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Occurrence of a plasmid co-carrying cfr(D) and poxtA2 linezolid resistance genes in *Enterococcus faecalis* and *Enterococcus casseliflavus* from porcine manure, Italy

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**Occurrence of a new plasmid co-carrying cfr(D) and poxtA2
linezolid resistance genes in Enterococcus faecalis and
Enterococcus casseliflavus from porcine manure, Italy**

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Brief report

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2 **Occurrence of a new plasmid co-carrying *cfrr(D)* and *poxtA2* linezolid**
3 **resistance genes in *Enterococcus faecalis* and *Enterococcus***
4 ***casseliflavus* from porcine manure, Italy**

5
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27

28 **Abstract**

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

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

37 **Results:** Three *E. faecalis* and two *E. casseliflavus* isolates besides *cfr(D)* gene also
38 carried the recently described *poxA2* variant. *cfr(D)* and *poxA2* were co-located on a
39 novel 33,480-bp plasmid, pV386, 95-100% identical (coverage 84%) to the Tn6349
40 transposon of *Staphylococcus aureus* AOUC-0915. Both genes also showed a
41 chromosomal location. Same sequence identities were found from the comparison with
42 currently known *poxA2* genetic elements. In pV386 *poxA2* gene, not bounded by two
43 IS1216, was closely associated to the *cfr(D)* and *fexA* genes in the new plasmid. pV386
44 was always transferred by filter mating to *E. faecium* 64/3 recipient.

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

48

49 **Introduction**

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

58 The abundance of *Enterococcus* spp. in animal and human faeces and their prolonged
59 survival in the environment have made them a well-established indicator of faecal
60 contamination in the environment³ and in the food.⁴ More recently, enterococci have
61 been also proposed for monitoring antibiotic resistance in food animals.⁵

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

69 Oxazolidinones, including linezolid and tedizolid, bind in the V domain of the 23S rRNA
70 of the 50S ribosomal subunit and inhibit protein synthesis.⁸ Besides the mutations in 23S
71 rRNA and ribosomal proteins,⁷ linezolid resistance can arise from the acquisition of
72 transferable resistance determinants: *cfr* and its variants, *optrA* or *poxtA* genes. Cfr and
73 Cfr-like methylases confer resistance to phenicols, lincosamides, oxazolidinones,
74 pleuromutilines and streptogramin A (PhLOPS_A phenotype),⁹ whereas the ABC-F
75 proteins OptrA and PoxTA⁹ leads to a decreased susceptibility to phenicols and
76 oxazolidinones (including tedizolid) by a ribosomal protection mechanism.¹⁰

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

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

89 In this study, we characterized a new transferable plasmid co-carrying *cfr*(D) and *poxA2*
90 genes detected in *E. faecalis* and two *Enterococcus casseliflavus* isolates from swine
91 manure.

92

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93 **Materials and methods**

94

95 **Bacterial strains**

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

104

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

111

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

119

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

122

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

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

133

134 **WGS and sequence analysis.** Bacterial genomic DNA was extracted by the QIAcube
135 automated extractor using DNeasy PowerLyzer PowerSoil Kit according to
136 manufacturer's instructions (Qiagen, Germany). Extracted DNA was subjected to WGS
137 by a hybrid approach using both short-read Illumina MiSeq platform (MicrobesNG,
138 Birmingham, UK) with a 2 x 250 paired end technology and a long-read sequencing
139 approach (MinION, Oxford Nanopore Technologies, Oxford, UK). SPAdes 3.15.2
140 software was used for the hybrid assembly of short and long reads
141 (<http://bioinf.spbau.ru/spades>).

142 In silico identification of acquired antimicrobial resistance genes and ribosomal
143 mutations involved in oxazolidinone resistance were carried out using dedicated tools
144 available at the Center for Genomic Epidemiology available at
145 <http://www.genomicepidemiology.org/> (MLST v.2.0, ResFinder v.3.2, LRE-finder v.1.0
146 and PlasmidFinder 2.1) and by the BLAST suite
147 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

148

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

151

152 **Results and discussion**

153

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

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

164 Sequencing also showed that all *poxtA*-positive isolates actually harboured the *poxtA2*
165 variant recently detected in a human linezolid-resistant *Enterococcus gallinarum* from
166 Colombia¹⁹ and in *E. faecalis* EFS0019 of swine origin in in Korea (Table 1).²⁰ Unlike
167 *poxtA*, *poxtA2* variant (accession no. MZ171245) was not truncated by an *IS1216*
168 insertion at the 3' end, thus eight new amino acid (TPEEEQKY) sequence replaced the
169 six-amino-acid (GSVAKF) of wild type protein. The Authors suggested that this variant
170 could be considered the ancestor of the *poxtA* gene.¹⁹

171

172 **Phylogenetic relatedness of the isolates.** All strains were typed by SmaI-PFGE (Table
173 1). Enterococcal isolates belonged to 4 different SmaI-PFGE types (A to D), and one
174 subtypes (A1). *E. faecalis* strains belonged to 2 different SmaI-PFGE types (A, B); *E.*
175 *faecalis* V359 and V386 were found to be closely related (A and A₁, respectively). *E.*
176 *casseliflavus* isolates exhibited two different pulsotypes (C and D) (Table 1).
177 *E. faecalis* strains were associated to ST32, a sequence type previously described in
178 enterococci isolated from chicken faeces (www.pubmlst.org), from faeces and carcass of
179 poultry and pigs²¹ and related to the occurrence of amyloidosis and septicaemia in
180 broilers.²²

181

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

188 Since *cfr(D)* and *poxA2* genes exhibited both chromosomal and plasmid location, we
189 analyzed by inverse PCR the presence of circular intermediates. No circular form was
190 detected for both *cfr(D)* and *poxA2* genes.

191

192 **Transferability of oxazolidinone resistance genes.** *cfr(D)* and *poxA2* genes were
193 successfully co-transferred from both *E. faecalis* and *E. casseliflavus* donors to *E.*
194 *faecium* 64/3 recipient with frequencies ranging from 2.3×10^{-1} to 8.0×10^{-4} per
195 recipient (Table 2). Transconjugants exhibited resistance to florfenicol and
196 chloramphenicol, reduced susceptibility or susceptibility to linezolid and resistance to
197 tedizolid. All transconjugants were susceptible to tetracycline and vancomycin (Table 2).
198 PCR and Sanger sequencing indicated that all transconjugants acquired both *cfr(D)* and
199 *poxA2* genes (Table 2).

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

204

205 **WGS analysis.** All five test strains were subjected to WGS analysis. Bioinformatic data
206 revealed that the *cfr(D)*- and *poxA2*-carrying plasmid, named pV386, was identical in *E.*
207 *faecalis* and in *E. casseliflavus* isolates. This 33,480-bp plasmid (34% GC content),
208 exhibited 36 ORFs encoding proteins ≥ 50 amino acids (Figure 1 and Table S2). BLASTN
209 analysis revealed that pV386 was 95-100% identical (coverage 84%) to the Tn6349
210 transposon (accession no. MH746818.1) carrying *cfr* and *poxA* in *S. aureus* AOUC-
211 0915.²³ Tn6349 was closely related to the pE35048-oc, a pRE25 derivative carrying *cfr*,
212 *erm(B)* and *optrA* resistance genes and described in a clinical *E. faecium* strain.²⁴
213 Comparison between the sequences of pV386 and those of the currently known *poxA2*
214 genetic elements showed a nucleotide identity of 99% (coverage 16%) with the pIB-BOL
215 plasmid (accession no. MZ171245) of *E. gallinarum* Eg-IV02¹⁹ and of 96-100%
216 (coverage 37%) with the contigs #20 and #26 (accession no. QUSQ00000000) from *E.*
217 *faecalis* EFS0019 draft genome (Figure 1).²⁰

218 Unlike detected in pIB-BOL, in pV386 *poxA2* gene was not bounded by two *IS1216*
219 arranged in the opposite orientation but was closely associated to the *cfr(D)* gene.

220 Upstream of *poxA2* we found the *fexA* gene in turn bracketed by two *IS1216* with the
221 same polarity, as previously described by Jung *et al.* (Figure 1).²⁰

222 A complete conjugation region, *repE* (belonging to the *rep1* family) and *parA* genes
223 (responsible for plasmid replication and partitioning, respectively), and a toxin-antitoxin

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

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

228

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229 **Conclusions**

230 To the best of our knowledge, this is the first detection of the *poxA2* variant in *E.*
231 *casseliflavus* species.

232 Occurrence of the pV386 – a new conjugative plasmid co-carrying *cfr*(D) and *poxA2*
233 genes – both in *E. faecalis* and *E. casseliflavus* isolates from swine manure,
234 demonstrates that intense genetic exchanges between enterococci occur promoting the
235 spread of oxazolidinone resistance determinants.

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

240

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248 Transparency declarations

249 None to declare

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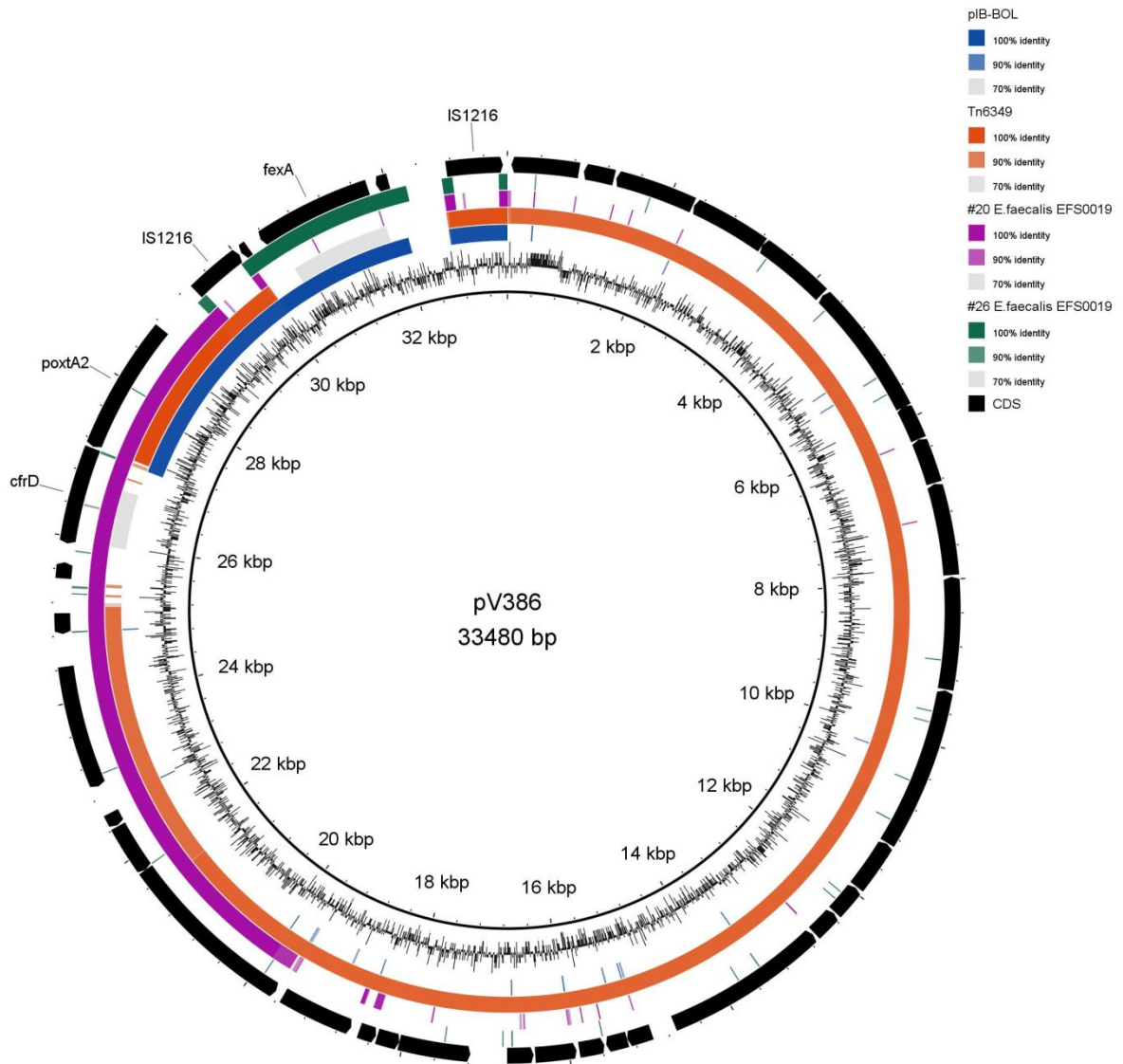
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317 plasmid carrying the *optrA* and *cfr* resistance genes from an *Enterococcus faecium*
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- 319

320 **Figures**

321 **Figure 1.** Circular map of the pV386 plasmid in comparison with other genetic elements and
 322 contigs from draft genome using BRIG software. Plasmids, transposons and contigs included in
 323 the analysis were as follows: (inner to outer circles) pIB-BOL of *E. gallinarum* Eg-IV02
 324 (accession no. MZ171245), Tn6349 of *S. aureus* AOUC-0915 (accession no. MH746818.1), and
 325 #20 and #26 contigs of *E. faecalis* EFS0019 (accession no. QUSQ00000000).
 326 Black arrows indicate the positions and orientations of genes; some antibiotic resistance
 327 determinants and relevant genes described in this study are shown.
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Table 1. Oxazolidinone resistance genes, antimicrobial susceptibility profiles, typing data and gene location.

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

^aFFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TDZ, tedizolid; TE, tetracycline; VA, vancomycin.

^bEstimated plasmid size (in kb)

^cc, 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	
<i>E. faecalis</i> V359	<i>cfr</i> (D), <i>poxA2</i>	<i>E. faecium</i> 64/3	8.1 x 10 ⁻²	64	32	4	1	1	0.5	<i>cfr</i> (D), <i>poxA2</i>
<i>E. faecalis</i> V386	<i>cfr</i> (D), <i>poxA2</i>	<i>E. faecium</i> 64/3	2.3 x 10 ⁻¹	32	16	2	1	1	0.5	<i>cfr</i> (D), <i>poxA2</i>
<i>E. faecalis</i> V392	<i>cfr</i> (D), <i>poxA2</i>	<i>E. faecium</i> 64/3	8.0 x 10 ⁻²	128	64	4	2	1	0.5	<i>cfr</i> (D), <i>poxA2</i>
<i>E. casseliflavus</i> V308	<i>cfr</i> (D), <i>poxA2</i>	<i>E. faecium</i> 64/3	2.3 x 10 ⁻²	64	32	4	1	1	0.5	<i>cfr</i> (D), <i>poxA2</i>
<i>E. casseliflavus</i> V311	<i>cfr</i> (D), <i>poxA2</i>	<i>E. faecium</i> 64/3	9.5 x 10 ⁻⁴	128	64	4	1	1	0.5	<i>cfr</i> (D), <i>poxA2</i>

^aFFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TDZ, tedizolid; TE, tetracycline; VA, vancomycin.

^bND, 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).

371 **Supplementary materials**

372

373 **Table S1.** Primer pairs used in this study.

Gene	Primer		Reference	Product size (bp)
	Designation	Sequence (5'-3')		
<i>cfr(D)</i>	<i>cfrD</i> -FW ^a	TGGCTGGGAATCTTTTGTGTA	This study	304
	<i>cfrD</i> -RV	TAGTCGTTTTATTTTAGGAA		
<i>poxA</i>	<i>poxA</i> -FW ^a	GAACGCTTGGAGTATTTGACTTC	1	778
	<i>poxA</i> -RV	CTGGACTGAGAATACCCATC		
Detection of circular forms				
<i>poxA</i>	<i>poxA</i> -3	GACGAGCCGACCAACCACCT	2	
	<i>poxA</i> -4	TTGGATTTTTGTCCGCCTGAA		
<i>cfr(D)</i>	<i>cfrD</i> -3	TACAAAAAGATTCCCAGCCA	This study	
	<i>cfrD</i> -4	TTCCTAAAATAAAAACGACTA		

391 ^a These primer pairs were also used to obtain specific probes.

392

393 References:

- 394 1. Brenciani A, Fioriti S, Morroni G *et al.* Detection in Italy of a porcine *Enterococcus faecium* isolate
 395 carrying the novel phenicol-oxazolidinonetetracycline resistance gene *poxA* 2019; **74**: 817–8.
 396 2. Fioriti S, Morroni G, Coccitto SN, *et al.* Detection of oxazolidinone resistance genes and
 397 characterization of genetic environments in enterococci of swine origin, Italy. *Microorganisms* 2020; **8**:
 398 2021.
 399

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

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402 BLASTP analysis^a

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404	ORF	Start	Stop	Size	Predicted function	Most significant database match	Accession no.	% Amino acid
405		(bp)	(bp)	(amino				identity (% amino
406				acids)				acid similarity)
407								
408	<i>orf1</i>	861	49	270		LPXTG-domain-containing protein cell wall anchor domain, partial	EOI35867.1	100 (100)
409						[<i>Enterococcus faecium</i> EnGen0313]		
410	<i>orf2</i>	1289	921	122		TraN, a repressor of an <i>Enterococcus</i> conjugative type IV secretion system	6G1T_A	100 (100)
411						[<i>Enterococcus faecalis</i>]		
412	<i>orf3</i>	2289	1318	323	C-terminal domain of transfer protein TraM	Hypothetical protein [<i>E. faecalis</i>]	WP_141415573.1	99 (100)
413	<i>orf4</i>	3250	2306	314		TrsL [<i>E. faecalis</i>]	AFW17916.1	99 (100)
414	<i>orf5</i>	4173	3250	307		Hypothetical protein [<i>Listeria monocytogenes</i>]	HAC2068483.1	99 (99)
415	<i>orf6</i>	5846	4191	551	Type IV secretory system Conjugative	TrsK [<i>Enterococcus thailandicus</i>]	AFW17886.1	99 (100)
416					DNA transfer			
417	<i>orf7</i>	6270	5839	143		Hypothetical protein P025_02896 [<i>E. faecalis</i> EnGen0425]	ETU59397.1	98 (99)
418	<i>orf8</i>	6826	6275	183		Hypothetical protein [<i>E. faecalis</i>]	WP_115253299.1	97 (100)
419	<i>orf9</i>	7948	6839	369	CwlT-like N-terminal lysozyme domain	CHAP domain-containing protein [<i>L. monocytogenes</i>]	EAF8635200.1	100 (100)
420	<i>orf10</i>	9322	7970	450		Conjugal transfer protein TraF [<i>E. faecalis</i>]	RNA46706.1	100 (100)
421	<i>orf11</i>	11297	9336	653	Type IV secretory pathway, VirB4 component	TrsE protein [<i>E. faecalis</i>]	MBF0654564.1	100 (100)
422	<i>orf12</i>	11937	11308	209		Hypothetical protein [<i>E. faecalis</i>]	WP_010783405.1	100 (100)
423	<i>orf13</i>	12337	11954	127		TrsC [<i>E. thailandicus</i>]	AFW17880.1	100 (100)
424	<i>orf14</i>	12688	12356	110		TrsB [<i>E. thailandicus</i>]	AFW17858.1	100 (100)
425	<i>orf15</i>	14697	12712	661	MobA/MobL family protein	MobA/MobL family protein [<i>E. faecalis</i>]	WP_194187770.1	100 (100)
426	<i>orf16</i>	14989	15288	99		Hypothetical protein [<i>E. faecalis</i>]	WP_010783409.1	98 (99)
427	<i>orf17</i>	15291	15548	85		Hypothetical protein [<i>E. faecalis</i>]	ADM24844.1	100 (100)
428	<i>orf18</i>	15876	15571	101		Hypothetical protein [<i>Enterococcus</i> sp. HMSC063D12]	WP_070544069.1	100 (100)
429	<i>orf19</i>	16407	15910	165		Molecular chaperone DnaJ [<i>E. faecium</i>]	EGP1922740.1	100 (100)
430	<i>orf20</i>	16744	16427	105		ssDNA binding protein [<i>E. thailandicus</i>]	AFW17850.1	100 (100)
431	<i>orf21</i>	18048	17185	287	Zeta toxin protein	Zeta toxin family protein [<i>E. faecium</i>]	WP_080333884.1	100 (100)

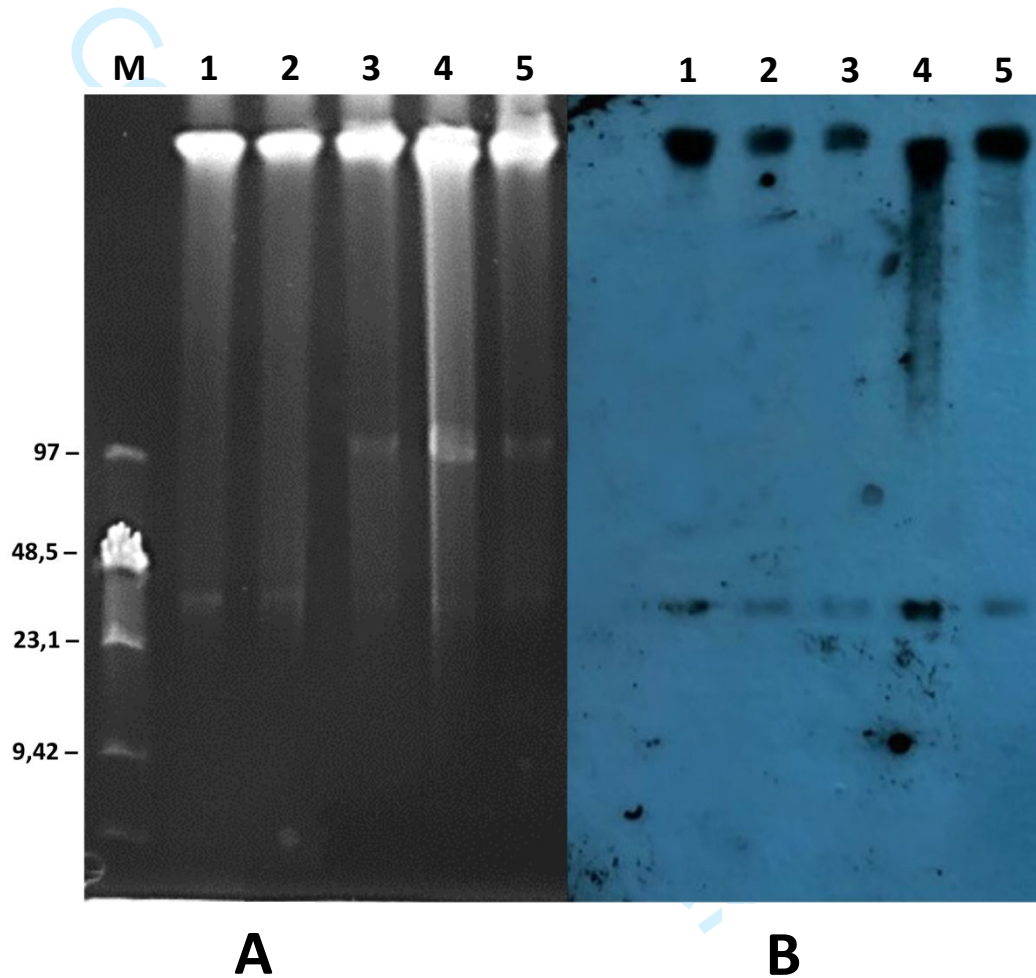
432	<i>orf22</i>	18322	18050	90	Epsilon antitoxin protein	Bacterial epsilon antitoxin family protein [<i>E. faecalis</i> 62]	ADX79060.1	99 (100)
433	<i>orf23</i>	18548	18339	69	Omega Transcriptional Repressor	Omega Transcriptional Repressor [<i>Clostridioides difficile</i>]	AXU58284.1	100 (100)
434	<i>orf24</i>	19549	18653	298	ParA family protein	Chromosome partitioning protein ParA [<i>L. monocytogenes</i>]	EAC2977794.1	100 (100)
435	<i>orf25</i>	21796	19652	714	DNA topoisomerase IA	Type IA DNA topoisomerase [<i>Lactococcus raffinolactis</i>]	WP_172507152.1	100 (100)
436	<i>orf26</i>	22413	21796	205	Serine Recombinase family protein	Resolvase, N-terminal domain protein [<i>E. faecalis</i> EnGen0297]	EEI55824.1	100 (100)
437	<i>orf27</i>	22597	22427	56		Hypothetical protein [<i>E. faecalis</i>]	ADM24823.1	100 (100)
438	<i>orf28</i>	24435	22945	496	Primase C terminal 1 (PriCT-1)	RepE protein [<i>E. faecium</i> EnGen0035]	ELB18014.1	100 (100)
439	<i>orf29</i>	25075	24827	82	Transcriptional regulator	XRE family transcriptional regulator [<i>E. faecalis</i>]	EGO8392756.1	100 (100)
440	<i>orf30</i>	25491	25685	64	Site-specific recombinase XerD	Tyrosine-type recombinase/integrase [<i>E. faecalis</i>]	WP_194187778.1	100 (100)
441	<i>orf31</i>	26986	25913	357	23S rRNA methyltransferase	23S rRNA (adenine(2503)-C(8))-methyltransferase Cfr(D) [<i>E. faecalis</i>]	WP_194187780.1	100 (100)
442	<i>orf32</i>	28747	27113	544	ABC-F type ribosomal protection protein	ARE-ABC-F family resistance factor PoxA2 [<i>Enterococcus gallinarum</i>]	QWE90340.1	100 (100)
443	<i>orf33</i>	29357	30037	226	IS6 family transposase	IS1216 transposase [<i>E. faecalis</i>]	ADN34762.1	100 (100)
444	<i>orf34</i>	31776	30349	475	Chloramphenicol/florfenicol efflux pump	Chloramphenicol/florfenicol efflux MFS transporter FexA [<i>E. faecium</i>]	HAP7627980.1	100 (100)
445	<i>orf35</i>	32035	31883	50		Hypothetical protein [<i>E. gallinarum</i>]	QWE90344.1	100 (100)
446	<i>orf36</i>	32746	33426	226	IS6 family transposase	IS6-like element IS1216 family transposase [<i>Enterococcus</i>]	WP_086558022.1	100 (100)

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For each ORF, only the most significant identity detected is listed.

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



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