

Novel Plasmid-Borne Multidrug Resistance Gene Cluster Including *lsa(E)* from a Linezolid-Resistant *Enterococcus faecium* Isolate of Swine Origin

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A novel nonconjugative plasmid of 28,489 bp from a porcine linezolid-resistant *Enterococcus faecium* isolate was completely sequenced. This plasmid harbored a novel type of multiresistance gene cluster that comprised the resistance genes *lnu(B)*, *lsa(E)*, *spw*, *aadE*, *aphA3*, and two copies of *erm(B)*, which account for resistance to macrolides, lincosamides, streptogramins, pleuromutilins, streptomycin, spectinomycin, and kanamycin/neomycin. Structural comparisons suggested that this plasmid might have developed from other enterococcal plasmids by insertion element (IS)-mediated interplasmid recombination processes.

During recent years, several ABC transporters were identified in staphylococci, streptococci, and enterococci that confer combined resistance to pleuromutilins, lincosamides, and streptogramin A antibiotics (PLS_A). The corresponding genes are *vga(A)* and *vga(A)v* (1), *vga(C)* (2), *vga(E)* (3), *vga(E)v* (4), *eat(A)v* (5), *sal(A)* (6, 7), *lsa(A)* (8), *lsa(C)* (9), and *lsa(E)* (10). In contrast to the aforementioned genes, the gene *lsa(B)* confers only elevated MICs to lincosamides which, however, are below the clinical breakpoints for resistance (11). The *lsa(E)* gene has been identified as part of plasmid-borne or chromosomal multiresistance gene clusters in methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) *Staphylococcus aureus* (12–14), coagulase-negative or -variable staphylococci (7, 15), and *Enterococcus* spp. (16) of human and animal origin in Europe and Asia; in human *Streptococcus agalactiae* from South America (17); and, most recently, in *Erysipelothrix rhusiopathiae* of swine origin in China (18). It is believed that the basic type of these multiresistance gene clusters, which comprises the resistance genes *aadE*, *spw*, *lsa(E)*, and *lnu(B)*, has developed in *Enterococcus* spp. (10, 12, 13). In the present study, a nonconjugative plasmid from *Enterococcus faecium* that harbors a novel *lsa(E)*-carrying multiresistance gene cluster was identified and completely sequenced to gain insight into its structure and the genetic environment of *lsa(E)*.

Thirty-five enterococcal strains, including *Enterococcus faecalis* ($n = 21$), *Enterococcus faecium* ($n = 13$), and *Enterococcus gallinarum* ($n = 1$), were isolated from a pig farm in Guangxi province, China. These isolates were investigated for the presence of the *lsa(E)* gene by PCR using previously described primers (13). The *lsa(E)* gene was detected in five *E. faecalis* isolates and one isolate each of *E. faecium* and *E. gallinarum*. The *lsa(E)* nucleotide sequences of the seven isolates were identical to those of *lsa(E)* on plasmids pV7037 from MRSA ST9 and pXD4 from *E. faecium* (13, 16). The six *E. faecalis* and *E. faecium* isolates were analyzed by multilocus sequence typing (MLST; <http://www.mlst.net/>). Antimicrobial susceptibility testing by broth microdilution (19, 20) revealed that except for the PLS_A phenotype, all isolates were also resistant to erythromycin, tetracycline, streptomycin, gentamicin, kanamycin, and ciprofloxacin but susceptible to ampicillin and vancomycin. Additional resistance (or elevated MICs) to rifampin, florfenicol, or linezolid was seen in five or six of the isolates

(Table 1). Linezolid resistance was due to the previously described mutation at position 2576 (G2576T) in the 23S rRNA gene (21), while mutations in genes for the ribosomal proteins L3 and L4 or the *cfr* gene were detected by PCR and sequence analysis (22, 23).

Conjugations by filter mating and transformation experiments were conducted using *E. faecalis* JH2-2 as the recipient (24). The two *E. faecium* plasmids pXD4 and pN39 served as positive controls in transformation and conjugation experiments (16). Transconjugants and transformants were selected on brain heart infusion (BHI) agar supplemented with 10 mg/liter valnemulin and 50 mg/liter rifampin. Only one of the *lsa(E)*-positive enterococcal isolates, namely, *E. faecium* Y13, yielded a transformant after electrotransformation. This transformant (designated TY13) exhibited high MICs not only for tiamulin, valnemulin, lincomycin, and virginiamycin M1 but also for erythromycin, kanamycin, and streptomycin (Table 1). S1 nuclease pulsed-field gel electrophoresis (PFGE) combined with Southern blot analysis revealed that the *lsa(E)* gene was located on a ca. 28-kb plasmid, designated pY13. This plasmid was sequenced using the 454 Life Sciences GS FLX system (Roche), and sequence assembly was further confirmed by nine overlapping PCR assays (PCR1 to PCR9) (see Table S1 in the supplemental material). Plasmid sequence analyses, comparisons, and annotations were performed as previously described (23). The complete plasmid pY13 from *E. faecium* Y13 was 28,489 bp in size and contained 30 putative open reading frames

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TABLE 1 Antimicrobial susceptibility of the seven *lsa(E)*-carrying enterococcal isolates, a transformant, and a recipient strain used in this study

Strain	MLST type	MIC (mg/liter) for ^a :															
		GEN	KAN	VIR M1 ^b	STR	LIN	TIA	VAL	ERY	FFC	TET	CIP	AMP	RIF	VAN	LZD	
<i>E. faecalis</i> 11-27	ST169	128	>256	>128	128	256	128	128	128	>256	8	64	16	1	8	1	1
<i>E. faecalis</i> 12-7	ST220	>256	>256	>128	256	256	128	128	128	>256	128	64	32	1	4	1	8
<i>E. faecalis</i> 14-1	ST146	256	>256	>128	256	128	128	128	128	>256	128	128	64	1	16	2	16
<i>E. faecalis</i> E15	ST283	128	>256	>128	256	128	128	256	>256	128	128	64	2	32	2	16	
<i>E. faecalis</i> D12-2	ST553	128	>256	>128	128	128	>128	256	>256	128	64	64	1	2	2	16	
<i>E. gallinarum</i> Y15	NA ^c	256	>256	128	128	128	128	64	>256	128	64	64	0.5	8	2	8	
<i>E. faecium</i> Y13	ST29	>256	>256	128	256	128	>128	128	>256	64	64	64	1	2	0.5	16	
<i>E. faecalis</i> TY13 ^d		2	>256	128	256	128	>128	128	>256	4	4	1	1	1	≤0.5	2	
<i>E. faecalis</i> JH2-2		2	4	2	2	4	2	2	0.5	4	2	2	1	2	1	2	

^a GEN, gentamicin; STR, streptomycin; KAN, kanamycin; VIR M1, virginiamycin M1; LIN, lincomycin; TIA, tiamulin; VAL, valnemulin; ERY, erythromycin; FFC, florfenicol; TET, tetracycline; CIP, ciprofloxacin; AMP, ampicillin; RIF, rifampin; VAN, vancomycin; LZD, linezolid.

^b Note that *E. faecalis* is intrinsically resistant to streptogramins.

^c NA, not applicable.

^d TY13 was the transformant derived from transformation of plasmid pY13 from *E. faecium* Y13 into *E. faecalis* JH2-2.

(ORFs) (see Table S2 in the supplemental material). Based on the classification system for plasmids from enterococci and other Gram-positive bacteria described by Jensen et al. (25), plasmid pY13 is a member of the rep1 family of plasmids.

Sequence comparisons revealed a 12,461-bp region in pY13 that contained ORFs involved in plasmid replication, maintenance, and transfer and exhibited >99% identity to plasmid pM7M2 from a dairy-derived *E. faecium* isolate (26) (Fig. 1). Within this region, a 7,118-bp segment showed >99% identity to

the corresponding sequence of plasmid p3 from a vancomycin-resistant *E. faecium* ST203 isolate of human origin (27). Upstream of an *ISEfa8* element, a *prgOPN* gene cluster was detected. Previous studies demonstrated that the *prgOPN* gene cluster may represent a toxin-antitoxin-independent stabilization mechanism and may be involved in the persistence and distribution of antibiotic resistance plasmids (28, 29). The *prgP-prgO* genes may represent the most common partition cassettes in enterococci (30).

A region of 8,705 bp in pY13, which is bracketed on the left-

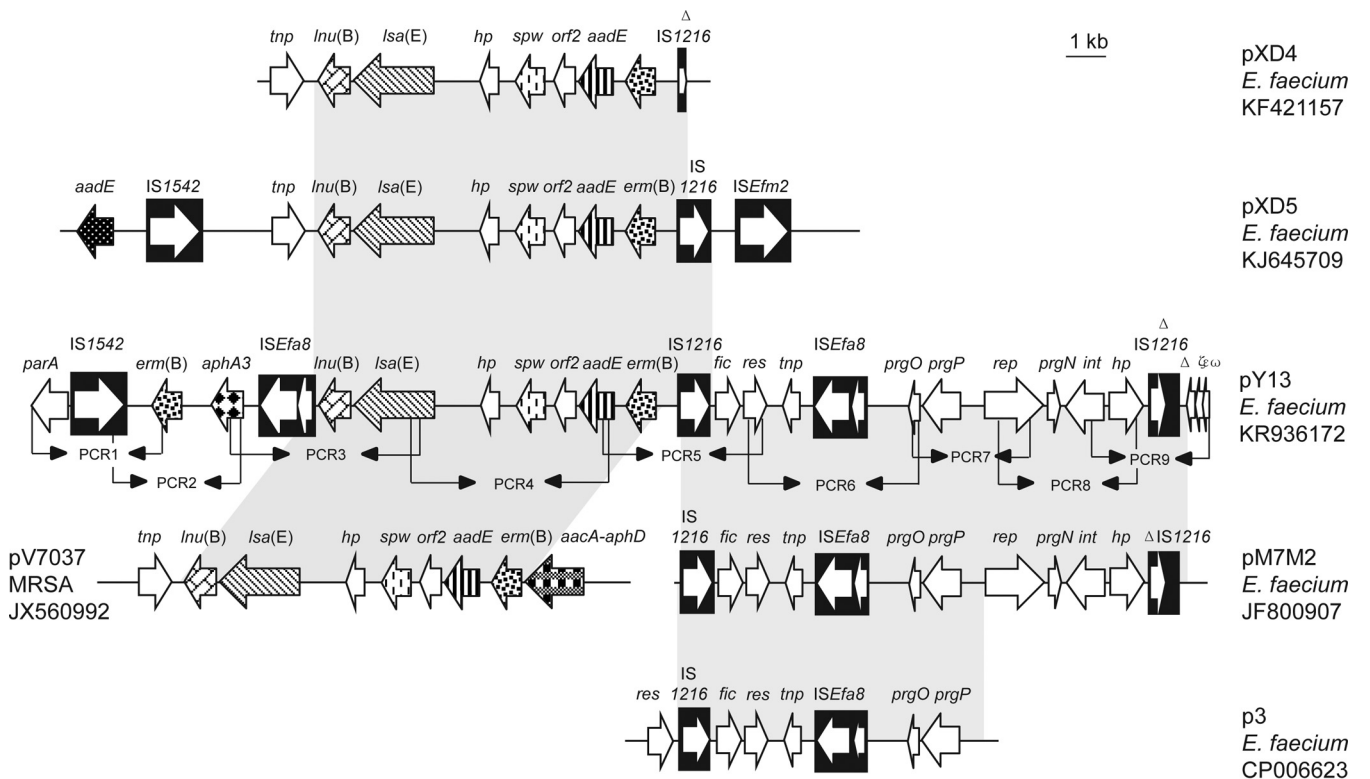


FIG 1 Linear comparison of the *lsa(E)*-carrying plasmid pY13 (GenBank accession no. KR936172) with enterococcal and staphylococcal plasmids pXD4, pXD5, pM7M2, pV7037, and p3. Arrows, positions and directions of transcription of the genes; shading, regions of >99% nucleotide sequence identity in the different plasmids; black arrowheads, the nine overlapping PCRs (PCR1 to PCR9) designed to confirm the pY13 plasmid and the genetic environment of the *lsa(E)* gene; Δ, a truncated gene.

hand side by an *ISEfa8* element and on the right-hand side by an *IS1216* element, contains the five antimicrobial resistance genes *lnu(B)* (lincosamide resistance), *Isa(E)* (PLS_A resistance), *spw* (spectinomycin resistance), *aadE* (streptomycin resistance), and *erm(B)* (macrolide-lincosamide-streptogramin B resistance). This region corresponded closely to the regions identified on plasmid pV7037 from MRSA ST9 (13) and on plasmids pXD4 (16) and pXD5 (31) from *E. faecium* (Fig. 1). Further downstream of *lnu(B)*, an *aphA3* gene for kanamycin/neomycin resistance and another copy of the *erm(B)* gene were detected in the pY13 sequence (Fig. 1). The MICs of the transformant TY13 (Table 1) indicated that all six antimicrobial resistance genes of plasmid pY13 are functionally active. Structural comparisons suggested that pY13 is composed of various segments, which have been found, at least in part, on other enterococcal or staphylococcal plasmids (Fig. 1). Since all of these segments are flanked by intact or truncated insertion sequences, it is possible that pY13 developed from interplasmidic recombination events in which insertion sequences, such as *ISEfa8*, *IS1216*, and *IS1542*, have been involved.

S1 nuclease-PFGE combined with Southern blotting revealed that the *Isa(E)* gene was also located on plasmids of ca. 28 kb in the remaining five *E. faecalis* isolates and on a plasmid of ca. 40 kb in the *E. gallinarum* isolate (see Fig. S1 in the supplemental material). To investigate whether a pY13-like plasmid is present in these isolates, eight overlapping PCRs were designed (Fig. 1, PCR1 to PCR9; see also Table S1 in the supplemental material) to amplify eight partly overlapping regions covering the entire sequence of pY13. Subsequently, the purified PCR products were cloned into the vector pEASY-T1 and then sequenced by primer walking (Invitrogen, Beijing, China). Results showed that all five *E. faecalis* strains carried virtually the same pY13-like plasmid except for a few nucleotide substitutions, whereas the *E. gallinarum* strain was only positive for PCR3 to PCR6. Although the reason(s) that these pY13-like plasmids were not transferrable by electrotransformation into *E. faecalis* JH2-2 remains to be elucidated, these observations suggested that pY13-like plasmids have spread among different *E. faecalis* strains and play a role in the dissemination of *Isa(E)*-carrying multiresistance gene clusters.

In conclusion, we report the first complete sequence of a *Isa(E)*-harboring plasmid from an *E. faecium* isolate of swine origin. Although nonconjugative, pY13-like plasmids may act as vectors in the dissemination of antimicrobial multiresistance within the Gram-positive gene pool.

Nucleotide sequence accession number.

The sequence of plasmid pY13 has been deposited in GenBank under accession no. KR936172. The sequences of the five pY13-like plasmids from *E. faecalis*, i.e., p11-27 (28,489 bp) (KT448817), pE15 (28,490 bp) (KT448821), p12-7 (28,491 bp) (KT448818), pD12 (28,491 bp) (KT448820), and p14-1 (28,492 bp) (KT448819), as well as the sequence of a 15,464-bp segment of plasmid pY15 (KT448822) from *E. gallinarum*, have also been deposited in GenBank.

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