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Linezolid-resistant *Enterococcus gallinarum* isolate of swine origin carrying cfr, optrA and poxtA genes

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1
2 **Linezolid-resistant *Enterococcus gallinarum* isolate of swine origin**
3 **carrying *cfr*, *optrA* and *poxxA* genes**
4

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21 **Running title:**

22 *cfr*, *optrA* and *poxxA* genes in a porcine *Enterococcus gallinarum*
23 -----
24

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32 **Abstract**

33 **Objectives:** To characterize a linezolid-resistant *Enterococcus gallinarum* isolate of
34 porcine origin co-carrying *cfr*, *optrA* and *poxxA* genes.

35 **Methods:** The plasmidome was evaluated by S1-PFGE/hybridisation. The genome was
36 sequenced using the Illumina platform. The presence of circular intermediates was
37 examined by inverse PCR. Transferability of oxazolidinone resistance genes was
38 investigated by transformation and conjugation.

39 **Results:** Two plasmids, the *cfr*- and *optrA*-carrying pEgFS4-1 (35 kb) and the *poxxA*-
40 harbouring pEgFS4-2 (38 kb), were identified. pEgFS4-1 disclosed a distinctive mosaic
41 structure with two cargo regions bounded by identical IS1216 elements interpolated into
42 a backbone related to that of *E. faecium* *vanA*-containing pVEF2. The first cargo region
43 included the *cfr* and *optrA* contexts, whereas the second one carried a Tn554 remnant and
44 the *Inu(A)* gene. Both regions were able to excise in circular form as a unique
45 translocable unit. pEgFS4-2 plasmid was 99% identical to a not fully described *E.*
46 *faecium* pSBC1 plasmid. The *poxxA* environment, flanked by IS1216, was proved to be
47 instable. pEgFS4-2 also exhibited another cargo region containing the *tet(M)*-*tet(L)*
48 genes arranged in tandem and its circular form was detected. Transformation and
49 conjugation experiments failed to demonstrate the transferability of both plasmids to
50 enterococcal recipients. Both plasmids persisted in the absence of selective pressure.
51 **Conclusions:** To the best of our knowledge, this is the first description of a linezolid-
52 resistant *E. gallinarum* isolate of swine origin carrying *cfr*, *optrA* and *poxxA* genes.
53 The co-presence of three linezolid resistance determinants in an intrinsically
54 vancomycin-resistant enterococcal species is cause of concern.

55 **Introduction**

56 Enterococci are traditionally regarded as human opportunistic bacteria and continue to
57 be important nosocomial pathogens; particularly the clinically relevant species
58 *Enterococcus faecalis* and *Enterococcus faecium* are globally considered as leading
59 cause of healthcare-associated infections. Most of their success as nosocomial pathogens
60 is due to their capability to survive in the environment and tolerate disinfectants, as well
61 as to intrinsic resistance to many drugs and their ability to acquire new antibiotic
62 resistance genes. Multidrug resistance is common in enterococcal isolates, which
63 prolongs hospitalization and increases costs and the risk of therapeutic failure. Few
64 options are still available to treat infections due to antibiotic-resistant enterococci.¹
65 Oxazolidinones, including linezolid and tedizolid, are effective antimicrobial agents for
66 the treatment of clinical infections caused by MDR Gram-positive pathogens, including
67 VRE. However, enterococci can also develop resistance to linezolid through acquisition
68 of the *cfr*, *cfr(B)* and *cfr(D)* genes,²⁻⁴ which encode methyltransferases conferring
69 resistance to five classes of antimicrobial agents, including phenicols, lincosamides,
70 oxazolidinones, pleuromutilins, and streptogramin A (PhLOPS_A phenotype).⁵
71 Oxazolidinone resistance can also be due to the acquisition of *optrA* and *poxxA* genes
72 encoding protein of ABC-F family.^{6,7} *Optra* and *PoxxA* lead to decreased susceptibility
73 to phenicols, oxazolidinones (including tedizolid), and tetracyclines (*PoxxA* protein
74 only) by a ribosomal protection mechanism.⁸ Oxazolidinone resistance determinants are
75 commonly located on conjugative plasmids, the main mobile genetic elements
76 responsible for the spread of antibiotic resistance within the enterococcal population.⁹
77 Although oxazolidinones have been approved only for human use, *cfr*, *optrA*, and *poxxA*
78 genes have been detected in enterococcal isolates of animal and environmental origin,¹⁰
79 and very recently even in enterococci from coastal seawaters samples.¹¹
80 *Enterococcus gallinarum*, intrinsically resistant to vancomycin, is only occasionally
81 found in human gut and bovine or swine intestinal microbiota.¹² *E. gallinarum* is
82 considered an opportunistic human pathogen and in recent years, severe infections,
83 including bacteremia, endocarditis, and meningitis, as well as several hospital-acquired
84 outbreaks caused by this enterococcal species, have gradually increased.¹³
85 To date, the *cfr* and *optrA* genes have been identified in *E. gallinarum*, individually^{14,15}
86 or in combination.¹⁶ Very recently, a *poxxA2* variant was detected in a linezolid-resistant
87 *E. gallinarum*.¹⁷
88 To the best of our knowledge, this is the first characterization of a linezolid-resistant *E.*
89 *gallinarum* isolate of swine origin carrying *cfr*, *optrA* and *poxxA* genes.

90 **Materials and methods**

91

92 **Bacterial strain.** The strain *E. gallinarum* FS4 was isolated in the framework of a study
93 (data unpublished) aimed at the detection of resistant enterococci from fecal samples (of
94 healthy animals) collected at swine farms. The strain selected on Slanetz-Bartley agar
95 plates supplemented with florfenicol (10 mg/L) was positive for the presence of the *cfr*,
96 *optrA* and *poxxA* genes using primer pairs previously described (Table S1).¹⁸ Since the
97 isolate FS4 belonged to *E. gallinarum* species and harbored all three *cfr*, *optrA* and
98 *poxxA* genes, a deeper genetic investigation of the strain was carried out in this study.

99

100 **Susceptibility tests.** Susceptibility to florfenicol, chloramphenicol, linezolid,
101 tetracycline, erythromycin and vancomycin (Sigma-Aldrich, St. Louis, MO) by standard
102 broth microdilution assays according to the CLSI,¹⁹ and to tedizolid using Etest strips
103 (Liofilchem, Roseto degli Abruzzi, Italy). The results were interpreted according to the
104 EUCAST clinical breakpoint tables (version 10.0, www.eucast.org). *E. faecalis*
105 ATCC29212 and *S. aureus* ATCC29213 were used for quality control in susceptibility
106 tests.

107

108 **S1-PFGE, Southern blotting, and hybridisation assays.** Genomic DNA embedded in
109 agarose gel plugs was digested with S1 nuclease (Thermo Fisher Scientific, Milan,
110 Italy), and chromosomes and plasmids separated by PFGE as described previously.²⁰
111 After S1 PFGE, total DNA was blotted onto positively charged nylon membranes
112 (Ambion-Celbio, Milan, Italy) and hybridized with biotin-labelled *cfr*, *optrA*, and *poxxA*
113 DNA probes as described elsewhere (Table S1).²¹

114

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

123 In silico identification of acquired antimicrobial resistance genes and ribosomal
124 mutations involved in oxazolidinone resistance were carried out using dedicated tools
125 available at the Center for Genomic Epidemiology available at

126 <http://www.genomicepidemiology.org/> (ResFinder v.3.2, LRE-finder v.1.0) and by the
127 BLAST suite (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

128

129 **Plasmid isolation.** Total plasmid DNA was isolated using the GeneElute Plasmid
130 Miniprep Kit (Sigma), with the addition of 18 mg/ml lysozyme (Sigma) to the buffer.

131

132 **Transformation and conjugation experiments.** Purified plasmid DNA extracted from
133 *E. gallinarum* FS4 was transformed into *E. faecalis* JH2-2 recipient by
134 electrotransformation.²² Transformants were selected on plates supplemented with
135 florfenicol (10 mg/L).

136 Conjugal transfer was performed on a membrane filter as described previously²¹ using *E.*
137 *gallinarum* FS4 as the donor and the florfenicol-susceptible *E. faecium* 64/3²³ as
138 recipient. Transconjugants were selected on brain heart infusion agar (BHIA; Oxoid,
139 Basingstoke, UK) plates containing fusidic acid, rifampicin, and florfenicol (all at 10
140 mg/L). The transfer frequency was expressed as the ratio of the cell number (CFU/mL)
141 of the transconjugant to that of the recipient.

142

143 **Detection of circular forms.** Excision of the genetic contexts was detected using both
144 outward-directed primer pairs targeting DNA flanking the cargo regions and primer pairs
145 bounding the empty excision sites (Table S1 and Figure S1).

146

147 **Plasmid stability.** Over time plasmid stability was evaluated by daily serial passages of
148 *E. gallinarum* FS4 on antibiotic-free BHIA. Weekly, some randomly chosen colonies
149 were tested for their susceptibility to oxazolidinones and phenicols and their DNA was
150 extracted and screened for the presence of the oxazolidinone resistance genes by PCR
151 (Table S1). In case of negative testing, the strain was regarded as possibly cured and
152 subjected to WGS for confirmation.

153

154 **Nucleotide sequence accession numbers.** The nucleotide sequences of plasmids
155 pEgFS4-1 and pEgFS4-2 have been deposited in GenBank under accession numbers
156 MZ291452 and MZ291453, respectively.

157 **Results and discussion**

158

159 **Susceptibility testing.** *E. gallinarum* FS4 exhibited an MDR phenotype, including
160 resistance to florfenicol (MIC, 128 mg/L), chloramphenicol (MIC, 32 mg/L), linezolid
161 (MIC, 8 mg/L), tedizolid (MIC, 4 mg/L), tetracycline (MIC, >128 mg/L), erythromycin
162 (MIC, >128 mg/l), and vancomycin (MIC, 8 mg/L).

163

164 **Location of oxazolidinone resistance genes.** S1-PFGE/hybridization assays showed that
165 a *poxTA* gene probe hybridized with a ~35-kb plasmid (Figure S2), whereas experiments
166 performed with *cfr* and *optrA* gene probes were unsuccessful probably due to the low
167 number of plasmid copies (see below).

168

169 **WGS analysis.** Sequencing analysis of *E. gallinarum* FS4 revealed a genome consisting
170 of 3,299,121 bp (40,5% GC content) and the presence of a 34,679-bp plasmid, designated
171 pEgFS4-1 (accession no. MZ291452), and a 38,387-bp plasmid named pEgFS4-2
172 (accession no. MZ291453), both with a 36% GC content.

173

174 In addition to the *vanC* cluster conferring intrinsic vancomycin resistance, ResFinder
175 analysis revealed *E. gallinarum* FS4 strain carried multiple acquired antibiotic resistance
176 genes (ARGs). *vanC* cluster was typically located on the chromosome and showed 100%
177 coverage and 99.82% identity to that of the reference strain *E. gallinarum* BM4174
178 (GenBank accession no. AF162694.1), whereas ARGs were all plasmid-associated. As
179 regards oxazolidinone resistance genes, WGS analysis confirmed that the *cfr* and *optrA*
180 genes were located on pEgFS4-1, whereas the *poxTA* gene was part of the pEgFS4-2.
181 The maps of the two plasmids with all the detected ARGs, are shown in Figures 1 and 2.
182 The major characteristics of the ORFs of pEgFS4-1 and pEgFS4-2 are detailed in Tables
183 S2 and S3, respectively.

183

184 WGS analysis also ruled out the presence of *cfr* variants and mutations in 23S rRNA and
185 L3/L4 ribosomal proteins.

185

186 **pEgFS4-1 plasmid**

187 Sequence analysis of pEgFS4-1 plasmid identified 38 ORFs encoding proteins ≥ 50 amino
188 acids. BLASTN analysis revealed a mosaic structure consisting of a backbone and two
189 cargo regions (Figure 1 and Table S2).

190

191 The backbone region (GC content of 34.0%), spanning from *orf1* to *orf9* and from *orf31*
192 to *orf38*, had high-level identity (from 90 to 99%) and wide synteny to the scaffold of
193 the non-conjugative *vanA*-containing plasmid pVEF2 (accession number AM410096.1),
194 responsible for glycopeptide resistance in *E. faecium* 399/F99/A9 of poultry origin in
195 Norway.²⁴ These two regions contained several genes involved in pEgFS4-1 replication,

195 partitioning and persistence (such as the δ - ω - ε - ζ toxin/antitoxin genetic cluster).
196 However, compared to pVEF2, pEgFS4-1 lacked the Tn1546 transposon and harboured
197 the *erm*(B) gene (*orf9*) responsible for macrolide, lincosamide and streptogramin group B
198 resistance.

199 The two cargo regions were distinguished by their GC content and similarity to portions
200 of known genetic elements:

201 The first cargo region (GC content 38%), spanning from *orf9* to *orf23* and including the
202 *cfr* and *optrA* genetic contexts, disclosed high-level nucleotide identity (from 90 to 99%)
203 to the corresponding region of pWo27-9 plasmid of *Staphylococcus sciuri* (accession no.
204 KX982169.1) (Figure 1).²⁵ Unlike pWo27-9, in pEgFS4-1 this region displayed the
205 insertion of two transposases: the former, belonging to IS30 family, was inserted
206 between *cfr* and *istAS-istBS* genes (encoding transposases belonging to the IS21 family);
207 the latter, belonging to Tn3 family, was truncated and located between the *ble*
208 (responsible for bleomycin resistance) and the *optrA* genes. Upstream of *ant*(4')-Ia
209 (*orf22*), we also detected the insertion of the *aph*(2'')-IIIa, both genes are responsible for
210 aminoglycoside resistance.

211 The *optrA* gene was 99.0% identical to the DNA reference sequence (GenBank accession
212 no. KP399637.1). Furthermore, three amino acid changes (K3E, N12Y, Y176D) were
213 detected in the protein sequence compared to OptrA_{E349} (99% amino acid identity and
214 similarity). Interestingly, the OptrA_{EYD} variant was previously detected in porcine *E.*
215 *faecalis* and *Enterococcus avium* isolates recovered during an antibiotic surveillance in
216 central Italy.¹⁶

217 The second cargo region (GC content 35%), showing high-level nucleotide identity (from
218 90 to 99%) to the corresponding portion of *Lactobacillus johnsonii* UMNLJ22
219 chromosome (accession no. CP021704.1), displayed a mosaic structure with the plasmid
220 replication *repB* gene (*orf24*), a truncated *mobM* relaxase gene (*orf27*), and *lnu*(A)
221 (*orf28*) responsible for lincosamide resistance. This region was disrupted due to
222 insertion in *mobM* gene of a 1.3-kb Tn554 remnant showing *ant*(9)-Ia gene (responsible
223 for spectinomycin resistance), and a truncated *erm*(A) gene lacking 270-bp at 5' end.

224 Both cargo regions were bounded by identical IS1216 elements with the same
225 orientation: the *cfr-optrA* genetic context was flanked by *orf9* and *orf23*, while the
226 Δ Tn554/*lnu*(A) genetic environment was bracketed by *orf23* and *orf31*. The presence of
227 specific direct repeats flanking IS1216 elements was not detected.

228 A PCR assay showed that these two cargo regions were able to excise in circular form as
229 a unique translocable unit (TU). Sequencing data displayed an IS1216 element in the
230 circular form and an empty excision site devoid of IS element (Table S2 and Figure S1).

231 Very recently, Harmer *et al.* proposed a novel nomenclature for genetic elements that are
232 bounded by members of the IS26 family orientated in the same direction defining them
233 as “pseudo-compound transposons (PCTs)”.²⁶ Since the TU contains the *cfp* and *optrA*
234 genes and IS1216 elements (belongs to the IS26 family) with the same orientation, it
235 could be classified actually as a PCT.

236 Transfer experiments of the pEgFS4-1 to *E. faecalis* JH2-2 and *E. faecium* 64/3
237 recipients were unsuccessful. Sequencing analysis showed the lack in pEgFS4-1 of a
238 complete transfer machinery, which explains the failure of the conjugation experiments.

239

240 **pEgFS4-2 plasmid**

241 pEgFS4-2 plasmid exhibited 99% DNA identity (coverage, 98%) with the conjugative
242 plasmid pSBC1 (accession no. CP038169), partially described in a porcine *E. faecium*
243 (Figure 2).²⁷ Moreover, pEgFS4-2 displayed 99% DNA identity (coverage, 40%) with a
244 region of Tn6657 transposon (containing *fexB* and *poxTA* genes). Tn6657 is part of
245 Tn6349 (accession no. MH746818.1), the first *poxTA*-carrying element characterized in
246 the chromosome of the clinical *S. aureus* AOUC-0915 isolate.²⁸ In pEgFS4-2, the *poxTA*
247 genetic context, flanked by IS1216 with the same polarity, was highly conserved; inverse
248 PCR experiments and sequencing showed that a circular form of *poxTA* genetic context
249 was detectable. On the other hand, the *poxTA* context excision due to an IS1216-mediated
250 recombination has already been established^{10,11,16,29,30} suggesting that the circularisation
251 can facilitate the spreading of this oxazolidinone resistance gene.

252 Interestingly, pEgFS4-2 exhibited another 19,337-bp cargo region (from *orf11* to *orf30*)
253 containing the tetracycline resistance *tet(M)* and *tet(L)* genes arranged in tandem (*orf25*
254 and *orf26*), and several genes involved in transposition and plasmid replication (Table
255 S3). This genetic context, bounded by two identical IS1216 insertion sequences (*orf11*
256 and *orf30*) with the same orientation, was integrated within *orf10*, encoding a DNA
257 topoisomerase, splitting the gene into two portions. Remarkably, the *tet(M)-tet(L)*
258 genetic context was flanked by 8 bp direct repeats (5'-CAAAAAG-3'), apparently
259 resulting from target site duplication following IS1216-mediated transposition at this
260 site. Inverse PCR assays, with primers targeting the flanking regions the insertion site
261 (Figure S1 and Table S3), indicated the circularisation of *tet(M)-tet(L)* genetic
262 environment confirming the mobility of this region. Sequencing analysis demonstrated
263 the presence of a IS1216 element in the circular form and a single IS1216 copy at the
264 excision site. Interestingly, this genetic context displayed 99% nucleotide identity with a
265 mobile *tet(M)-tet(L)* locus, flanked by two copies of IS1216E elements in the same
266 orientation, detected in pFas4-1 plasmid of the *Enterococcus hirae* of swine origin.³¹ As

267 indicated above for the *cfr-optrA* TU, also the cargo region carrying the *tet(M)-tet(L)*
268 genes could be actually considered as a PTC.²⁶

269 Unlike Lei *et al.* which demonstrated the pSCBC1 transferability,²⁷ we were unable to
270 transfer pEgFS4-2 to enterococcal recipients despite the presence of several genes
271 involved in plasmid conjugation. It should nevertheless be underlined that sequencing
272 analysis displayed that some key genes involved in conjugation were truncated [*traG*
273 (*orf1*), *topA* (*orf10*) and *mobM* (*orf27* and *orf28*), Table S3], which may explain the non-
274 transferability of pEgFS4-2 plasmid. Also transfer to *E. faecalis* JH2-2 via
275 electrotransformation was not observed.

276

277 **Plasmids stability assays.** *E. gallinarum* FS4 was maintained for 30 days in antibiotic-
278 free BHIA. During this time, both a modification in oxazolidinone and phenicols
279 susceptibility and a loss of pEgFS4-1 and pEgFS4-2 plasmids have not been observed.

280 **Conclusions**

281 This is – to the best of our knowledge – the first characterization of a linezolid-resistant
282 *E. gallinarum* isolate of swine origin carrying *cfr*, *optrA* and *poxTA* genes.

283 Although the oxazolidinone resistance genes were carried by non-transferable plasmids
284 their location in PCTs, able to excise as TUs, suggests that they could be mobilized to
285 other conjugative plasmids and spread among Gram-positive cocci.

286 Despite its broad environmental diffusion and low prevalence in clinical specimens, *E.*
287 *gallinarum* species is increasingly implicated in human opportunistic infections and
288 especially patients with concurrent hepatobiliary or onco-hematological diseases seem to
289 be more susceptible to severe invasive infections.¹³ Typically, *E. gallinarum* display
290 low-level resistance to vancomycin due to chromosomally encoded non-transferable *vanC*
291 gene cluster and is relatively susceptible to teicoplanin and linezolid. Although *vanA* and
292 *vanB* genes are typically observed in *E. faecium* and *E. faecalis* species, detection of *E.*
293 *gallinarum* isolates harboring different genotypes of vancomycin resistance (including a
294 dual *vanA* and *vanB* cassette) is worrisome.³² Detection in this species of oxazolidinones
295 resistance genes limits the therapeutic choices in cases of invasive infections causing
296 further concern.

297 Consideration should be given to possibility that *E. gallinarum* isolates full resistant to
298 both glycopeptides and oxazolidinones may emerge in the future.

299

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304

305

306 **Transparency declarations**

307 None to declare

Confidential: for peer review only

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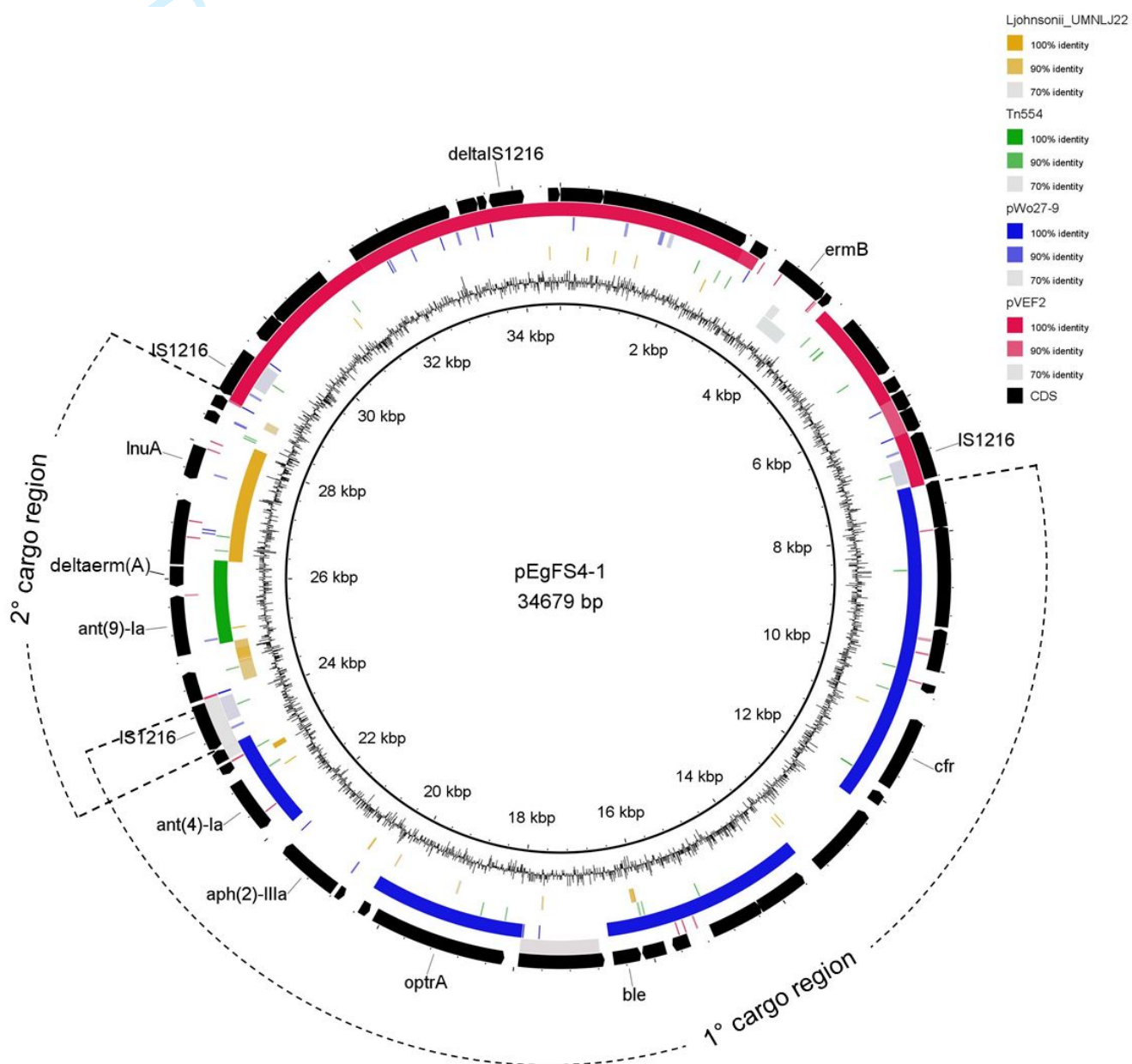
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406 **Figures**

407 **Figure 1.** Circular map of the pEgFS4-1 plasmid in comparison with similar reported
 408 plasmids using BRIG software. Plasmids, transposons and chromosomal regions included
 409 in the analysis were as follows: (inner to outer circles) chromosomal segment of *L.*
 410 *johnsonii* (CP021704.1), Tn554 (X03216.1), pWo27-9 (KX982169.1) and pVEF2
 411 (GenBank ID AM410096.1).

412 Black arrows indicate the positions and orientations of genes; some antibiotic resistance
 413 determinants and relevant genes described in this study are shown.

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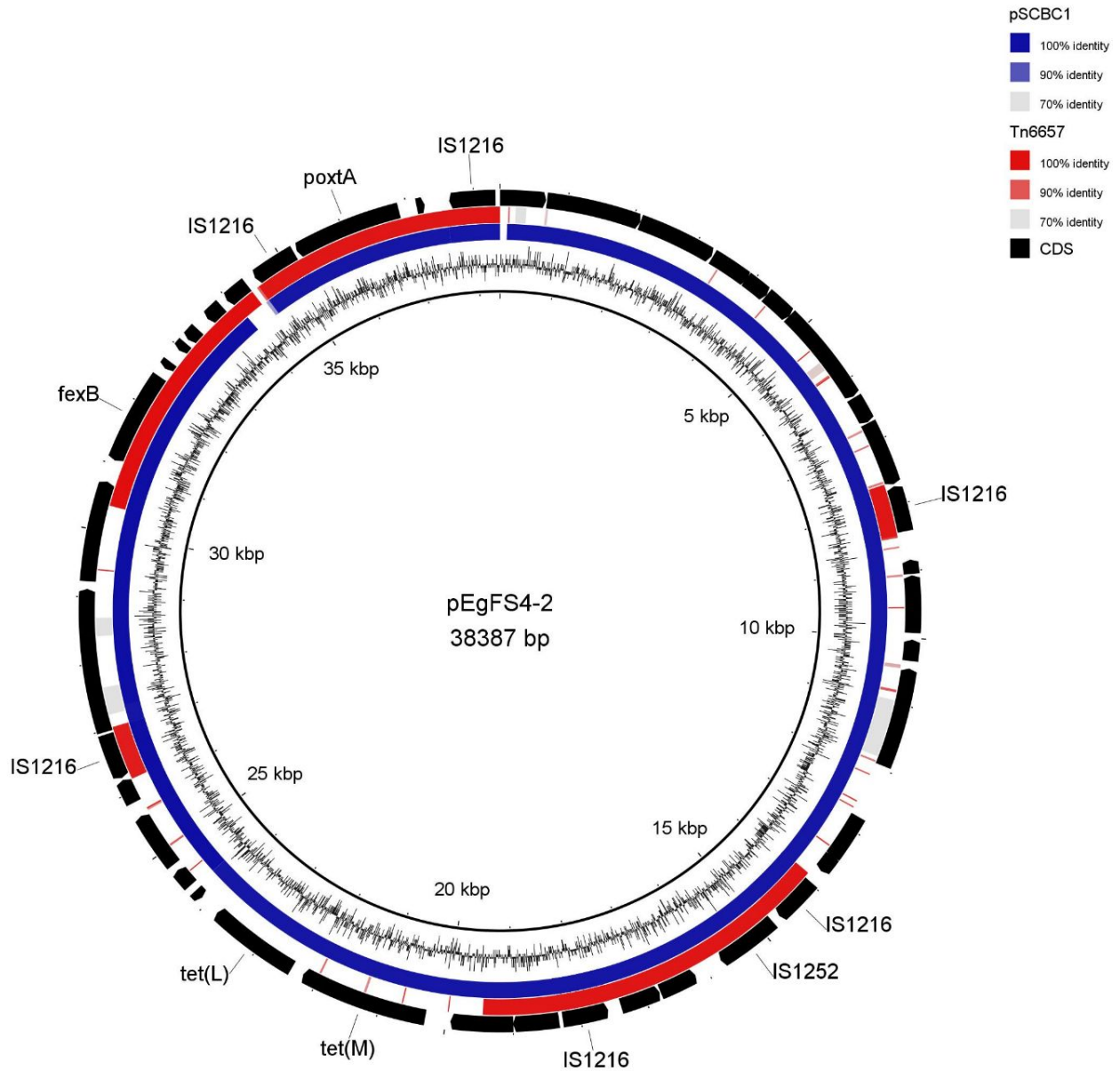
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416 **Figure 2.** Circular map of the pEgFS4-2 plasmid in comparison with similar reported
 417 plasmids using BRIG software. Plasmids and transposons included in the analysis were
 418 as follows: (inner to outer circles) pSBC1 (CP038169) and Tn6657 (MH746818.1).
 419 Black arrows indicate the positions and orientations of genes; some antibiotic resistance
 420 determinants and relevant genes described in this study are shown.

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424 **Supplementary materials**

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

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440 **Detection of circular forms and analysis of the excision sites in pEgFS4-1**

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446 **Detection of circular forms and analysis of the excision sites in pEgFS4-2**

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

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Gene	Primer		Reference	Product size (bp)
	Designation	Sequence (5'-3')		
<i>cfr</i>	<i>cfr</i> -FW ^a	TGAAGTATAAAGCAGGTTGGGAGTCA	1	746
	<i>cfr</i> -RV ^a	ACCATATAATTGACCACAAGCAGC		
<i>optrA</i>	<i>optrA</i> -FW ^a	TACTTGATGAACCTACTAACCA	1	422
	<i>optrA</i> -RV ^a	CCTTGAACTACTGATTCTCGG		
<i>poxA</i>	<i>poxA</i> -FW ^a	GAACGCTTGGAGTATTTGACTTC	1	778
	<i>poxA</i> -RV ^a	CTGGACTGAGAATACCCATC		
<i>orf10</i>	<i>orf10</i> inv	CGAAAAACGGTTGGCACGGTA	This study	
<i>orf30</i>	<i>orf30</i> -fw	CGTTTATTGTGTATCCAGAA		
<i>orf8</i>	<i>orf8</i> -fw	GGGCAACCAGGGTCAGGGAAAA	This study	
<i>orf32</i>	<i>orf32</i> -inv	AAGAAAGAAAAAAGGAAGAAGA		
<i>orf12</i>	<i>orf12</i> inv	AAACTGATTTTTTGTGATTTCG	This study	
<i>orf29</i>	<i>orf29</i> -fw	AAAGGCTGAAAGAGTAAAAGA		
<i>orf9</i>	<i>orf9</i> -fw	CCAAAGGAGCAGGACGGT	This study	
<i>orf10</i>	<i>orf10</i> -rv	TTCAGGGAAAATGGGTAAAT		
<i>poxA</i>	<i>poxA</i> -3	GACGAGCCGACCAACCACCT	2	
	<i>poxA</i> -4	TTGGATTTTTGTCCGCCTGAA		

References:

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463 **Table S2.** Amino acid sequence identities/similarities of putative proteins encoded by the pEgFS4-1 (GenBank accession no. MZ291452).

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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)
<i>orf1</i>	2	625	207	Serine Recombinase	Recombinase family protein [<i>Enterococcus faecium</i>]	EGP4902831.1	99 (100)
<i>orf2</i>	625	2,769	714	DNA topoisomerase IA	DNA topoisomerase III [<i>Enterococcus faecium</i> Ef_ aus0100]	HAQ1370444.1	99 (99)
<i>orf3</i>	2,895	3,128	77	Omega transcriptional repressor	Omega2 protein [<i>Enterococcus faecalis</i> HIP11704]	EEU72717.1	100 (100)
<i>orf4</i>	3,397	4,134	245	Ribosomal RNA adenine demethylase	23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(B) [<i>E. faecalis</i>]	EGO2511360.1	100 (100)
<i>orf5</i>	4,667	5,563	298	Plasmid-partitioning protein	ParA family protein [<i>Enterococcus gallinarum</i>]	WP_096711234.1	100 (100)
<i>orf6</i>	5,661	5,870	69	Omega Transcriptional Repressor	Omega Transcriptional Repressor family protein [<i>E. faecalis</i> 62]	ADX79061.1	100 (100)
<i>orf7</i>	5,888	6,160	90	Epsilon antitoxin	Bacterial epsilon antitoxin family protein [<i>E. faecalis</i> 62]	ADX79060.1	100 (100)
<i>orf8</i>	6,162	6,491	109	Zeta toxin	Zeta toxin family protein [<i>E. faecium</i>]	WP_172689917.1	94 (95)
<i>orf9</i>	7,207	6,521	228	IS6 family transposase	IS1216 transposase [<i>E. faecalis</i>]	ARQ19110.1	100 (100)
<i>orf10</i>	7,929	7,255	224	AAA family ATPase	AAA family ATPase [<i>Staphylococcaceae</i>]	WP_119881052.1	99 (99)
<i>orf11</i>	9,379	7,922	482	Integrase core domain	DDE-type integrase/transposase/recombinase [<i>Staphylococcaceae</i>]	WP_119890407.1	100 (100)
<i>orf12</i>	10,017	9,403	204	Serine Recombinase	Recombinase family protein [<i>Staphylococcaceae</i>]	WP_032495683.1	100 (100)
<i>orf13</i>	11,810	10,752	352	23S rRNA methyltransferase	rRNA methylase [<i>Staphylococcus aureus</i>]	AFF18416.1	100 (100)
<i>orf14</i>	12,099	11,920	59		Hypothetical protein [<i>S. epidermidis</i>]	AJW29121.1	100 (100)
<i>orf15</i>	13,319	12,285	344	IS30 family transposase	IS30 family transposase [<i>Oceanobacillus</i> sp. AG]	WP_156858118.1	99 (99)
<i>orf16</i>	14,329	13,577	250	DNA replication protein DnaC	Integrase/resolvase B [<i>Staphylococcus saprophyticus</i>]	AEP69231.1	100 (100)
<i>orf17</i>	15,104	14,322	518	Integrase core domain	Integrase/resolvase A [<i>S. saprophyticus</i>]	AVE17230.1	100 (100)
<i>orf18</i>	16,577	16,173	134	Bleomycin binding protein	Bleomycin resistance protein [<i>S. saprophyticus</i>]	AVE17232.1	100 (100)
<i>orf19</i>	17,944	16,703	413	Truncated Tn3 transposase DDE domain	Tn3 family transposase, partial [<i>E. faecalis</i>]	EGO2516840.1	100 (100)
<i>orf20</i>	20,117	18,150	655	ABC-F type ribosomal protection protein OptrA	ABC-F type ribosomal protection protein OptrA [<i>Streptococcus suis</i>]	WP_050571857.1	100 (100)
<i>orf21</i>	20,815	21,735	306	Aminoglycoside 2"-phosphotransferase	Aminoglycoside 2"-phosphotransferase-IIIa [<i>E. gallinarum</i>]	3TDV_A	100 (100)
<i>orf22</i>	22,906	22,136	256	Aminoglycoside 4'-nucleotidyltransferase	Aminoglycoside O-nucleotidyltransferase ANT(4')-Ia [<i>S. aureus</i>]	WP_137075613.1	100 (100)
<i>orf23</i>	24,153	23,467	228	IS6 family transposase	IS1216 transposase [<i>E. faecalis</i>]	ARQ19110.1	100 (100)
<i>orf24</i>	24,209	24,646	145	Plasmid replication protein	Replication protein Rep [<i>Lactobacillales</i>]	WP_011117198.1	98 (100)

496	Δ orf25	24,897	24,970	24	Truncated relaxase domain of MobM	Plasmid recombination protein, partial [<i>Lactobacillus</i>]	WP_089143251.1	99 (99)
497	orf26	24,981	25,799	272	Aminoglycoside 9-nucleotidyltransferase	Aminoglycoside nucleotidyltransferase ANT(9) [<i>S. aureus</i>]	MVJ61453.1	100 (100)
498	Δ orf27	26,298	25,889	97	Truncated 23S rRNA dimethyltransferase	rRNA adenine N-6-methyltransferase, partial [<i>S. aureus</i> M1150]	EWN25939.1	97 (97)
499	Δ orf25	26,287	27,154	313	Truncated relaxase domain of MobM	Plasmid recombination protein, partial [<i>Lactobacillus</i>]	WP_089143251.1	99 (99)
500	orf28	27,946	27,461	161	Lincosamide nucleotidyltransferase	Lincosamide nucleotidyltransferase Lnu(A) [<i>E. faecalis</i>]	EGS7980767.1	100 (100)
501	orf29	28,337	28,501	54	Transcriptional repressor protein CopG	Ribbon-helix-helix protein, CopG family [<i>Aerococcus urinaeequi</i>]	WP_069286785.1	98 (100)
502	Δ orf30	28,555	28,746	63	Truncated replication initiation protein	Replication protein, partial [<i>E. faecalis</i>]	EGS7980764.1	100 (100)
503	orf31	29,466	28,780	228	IS6 family transposase	IS1216 transposase [<i>E. faecalis</i>]	ARQ19110.1	100 (100)
504	orf32	30,094	29,723	123		Hypothetical protein pEF-01_031 [<i>E. faecalis</i>]	ADN34780.1	100 (100)
505	orf33	31,007	30,096	303	Plasmid-partitioning protein	Chromosome partitioning protein ParA [<i>E. faecium</i>]	AWB15771.1	100 (100)
506	orf34	31,558	33,057	499	Primase C terminal 1 (PriCT-1)	Primase C-terminal domain-containing protein [<i>Enterococcus</i>]	WP_002333826.1	100 (100)
507	orf35	33,193	33,480	95	Replication control protein PrgN	PrgN protein [<i>E. faecalis</i>]	ADN34751.1	100 (100)
508	Δ orf36	33,646	34,002	118	Truncated IS6 family transposase	Truncated IS1216 [<i>E. faecalis</i>]	ADN34752.1	98 (99)
509	orf37	33,986	34,156	56		Hypothetical protein [<i>E. faecium</i>]	HAZ0642166.1	100 (100)
510	orf38	34,503	34,673	56		Hypothetical protein [<i>E. faecalis</i>]	ADM24823.1	100 (100)

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512 ^aFor each ORF, only the most significant identity detected is listed.513 ^b Δ represented a truncated ORF.

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515 **Table S3.** Amino acid sequence identities/similarities of putative proteins encoded by the pEgFS4-2 (GenBank accession no. MZ291453).

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517 BLASTP analysis^a

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519 ORF Start Stop Size Predicted function

520 (bp) (bp) (amino acids)

521 Most significant database match Accession no. % Amino acid

522 ----- identity (% amino acid similarity) -----

523	<i>Δorf1</i>	3	684	238	Truncated TrwB/TraG/TraD/VirD4 family	Conjugal transfer protein, partial [<i>Enterococcus faecium</i>]	HAP7631544.1	100 (100)
524					of bacterial conjugation proteins			
525	<i>orf2</i>	697	2,115	472	Protein secretion system	Conjugal transfer protein [<i>E. faecium</i>]	WP_159373493.1	100 (100)
526	<i>orf3</i>	2,117	3,286	389	Phage tail lysozyme	CHAP domain-containing protein [<i>E. faecium</i>]	WP_159373492.1	100 (100)
527	<i>orf4</i>	3,304	3,921	205		Hypothetical protein [<i>E. faecium</i>]	WP_159373491.1	100 (100)
528	<i>orf5</i>	3,899	4,282	127		Hypothetical protein [<i>Lactiplantibacillus plantarum</i>]	KZU18644.1	89 (96)
529	<i>orf6</i>	4,283	4,741	152		Conjugal transfer protein [<i>E. faecium</i>]	WP_159373490.1	100 (100)
530	<i>orf7</i>	4,738	6,264	508	Type IV secretory system Conjugative	Type IV secretory system conjugative DNA transfer family protein	WP_159373489.1	100 (100)
531					DNA transfer	[<i>E. faecium</i>]		
532	<i>orf8</i>	6,283	6,699	138		Hypothetical protein [<i>E. faecium</i>]	WP_159373488.1	100 (100)
533	<i>orf9</i>	6,712	7,581	289		Conjugal transfer protein [<i>Enterococcus faecalis</i>]	KII46943.1	100 (100)
534	<i>Δorf10</i>	7,598	7,668	24	Truncated DNA topoisomerase IA	Type IA DNA topoisomerase, partial [<i>E. faecalis</i>]	WP_033922359.1	100 (100)
535	<i>orf11</i>	8,409	7,723	228	IS6 family transposase	IS6-like element IS1216 family transposase [<i>E. faecium</i>]	WP_002354485.1	100 (100)
536	<i>orf12</i>	9,048	8,836	70		Hypothetical protein [<i>E. faecium</i>]	WP_002324171.1	100 (100)
537	<i>orf13</i>	9,920	9,069	283		Hypothetical protein [<i>E. faecium</i> Aus0085]	AGS77122.1	100 (100)
538	<i>orf14</i>	10,338	10,021	105		PrgN [<i>E. faecium</i>]	ABB46244.1	99 (99)
539	<i>orf15</i>	11,945	10,452	497	Primase C terminal 1 (PriCT-1)	Primase C-terminal domain-containing protein [<i>Bacteria</i>]	WP_000947691.1	100 (100)
540	<i>orf16</i>	12,557	13,510	317	Cellulose biosynthesis protein BcsQ;	Putative PrgP protein [<i>E. faecium</i> Aus0085]	AGS77160.1	100 (100)
541	<i>orf17</i>	13,482	13,757	91		Hypothetical protein EFAU085_p3041 [<i>E. faecium</i> Aus0085]	AGS77159.1	100 (100)
542	<i>orf18</i>	13,979	14,665	228	IS6 family transposase	IS6-like element IS1216 family transposase [<i>E. faecium</i>]	WP_002354485.1	100 (100)
543	<i>orf19</i>	14,793	15,752	319	IS30 family transposase	IS30-like element IS1252 family transposase [<i>Enterococcus</i> sp.]	MBC9710599.1	100 (100)
544	<i>orf20</i>	16,757	16,185	190	Serine Recombinase (SR) family	Recombinase family protein [<i>Enterococcus</i> sp.]	MBC9710598.1	100 (100)
545	<i>orf21</i>	17,378	16,773	201	Fic/DOC family	Fic family protein [<i>E. faecium</i> Aus0085]	AGS77151.1	100 (100)
546	<i>orf22</i>	18,262	17,576	228	IS6 family transposase	IS6-like element IS1216 family transposase [<i>E. faecium</i>]	WP_002354485.1	100 (100)
547	<i>orf23</i>	18,318	19,001	227	NlpC/P60 family	Lipoprotein, NLP/P60 family [<i>E. faecium</i>]	QDL89967.1	100 (100)

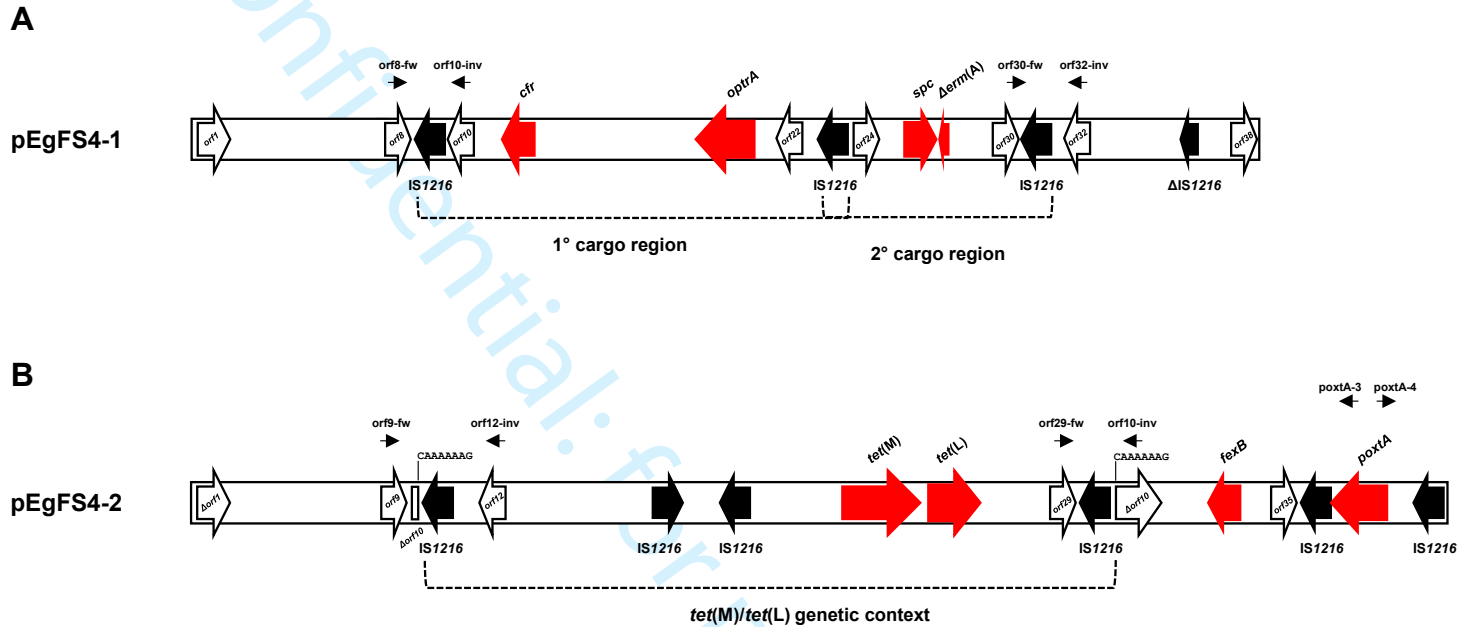
548	<i>orf24</i>	18,998	19,930	310	Conjugative transposon protein TcpC	Conjugal transfer protein [<i>Bacteria</i>]	WP_001224319.1	100 (100)
549	<i>orf25</i>	20,307	22,226	639	Tetracycline resistance, ribosomal protection Tet(M)	Tetracycline resistance ribosomal protection protein Tet(M) [<i>E. faecium</i> Ef_ aus0098]	HAQ1362153.1	100 (100)
551	<i>orf26</i>	22,420	23,796	458	Tetracycline resistance, MFS efflux pump	Tetracycline efflux MFS transporter Tet(L) [<i>E. faecalis</i>]	EHA4049641.1	100 (100)
552	Δ <i>orf27</i>	24,360	24,656	98	MobM_relaxase	Plasmid recombination enzyme, partial [<i>E. faecium</i>]	QDL89972.1	100 (100)
553	Δ <i>orf28</i>	24,756	25,622	288	MobM_relaxase	Plasmid recombination protein, partial [<i>E. faecium</i>]	WP_192423965.1	100 (100)
554	<i>orf29</i>	25,846	26,217	123	Replication protein	Truncated replication protein [<i>E. faecium</i>]	QRN45573.1	100 (100)
555	<i>orf30</i>	26,937	26,251	228	IS6 family transposase	IS6-like element IS1216 family transposase [<i>E. faecium</i>]	WP_002354485.1	100 (100)
556	Δ <i>orf10</i>	27,014	29,117	714	Truncated DNA topoisomerase IA	Type IA DNA topoisomerase, partial [<i>E. faecalis</i>]	WP_033922359.1	98 (98)
557	<i>orf31</i>	29,231	30,736	501		Hypothetical protein [<i>Enterococcus</i>]	WP_013330743.1	100 (100)
558	<i>orf32</i>	32,475	31,066	469	Chloramphenicol/florfenicol MFS transporter	Chloramphenicol/florfenicol exporter [<i>E. faecium</i>]	AKU20099.1	100 (100)
559	<i>orf33</i>	33,376	33,164	70		Hypothetical protein HMPREF1327_00680 [<i>E. faecalis</i> 599]	EJU93012.1	100 (100)
560	<i>orf34</i>	33,843	33,586	85		Putative transposase [<i>E. faecalis</i>]	ADN34760.1	100 (100)
561	<i>orf35</i>	34,357	33,980	125		Hypothetical conserved protein [<i>E. faecalis</i>]	ADN34761.1	100 (100)
562	<i>orf36</i>	35,200	34,514	228	IS6 family transposase	IS1216 transposase [<i>E. faecalis</i>]	ADN34762.1	100 (100)
563	<i>orf37</i>	36,876	35,248	542	Ribosomal protection protein	ARE-ABC-F family resistance factor PoxTA [<i>Staphylococcus aureus</i>]	AVI44920.1	100 (100)
564	<i>orf38</i>	38,319	37,633	228	IS6 family transposase	IS6-like element IS1216 family transposase [<i>E. faecalis</i>]	TKN59864.1	100 (100)

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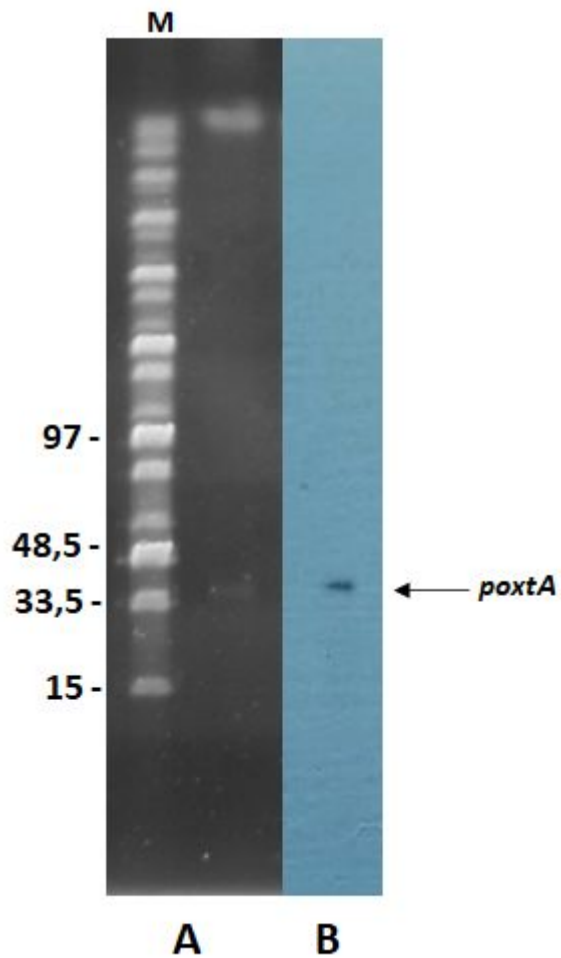
566 ^aFor each ORF, only the most significant identity detected is listed.567 ^b Δ represented a truncated ORF.

568

569 **Figure S1.** Schematic representation, not in scale, of the pEgFS4-1 (A) and pEgFS4-2 (B)
 570 plasmids from *E. gallinarum* FS4. IS1216 elements are represented as black arrows,
 571 antibiotic resistance genes were indicated as red arrows and other plasmid ORFs are
 572 specified as white arrows. Thin arrows indicate the primer pairs used for the stability tests
 573 of each cargo region. In pEgFS4-2 the target site duplication (CAAAAAG) of the
 574 *tet(M)/tet(L)* genetic context was also indicated. Δ symbol represented a truncated ORF.



577 **Figure S2.** *E. gallinarum* FS4 plasmid profile after S1-PFGE (A). Hybridization with a
578 *poxA* probe (B). DNA molecular weight mid-range PFG marker (New England Biolabs,
579 Ipswich, MA), with the size of fragments reported on the left, is shown in the first
580 column (M).
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