Identification of plasmids co-carrying cfr(D)/optrA and cfr(D2)/poxtA linezolid resistance genes in two Enterococcus avium isolates from swine brain

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Abstract:	Oxazolidinones are critically important antibiotics to treat human infections caused by multidrug-resistant bacteria, therefore the occurrence of linezolid-resistant enterococci from food-producing animals poses a serious risk to human health. In this study, Enterococcus avium 38157 and 44917 strains, isolated from the brain of two unrelated piglets, were found to carry the linezolid resistance genes cfr(D)-optrA, and cfr(D2)-poxtA, respectively. Whole genome sequencing analysis of E. avium 38157 revealed that the genes were co-located on the 36.5-kb pEa_cfr(D)-optrA plasmid showing high identity with the pAT02-c of Enterococcus faecium AT02 from pet food. The optrA region, was 99% identical to the one of the pAv-optrA plasmid from a bovine Aerococcus viridans strain, whereas the cfr(D) genetic context was identical to that of the plasmid 2 of E. faecium 15-307.1. pEa_cfr(D)-optrA was not transferable to enterococcal recipients. In E. avium 44917 a cfr(D)-like gene, named cfr(D2), and the poxtA gene were co-located on the transferable 42.6-kb pEa-cfr(D2)-poxtA plasmid 97% identical to the Tn6349 transposon of the human MRSA AOUC-0915. The cfr(D2) genetic context, fully replaced the Tn6644 that in S. aureus AOUC-0915 harbor the cfr gene. In conclusion, this is, the best of our knowledge, the first report of the new cfr(D2) gene variant. The occurrence of plasmids co-carrying two linezolid resistance genes in enterococci from food-producing animals needs close surveillance to prevent their spread to human pathogens.
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Dear Sir,

Please find attached a manuscript, submitted electronically via the Journal's online submission system, that I should like to be considered for publication as Short communication in the *Veterinary Microbiology* journal.

The manuscript contains a colour figure that, if the article is accepted for publication, can appear in black and white in the printed version and in colour in the online version.

Thank you very much for your time and consideration.

Yours sincerely,

Andrea Brenciani, Ph.D.
Assistant Professor in Microbiology

Highlights

1- *Enterococcus avium* strains isolated from two different swine brain
2- Characterization of 2 plasmids containing the *cfr*(D)/*optrA* and *cfr*(D2)/*poxtA* genes
3- Identification of a new *cfr*(D) variant, here named *cfr*(D2)
4- Spread of plasmids co-carrying oxazolidinones resistance genes in enterococci

Short communication

Identification of plasmids co-carrying *cfr*(D)/*optrA* and *cfr*(D2)/*poxtA* linezolid resistance genes in two *Enterococcus avium* isolates from swine brain

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ABSTRACT

Oxazolidinones are critically important antibiotics to treat human infections caused by multidrug-resistant bacteria, therefore the occurrence of linezolid-resistant enterococci from food-producing animals poses a serious risk to human health.

In this study, *Enterococcus avium* 38157 and 44917 strains, isolated from the brain of two unrelated piglets, were found to carry the linezolid resistance genes *cfr*(D)-*optrA*, and *cfr*(D2)-*poxtA*, respectively.

Whole genome sequencing analysis of *E. avium* 38157 revealed that the genes were co-located on the 36.5-kb pEa_*cfr*(D)-*optrA* plasmid showing high identity with the pAT02-c of *Enterococcus faecium* AT02 from pet food. The *optrA* region, was 99% identical to the one of the pAv-*optrA* plasmid from a bovine *Aerococcus viridans* strain, whereas the *cfr*(D) genetic context was identical to that of the plasmid 2 of *E. faecium* 15-307.1. pEa_*cfr*(D)-*optrA* was not transferable to enterococcal recipients.

In *E. avium* 44917 a *cfr*(D)-like gene, named *cfr*(D2), and the *poxtA* gene were co-located on the transferable 42.6-kb pEa-*cfr*(D2)-*poxtA* plasmid 97% identical to the Tn*6349* transposon of the human MRSA AOUC-0915. The *cfr*(D2) genetic context, fully replaced the Tn*6644* that in *S. aureus* AOUC-0915 harbor the *cfr* gene.

In conclusion, this is, the best of our knowledge, the first report of the new *cfr*(D2) gene variant. The occurrence of plasmids co-carrying two linezolid resistance genes in enterococci from food-producing animals needs close surveillance to prevent their spread to human pathogens.

1. Introduction

Members of the genus *Enterococcus*, are Gram-positive bacteria, common inhabitants of the gastrointestinal tract of healthy humans and animals. *Enterococcus faecium* and *Enterococcus faecalis* are recognized as the leading cause of nosocomial infections worldwide (Arias et al., 2012). Other enterococcal species, including *Enterococcus avium*, are only sporadically associated with human infections. *E. avium*, commonly isolated from chicken feces (Patel et al., 1993), can be responsible for human infections including bacteremia, peritonitis, intracranial suppurative infection and osteomyelitis (Yu et al., 2019).

Enterococci often cause severe human infections difficult to treat due to the spread of resistance to several antibiotics including last-resort ones such as oxazolidinones with serious implications for human health.

Oxazolidinones − linezolid and tedizolid − inhibit bacterial protein synthesis by interacting with the 23S rRNA of the 50S ribosomal subunit (Wilson et al., 2008). Linezolid resistance can develop through mutations in the 23S rRNA and ribosomal proteins L3 and L4, but also as a result of the acquisition of transferable resistance genes: *cfr*, *cfr*(B), *cfr*(C), *cfr*(D), *cfr*(E), *optrA*, *poxtA* and *poxtA2* (Brenciani et al., 2022). The MDR *cfr* gene and its variants encode methyltransferases that add a methyl group to the 23S rRNA; this post-transcriptional methylation confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilines and streptogramin A ($PhLOPS_A$ phenotype) (Long et al., 2006). *oprtA*, *poxtA*, and *poxtA2* encode ABC-F proteins resulting in resistance to phenicols and oxazolidinones (including tedizolid) by a ribosomal protection mechanism (Crowe-McAuliffe et al., 2022).

The linezolid resistance genes are often located on mobile genetic elements responsible for their spread in enterococci (Brenciani et al., 2022).

Despite oxazolidinones are never been licensed for use in veterinary medicine, the occurrence of linezolid resistance, potentially transmissible to humans through the food chain, was increasingly reported in enterococci from food-producing animals (Schwarz et al., 2021; Fioriti et al.; 2020; Coccitto et al., 2022). The spread of linezolid resistance genes in the animal setting could occurred owing to extensive use of phenicols in veterinary medicine with serious consequences for human health (Wang et al., 2020; Cinthi et al., 2022).

Few reports are available in literature on oxazolidinone resistance in *E. avium* species (Fioriti et al., 2020; Chen et al., 2020).

In this study, we characterised two plasmids co-carrying *cfr*(D)/*optrA* and *cfr*(D2) /*poxtA* linezolid resistance genes from two *E. avium* strains isolated from swine brain. To the best of our knowledge this is the first detection of the *cfr*(D2) gene, a new *cfr*(D) variant in enterococci.

2. Materials and Methods

2.1 Bacterial strains

The two *E. avium* isolates originated from two cases of sudden death in piglets (38157 and 44917). The piglets belonged to two unrelated farms in Umbria, central Italy, and were sent to IZSUM for diagnostic purposes. In the first case, 38157, mild hepatosplenomegaly and moderate pericarditis were observed after necropsy while in the second case, 44917, no lesions were observed except for moderate congestion of the internal organs. Culture tests were carried out on the spleen, heart, liver, and brain. The samples were plated on MacConkey, mannitol salt agar, and blood agar plates and incubated at 37°C for 2 days. Colonies were identified using a MALDI-TOF MS instrument (Microflex LT Smart Biotyper with FlexControl Biotyper 3.4 software, Bruker Daltonics, Bremen, Germany). From

both piglets, *E. avium* strains were isolated from the brain. No other pathogen was found in other organs/tissues except for *Streptococcus dysgalactiae* subsp. *equisimilis*, which was isolated from the heart of the first piglet, 38157.

2.2 Genotypic and phenotypic characterization

E. avium 38157 and *E. avium* 44917 were screened by PCR for the presence of known transferable oxazolidinone resistance genes (Cinthi et al., 2022). Susceptibility tests, performed using Etest strips (Liofilchem, Roseto degli Abruzzi, Italy) for tedizolid and by standard broth microdilution assay for florfenicol, chloramphenicol, linezolid, tetracycline, erythromycin and vancomycin (Sigma Aldrich, St. Louis, MI), were interpreted according to clinical breakpoints (EUCAST, version 10.0, 2020. http://www.eucast.org and CLSI, https://clsi.org/standards/products/free-resources/access-our-free-resources/). *Enterococcus faecalis* ATCC 29212 was used as quality control (EUCAST QC tables v 10.0, 2020. http://www.eucast.org).

2.3. Detection of circular forms

To investigate the excision of the linezolid resistance genes contexts, PCR mapping assays were performed using outward-directed primer pairs targeting the linezolid resistance genes: *cfr*(D) (5'-TTCCTAAAATAAAACGACTA-3'and 5'-TACAAAAAGATTCCCAGCCA-3'), *optrA* (5'-GAAAAATAACACAGTAAAAGGC-3'and 5'-TTTTTCCACATCCATTTCTACC-3'), and *poxtA* (5'-GACGAGCCGACCAACCACCT-3' and 5'-TTCAGGCGGACAAAAATCCAA-3').

2.4 WGS and sequence analysis

The genomes of *E. avium* 38157 and *E. avium* 44917 were extracted by the QIAcube automated extractor using DNeasy PowerLyzer PowerSoil Kit, according to the manufacturer's instructions (Qiagen, Germany). Extracted DNA was subjected to WGS by a hybrid approach using both short-read Illumina MiSeq platform (MicrobesNG, Birmingham, UK) with a 2x250 bp paired-end technology and a long-read sequencing approach (MinION,Oxford Nanopore Technologies, Oxford, UK). Hybrid assembly was performed with Unicycler v. 0.4.8 (https://github.com/rrwick/Unicycler). *In silico* identification of acquired antimicrobial resistance genes, ribosomal mutations involved in oxazolidinone resistance, plasmid replicon type and virulome were carried out using dedicated tools of the Center for Genomic Epidemiology available at http://www.genomicepidemiology.org/ (ResFinder v.3.2, VirulenceFinder 2.0, LRE-finder v.1.0, PlasmidFinder 2.1) and using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 Mating and transformation experiments

Conjugal transfer was performed on membrane filter as described previously (Brenciani et al., 2016) using the florfenicol-susceptible *E. faecium* 64/3 and *E. faecalis* JH2-2 as recipients. Transconjugants were selected on plates containing chloramphenicol (8 mg/L). The transfer frequency was expressed as the ratio of the cell number (CFU/ml) of the transconjugant to that of the recipient. SmaI-PFGE was carried out and patterns analysed in order to confirm the genetic background of transconjugants.

Transconjugants were tested for the presence of linezolid resistance genes by PCR and for their susceptibility to florfenicol, chloramphenicol, linezolid, tedizolid, tetracycline, and erythromycin.

2.7 Nucleotide sequence accession numbers

The nucleotide sequences of the pEa_*cfr*(D)-*optrA* and pEa_*cfr*(D2)-*poxtA* plasmids have been deposited in GenBank under accession numbers OQ298926 and OQ298927, respectively.

2. Results and discussion

2.1 Detection of oxazolidinone resistance genes and antimicrobial susceptibility testing

E. avium 38157 was found positive for the presence of *cfr*(D) and *optrA* genes. The isolate was resistant to erythromycin (MIC, >128 mg/L) and tetracycline (MIC, >128 mg/L), had reduced susceptibility to linezolid (MIC, 4 mg/L) and was susceptible to tedizolid (MIC, 0.5 mg/L), florfenicol (MIC, 8 mg/L), chloramphenicol (MIC, 16 mg/L), and vancomycin (MIC, 2 mg/L).

PCR screening showed that *E. avium* 44917 was *cfr*(D)- and *poxtA*-positive. The isolate was resistant to florfenicol (MIC, 64 mg/L), chloramphenicol (MIC, 64 mg/L), tetracycline (MIC, 64 mg/L) and erythromycin (MIC, > 128 mg/L) and susceptible to linezolid (MIC, 2 mg/L), tedizolid (MIC, 0.25 mg/L) and vancomycin (MIC, 0.5 mg/L).

2.2 WGS and bioinformatic analysis

The *E. avium* 38157 genome consisted of one chromosome (4,398,426 bp) and two plasmids of 36,573 bp and 27,778 bp in size.

ResFinder analysis revealed the presence of *erm*(B) (resistance to macrolides, lincosamides and streptogramins group B), *tet*(M) (resistance to tetracyclines), and *dfrG* (resistance to trimethoprim) genes, in addition to *cfr*(D) and *optrA*.

No mutations were detected in the genes encoding the 23S rRNA or ribosomal proteins.

Virulome analysis excluded the presence of known acquired virulence genes.

WGS analysis indicated that the *cfr*(D) and *optrA* genes were co-located (3,624 bp far apart) on a 36,573-bp plasmid (34% GC content) designated pEa_*cfr*(D)-*optrA* (accession no. OQ298926) containing 36 ORFs (Figure 1, Table S1). The pEa_*cfr*(D)-*optrA* plasmid belonged to the Rep1 replicon type. The plasmid showed high nucleotide identity (99,82%; coverage 91%) with the 33.2-kb pAT02-c plasmid (accession no. CP097064) of *E. faecium* AT02 isolate from pet food. Interestingly, the region of pEa_*cfr*(D)-*optrA* harboring the *optrA* and the closely associated *erm*(B) gene was 99% identical (coverage 14%) to the one of the pAv-*optrA* plasmid detected in the *Aerococcus viridans* 1417-4A from bovine faeces in Italy (accession no. MW364930) (Coccitto et al., 2021).

Since the pAT02-c plasmid only carries the *cfr*(D) gene, it can be assumed that the *optrA* genetic context integration (Figure 1, Table S1), resulting in the pEa_*cfr*(D)-*optrA* plasmid, occurred later on. Although, in pEa_*cfr*(D)-*optrA* insertion sequences bounding the *optrA* genetic context have not been found, an in-depth analysis of the 3,382-bp *optrA* region revealed the presence of two 48-bp direct repeats (DRs) (ATACCTAATAATTTATCTACATTCCCTTTAGTAACGTGTAAC) flanking this genetic contest. These DRs, also detected in pAv-*optrA* plasmid from *A. viridans* (Coccitto et al., 2021) (unpublished data), could be involved in the mobilization of the *optrA* gene. On the other hand, a previously study demonstrated that unconventional circularizable structures (UCS) − though lacking their own recombinase genes − can be excised in circular form through DRs flanking the DNA segment undergoing excision (Palmieri et al., 2013). Nevertheless, inverse

PCR experiments, using outward-directed primer pairs targeting the *optrA* gene, showed that its genetic context was unable to excise in circular form suggesting a stable acquisition.

The *optrA* gene shared 98% nucleotide and 97% amino acid identities with the wildtype *optrA*$_{E349}$ (Wang et al., 2015). *E. avium* 38157 exhibited an OptrA variant (EYKWDVDASKELYNKQLEIG) previously described in enterococci from human and pig origin in China and in Italy, respectively (Cai et al., 2019; Fioriti et al., 2020).

In pEa_*cfr*(D)-*optrA*, the *cfr*(D) genetic context, flanking by two IS*1216* elements with opposite orientation, was identical to that of the plasmid 2 of *E. faecium* 15-307.1, where the *cfr*(D) is closely associated with a truncated *guaA* gene (Guerin et al., 2020). No circular intermediate was detected.

The *E. avium* 44917 genome consisted of a 4,737,938-bp chromosome, a *cfr*(D)- and *poxtA*-carrying plasmid (42,657 bp), designated pEa_*cfr*(D2)-*poxtA* (accession no. OQ298927), and two other plasmids of 22,140 bp and 11,162 bp in size.

ResFinder analysis revealed a complex resistome for the presence of several acquired antibiotic resistance genes in addition to *cfr*(D) and *poxtA*: *erm*(B), *tet*(M) and *tet*(L) (resistance to tetracyclines), *aph*(3')-III, *ant*(6)-Ia, and *aac*(6')-*aph*(2'') (resistance to aminoglycosides), *fexB* (resistance to florfenicol), *cat* (resistance to chloramphenicol), and *dfrE* (resistance to trimethoprim).

No mutations were detected in the genes encoding the 23S rRNA or ribosomal proteins.

Virulome analysis displayed the presence of the *esp*$_{fm}$ gene encoding an enterococcal surface protein.

Sequence analysis showed that the *poxtA* gene was identical to that first described in a clinical MRSA strain (Antonelli et al., 2018).

pEa_*cfr*(D2)-*poxtA* was 97% identical (coverage 85%) to the MDR composite transposon Tn*6349* (48 kb) − also including the *poxtA*- and *fexB*-carrying Tn*6657* − first described in the human MRSA AOUC-0915 (D'Andrea et al., 2019). However, in pEa_*cfr*(D2)-*poxtA*, the Tn*6657* was 2.7 kb shorter (11,674 bp *vs* 14,396 bp), and the *poxtA* and *fexB* genes, showing opposite orientation, were 5,061 bp far apart compared to the staphylococcal Tn*6657* (2,375 bp) (Figure 1). The *poxtA* genetic context, flanked by IS*1216* with the same polarity, was highly conserved; inverse PCR experiments and sequencing showed that a circular form was detectable.

Interestingly, bioinformatic analysis revealed the presence in pEa_*cfr*(D2)-*poxtA* of a *cfr*(D)-like gene, named *cfr*(D2), which was shorter than *cfr*(D) wildtype (1,053 bp *vs* 1,074 bp) due to the loss of 21 bp to the 3'-end. Therefore, Cfr(D2) (352 amino acids) differed from wildtype (357 amino acids) by the presence of a histidine (H) that replaced the last six amino acids (TIQVND).

The *cfr*(D2) genetic environment, not flanked by DRs, included the IS*1216* (*orf24*) and IS*Seq2* (*orf25*) transposases as previously detected in plasmid 4 of *E. faecium* E8014 (Guerin et al., 2020), but it was devoid of the *guaA* gene (Figure 1). No circular intermediate was detected. Moreover, the *cfr*(D2) context, inserted upstream of the Tn*6657*-like transposon, fully replaced the Tn*6644* transposon that in *S. aureus* AOUC-0915 harbor the *cfr* gene flanking by two IS*Enfa5* transposases (D'Andrea et al., 2019) (Figure 1).

The pEa_*cfr*(D2)-*poxtA* plasmid, belonged to the Rep1 replicon type, had a complete transfer machinery (from *orf4* to *orf23*) responsible of the conjugation process (Figure 1, Table S2).

2.3 Transfer experiments

Despite several attempts, *E. avium* 38157 was unable to transfer the pEa_*cfr*(D)-*optrA* plasmid to *E. faecalis* JH2-2 and *E. faecium* 64/3 recipients.

Conversely, *E. avium* 44917 was able to move the pEa_*cfr*(D2)-*poxtA* plasmid to both *E. faecalis* JH2-2 and *E. faecium* 64/3 recipients at frequencies of 8.2×10^{-4} and 4.5×10^{-6}, respectively. For each mating experiment, two randomly selected transconjugants were analysed for their genotype, phenotype and genetic background by SmaI-PFGE.

Transconjugants exhibited resistance to florfenicol, chloramphenicol and tedizolid and susceptibility to linezolid, erythromycin and tetracycline. PCR and Sanger sequencing indicated that all transconjugants acquired both *cfr*(D2) and *poxtA* genes (Table 1).

Conclusion

We report, to the best of our knowledge, the first identification of a new *cfr*(D)-like gene, mobilizable to clinically relevant enterococci and characterize two plasmids co-carrying *cfr*(D)/*oprtA* and *cfr*(D2)/*poxtA* linezolid resistance genes from *E. avium* swine isolates. Since the members of this bacterial genus are known for their ability to transfer antibiotic resistance determinants to human pathogens, the plasmid co-carriage of linezolid resistance genes in enterococci from food-producing animals, is concerning and needs surveillance.

Ethics statement

Not required

Declaration of Competing Interest

None to declare.

Data Availability

The data that support the findings of this study are openly available in this manuscript and in the Supporting Information attached.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version.

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Figure 1. Alignment of the pEa_*cfr*(D)-*optrA* from *E. avium* 38157 and pEa_*cfr*(D2)-*optrA* from *E. avium* 44917 with the pAT02-c plasmid from *E. faecium* AT02 and Tn*6349* transposon of the *S. aureus* AOUC 09-15. Green box indicates the *optrA* genetic context, light blue box indicates the *cfr*(D2) genetic context, pink box depicts the Tn*6657* transposon and light yellow box represents the Tn*6644* transposon. Direct repeats (DRs) (ATACCTAATAATTTATCTACATTCCCTTTAGTAACGTGTAAC) flanking the *optrA* genetic context. The gray shading indicates regions of shared homology (ranging from 66 to 100%).

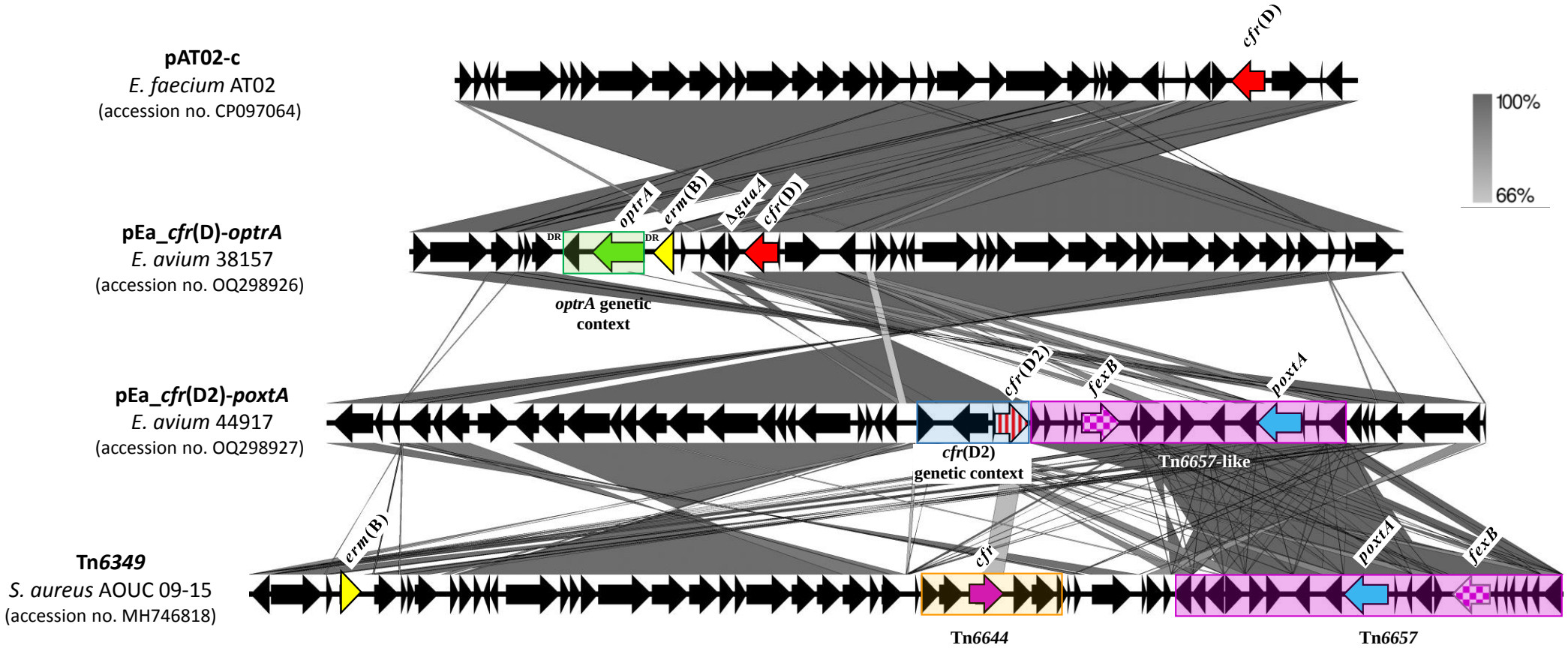

Table 1. Genotypes and MIC values for *E. avium* 44987, relevant transconjugants and enterococcal recipients.

Strain	Genotype	MIC (mg/L)					
		CHL[a]	FFC	LZD	TZD	TET	ERY
E. faecium 44987 (donor)	*cfr*(D2) *poxtA fexB tet*(M) *tet*(L) *erm*(B)	64	64	2	0.25	64	>128
TCJH-1 (transconjugant)	*cfr*(D2), *poxtA*	32	32	2	1	1	0.125
TCJH-2 (transconjugant)	*cfr*(D2), *poxtA*	32	32	2	1	1	0.125
E. faecalis JH2-2 (recipient)	-	0.25	1	0.5	0.125	0.125	1
TC64/3-1 (transconjugant)	*cfr*(D2), *poxtA*	32	32	2	1	1	0.125
TC64/3-2 (transconjugant)	*cfr*(D2), *poxtA*	32	32	2	1	1	0.125
E. faecium 64/3 (recipient)	-	4	4	1	1	0.25	1

[a] Abbreviations: CHL, chlormaphenicol; FFC, florfenicol; LZD, linezolid; TZD, tedizolid; TET, tetracycline; ERY, erythromycin

Supplementary materials

Table S1. Amino acid sequence identities/similarities of putative proteins encoded by the pEa_*cfr*(D)-*optrA* (GenBank accession no. OQ298926).

ORF	Start (bp)	Stop (bp)	Size (amino acids)	BLASTP analysis[a]			
				Predicted function	Most significant database match	Accession no.	% Amino acid identity (% amino acid similarity)
orf1	156	773	205	Recombinase family protein	Recombinase family protein [*Enterococcus faecalis*]	MCD5118149.1	100 (100)
orf2	773	2917	714	DNA topoisomerase III	DNA topoisomerase 3 [*E. faecalis*]	MCD5118148.1	100 (100)
orf3	3020	3916	298	ParA family protein	ParA family protein [*E. faecalis*]	MCD5118147.1	100 (100)
orf4	4014	4223	69	Omega transcriptional repressor	Omega transcriptional repressor [uncultured bacterium]	APO31094.1	100 (100)
orf5	4241	4513	90	Epsilon antitoxin	Epsilon-antitoxin [*Enterococcus avium*]	QXF69004.1	100 (100)
orf6	4515	5378	287	Zeta toxin	Zeta toxin [*E. avium*]	QXF69005.1	100 (100)
orf7	6249	5596	217		HNH endonuclease [*Enterococcus thailandicus*]	QXF68964.1	100 (100)
orf8	8624	6711	655	ABC-F type ribosomal protection protein	ABC-F type ribosomal protection protein OptrA [*Enterococcus hirae*]	MCD4950369.1	99 (100)
orf9	9718	8981	245	Ribosomal RNA adenine dimethylase	23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(B) [Firmicutes]	WP_001038790.1	100 (100)
orf10	11621	10941	226	IS6 family transposase	IS6 family transposase [*E. faecalis*]	MCD5118221.1	100 (100)
Δ*orf11*	11692	12219	175	GMP synthase C terminal domain	Glutamine-hydrolyzing GMP synthase [*E. faecium*]	HBH5625399.1	100 (100)
cfr(D)	13376	12303	357	Radical SAM superfamily	23S rRNA methyltransferase Cfr(D) [*Lactobacillales*]	WP_105459893.1	100 (100)
orf13	13821	15206	461	ISNCY family transposase	ISSeq2 [*E. avium*]	QXF68955.1	100 (100)
orf14	16414	15734	226	IS6 family transposase	IS6 family transposase [*Enterococcus*]	WP_127821369.1	100 (100)
orf15	17174	17671	165		Molecular chaperone DnaJ [*E. faecalis*]	HAP5618431.1	99 (99)
orf16	17705	18010	101		Hypothetical protein [*E. faecalis*]	EGO9399619.1	100 (100)
orf17	18290	18033	85		Hypothetical protein EGCR1_18680 [*Enterococcus gilvus*]	AXG40745.1	100 (100)
orf18	18592	18293	99		Conserved hypothetical protein [*E. faecium* E1679]	EFF25713.1	100 (100)
orf19	18883	20868	661	Mob family protein	MobA/MobL family protein [*E. faecalis*]	WP_233700909.1	100 (100)
orf20	20892	21224	110		Hypothetical protein [*Enterococcus* sp.]	NLM66958.1	100 (100)
orf21	21243	21626	127		Glycosyltransferase [*E. faecium* CRL1879]	ERK33173.1	100 (100)

orf22	21643	22272	209		Hypothetical protein [*E. faecalis*]	HAP2865558.1	99 (99)
orf23	22283	24244	653		TrsE protein [*Enterococcus*]	WP_231428285.1	100 (100)
orf24	24258	25610	450		Conjugal transfer protein TraF [*Enterococcus*]	WP_231428284.1	100 (100)
orf25	25632	26741	369	Lipoprotein, NLP/P60 family	Lysozyme family protein [*E. faecalis*]	MCD5118161.1	100 (100)
orf26	26754	27305	183		Hypothetical protein [*E. faecalis*]	WP_156188637.1	99 (100)
orf27	27310	27741	143		Hypothetical protein [*E. faecalis*]	HBI2071430.1	99 (100)
orf28	27734	29389	551	Type IV secretory system protein	Type IV secretory system conjugative DNA transfer protein [*Enterococcus*]	WP_155282209.1	100 (100)
orf29	29407	30330	307		Hypothetical protein [*E. faecalis*]	EGO9014235.1	99 (99)
orf30	30332	31264	310		TrsL protein [*E. thailandicus*]	AFW17868.1	100 (100)
orf31	31281	32249	322		Hypothetical protein [*Enterococcus*]	WP_231428281.1	100 (100)
orf32	32278	32646	122		Hypothetical protein [*Enterococcus*]	WP_231428280.1	100 (100)
orf33	32706	33503	265	LPXTG protein	LPXTG cell wall anchor domain-containing protein [*E. faecium*]	HAP7639615.1	100 (100)
orf34	33820	34092	90	XRE family transcriptional regulator	Plasmid copy control protein [*E. faecium*]	ARQ19309.1	100 (100)
orf35	34458	34757	99		Hypothetical protein [*Bacilli*]	WP_002325779.1	99 (99)
orf36	34800	36290	496	Replication protein	Replication protein RepR [*E. faecalis*]	RXF24396.1	100 (100)

[a]For each ORF, only the most significant identity detected is listed.
Δtruncated ORFs.

Table S2. Amino acid sequence identities/similarities of putative proteins encoded by the pEa_*cfr*(D2)-*poxtA* (GenBank accession no. OQ298927).

ORF	Start (bp)	Stop (bp)	Size (Amino acids)	BLASTP analysis[a]			
				Predicted function	Most significant database match	Accession no.	% Amino acid identity (% amino acid similarity)
orf1	1708	218	496	Replication protein	Replication protein RepR [*Enterococcus faecium*]	HAP7628643.1	100 (100)
orf2	2050	1751	99		Hypothetical protein [*Bacilli*]	WP_002325779.1	100 (100)
orf3	2688	2416	90	XRE family transcriptional regulator	Plasmid copy control protein [*E. faecium*]	ARQ19309.1	100 (100)
orf4	3820	3005	271	Cell wall surface anchor family protein	LPXTG cell wall anchor domain, partial [*Enterococcus faecalis*]	UQF47390.1	100 (100)
orf5	4248	3880	122		Hypothetical protein M2911_13870 [*E. faecalis*]	UQF47389.1	100 (100)
orf6	5245	4277	322		Hypothetical protein [*Enterococcus*]	WP_231428281.1	100 (100)
orf7	5568	6755	395	IS110 family transposase	IS*110* family transposase [*E. faecium* Ef_aus0098]	HAQ1360039.1	100 (100)
orf8	7814	6882	310		Hypothetical protein [*Bacilli*]	WP_011266105.1	99 (99)
orf9	8739	7816	307		Hypothetical protein [*Enterococcus hirae*]	MCD4901252.1	99 (99)
orf10	10412	8757	551	Type IV secretory system	Type IV secretory system [*E. faecalis*]	QTO65517.1	100 (100)
orf11	10836	10405	143		Hypothetical protein [*Bacilli*]	WP_001085135.1	100 (100)
orf12	11392	10841	183		Hypothetical protein [*Lactobacillales*]	WP_015543618.1	100 (100)
orf13	12514	11405	369	Lipoprotein, NLP/P60 family	Lysozyme family protein [*E. faecalis*]	MCD5118161.1	100 (100)
orf14	13888	12536	450	Conjugal transfer protein	Conjugal transfer protein TraF [*E. faecalis*]	MCD5118162.1	100 (100)
orf15	15863	13902	653		TrsE protein [*E. faecalis*]	HAP3444496.1	100 (100)
orf16	16503	15874	209		Hypothetical protein QQ23_07020 [*E. faecalis*]	KII51102.1	100 (100)
orf17	16903	16520	127		TrsC [*Enterococcus thailandicus*]	AFW17859.1	100 (100)
orf18	17254	16922	110	Conjugation protein	TrsB [*E. thailandicus*]	AFW17858.1	100 (100)
orf19	19263	17278	661	Mobilization protein	MobA/MobL family protein [*E. faecalis*]	WP_233700909.1	100 (100)
orf20	19554	19853	99		Hypothetical protein [*Bacilli*]	WP_002301627.1	99 (99)
orf21	19856	20113	85		Hypothetical protein [*Firmicutes*]	WP_002325623.1	100 (100)
orf22	20441	20136	101		Hypothetical protein [*E. faecalis*]	EHG5940617.1	100 (100)
orf23	20972	20475	165		Molecular chaperone DnaJ [*E. faecium* CRL1879]	ERK33167.1	100 (100)
orf24	21732	22412	226	IS6 family transposase	IS*6* family transposase [*E. faecalis*]	MCD5118221.1	100 (100)

orf25	24346	22940	468	Mobile element protein	IS*Seq2* [*Enterococcus avium*]	QXF68955.1	100 (100)
cfr(D)2	24770	25828	352	23S rRNA methyltransferase	23S rRNA (adenine(2503)-C(8))-methyltransferase Cfr(D) [*E. faecalis*]	MBW4162193.1	100 (100)
orf27	25897	26274	125		Hypothetical protein [*Enterococcus gallinarum*]	UKC63197.1	100 (100)
orf28	26411	26668	85		Putative transposase [*E. faecalis*]	ADN34760.1	100 (100)
fexB	27779	29188	469	Chloramphenicol/florfenicol efflux protein	FexB MFS transporter [*Pediococcus pentosaceus*]	NVZ01872.1	99 (99)
orf30	29835	29518	105		Hypothetical protein [*E. faecalis*]	MCD5000849.1	100 (100)
orf31	29925	30605	226	IS6 family transposase	DDE transposase of IS*1216E* [*E. hirae*]	QEO73343.1	100 (100)
orf32	30803	31408	201	Fic domain protein	Fic family protein [*Bacteria*]	WP_000599739.1	100 (100)
orf33	31424	31996	190	Site-specific recombinase, resolvase family	Recombinase family protein [*Bacteria*]	WP_000170424.1	100 (100)
orf34	33043	32429	204	Transposase IS30 family	Integrase catalytic region [*E. faecium*]	UBL09667.1	100 (100)
orf35	34196	33516	226	IS6 family transposase	IS*6* family transposase [*E. faecium*]	WP_181040921.1	99 (99)
poxtA	35878	34250	542	Ribosomal protection protein	ARE-ABC-F family resistance factor PoxtA [*Staphylococcus aureus*]	AVI44920.1	100 (100)
orf37	37517	36837	226	IS6 family transposase	IS6 family transposase [*E. faecalis*]	WP_048961587.1	99 (100)
Δ*orf38*	38077	37571	168	Zeta toxin	Zeta toxin [*Streptococcus parauberis* KRS-02083]	EMG24378.1	99 (98)
orf39	38351	38079	90	Epsilon antitoxin	Epsilon-antitoxin [*E. avium*]	WP_005237730.1	100 (100)
orf40	38578	38369	69	Transcriptional Repressor	Peptide-binding protein [*Bacteria*]	WP_000527318.1	100 (100)
orf41	39572	38676	298	Partitioning plasmid protein	ParA family protein [*E. faecalis*]	MCD5118147.1	100 (100)
orf42	41819	39675	714	DNA topoisomerase III	DNA topoisomerase 3 [*Enterococcus*]	WP_231428277.1	100 (100)
orf43	42436	41819	205	Resolvase	Recombinase family protein [*Enterococcus*]	WP_126263923.1	100 (100)

[a]For each ORF, only the most significant identity detected is listed.
Δtruncated ORFs.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Nucleotide sequence of the pEa_cfr(D2)-poxtA plasmid

Nucleotide sequence of the pEa_cfr(D)-optrA plasmid