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Emergence of plasmid-mediated oxazolidinone resistance gene *poxtA* from CC17 *Enterococcus faecium* of pig origin

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Objectives: To characterize the oxazolidinone resistance gene *poxtA* on broad-host-range Inc18 plasmids from CC17 *Enterococcus faecium* of pig origin.

Methods: Oxazolidinone-resistant *E. faecium* isolates were screened for the presence of *poxtA*. The *poxtA*-carrying isolates were characterized by antimicrobial susceptibility testing, conjugation, S1-PFGE and hybridization. The *poxtA*-carrying plasmids were completely sequenced and their instability was verified.

Results: Two individual CC17 *E. faecium* strains were positive for *poxtA*. S1-PFGE and hybridization revealed the presence of a *poxtA*-carrying plasmid of ~62 kb in both WZ27-2 and the transconjugant, while *poxtA*-carrying plasmids of different sizes were observed in QF25-1 and the transconjugant. The two *poxtA*-carrying plasmids, pC25-1 and pC27-2, belonged to the broad-host-range plasmids of the Inc18 family and carried *dfrG*, *aadE*, Δ*sat4*, *aph(3′)-III*, *erm*(B), *tet*(M), *tet*(L) and *fexB*. Plasmid pC27-2 was virtually identical to pC25-1, with minor differences. The calculated transfer frequency was $\sim 0.87\times10^{-8}$ and $\sim 1.03\times10^{-7}$ per recipient to plasmids pC25-1 and pC27-2, respectively. Instability assays of the region with four adjacent IS*1216E*s, which forms three IS*1216E* translocatable units, revealed the formation of a series of mosaic circular intermediates.

Conclusions: We report the emergence of the plasmid-mediated oxazolidinone resistance gene *poxtA* in *E. faecium* from different farms in China. Comparison of the *poxtA* genetic context suggests that IS*1216E* elements play an important role in the dissemination of *poxtA*. The co-occurrence of *poxtA* with other antimicrobial and heavy metal resistance genes on the broad-host-range plasmids of the Inc18 family may lead to the co-selection of *poxtA*, contributing to its persistence and accelerating its dissemination.

Introduction

Enterococcus faecium is one of the leading causes of nosocomial infections; it is known as an ESKAPE pathogen, together with *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species.[1] The hospital-associated lineage of *E. faecium*, known as CC17, has been responsible for the majority of the hospital burden since the 1980s and likely emerged from animal strains ~80 years ago as a result of the use of antimicrobials in animal feed.[2–4] Resistance to ampicillin and fluoroquinolones is quite common and vancomycin-resistant *E. faecium* represents up to 80% of CC17 *E. faecium* strains in some hospitals.[4–6]

Oxazolidinones, including linezolid and tedizolid, are highly effective against infections of clinically important Gram-positive pathogens exemplified by VRE and MRSA. However, mutation in domain V of the 23S rRNA, most frequently as G2576U, was found to be associated with resistance to linezolid.[7] Moreover, the multiresistance gene *cfr* was reported to confer resistance to linezolid as well as phenicols, lincosamides, pleuromutilins and streptogramin A in enterococci and other bacteria.[8,9] Subsequently, two *cfr* variant genes, *cfr*(B) and *cfr*(C), were identified in *Clostridioides* (*Clostridium*) *difficile*, *E. faecium* and *Campylobacter*.[10–12] In 2015, the second plasmid-borne transferable oxazolidinone resistance gene *optrA* was found to mediate resistance to oxazolidinones, both linezolid and tedizolid, and phenicols, such as chloramphenicol and florfenicol, in enterococci of both human and animal origin in China.[13] Shortly after, the *optrA* gene was disseminated among enterococci and other Gram-positive bacteria worldwide.[14–16]

The recently described *poxtA* gene in a clinical MRSA strain was characterized to be related to decreased susceptibility to linezolid, phenicols and tetracyclines.[17] The *poxtA* gene encodes an ABC superfamily protein of F lineage associated with antibiotic resistance (ARE ABC-F) and distantly related to OptrA.[17] As reported for *cfr*, *optrA* and other genes mediating resistance to phenicols and

other ribosomal-targeted drugs that are broadly used in veterinary medicine,[9,15,18] the *poxtA* gene was suggested to have occurred in the animal setting under selection.[17,19]

In this study, we investigated florfenicol- and linezolid-resistant *E. faecium* isolates with unidentified resistance mechanisms. Two strains of CC17 from different farms were found to harbour the *poxtA* gene on Inc18 plasmids and to be able to undergo conjugative transfer to other *Enterococcus* strains under laboratory conditions.

Materials and methods

Bacterial isolations and detection of oxazolidinone resistance genes

E. faecium isolates QF25-1 and WZ27-2 were recovered from swine faecal samples from two different farms in Jiangsu, China, 2017. Strains were identified by PCR using primers targeting the 16S rRNA gene as previously described.[20] PCR was used to determine the presence of oxazolidinone resistance genes *cfr*, *cfr*(B), *cfr*(C) and *optrA*. An internal PCR was designed to detect the presence of the *poxtA* gene using specific primers *poxtA*-F and *poxtA*-R with an expected PCR product 1336 bp in size (Table S1, available as Supplementary data at *JAC* Online).

Antimicrobial susceptibility testing

MICs of 16 antibiotics for the two isolates and their transconjugants were determined by the broth microdilution method using *S. aureus* ATCC 25923 as quality control. The results were interpreted according to the EUCAST guideline (http://www.eucast.org) and CLSI document VET08. The antimicrobial agents used in this study are shown in Table 1.

Conjugation assays

Conjugation assays were performed by filter mating as described previously[21] and *Enterococcus faecalis* JH2-2 (linezolid MIC=0.5 mg/L) was used as the recipient. Donor and recipient strains were mixed at a ratio of 1:5 on a nitrocellulose membrane. Selection of transconjugants was performed on tryptic soy agar (TSA, Qingdao Hope Bio-Technology) containing 2 mg/L linezolid, 100 mg/L rifampicin and 100 mg/L fusidic acid. The transfer frequency in conjugation was calculated based on the number of observed transconjugants divided by the initial number of recipients.

S1-PFGE and hybridization

To determine the location of the *poxtA* gene and the sizes of the *poxtA*-carrying plasmids, both the donors and the related transconjugants were subjected to S1-nuclease PFGE,[22] followed by DNA hybridization analysis using a *poxtA* probe with specific primers (Table S1).

Genome sequencing and other analyses

The genomic DNA was purified and subjected to sequencing with the Illumina HiSeq2000 platform and nanopore long-read sequencing technology at Shanghai Biozeron Co., Ltd. The plasmids were annotated using the Rapid Annotation of microbial genomes using Subsystems Technology (RAST) annotation server (http://rast.nmpdr.org/).[23] Acquired resistance genes were identified in the genomes using ResFinder 3.1.[24] A linear schematic diagram for the two plasmids and genetic comparison of the *poxtA* gene from different species was generated using Easyfig 2.2.2.[25]

Plasmid instability assay

To determine the formation of potential translocatable units (TUs) resulting from IS*1216E*-mediated excision, a series of PCR and inverse PCR experiments were performed using specifically designed primers P1–P8 (Table S1).

Nucleotide sequence accession number

The complete sequences of plasmids pC25-1 and pC27-2 have been deposited in GenBank under accession numbers MH784601 and MH784602, respectively.

Results and discussion

Among the linezolid-resistant *E. faecium* isolates, seven strains showed no chromosome mutations for linezolid and were negative for *cfr*, *cfr*(B), *cfr*(C) and *optrA*. Four of the isolates were positive for *poxtA*. A conjugation assay was performed for the four *poxtA*-positive strains. Two strains, QF25-1 and WZ27-2, were able to transfer linezolid resistance to *E. faecalis* JH2-2. PCR and WGS analysis confirmed the presence of *poxtA* in both donors and transconjugants. S1-PFGE and Southern blot analysis revealed the presence of a *poxtA*-carrying plasmid of ~62 kb, designated pC27-2, in both the original WZ27-2 and the transconjugant *E. faecalis* JH2-2/pC27-2 (Figure 1). However, two *poxtA*-carrying plasmids of different sizes, ~22 and ~68 kb, the latter designated pC25-1, were observed in QF25-1 and the transconjugant *E. faecalis* JH2-2/pC25-1, respectively (Figure 1). These results suggest that the transfer of pC25-1 was associated with recombination events.[26] Our study showed that the *poxtA* gene is located in conjugative plasmids in *E. faecium* of porcine origin.[19]

In addition, the two *poxtA*-transferable *E. faecium* isolates exhibited resistance or reduced susceptibility to chloramphenicol, florfenicol, linezolid, tetracycline, doxycycline, streptomycin, kanamycin, erythromycin, sulfadimidine, bacitracin, penicillin and ampicillin, with QF25-1 additionally resistant to gentamicin (Table 1). WGS analysis of QF25-1 revealed the presence of additional resistance genes, including the phenicol exporter gene *fexB* and inactivating enzyme gene *cat*, the tetracycline resistance genes *tet*(M) and *tet*(L), the aminoglycoside-modifying enzyme genes *aac(6′)-Ii*, *aac(6′)-aph(2″)*, *aadE*, Δ*sat4* and *aph(3′)-III*, the macrolide-lincosamide-streptogramin B resistance gene *erm*(B), the sulphonamide resistance gene *dfrG* and the bacitracin resistance locus *bcrABDR* (Table 1). Although isolate WZ27-2 lacked resistance genes *aac(6′)-aph(2″)*, *cat* and *bcrABDR*, it carried another phenicol exporter gene, *fexA*, when compared with strain QF25-1 (Table 1). MLST showed that both isolates were assigned to the same sequence type, ST29, which belonged to the hospital-adapted CC17 *E. faecium*, posing an increased risk of zoonotic transfer to humans.[5] WGS phylogenetic analysis showed that QF25-1 and WZ27-2 were grouped in the CC17 clade but located in different small clusters, although they were highly similar, with the same MLST ST (Figure S1).

Antimicrobial susceptibilities of the transconjugants *E. faecalis* JH2-2/pC25-1 and *E. faecalis* JH2-2/pC27-2 revealed drastically increased MICs of chloramphenicol, florfenicol, linezolid, tetracycline, doxycycline, streptomycin, kanamycin and erythromycin (Table 1). Consistent with the resistance phenotypes, we observed co-transfer of *poxtA* with *dfrG*, *aadE*, Δ*sat4*, *aph(3′)-III*, *erm*(B), *tet*(M), *tet*(L) and *fexB* by plasmids pC25-1 and pC27-2 to *E. faecalis*

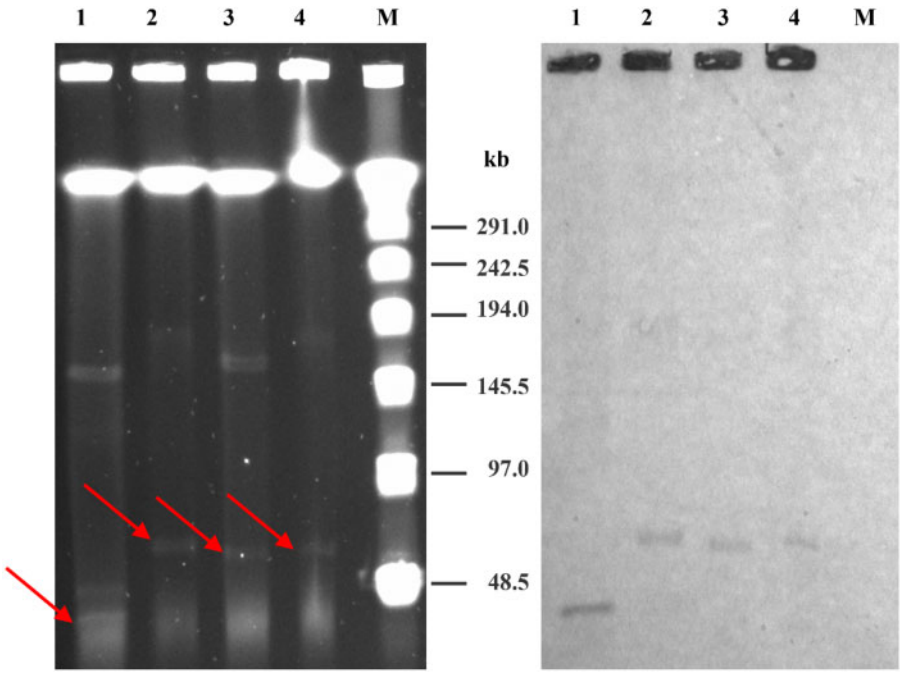

Figure 1. S1-PFGE (left panel) and Southern-blot hybridization (right panel) of the *poxtA*-carrying *E. faecium* isolates and the transconjugants. Locations of the *poxtA*-carrying plasmids are highlighted by red arrows in the left-hand panel. Lane 1, *E. faecium* QF25-1; lane 2, transconjugant *E. faecalis* JH2-2/pC25-1; lane 3, *E. faecium* WZ27-2; lane 4, transconjugant *E. faecalis* JH2-2/pC27-2. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1. Characterization of *poxtA*-positive strains and their transconjugants

Antimicrobial group/agent	JH2-2[a]	QF25-1[a]	Transconjugant C25-1 (JH2-2/pC25-1)[a,b]		WZ27-2[a]	Transconjugant C27-2 (JH2-2/pC27-2)[a,b]	
Phenicols				*cat*, ***fexB***, ***poxtA***			*fexA*, ***fexB***, ***poxtA***
chloramphenicol	2	32	8		32	8	
florfenicol	2	32	16		32	8	
Oxazolidinones				***poxtA***			***poxtA***
linezolid	0.5	4	4		16	8	
tedizolid	0.25	0.25	0.25		0.5	0.25	
Tetracyclines				***tet*(M)**, ***tet*(L)**, ***poxtA***			***tet*(M)**, ***tet*(L)**, ***poxtA***
tetracycline	2	128	128		256	128	
doxycycline	0.5	64	64		64	64	
Aminoglycosides				*aac(6′)-aph(2″)*, *aac(6′)-Ii*,			*aac(6′)-Ii*, ***aadE***,
gentamicin[c]	–	HLGR	–	***aadE***, **Δ*sat4***, ***aphA-3***	–	–	**Δ*sat4***, ***aphA-3***
streptomycin[c]	–	HLSR	HLSR		HLSR	HLSR	
kanamycin	64	>256	>256		>256	>256	
Erythromycin	0.5	>256	>256	***erm*(B)**	>256	>256	***erm*(B)**
Sulfadimidine	>256	>256	>256	***dfrG***	>256	>256	***dfrG***
Bacitracin	32	>256	32	*bcrABDR*	32	32	
Penicillin	1	32	1		16	1	
Ampicillin	4	256	4		32	8	
Fusidic acid	>256	4	>256		8	>256	
Rifampicin	>256	2	>256		0.5	>256	

[a]MICs (mg/L) shaded grey represent strains that were resistant to the corresponding antimicrobial agents.
[b]Antimicrobial resistance genes transferred by plasmids are shown in bold.
[c]HLGR, high-level gentamicin resistance; HLSR, high-level streptomycin resistance; –, not HLGR/HLSR.

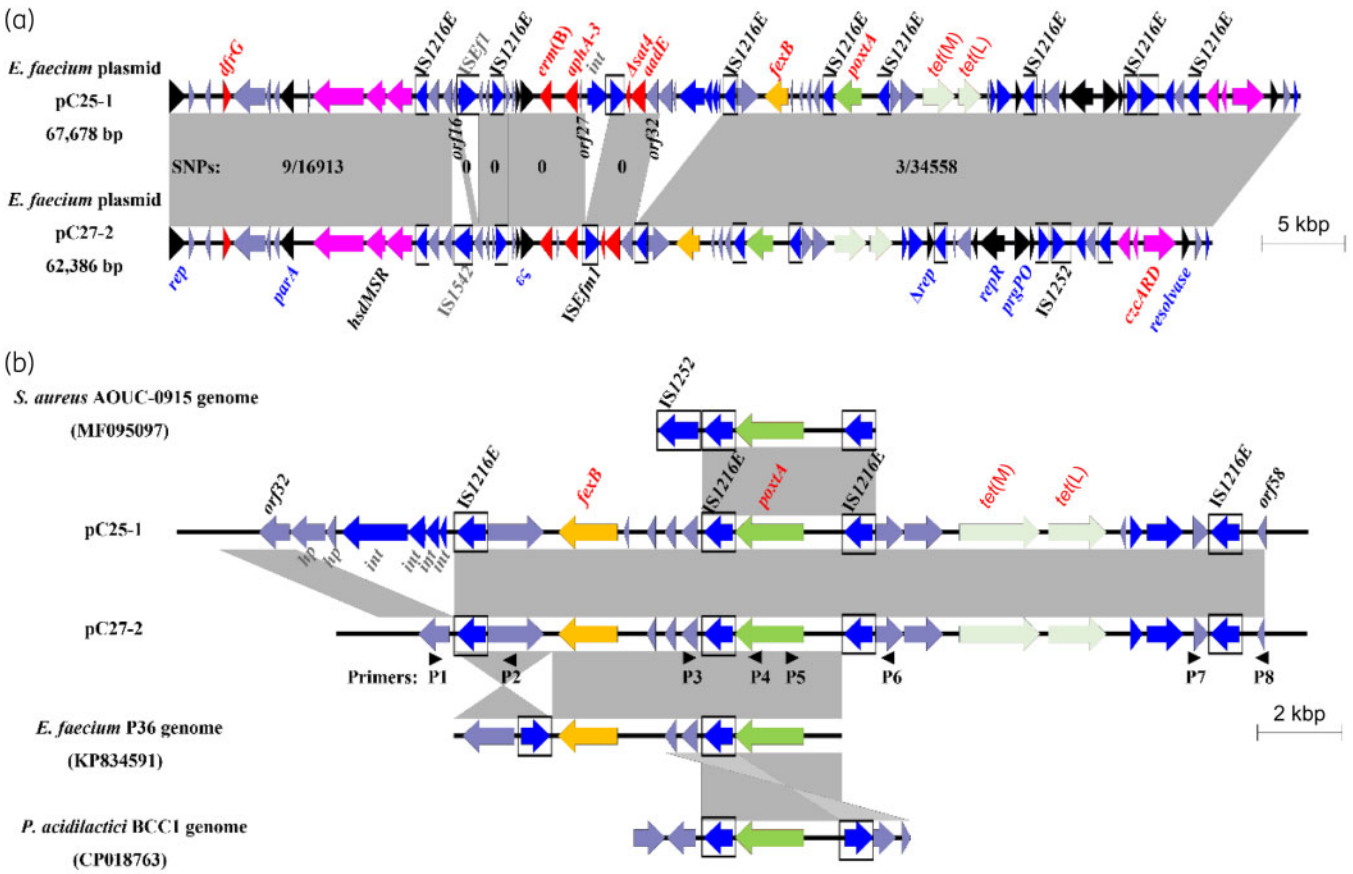

Figure 2. (a) Linear schematic diagram of plasmids pC25-1 and pC27-2. (b) Comparison of the genetic contexts of *poxtA* in plasmids pC25-1 and pC27-2 with corresponding sequences in the genomes of *S. aureus* AOUC-0915 (MF095097) and *E. faecium* P36 (KP834591.1). Arrows indicate the positions and orientations of the genes. Blue arrows represent genes for transposase/integrase/recombinase, black arrows represent genes conserved in Inc18 plasmids or having other functions, red/orange/green/light green arrows represent resistance genes, pink arrows represent the restriction-modification genes *hsdMSR* and the heavy metal resistance locus *czcARD*, and grey arrows represent other genes. Areas shaded grey represent regions of >80% nucleotide sequence identity. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

JH2-2 (Table 1). The calculated transfer frequency was $\sim 0.87\times10^{-8}$ and $\sim 1.03\times10^{-7}$ per recipient to plasmids pC25-1 and pC27-2, respectively.

To determine the complete sequences of these *poxtA*-bearing plasmids, the transconjugants were further sequenced using MinION long-read sequencing technology. Plasmid pC25-1 is 67678 bp in length and encodes 74 predicted ORFs (Table S2). Plasmid pC27-2 has a size of 62386 bp and is virtually identical to pC25-1, with minor differences (Figure 2a). An IS*1542* gene was absent downstream of *orf16* in pC25-1, but present in pC27-2, and three fragments of IS*Ef1*, an integrase gene and six genes (four integrases and two genes encoding hypothetical proteins) were inserted upstream of *orf16*, *orf27* and *orf32*, respectively, in pC25-1 when compared with pC27-2. BLASTP analysis of the pC25-1/pC27-2 ORFs showed the presence of a replication initiator protein (RIP) RepR carrying a primase domain, PriCT_1 (pfam08708). The *repR* gene (nt 48466–49959 in pC27-2) was identical to *repR* (CDS1) of pRE25 and belongs to the rep_2 family, allowing identification of pC25-1 and pC27-2 as broad-host-range Inc18-family plasmids.[27] Both plasmids contained two extra RIPs belonging to the Rep_3 family (pfam01051) and truncated Rep_1 family (COG5655), possessing a mosaicism of Inc18 plasmid structure.[28] Other characteristic proteins of the Inc18 family, e.g. the post-segregational killing systems, $\epsilon\varsigma$, which could be responsible for maintaining the plasmid in the enterococcal population, and type I partition cassette *prgPO* were also present (Figure 2a). Inc18 plasmids have contributed to widespread antimicrobial resistance to macrolides, chloramphenicol, aminoglycosides and glycopeptides among enterococci and other Gram-positive and Gram-negative bacteria.[28,29] However, the transfer system genes were absent in these two plasmids. Nevertheless, S1-PFGE revealed the presence of two additional plasmids of ~167 and ~46 kb in QF25-1 and one plasmid of ~172 kb in WZ27-2 (Figure 1). Further genome sequencing analysis showed that the ~167 kb plasmid in QF25-1 and the ~172 kb plasmid in W27-2 contained the conjugation transfer system. These results suggest the transfer of pC25-1/pC27-2 to *E. faecalis* JH2-2 was mobilized by other conjugative plasmids present in the donor strains.[26]

The plasmid-borne *poxtA* gene in pC25-1/pC27-2 was 100% identical to the corresponding gene in the genomes of *S. aureus* AOUC-0915 (MF095097), *E. faecium* P36 (KP834591), *E. faecalis* 599 (JH805640), *E. faecalis* 12 (JTKT01000026) and *Pediococcus*

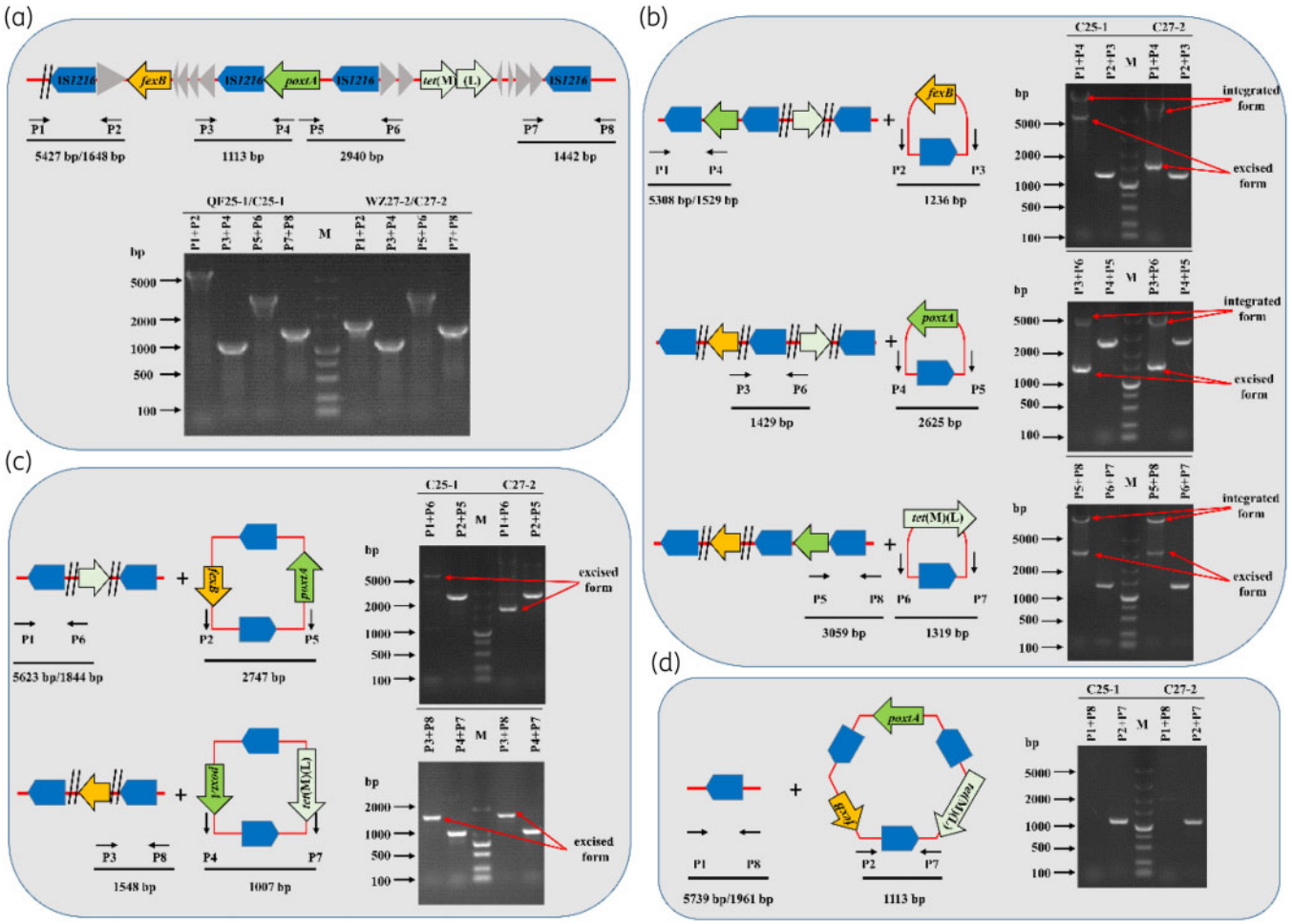

Figure 3. Instability assays of the region with four adjacent IS*1216E*s, which forms three IS*1216E* TUs and carried the resistance genes *fexB*, *poxtA*, *tet*(M) and *tet*(L). (a) Integrated form of IS*1216E* TUs. (b) Excised form and the circular intermediates of three simple IS*1216E* TUs. (c) Excised form and the circular intermediates of the two adjacent IS*1216E* TUs. (d) Excised form and the circular intermediate of all three IS*1216E* TUs. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

acidilactici BCC1 (CP018763). Analysis of the genetic context of *poxtA* revealed the presence of two IS*1216E* ISs flanking it both upstream and downstream in the same orientation, which is conserved in other available sequences containing *poxtA* (Figure 2b).

Upstream of the IS*1216E*-*poxtA*-IS*1216*E segment, a 7866 bp segment that contained *tet*(M) and *tet*(L) exhibited >99% identity to a variety of plasmids [e.g. *E. faecalis* pLAG (KY264168)] and chromosomal sequences [e.g. *S. aureus* AR_0470 (CP029653) and *Streptococcus suis* BM407 (FM252032.1)]. A heavy metal (cobalt, zinc and cadmium) resistance locus, *czcARD*,[30] was found located ~20 kb upstream of the *poxtA* gene (Figure 2a). The presence of heavy metal resistance genes could be responsible for the co-selection in the enterococcal population of the *poxtA* gene, even in the absence of antibiotics belonging to the families of the phenicols, oxazolidinones and tetracyclines. Downstream of the IS*1216E*-*poxtA*-IS*1216*E segment, a 5859 bp segment carrying *fexB* was virtually identical to *E. faecalis* pEF-01 (CP002208) and *E. faecium* pEFM-1 (JN201336). Further downstream, an 8369 bp segment that harboured *aadE*, Δ*sat4*, *aph(3′)-III* and *erm*(B) showed best identity (>99%) to a region found in *E. faecium* plasmid p3 (CP006623). A 3291 bp region containing *dfrG*, which is responsible for sulphonamide resistance, exhibited >99.9% identity to corresponding sequences in a variety of Gram-positive bacteria.[31,32]

IS*1216E* belongs to the IS*6* family and is often enriched in Inc18-family plasmids, which have contributed remarkably to the spread of the macrolide–lincosamide–streptogramin B resistance genes *erm*(B) and *erm*(T), the tetracycline resistance gene *tet*(S), the aminoglycoside resistance genes *aadE-sat4-aphA3*, the vancomycin resistance gene *vanA* and, more recently, the oxazolidinone resistance gene *optrA*.[28,29,33] Moreover, IS*1216E* is also responsible for the development of mosaic plasmids.[26] In the two *poxtA*-carrying plasmids, there was a total of eight copies of IS*1216E* in the same orientation, highlighting the important role of IS*1216E* in the spread of *poxtA* and pC25-1/pC27-2 mosaicism. Therefore, we first performed an inverse PCR using TU-*poxtA*-inv-F and TU-*poxtA*-inv-R and amplified a product of 2625 bp. Sequence analysis confirmed the formation of a circular intermediate of IS*1216E*-*poxtA* in both plasmids (Figure 3). This observation indicates that the IS*1216E*-*poxtA* segment could be excised via

IS*1216E*-mediated recombination and may facilitate dissemination to different genetic elements and different bacterial species.[17] We then performed a series of PCR and inverse PCR experiments to verify the instability of the region with four adjacent IS*1216E*s, which forms three IS*1216E* TUs[34] and carried the resistance genes *fexB*, *poxtA* and *tet*(M)(L) (Figure 2b). As expected, all three IS*1216E* TUs could be integrated into plasmids (Figure 3a) or excised from the plasmids (Figure 3b). What is perhaps even more surprising is that the adjacent two IS*1216E* TUs and all three IS*1216E* TUs could be excised from the plasmids as a whole (Figure 3c and d). Thus, the *poxtA* gene and other resistance genes may be disseminated among different Gram-positive bacteria via IS*1216*-mediated recombination events.

In conclusion, the isolation of oxazolidinone gene *poxtA* in hospital-adapted CC17 *E. faecium* of pig origin indicates an increased risk of zoonotic transfer to humans. Moreover, the co-occurrence of *poxtA* with other antimicrobial and heavy metal resistance genes on broad-host-range plasmids of the Inc18 family may lead to the co-selection of *poxtA*, contributing to its persistence and accelerating its dissemination. Attention should be paid to the potential risks of the transfer of the plasmid-borne *poxtA* from *E. faecium* to other Gram-positive bacteria, which warrants further evaluation of the use of phenicols and tetracyclines in food-producing animals and surveillance for prevalence of this gene.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online.

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