutation, which extended the reading frame for the regulatory peptide from 19 aa to 28 aa (Fig. S2). The results of the test for inducible clindamycin resistance showed that *S. suis* strains SC262 and SC117 were resistant to both erythromycin and clindamycin, which revealed that the expression of *erm(T)* in pSC262 and ICESsuSC117 was constitutive.

The *erm(T)* gene can be transmissible. Transformation experiments indicated that both the *erm(T)*-carrying plasmid pSC262 and ICESsuSC117 are transmissible. The transformants P1/7+pSC262 and P1/7+ICESsuSC117 displayed the elevated MICs to the respective antimicrobial agents compared with those of the recipient strain (Table 1). WGS analysis indicated that the *erm(T)*-carrying ICESsuSC117 was entirely integrated into the *rplL* gene in the recipient strain, with 15 bp target duplications (5'-TTATTTAAGAGTAAC-3') immediately up- and downstream of ICESsuSC117 (Fig. 3). The

TABLE 1 MICs of the *erm(T)*-carrying strains, the recipient strain *S. suis* P1/7, *S. aureus*, and their transformants

Strain	MIC (mg/L) ^a							
	FFC	ERY	LIN	CLI	GEN	CHL	TET	SPE
SC262	64	512	128	64	<1	32	32	2
SC117	16	512	128	64	2	32	64	>512
P1/7	<1	<1	<1	<1	2	<1	<1	16
P1/7+pSC262	<1	512	128	64	2	<1	<1	16
P1/7+ICESsuSC117	<1	512	128	64	2	<1	32	16
RN4220	4	<1	<1	<1	<1	4	<1	16
RN4220+pSC262	4	512	128	64	<1	4	<1	16
D39	<1	<1	<1	<1	2	<1	<1	2
D39+pSC262	<1	512	128	64	2	<1	<1	2

^aFFC, florfenicol; ERY, erythromycin; LIN, lincomycin; CLI, clindamycin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; SPE, spectinomycin.

recipient *S. suis* P1/7 (ST1) and the donor *S. suis* SC117 (ST1314) were distinguished by multilocus sequence type (MLST).

Furthermore, pSC262 was successfully transferred into the recipient strain *S. aureus* RN4220 (RN4220+pSC262) and *Streptococcus pneumoniae* D39 (D39+pSC262) by electrotransformation (19), confirmed by antimicrobial susceptibility testing (AST) (Table 1) and PCR. Compared with the recipient strains, the transformants RN4220+pSC262 and D39+pSC262 displayed elevated MICs of erythromycin, clindamycin, and lincomycin (Table 1), which indicated that an *erm(T)*-carrying plasmid can replicate and express macrolide-lincosamide resistance in heterologous hosts, including *S. aureus* and *S. pneumoniae*.

Fitness cost analyses. The growth kinetics of P1/7, P1/7+pSC262, and P1/7+ICESsuSC117 in the antibiotic-free Todd-Hewitt broth (THB) were determined (Fig. 4A). The results showed no significant difference for the strains in the absence of selective pressure. However, competition experiments offered a more discriminative and precise measurement of fitness. From the second day on, an obvious decrease in the proportion of pSC262-carrying and ICESsuSC117-carrying strains was observed. At the 7th generation, the pSC262-carrying strain could not be detected (Fig. 4B), and the ICESsuSC117-carrying strain disappeared at the third generation (Fig. 4C). These findings suggest that the *erm(T)*-carrying strain had a fitness cost compared to *S. suis* P1/7, which will allow a susceptible strain to outcompete the resistant strain in the absence of a macrolide.

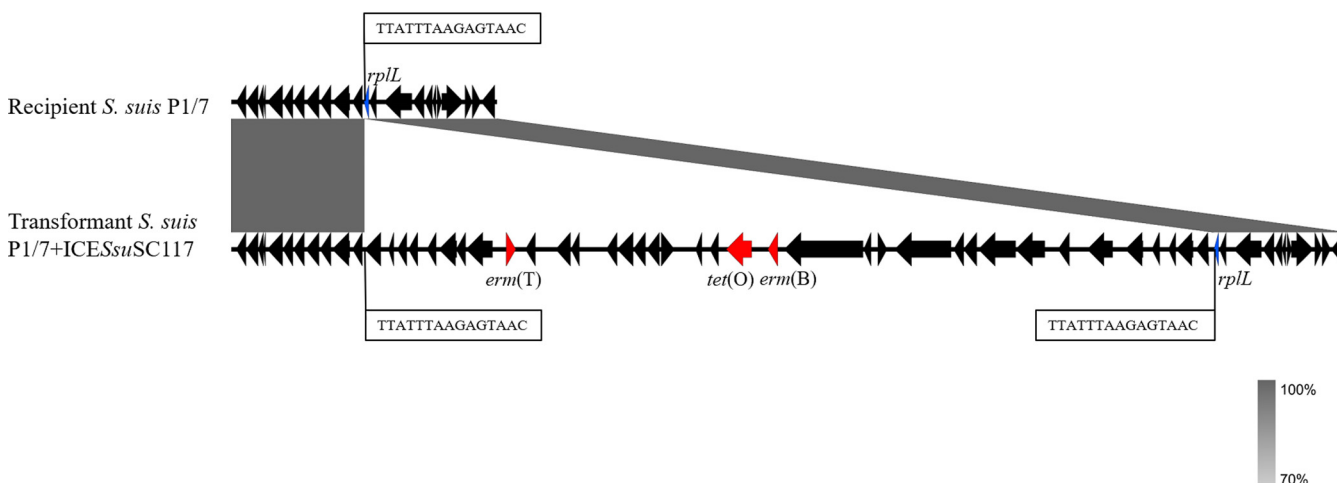


FIG 3 The structural comparison between the recipient *S. suis* P1/7 and the transformant *S. suis* P1/7+ICESsuSC117. The 15 bp target sites are shown in boxes. Regions with more than 70% nucleotide sequence identity are shaded gray. Resistance genes are shown in red, *rplL* genes are shown in blue, and other genes are shown in black.

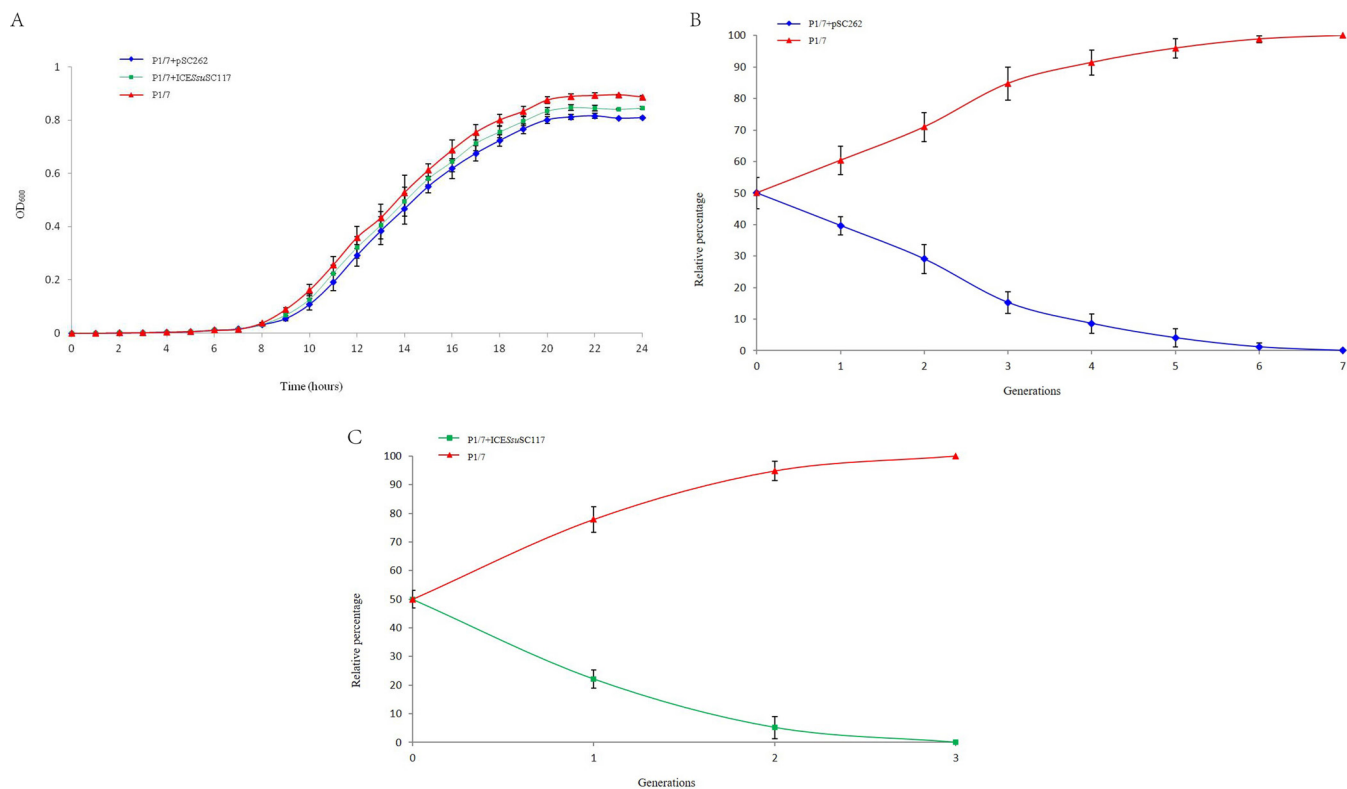


FIG 4 Fitness cost of pSC262 and ICESsuSC117 in *S. suis* in this study. (A) Comparison of the growth kinetics of strains *S. suis* P1/7, *S. suis* P1/7+pSC262, and *S. suis* P1/7+ICESsuSC117. (B) Growth competition between *S. suis* P1/7 and *S. suis* P1/7+pSC262. (C) Growth competition between *S. suis* P1/7 and *S. suis* P1/7+ICESsuSC117. The initial ratio of each strain in competition assay was 1:1.

The acquisition of resistance is generally thought to be accompanied by a fitness cost to the bacterium (20). Spread and maintenance of a resistance gene are directly linked to the fitness cost associated with the gene expression. The constitutive expression of *erm(T)* in both plasmid pSC262 and ICESsuSC117 observed in this study will increase the fitness cost of *erm(T)*-carrying mobile genetic elements in these *S. suis* isolates, which may explain the low detection rate of the *erm(T)* gene in the *S. suis* population.

MATERIALS AND METHODS

Bacterial strains and AST. A total of 237 nonduplicate *S. suis* strains were isolated and identified from individual diseased pigs in three provinces (Henan, Shanxi, and Guangdong) in China during 2010 to 2016. *S. suis* P1/7 and *S. pneumoniae* D39 served as the recipient strain in the transfer experiments (19). All strains were cultivated in THB at 37°C; the medium was supplemented with erythromycin (10 mg/L) for the selection of macrolide-resistant isolates. AST was performed by broth microdilution according to the recommendations given in the EUCAST breakpoint tables for interpretation of MICs and zone diameters, version 11.0 (21). The following antimicrobial agents were tested: florfenicol, erythromycin, lincomycin, clindamycin, gentamicin, chloramphenicol, tetracycline, and spectinomycin. *Streptococcus pneumoniae* ATCC 49619 served as the quality control strain. Simultaneously, the test for inducible clindamycin resistance in two *erm(T)*-positive *S. suis* isolates SC262 and SC117 was performed as described in CLSI document M100 to check whether the expression of *erm(T)* was inducible or constitutive (22).

PCR analysis. The *erm(T)* gene was detected in the *S. suis* strains by PCR using the primers *erm(T)*-fw, 5'-ATTGGTTCAGGGAAAGGTC-3', and *erm(T)*-rv, 5'-TGGATGAAAGTATTCTCTAGGG-3', and an annealing temperature of 53.5°C. The presence of circular intermediates in *S. suis* SC117 was detected by PCR using the primers ICE-circ-fw, 5'-TTGAACAGCCTAAAAGTGCCA-3', and ICE-circ-rv, 5'-GTAAAGACCAAACAAAGACTCCAG-3', and an annealing temperature of 59.0°C (Table S1).

WGS analysis. Whole-genome DNA of SC117 and SC262 was sequenced using the PacBio RS and Illumina MiSeq platforms (Shanghai Personal Biotechnology Co., Ltd., China). The PacBio sequence reads were assembled with HGAP4 and CANU (version 1.6) and then corrected by the Illumina MiSeq reads with pilon (version 1.22). The prediction of ORFs and their annotations were performed using Glimmer 3.0.

Intraspecies transformation. The transformation experiments were performed as described in a previous study (23). The peptide (GNWGTWVEE) was used as a pheromone for the transformation. The

detailed protocols for the transformation were as follows. The recipient strain P1/7 was grown to exponential phase at 37°C under 5% CO₂. Then, the logarithmic P1/7 strains were diluted 1:50 into Todd-Hewitt broth supplemented with yeast extract (THY) medium and grown to an optical density at 600 nm (OD₆₀₀) between 0.035 and 0.058 at 37°C without shaking. The donor DNA (chromosomal DNA, 1 μg or plasmid, 1.2 μg) and synthetic peptide (250 μM) were added to the 100 μL bacterial samples. After 2 h of incubation at 37°C under 5% CO₂, the samples were diluted, plated in THA plates with 5% sheep blood and 10 mg/L erythromycin, and incubated at 37°C overnight. Colonies were further confirmed by AST, 16s RNA sequencing, and MLST following harmonized protocols (<http://pubmlst.org/>) (Table S1).

Interspecies transformation. To investigate the replication ability of the *erm(T)*-carrying plasmid pSC262 in heterologous hosts, transformation assays were performed. Plasmid DNA was extracted by using the Qiagen plasmid extraction midi kit (Qiagen, Hilden, Germany) according to the following procedure. After the *S. suis* SC262 was suspended in buffer P1, lysozyme was added at a final concentration of 20 g/mL, and the mixture was incubated for 2 h at 37°C before buffer P2 was added. Transfer of the purified plasmid DNA was attempted with *S. aureus* RN4220 and *S. pneumoniae* D39 by electrotransformation. The transformants were selected on brain heart infusion (BHI) agar supplemented with 10 μg/mL erythromycin.

Fitness cost experiments. The growth kinetics of *S. suis* P1/7 and the two transformants P1/7+pSC262 and P1/7+ICESsuSC117 were determined. Cultures were grown for 24 h at 160 rpm and 37°C, and the absorbance at 600 nm was measured every hour.

The fitness cost of the plasmid pSC262 was determined by three independent competition experiments between P1/7 and P1/7+pSC262, and the fitness cost of the ICESsuSC117 was determined between P1/7 and P1/7+ICESsuSC117, as described previously (24). Strains were grown in THB for 16 h at 37°C. Then, 1 × 10⁸ CFU of P1/7 was mixed with 1 × 10⁸ CFU of P1/7+pSC262 or P1/7+ICESsuSC117, respectively. The mixtures were grown at 37°C and 160 rpm and diluted at 1:100 to fresh THB every 12 h. Before every dilution, samples were taken and plated onto antibiotic-free and erythromycin-containing THA plates simultaneously. The number of colonies growing on erythromycin plates was the number of drug-resistant bacteria in the mixed culture system. The number of colonies on the antibiotic-free plate minus the number of colonies on the erythromycin plate is the number of susceptible bacteria in the mixed culture system.

Data availability. The sequences of the plasmid pSC262 and the ICESsuSC117, determined in this study, have been deposited in GenBank under accession numbers CP06178 and [MW026423](https://doi.org/10.1101/026423), respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, DOCX file, 0.02 MB.

SUPPLEMENTAL FILE 2, DOCX file, 0.25 MB.

SUPPLEMENTAL FILE 3, DOCX file, 0.3 MB.

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