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Brief report

Occurrence of a new plasmid co-carrying *cfr*(D) and *poxtA*2 linezolid resistance genes in *Enterococcus faecalis* and *Enterococcus casseliflavus* from porcine manure, Italy

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Abstract

Objectives: To investigate the genetic elements and the transferability of linezolid resistance genes in five enterococci co-carrying *cfr*(D) and *poxtA* isolate from a manure of a swine farm in Central Italy.

Methods: Three *Enterococcus faecalis* and two *Enterococcus casseliflavus* isolates carrying both *cfr*(D) and *poxtA* genes were tested for their susceptibility to: florfenicol, chloramphenicol, linezolid, tedizolid, tetracycline and vancomycin. Linezolid resistance genes transfer (filter mating), localization (S1-PFGE/hybridization), genetic elements (WGS) were analyzed. Genetic relatedness was studied by PFGE and MLST.

Results: Three *E. faecalis* and two *E. casseliflavus* isolates besides *cfr*(D) gene also carried the recently described *poxtA2* variant. *cfr*(D) and *poxtA2* were co-located on a novel 33,480-bp plasmid, pV386, 95-100% identical (coverage 84%) to the Tn*6349* transposon of *Staphylococcus aureus* AOUC-0915. Both genes also showed a chromosomal location. Same sequence identities were found from the comparison with currently known *poxtA2* genetic elements. In pV386 *poxtA2* gene, not bounded by two IS*1216*, was closely associated to the *cfr*(D) and *fexA* genes in the new plasmid. pV386 was always transferred by filter mating to *E. faecium* 64/3 recipient.

Conclusions: The occurrence of the new pV386 plasmid in *E. faecalis* and *E. casseliflavus* from swine manure is of great concern and highlights the need of control measures to contain its spread to other enterococcal species.

Introduction

Livestock manures, widely used in farming practice as soil fertilizer worldwide, are major reservoirs of antibiotic-resistant bacteria and represent a potential hotspot for antibiotic resistance genes dissemination by horizontal gene transfer.[1] Moreover, after administration many antibiotics, increasingly used in food-producing animals, are poorly absorbed from the gut and are excreted into the environment as active metabolites. Therefore, several antibiotic residues, antibiotic resistance genes and antibiotic-resistant bacteria are spread into farmland thus posing significant potential risks to the environment and public health.[1,2]

The abundance of *Enterococcus* spp. in animal and human faeces and their prolonged survival in the environment have made them a well-established indicator of faecal contamination in the environment[3] and in the food.[4] More recently, enterococci have been also proposed for monitoring antibiotic resistance in food animals.[5]

Although regarded as commensals, some enterococcal species, namely *Enterococcus faecium* and *Enterococcus faecalis*, are important pathogens causing nosocomial infections in humans worldwide.[6] The increasing emergence of acquired resistances towards various antibiotics significantly limits therapeutic options and posing a serious challenge in the treatment of enterococcal diseases.[7] Oxazolidinones are among the few available last-resort antibiotics to treat severe infections caused by VRE and MDR enterococci.

Oxazolidinones, including linezolid and tedizolid, bind in the V domain of the 23S rRNA of the 50S ribosomal subunit and inhibit protein synthesis.[8] Besides the mutations in 23S rRNA and ribosomal proteins,[7] linezolid resistance can arise from the acquisition of transferable resistance determinants: *cfr* and its variants, *optrA* or *poxtA* genes. Cfr and Cfr-like methylases confer resistance to phenicols, lincosamides, oxazolidinones, pleuromutilines and streptogramin A ($PhLOPS_A$ phenotype),[9] whereas the ABC-F proteins OptrA and PoxtA[9] leads to a decreased susceptibility to phenicols and oxazolidinones (including tedizolid) by a ribosomal protection mechanism.[10]

Florfenicol, exclusively approved for use in veterinary medicine, is a broad-spectrum antimicrobial agent extensively used in livestock to prevent or cure bacterial infections.[9] Phenicols have a considerable impact on dissemination of florfenicol resistance genes including those also encoding resistance to linezolid.[11] Therefore, even though oxazolidinones have not been approved for veterinary use, linezolid resistance genes are increasingly detected in enterococci of animal and environmental origin.[9,12] Florfenicol administration also results in the release of antibiotic residues in the manure and soil which generate a selective pressure affecting the resistome of environmental bacterial communities.[11,13] The European Medicines Agency (EMA) has recently suggested using

florfenicol with caution in veterinary medicine, to mitigate the risk for public health (https://www.ema.europa.eu/en/news/categorisation-antibiotics-used-animals-promotes-responsible-use-protect-public-animal-health).

In this study, we characterized a new transferable plasmid co-carrying *cfr*(D) and *poxtA2* genes detected in *E. faecalis* and two *Enterococcus casseliflavus* isolates from swine manure.

Materials and methods

Bacterial strains

During a surveillance aimed at the detection of linezolid resistance genes in enterococci of animal origin (unpublished results), we isolated three *E. faecalis* and two *E. casseliflavus*, co-carrying *cfr*(D) and *poxtA* genes from a lagoon collecting raw manures from a swine farm via a drainpipe. The farm, located in Central Italy, raised approximately 1300 finishing pigs/year. Florfenicol was given to pigs by the parenteral route and tiamulin and lincomycin were orally administered to the animals. According to Italian legislation, these antibiotics were used for therapeutic purposes, under the supervision of the farm veterinarian.

Susceptibility tests. Isolates were tested for their susceptibility to florfenicol, chloramphenicol, linezolid, tetracycline and vancomycin (Sigma Aldrich, St. Louis, MI) by standard broth microdilution assay, and to tedizolid using Etest strips (Liofilchem, Roseto degli Abruzzi, Italy).[14] Susceptibility tests were interpreted according to EUCAST clinical breakpoints (version 10.0, 2020. http://www.eucast.org). *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC29213 were used as quality control.

SmaI-PFGE, S1-PFGE, Southern blotting and hybridisation assays. A preliminary typing was performed by SmaI-PFGE as described previously.[15]
Genomic DNA embedded in agarose gel plugs was digested with S1 nuclease (Thermo Fisher Scientific, Milan, Italy), and chromosomes and plasmids separated by PFGE as described previously.[16] After S1-PFGE, total DNA was blotted onto positively charged nylon membranes (Ambion-Celbio, Milan, Italy) and hybridized with biotin-labelled DNA probes as described elsewhere (Table S1).[17]

Detection of circular forms. Excision of the genetic contexts was detected using outward-directed primer pairs targeting the *cfr*(D) and *poxtA* resistance genes (Table S1).

Conjugation experiments. Conjugal transfer was performed on a membrane filter as described previously.[17] The florfenicol-susceptible *E. faecium* 64/3 was used as recipient. Transconjugants were selected on brain heart infusion agar (Oxoid, Basingstoke, UK) plates containing fusidic acid, rifampicin, and florfenicol (all at 10 mg/L). The transfer frequency was expressed as the ratio of the cell number (CFU/mL) of the transconjugant to that of the recipient.

Transconjugants were evaluated for their susceptibility to florfenicol, chloramphenicol, linezolid, tedizolid and tetracycline and tested by PCR for the presence of *cfr*(D) and *poxtA* genes. SmaI-PFGE was carried out and patterns analyzed to confirm the genetic background of the transconjugants.

WGS and sequence analysis. Bacterial genomic DNA was extracted by the QIAcube automated extractor using DNeasy PowerLyzer PowerSoil Kit according to 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 2 x 250 paired end technology and a long-read sequencing approach (MinION, Oxford Nanopore Technologies, Oxford, UK). SPAdes 3.15.2 software was used for the hybrid assembly of short and long reads (http://bioinf.spbau.ru/spades).
In silico identification of acquired antimicrobial resistance genes and ribosomal mutations involved in oxazolidinone resistance were carried out using dedicated tools available at the Center for Genomic Epidemiology available at http://www.genomicepidemiology.org/ (MLST v.2.0, ResFinder v.3.2, LRE-finder v.1.0 and PlasmidFinder 2.1) and by the BLAST suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide sequence accession number. The nucleotide sequence of the plasmid pV386 has been deposited in GenBank under accession number MZ603802.

Results and discussion

Phenotypic and genotypic characterization of isolates. All five enterococci, three *E. faecalis* and two *E. casseliflavus*, were resistant to florfenicol (MIC range, 64-128 mg/L), chloramphenicol (MIC range, 64-128 mg/L) and tedizolid (MIC range, 1-2 mg/L) and were either susceptible or resistant to linezolid (MIC range, 2-8 mg/L) and to tetracycline (MIC range, 1->128 mg/L). The *E. faecalis* isolates were susceptible to vancomycin (MIC range, 2-4 mg/L), while *E. casseliflavus* strains showed low-level resistance to vancomycin (MIC, 8 mg/L) (Table 1).

The PCR products obtained from *cfr*(D) and *poxtA* genes were subjected to Sanger sequencing. In all isolates, the *cfr*(D) gene was 99,9% identical to wild type (accession no. NG_067192).[18]

Sequencing also showed that all *poxtA*-positive isolates actually harboured the *poxtA2* variant recently detected in a human linezolid-resistant *Enterococcus gallinarum* from Colombia[19] and in *E. faecalis* EFS0019 of swine origin in in Korea (Table 1).[20] Unlike *poxtA*, *poxtA2* variant (accession no. MZ171245) was not truncated by an IS*1216* insertion at the 3' end, thus eight new amino acid (TPEEEQKY) sequence replaced the six-amino-acid (GSVAKF) of wild type protein. The Authors suggested that this variant could be considered the ancestor of the *poxtA* gene.[19]

Phylogenetic relatedness of the isolates. All strains were typed by SmaI-PFGE (Table 1). Enterococcal isolates belonged to 4 different SmaI-PFGE types (A to D), and one subtypes (A1). *E. faecalis* strains belonged to 2 different SmaI-PFGE types (A, B); *E. faecalis* V359 and V386 were found to be closely related (A and A_1, respectively). *E. casseliflavus* isolates exhibited two different pulsotypes (C and D) (Table 1).

E. faecalis strains were associated to ST32, a sequence type previously described in enterococci isolated from chicken faeces (www.pubmlst.org), from faeces and carcass of poultry and pigs[21] and related to the occurrence of amyloidosis and septicaemia in broilers.[22]

Location of the oxazolidinone resistance genes and detection of circular forms. S1-PFGE experiments revealed the presence of two plasmids of ~34 and ~100 kb in all three *E. faecalis* isolates, whereas in *E. casseliflavus* strains only a ~34-kb plasmid was detected (Figure S1). Hybridization assays showed that *cfr*(D) and *poxtA2* genes were co-located on the ~34-kb plasmid and that both determinants also had a chromosomal location (Table 1, Figure S1, and S2).

Since *cfr*(D) and *poxtA2* genes exhibited both chromosomal and plasmid location, we analyzed by inverse PCR the presence of circular intermediates. No circular form was detected for both *cfr*(D) and *poxtA2* genes.

Transferability of oxazolidinone resistance genes. *cfr*(D) and *poxtA2* genes were successfully co-transferred from both *E. faecalis* and *E. casseliflavus* donors to *E. faecium* 64/3 recipient with frequencies ranging from 2.3 X 10^{-1} to 8.0 X 10^{-4} per recipient (Table 2). Transconjugants exhibited resistance to florfenicol and chloramphenicol, reduced susceptibility or susceptibility to linezolid and resistance to tedizolid. All transconjugants were susceptible to tetracycline and vancomycin (Table 2). PCR and Sanger sequencing indicated that all transconjugants acquired both *cfr*(D) and *poxtA2* genes (Table 2).

E. casseliflavus species, very common in food-producing animals habitually treated with phenicols and tetracyclines, could be a reservoir of linezolid resistance genes potentially transmissible to human pathogens via different routes. Therefore, the high-frequency transfer of resistance genes from the *E. casseliflavus* to *E. faecium* is cause for concern.

WGS analysis. All five test strains were subjected to WGS analysis. Bioinformatic data revealed that the *cfr*(D)- and *poxtA2*-carrying plasmid, named pV386, was identical in *E. faecalis* and in *E. casseliflavus* isolates. This 33,480-bp plasmid (34% GC content), exhibited 36 ORFs encoding proteins ≥50 amino acids (Figure 1 and Table S2). BLASTN analysis revealed that pV386 was 95-100% identical (coverage 84%) to the Tn*6349* transposon (accession no. MH746818.1) carrying *cfr* and *poxtA* in *S. aureus* AOUC-0915.[23] Tn*6349* was closely related to the pE35048-oc, a pRE25 derivative carrying *cfr*, *erm*(B) and *optrA* resistance genes and described in a clinical *E. faecium* strain.[24] Comparison between the sequences of pV386 and those of the currently known *poxtA2* genetic elements showed a nucleotide identity of 99% (coverage 16%) with the pIB-BOL plasmid (accession no. MZ171245) of *E. gallinarum* Eg-IV02[19] and of 96-100% (coverage 37%) with the contigs #20 and #26 (accession no. QUSQ00000000) from *E. faecalis* EFS0019 draft genome (Figure 1).[20]

Unlike detected in pIB-BOL, in pV386 *poxtA2* gene was not bounded by two IS*1216* arranged in the opposite orientation but was closely associated to the *cfr*(D) gene. Upstream of *poxtA2* we found the *fexA* gene in turn bracketed by two IS*1216* with the same polarity, as previously described by Jung *et al.* (Figure 1).[20]

A complete conjugation region, *repE* (belonging to the rep_1 family) and *parA* genes (responsible for plasmid replication and partitioning, respectively), and a toxin-antitoxin

ώ-ε-ζ system (involved in the plasmid persistence in the enterococcal population), were also detected in pV386 (Figure 1).

The presence of pV386 in both *E. faecalis* and *E. casseliflavus* isolates could be result from HGT events between these enterococcal species.

Conclusions

To the best of our knowledge, this is the first detection of the *poxtA2* variant in *E. casseliflavus* species.

Occurrence of the pV386 − a new conjugative plasmid co-carrying *cfr*(D) and *poxtA2* genes – both in *E. faecalis* and *E. casseliflavus* isolates from swine manure, demonstrates that intense genetic exchanges between enterococci occur promoting the spread of oxazolidinone resistance determinants.

The environmental pollution via manure with linezolid-resistant enterococci from the feces of livestock animals with a history of administration of florfenicol, pleuromutilins or lincosamides, pose a significant risk to public health and and needs considerable attention.

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Transparency declarations

None to declare

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Figures

Figure 1. Circular map of the pV386 plasmid in comparison with other genetic elements and contigs from draft genome using BRIG software. Plasmids, transposons and contigs included in the analysis were as follows: (inner to outer circles) pIB-BOL of *E. gallinarum* Eg-IV02 (accession no. MZ171245), Tn*6349* of *S. aureus* AOUC-0915 (accession no. MH746818.1), and #20 and #26 contigs of *E. faecalis* EFS0019 (accession no. QUSQ00000000). Black arrows indicate the positions and orientations of genes; some antibiotic resistance determinants and relevant genes described in this study are shown.

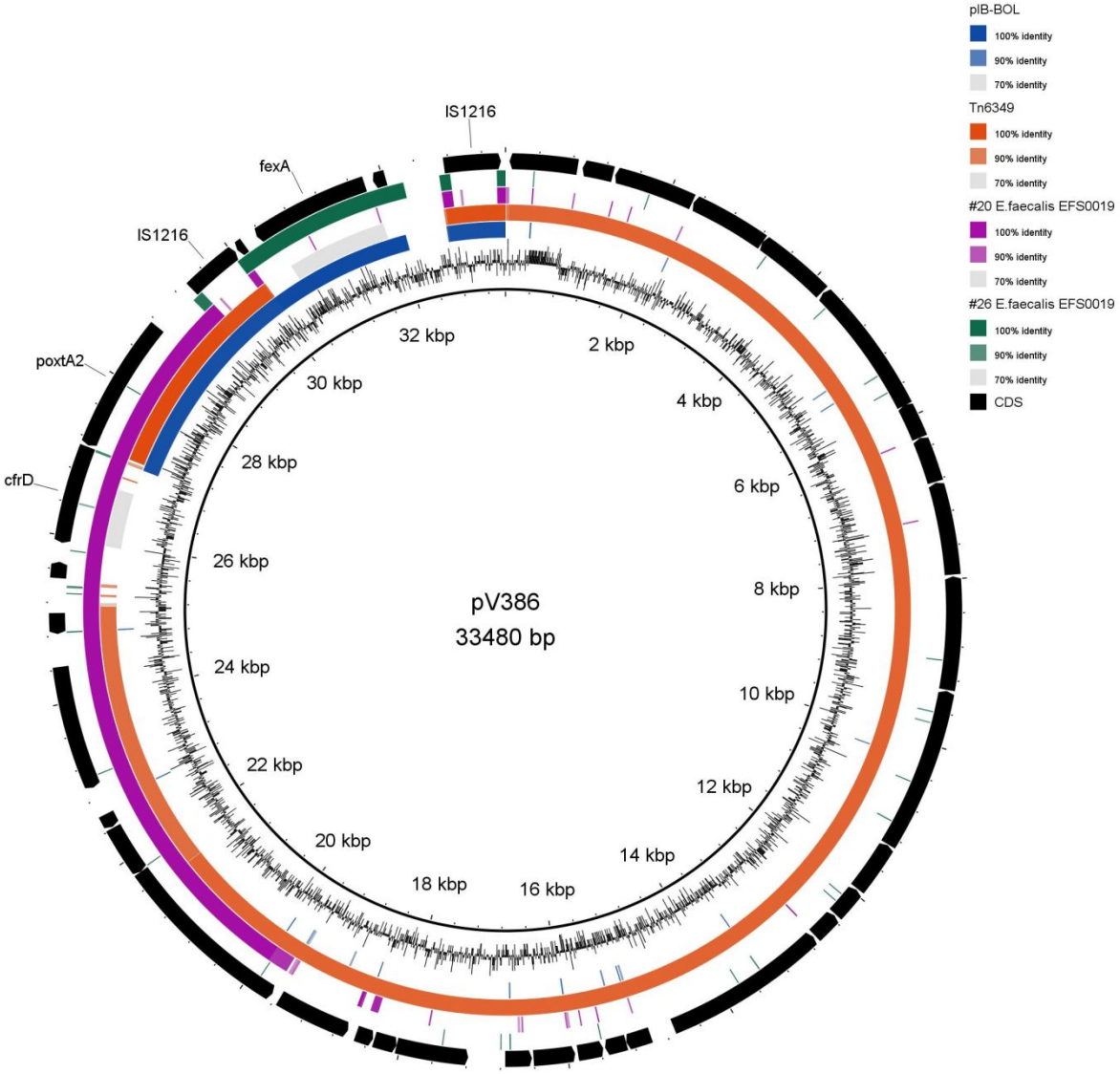

Table 1. Oxazolidinone resistance genes, antimicrobial susceptibility profiles, typing data and gene location.

Strain	Species	Oxazolidinone resistance genes		MIC (mg/L)						Typing		Hybridization assays	
		cfr(D)	*poxtA2*	FFC[a]	CHL	LZD	TZD	TE	VA	PFGE	MLST	*cfr*(D)	*poxtA2*
V359	*E. faecalis*	+	+	64	128	4	2	>128	4	A	ST32	~34[b], c[c]	~34, c
V386	*E. faecalis*	+	+	128	128	2	2	>128	2	A_1	ST32	~34, c	~34, c
V392	*E. faecalis*	+	+	64	128	4	2	>128	4	B	ST32	~34, c	~34, c
V308	*E. casseliflavus*	+	+	128	64	4	1	1	8	C	-	~34, c	~34, c
V311	*E. casseliflavus*	+	+	128	64	8	1	128	8	D	-	~34, c	~34, c

[a]FFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TDZ, tedizolid; TE, tetracycline; VA, vancomycin.
[b]Estimated plasmid size (in kb)
[c]c, chromosome

Table 2. Phenotypes and genotypes for enterococcal donors and relevant transconjugants.

Donors	Oxazolidinone resistance genotype	Recipient	Transfer Frequency	Transconjugants						
				MIC (mg/L)						Oxazolidinone resistance genotype
				FFC[a]	CHL	LZD	TZD	TE	VA	
E. faecalis V359	*cfr*(D), *poxtA2*	*E. faecium* 64/3	8.1×10^{-2}	64	32	4	1	1	0.5	*cfr*(D), *poxtA2*
E. faecalis V386	*cfr*(D), *poxtA2*	*E. faecium* 64/3	2.3×10^{-1}	32	16	2	1	1	0.5	*cfr*(D), *poxtA2*
E. faecalis V392	*cfr*(D), *poxtA2*	*E. faecium* 64/3	8.0×10^{-2}	128	64	4	2	1	0.5	*cfr*(D), *poxtA2*
E. casseliflavus V308	*cfr*(D), *poxtA2*	*E. faecium* 64/3	2.3×10^{-2}	64	32	4	1	1	0.5	*cfr*(D), *poxtA2*
E. casseliflavus V311	*cfr*(D), *poxtA2*	*E. faecium* 64/3	9.5×10^{-4}	128	64	4	1	1	0.5	*cfr*(D), *poxtA2*

[a]FFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TDZ, tedizolid; TE, tetracycline; VA, vancomycin.
[b]ND, not detectable.
E. faecium 64/3 MICs values: FFC (MIC, 4 mg/L), CHL (MIC, 4 mg/L), LZD (MIC, 1 mg/L), TZD (MIC, 1 mg/L), TE (MIC, 1 mg/L) and VA (MIC, 0.5 mg/L).

Supplementary materials

Table S1. Primer pairs used in this study.

Gene	Primer Designation	Sequence (5'-3')	Reference	Product size (bp)
cfr(D)	cfrD-FW[a]	TGGCTGGGAATCTTTTTGTA	This study	304
	cfrD-RV	TAGTCGTTTTATTTTAGGAA		
poxtA	poxtA-FW[a]	GAACGCTTGGAGTATTTCGACTTC	1	778
	poxtA-RV	CTGGACTGAGAATACCCATC		
Detection of circular forms				
poxtA	poxtA-3	GACGAGCCGACCAACCACCT	2	
	poxtA-4	TTGGATTTTTGTCCGCCTGAA		
cfr(D)	cfrD-3	TACAAAAAGATTCCCAGCCA	This study	
	cfrD-4	TTCCTAAAATAAAACGACTA		

[a] These primer pairs were also used to obtain specific probes.

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Table S2. Amino acid sequence identities/similarities of putative proteins encoded by the pV386 (GenBank accession no. MZ603802).

ORF	Start (bp)	Stop (bp)	Size (amino acids)	Predicted function	BLASTP analysis[a] Most significant database match	Accession no.	% Amino acid identity (% amino acid similarity)
orf1	861	49	270		LPXTG-domain-containing protein cell wall anchor domain, partial [*Enterococcus faecium* EnGen0313]	EOI35867.1	100 (100)
orf2	1289	921	122		TraN, a repressor of an *Enterococcus* conjugative type IV secretion system [*Enterococcus faecalis*]	6G1T_A	100 (100)
orf3	2289	1318	323	C-terminal domain of transfer protein TraM	Hypothetical protein [*E. faecalis*]	WP_141415573.1	99 (100)
orf4	3250	2306	314		TrsL [*E. faecalis*]	AFW17916.1	99 (100)
orf5	4173	3250	307		Hypothetical protein [*Listeria monocytogenes*]	HAC2068483.1	99 (99)
orf6	5846	4191	551	Type IV secretory system Conjugative DNA transfer	TrsK [*Enterococcus thailandicus*]	AFW17886.1	99 (100)
orf7	6270	5839	143		Hypothetical protein P025_02896 [*E. faecalis* EnGen0425]	ETU59397.1	98 (99)
orf8	6826	6275	183		Hypothetical protein [*E. faecalis*]	WP_115253299.1	97 (100)
orf9	7948	6839	369	CwlT-like N-terminal lysozyme domain	CHAP domain-containing protein [*L. monocytogenes*]	EAF8635200.1	100 (100)
orf10	9322	7970	450		Conjugal transfer protein TraF [*E. faecalis*]	RNA46706.1	100 (100)
orf11	11297	9336	653	Type IV secretory pathway, VirB4 component	TrsE protein [*E. faecalis*]	MBF0654564.1	100 (100)
orf12	11937	11308	209		Hypothetical protein [*E. faecalis*]	WP_010783405.1	100 (100)
orf13	12337	11954	127		TrsC [*E. thailandicus*]	AFW17880.1	100 (100)
orf14	12688	12356	110		TrsB [*E. thailandicus*]	AFW17858.1	100 (100)
orf15	14697	12712	661	MobA/MobL family protein	MobA/MobL family protein [*E. faecalis*]	WP_194187770.1	100 (100)
orf16	14989	15288	99		Hypothetical protein [*E. faecalis*]	WP_010783409.1	98 (99)
orf17	15291	15548	85		Hypothetical protein [*E. faecalis*]	ADM24844.1	100 (100)
orf18	15876	15571	101		Hypothetical protein [*Enterococcus* sp. HMSC063D12]	WP_070544069.1	100 (100)
orf19	16407	15910	165		Molecular chaperone DnaJ [*E. faecium*]	EGP1922740.1	100 (100)
orf20	16744	16427	105		ssDNA binding protein [*E. thailandicus*]	AFW17850.1	100 (100)
orf21	18048	17185	287	Zeta toxin protein	Zeta toxin family protein [*E. faecium*]	WP_080333884.1	100 (100)

432	*orf22*	18322	18050	90	Epsilon antitoxin protein	Bacterial epsilon antitoxin family protein [*E. faecalis* 62]	ADX79060.1	99 (100)
433	*orf23*	18548	18339	69	Omega Transcriptional Repressor	Omega Transcriptional Repressor [*Clostridioides difficile*]	AXU58284.1	100 (100)
434	*orf24*	19549	18653	298	ParA family protein	Chromosome partitioning protein ParA [*L. monocytogenes*]	EAC2977794.1	100 (100)
435	*orf25*	21796	19652	714	DNA topoisomerase IA	Type IA DNA topoisomerase [*Lactococcus raffinolactis*]	WP_172507152.1	100 (100)
436	*orf26*	22413	21796	205	Serine Recombinase family protein	Resolvase, N-terminal domain protein [*E. faecalis* EnGen0297]	EEI55824.1	100 (100)
437	*orf27*	22597	22427	56		Hypothetical protein [*E. faecalis*]	ADM24823.1	100 (100)
438	*orf28*	24435	22945	496	Primase C terminal 1 (PriCT-1)	RepE protein [*E. faecium* EnGen0035]	ELB18014.1	100 (100)
439	*orf29*	25075	24827	82	Transcriptional regulator	XRE family transcriptional regulator [*E. faecalis*]	EGO8392756.1	100 (100)
440	*orf30*	25491	25685	64	Site-specific recombinase XerD	Tyrosine-type recombinase/integrase [*E. faecalis*]	WP_194187778.1	100 (100)
441	*orf31*	26986	25913	357	23S rRNA methyltransferase	23S rRNA (adenine(2503)-C(8))-methyltransferase Cfr(D) [*E. faecalis*]	WP_194187780.1	100 (100)
442	*orf32*	28747	27113	544	ABC-F type ribosomal protection protein	ARE-ABC-F family resistance factor PoxtA2 [*Enterococcus gallinarum*]	QWE90340.1	100 (100)
443	*orf33*	29357	30037	226	IS*6* family transposase	IS*1216* transposase [*E. faecalis*]	ADN34762.1	100 (100)
444	*orf34*	31776	30349	475	Chloramphenicol/florfenicol efflux pump	Chloramphenicol/florfenicol efflux MFS transporter FexA [*E. faecium*]	HAP7627980.1	100 (100)
445	*orf35*	32035	31883	50		Hypothetical protein [*E. gallinarum*]	QWE90344.1	100 (100)
446	*orf36*	32746	33426	226	IS*6* family transposase	IS6-like element IS*1216* family transposase [*Enterococcus*]	WP_086558022.1	100 (100)

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

Figure S1. S1-PFGE **(A)**, and hybridization with the *poxtA2* probe **(B)**.
M, DNA molecular weight low-range PFG marker (New England Biolabs, Ipswich, MA, USA). The size (kb) of fragments is reported on the left; lane 1, *E. casseliflavus* V308; lane 2, *E. casseliflavus* V311; lane 3, *E. faecalis* V359; lane 4, *E. faecalis* V386; lane 5, *E. faecalis* V392.

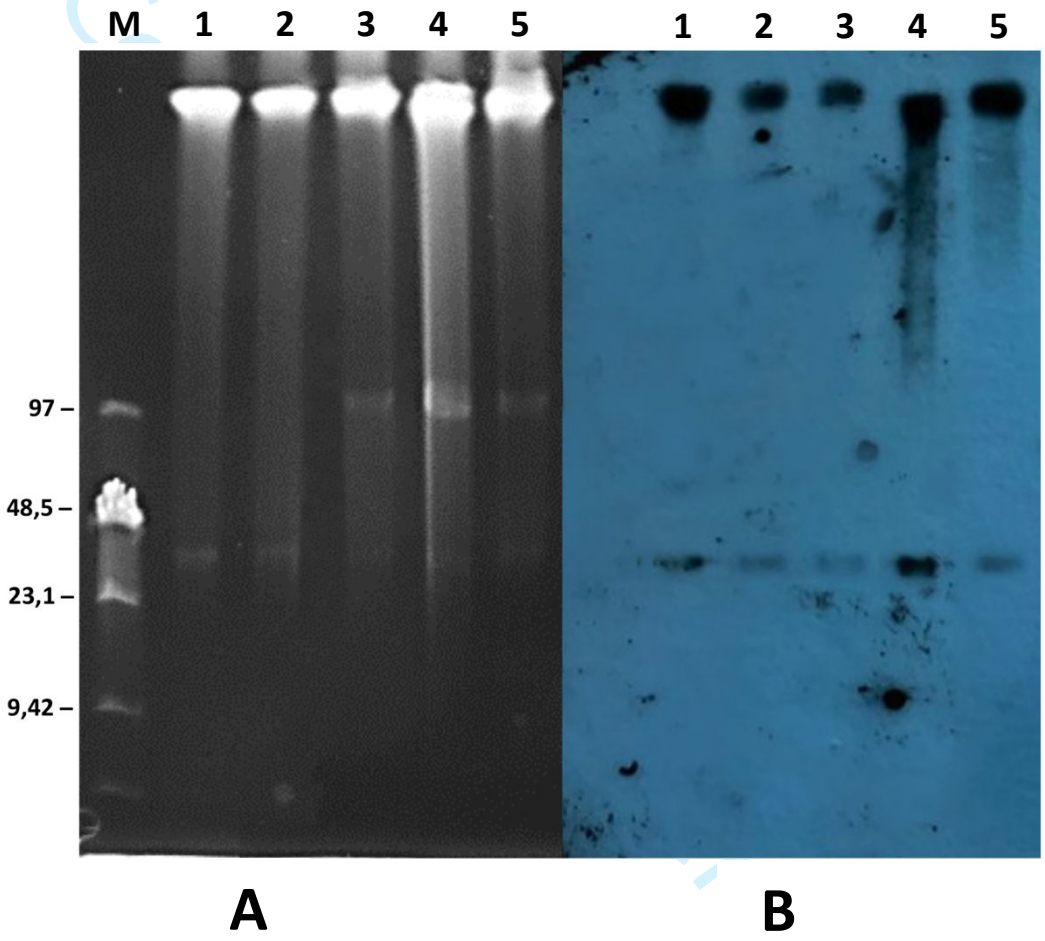

Figure S2. S1-PFGE **(A)**, and hybridization with the *cfr*(D) probe **(B)**.
M, DNA molecular weight low-range PFG marker (New England Biolabs, Ipswich, MA, USA). The size (kb) of fragments is reported on the left; lane 1, *E. casseliflavus* V308; lane 2, *E. casseliflavus* V311; lane 3, *E. faecalis* V359; lane 4, *E. faecalis* V386; lane 5, *E. faecalis* V392.