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Original research

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

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Running title:
cfr, *optrA* and *poxtA* genes in a porcine *Enterococcus gallinarum*

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Abstract

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

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

Results: Two plasmids, the *cfr*- and *optrA*-carrying pEgFS4-1 (35 kb) and the *poxtA*-harbouring pEgFS4-2 (38 kb), were identified. pEgFS4-1 disclosed a distinctive mosaic structure with two cargo regions bounded by identical IS*1216* elements interpolated into a backbone related to that of *E. faecium vanA*-containing pVEF2. The first cargo region included the *cfr* and *optrA* contexts, whereas the second one carried a Tn*554* remnant and the *lnu*(A) gene. Both regions were able to excise in circular form as a unique translocable unit. pEgFS4-2 plasmid was 99% identical to a not fully described *E. faecium* pSBC1 plasmid. The *poxtA* environment, flanked by IS*1216,* was proved to be instable. pEgFS4-2 also exhibited another cargo region containing the *tet*(M)-*tet*(L) genes arranged in tandem and its circular form was detected. Transformation and conjugation experiments failed to demonstrate the transferability of both plasmids to enterococcal recipients. Both plasmids persisted in the absence of selective pressure.

Conclusions: To the best of our knowledge, this is the first description of a linezolid-resistant *E. gallinarum* isolate of swine origin carrying *cfr*, *optrA* and *poxtA* genes. The co-presence of three linezolid resistance determinants in an intrinsically vancomycin-resistant enterococcal species is cause of concern.

Introduction

Enterococci are traditionally regarded as human opportunistic bacteria and continue to be important nosocomial pathogens; particularly the clinically relevant species *Enterococcus faecalis* and *Enterococcus faecium* are globally considered as leading cause of healthcare-associated infections. Most of their success as nosocomial pathogens is due to their capability to survive in the environment and tolerate disinfectants, as well as to intrinsic resistance to many drugs and their ability to acquire new antibiotic resistance genes. Multidrug resistance is common in enterococcal isolates, which prolongs hospitalization and increases costs and the risk of therapeutic failure. Few options are still available to treat infections due to antibiotic-resistant enterococci.[1] Oxazolidinones, including linezolid and tedizolid, are effective antimicrobial agents for the treatment of clinical infections caused by MDR Gram-positive pathogens, including VRE. However, enterococci can also develop resistance to linezolid through acquisition of the *cfr*, *cfr*(B) and *cfr*(D) genes,[2-4] which encode methyltransferases conferring resistance to five classes of antimicrobial agents, including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (PhLOPS$_A$ phenotype).[5] Oxazolidinone resistance can also be due to the acquisition of *optrA* and *poxtA* genes encoding protein of ABC-F family.[6,7] OptrA and PoxtA lead to decreased susceptibility to phenicols, oxazolidinones (including tedizolid), and tetracyclines (PoxtA protein only) by a ribosomal protection mechanism.[8] Oxazolidinone resistance determinants are commonly located on conjugative plasmids, the main mobile genetic elements responsible for the spread of antibiotic resistance within the enterococcal population.[9] Although oxazolidinones have been approved only for human use, *cfr*, *optrA*, and *poxtA* genes have been detected in enterococcal isolates of animal and environmental origin,[10] and very recently even in enterococci from coastal seawaters samples.[11]

Enterococcus gallinarum, intrinsically resistant to vancomycin, is only occasionally found in human gut and bovine or swine intestinal microbiota.[12] *E. gallinarum* is considered an opportunistic human pathogen and in recent years, severe infections, including bacteremia, endocarditis, and meningitis, as well as several hospital-acquired outbreaks caused by this enterococcal species, have gradually increased.[13]

To date, the *cfr* and *optrA* genes have been identified in *E. gallinarum*, individually[14,15] or in combination.[16] Very recently, a *poxtA2* variant was detected in a linezolid-resistant *E. gallinarum*.[17]

To the best of our knowledge, this is the first characterization of a linezolid-resistant *E. gallinarum* isolate of swine origin carrying *cfr*, *optrA* and *poxtA* genes.

Materials and methods

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

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

S1-PFGE, Southern blotting, and hybridisation assays. 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.[20] After S1 PFGE, total DNA was blotted onto positively charged nylon membranes (Ambion-Celbio, Milan, Italy) and hybridized with biotin-labelled *cfr*, *optrA*, and *poxtA* DNA probes as described elsewhere (Table S1).[21]

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/ (ResFinder v.3.2, LRE-finder v.1.0) and by the BLAST suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

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

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

Conjugal transfer was performed on a membrane filter as described previously[21] using *E. gallinarum* FS4 as the donor and the florfenicol-susceptible *E. faecium* 64/3[23] as recipient. Transconjugants were selected on brain heart infusion agar (BHIA; 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.

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

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

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

Results and discussion

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

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

WGS analysis. Sequencing analysis of *E. gallinarum* FS4 revealed a genome consisting of 3,299,121 bp (40,5% GC content) and the presence of a 34,679-bp plasmid, designated pEgFS4-1 (accession no. MZ291452), and a 38,387-bp plasmid named pEgFS4-2 (accession no. MZ291453), both with a 36% GC content.
In addition to the *vanC* cluster conferring intrinsic vancomycin resistance, ResFinder analysis revealed *E. gallinarum* FS4 strain carried multiple acquired antibiotic resistance genes (ARGs). *vanC* cluster was typically located on the chromosome and showed 100% coverage and 99.82% identity to that of the reference strain *E. gallinarum* BM4174 (GenBank accession no. AF162694.1), whereas ARGs were all plasmid-associated. As regards oxazolidinone resistance genes, WGS analysis confirmed that the *cfr* and *optrA* genes were located on pEgFS4-1, whereas the *poxtA* gene was part of the pEgFS4-2.
The maps of the two plasmids with all the detected ARGs, are shown in Figures 1 and 2. The major characteristics of the ORFs of pEgFS4-1 and pEgFS4-2 are detailed in Tables S2 and S3, respectively.
WGS analysis also ruled out the presence of *cfr* variants and mutations in 23S rRNA and L3/L4 ribosomal proteins.

pEgFS4-1 plasmid

Sequence analysis of pEgFS4-1 plasmid identified 38 ORFs encoding proteins ≥50 amino acids. BLASTN analysis revealed a mosaic structure consisting of a backbone and two cargo regions (Figure 1 and Table S2).
The backbone region (GC content of 34.0%), spanning from *orf1* to *orf9* and from *orf31* to *orf38*, had high-level identity (from 90 to 99%) and wide synteny to the scaffold of the non-conjugative *vanA*-containing plasmid pVEF2 (accession number AM410096.1), responsible for glycopeptide resistance in *E. faecium* 399/F99/A9 of poultry origin in Norway.[24] These two regions contained several genes involved in pEgFS4-1 replication,

partitioning and persistence (such as the δ-ώ-ε-ζ toxin/antitoxin genetic cluster). However, compared to pVEF2, pEgFS4-1 lacked the Tn*1546* transposon and harboured the *erm*(B) gene (*orf9*) responsible for macrolide, lincosamide and streptogramin group B resistance.

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

The first cargo region (GC content 38%), spanning from *orf9* to *orf23* and including the *cfr* and *optrA* genetic contexts, disclosed high-level nucleotide identity (from 90 to 99%) to the corresponding region of pWo27-9 plasmid of *Staphylococcus sciuri* (accession no. KX982169.1) (Figure 1).[25] Unlike pWo27-9, in pEgFS4-1 this region displayed the insertion of two transposases: the former, belonging to IS*30* family, was inserted between *cfr* and *istAS-istBS* genes (encoding transposases belonging to the IS*21* family); the latter, belonging to Tn*3* family, was truncated and located between the *ble* (responsible for bleomycin resistance) and the *optrA* genes. Upstream of *ant*(4')-Ia (*orf22*), we also detected the insertion of the *aph*(2")-IIIa, both genes are responsible for aminoglycoside resistance.

The *optrA* gene was 99.0% identical to the DNA reference sequence (GenBank accession no. KP399637.1). Furthermore, three amino acid changes (K3E, N12Y, Y176D) were detected in the protein sequence compared to $OptrAE_{349}$ (99% amino acid identity and similarity). Interestingly, the $OptrA_{EYD}$ variant was previously detected in porcine *E. faecalis* and *Enterococcus avium* isolates recovered during an antibiotic surveillance in central Italy.[16]

The second cargo region (GC content 35%), showing high-level nucleotide identity (from 90 to 99%) to the corresponding portion of *Lactobacillus johnsonii* UMNLJ22 chromosome (accession no. CP021704.1), displayed a mosaic structure with the plasmid replication *repB* gene (*orf24*), a truncated *mobM* relaxase gene (*orf27*), and *lnu*(A) (*orf28*) responsible for lincosamide resistance. This region was disrupted due to insertion in *mobM* gene of a 1.3-kb Tn*554* remnant showing *ant*(9)-Ia gene (responsible for spectinomycin resistance), and a truncated *erm*(A) gene lacking 270-bp at 5'end. Both cargo regions were bounded by identical IS*1216* elements with the same orientation: the *cfr-optrA* genetic context was flanked by *orf9* and *orf23*, while the ΔTn*554*/*lnu*(A) genetic environment was bracketed by *orf23* and *orf31*. The presence of specific direct repeats flanking IS*1216* elements was not detected.

A PCR assay showed that these two cargo regions were able to excise in circular form as a unique translocable unit (TU). Sequencing data displayed an IS*1216* element in the circular form and an empty excision site devoid of IS element (Table S2 and Figure S1).

Very recently, Harmer *et al*. proposed a novel nomenclature for genetic elements that are bounded by members of the IS*26* family orientated in the same direction defining them as "pseudo-compound transposons (PCTs)".[26] Since the TU contains the *cfr* and *optrA* genes and IS*1216* elements (belongs to the IS*26* family) with the same orientation, it could be classified actually as a PCT.

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

pEgFS4-2 plasmid

pEgFS4-2 plasmid exhibited 99% DNA identity (coverage, 98%) with the conjugative plasmid pSBC1 (accession no. CP038169), partially described in a porcine *E. faecium* (Figure 2).[27] Moreover, pEgFS4-2 displayed 99% DNA identity (coverage, 40%) with a region of Tn*6657* transposon (containing *fexB* and *poxtA* genes). Tn*6657* is part of Tn*6349* (accession no. MH746818.1), the first *poxtA*-carrying element characterized in the chromosome of the clinical *S. aureus* AOUC-0915 isolate.[28] In pEgFS4-2, 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 of *poxtA* genetic context was detectable. On the other hand, the *poxtA* context excision due to an IS*1216*-mediated recombination has already been established[10,11,16,29,30] suggesting that the circularisation can facilitate the spreading of this oxazolidinone resistance gene.

Interestingly, pEgFS4-2 exhibited another 19,337-bp cargo region (from *orf11* to *orf30*) containing the tetracycline resistance *tet*(M) and *tet*(L) genes arranged in tandem (*orf25* and *orf26*), and several genes involved in transposition and plasmid replication (Table S3). This genetic context, bounded by two identical IS*1216* insertion sequences (*orf11* and *orf30*) with the same orientation, was integrated within *orf10*, encoding a DNA topoisomerase, splitting the gene into two portions. Remarkably, the *tet*(M)-*tet*(L) genetic context was flanked by 8 bp direct repeats (5'-CAAAAAAG-3'), apparently resulting from target site duplication following IS*1216*-mediated transposition at this site. Inverse PCR assays, with primers targeting the flanking regions the insertion site (Figure S1 and Table S3), indicated the circularisation of *tet*(M)-*tet*(L) genetic environment confirming the mobility of this region. Sequencing analysis demonstrated the presence of a IS*1216* element in the circular form and a single IS*1216* copy at the excision site. Interestingly, this genetic context displayed 99% nucleotide identity with a mobile *tet*(M)-*tet*(L) locus, flanked by two copies of IS*1216E* elements in the same orientation, detected in pFas4-1 plasmid of the *Enterococcus hirae* of swine origin.[31] As

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

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

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

Conclusions

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

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

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

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

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Transparency declarations

None to declare

References

1. García-Solache M, Rice LB. The enterococcus: a model of adaptability to its environment. *Clin Microbiol Rev* 2019; **32**: e00058–18.
2. Schwarz S, Werckenthin C, Kehrenberg C. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. *Antimicrob Agents Chemother* 2000; **44**: 2530–3.
3. Deshpande LM, Ashcraft DS, Kahn HP *et al*. Detection of a new cfr-like gene, *cfr*(B), in *Enterococcus faecium* isolates recovered from human specimens in the United States as part of the SENTRY Antimicrobial Surveillance Program. *Antimicrob Agents Chemother* 2015; **59**: 6256–61.
4. Guerin F, Sassi M, Dejoies L *et al*. Molecular and functional analysis of the novel *cfr*(D) linezolid resistance gene identified in *Enterococcus faecium*. *J. Antimicrob Chemother* 2020; **75**: 1699–1703.
5. Long KS, Poehlsgaard J, Kehrenberg C *et al*. The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. *Antimicrob Agents Chemother*. 2006; **50**: 2500–5.
6. Wang Y, Lv Y, Cai J *et al*. A novel gene, *optrA*, that confers transferable resistance to oxazolidinones and phenicols and its presence in *Enterococcus faecalis* and *Enterococcus faecium* of human and animal origin. *J Antimicrob Chemother* 2015; **70:** 2182–90.
7. Antonelli A, D'Andrea MM, Brenciani A *et al*. Characterization of *poxtA*, a novel phenicol-oxazolidinone-tetracycline resistance gene from an MRSA of clinical origin. *J Antimicrob Chemother* 2018; **73:** 1763–9.
8. Ero R, Kumar V, Su W *et al*. Ribosome protection by ABC-F proteins—molecular mechanism and potential drug design. *Protein science* 2019; **28**: 684–93.
9. Partridge SR, Kwong SM, Firth N *et al*. Mobile genetic elements associated with antimicrobial resistance. *Clin Microbiol Rev* 2018; **31**: e00088–17.
10. Schwarz SS, Zhang W, Du XD *et al*. Mobile oxazolidinone resistance genes in Gram-positive and Gram-negative bacteria. *Clin Microbiol Rev* 2021; **34**: e00188–20.
11. Fioriti S, Coccitto SN, Cedraro N *et al*. Linezolid Resistance Genes in Enterococci Isolated from Sediment and Zooplankton in Two Italian Coastal Areas. *Appl and Environ Microbiol* 2021; **87**: e02958–20.
12. Torres C, Alonso CA, Ruiz-Ripa L *et al*. Antimicrobial resistance in *Enterococcus* spp. of animal origin. *Microbiol Spectr* 2018; **6**: 185–227.
13. Monticelli J, Knezevich A, Luzzati R, *et al*. Clinical management of non-faecium non-faecalis vancomycin-resistant enterococci infection. Focus on *Enterococcus*

gallinarum and *Enterococcus casseliflavus/flavescens*. *J Infect Chemother* 2018; **24**: 237–246.

14. Liu Y, Wang Y, Dai L *et al*. First report of multiresistance gene *cfr* in *Enterococcus* species *casseliflavus* and *gallinarum* of swine origin. *Vet Microbiol* 2014; **170**: 352–7.
15. Yao TG, Li BY, Luan RD *et al*. Whole genome sequence of *Enterococcus gallinarum* EG81, a porcine strain harbouring the oxazolidinone-phenicol resistance gene *optrA* with chromosomal and plasmid location. *J Glob Antimicrob Res* 2020; **22**: 598–600.
16. Fioriti S, Morroni G, Coccitto SN *et al*. Detection of oxazolidinone resistance genes and characterization of genetic environments in enterococci of swine origin, Italy. *Microorganims* 2020; **8**: 2021.
17. Baccani I, Antonelli A, Di Pilato V *et al*. Detection of *poxtA2*, a presumptive *poxtA* ancestor, in a plasmid from a linezolid-resistant *Enterococcus gallinarum*. *Antimicrob Agents Chemother* 2021; In press.
18. Brenciani A, Fioriti S, Morroni G *et al*. Detection in Italy of a porcine *Enterococcus faecium* isolate carrying the novel phenicol-oxazolidinone-tetracycline resistance gene *poxtA*. *J Antimicrob Chemother* 2019; **74**: 817–8.
19. Clinical and Laboratory Standards Institute (CLSI). 2020. Performance standards for antimicrobial susceptibility testing. CLSI M100. CLSI, Wayne, PA.
20. Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. *Anal Biochem* 1995; **226**: 235–40.
21. Brenciani A, Morroni G, Pollini S *et al*. Characterization of novel conjugative multiresistance plasmids carrying *cfr* from linezolid-resistant *Staphylococcus epidermidis* clinical isolates from Italy. *J Antimicrob Chemother* 2016; **71**: 307–313.
22. Massidda O, Mingoia M, Fadda D *et al*. Analysis of the ß-lactamase plasmid of borderline methicillin-susceptible *Staphylococcus aureus*: focus on *bla* complex genes and cadmium resistance determinants *cadD* and *cadX*. *Plasmid* 2006; **55**: 114–27.
23. Werner G, Klare I, Witte W. Arrangement of the *vanA* gene cluster in enterococci of different ecological origin. *FEMS Microbiol Lett* 1997; **155**: 55–61.
24. Sletvold H, Johnsen PJ,1 Simonsen GS *et al*. Comparative DNA analysis of two *vanA* plasmids from *Enterococcus faecium* strains isolated from poultry and a poultry farmer in Norway. *Antimicrob Agents Chemother* 2007; **51**: 736–9.
25. Fan R, Li D, Feßler AT *et al*. Distribution of *optrA* and *cfr* in florfenicol-resistant *Staphylococcus sciuri* of pig origin. *Vet Microbiol* 2017; **210**: 43–8.

26. Harmer CJ, Pong CH, Hall RM. Structures bounded by directly-oriented members of the IS*26* family are pseudo-compound transposons. *Plasmid* 2020; **111**: 102530.
27. Lei CW, Kang ZZ, Wu SK *et al*. Detection of the phenicol–oxazolidinone–tetracycline resistance gene *poxtA* in *Enterococcus faecium* and *Enterococcus faecalis* of food producing animal origin in China. *J Antimicrob Chemother* 2019; **74**: 2459–61.
28. D'Andrea, MM, Antonelli A, Brenciani A *et al*. Characterization of Tn*6349*, a novel mosaic transposon carrying *poxtA*, *cfr* and other resistance determinants, inserted in the chromosome of an ST5-MRSA-II strain of clinical origin. *J Antimicrob Chemother* 2019; **74**: 2870–5.
29. Huang J, Wang M, Gao Y *et al*. Emergence of plasmid-mediated oxazolidinone resistance gene *poxtA* from CC17 *Enterococcus faecium* of pig origin. *J Antimicrob Chemother* 2019; **74**: 2524–30.
30. Freitas AR, Tedim AP, Duarte B *et al*. Linezolid-resistant (Tn*6246*::*fexB-poxtA*) *Enterococcus faecium* strains colonizing humans and bovines on different continents: similarity without epidemiological link. *J Antimicrob Chemother* 2020; **75**: 2416–23.
31. Li D, Cheng Y, Schwarz S *et al*. Identification of a *poxtA*- and *cfr*-carrying multiresistant *Enterococcus hirae* strain. *J Antimicrob Chemother* 2020; **75**: 482-4.
32. Eshaghi A, Shahinas D, Li A *et al*. Characterization of an *Enterococcus gallinarum* isolate carrying a dual *vanA* and *vanB* cassette. *J Clin Microbiol* 2015; **53**: 2225-29.

Figures

Figure 1. Circular map of the pEgFS4-1 plasmid in comparison with similar reported plasmids using BRIG software. Plasmids, transposons and chromosomal regions included in the analysis were as follows: (inner to outer circles) chromosomal segment of *L. jonhsonii* (CP021704.1), Tn*554* (X03216.1), pWo27-9 (KX982169.1) and pVEF2 (GenBank ID AM410096.1).
Black arrows indicate the positions and orientations of genes; some antibiotic resistance determinants and relevant genes described in this study are shown.

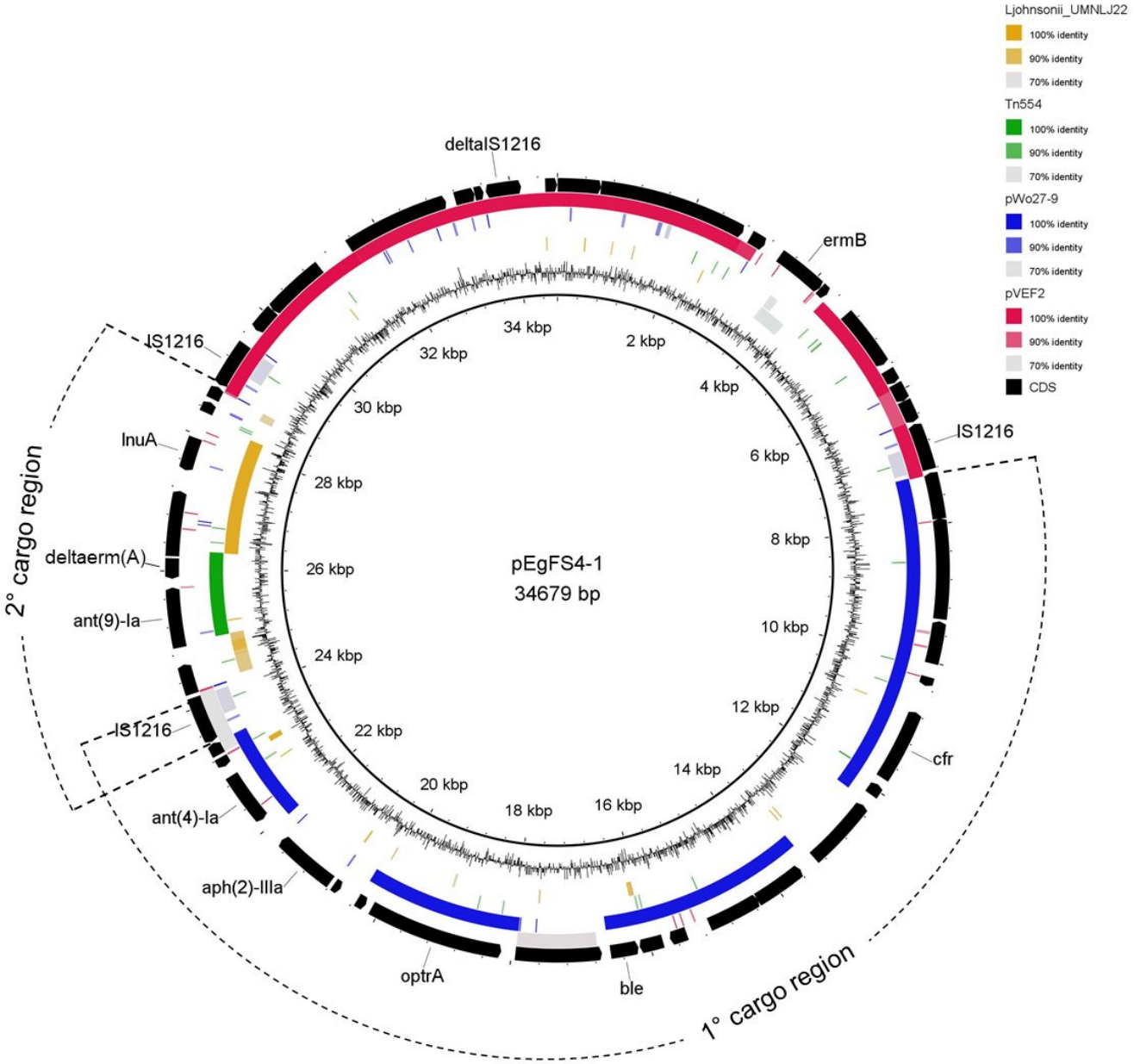
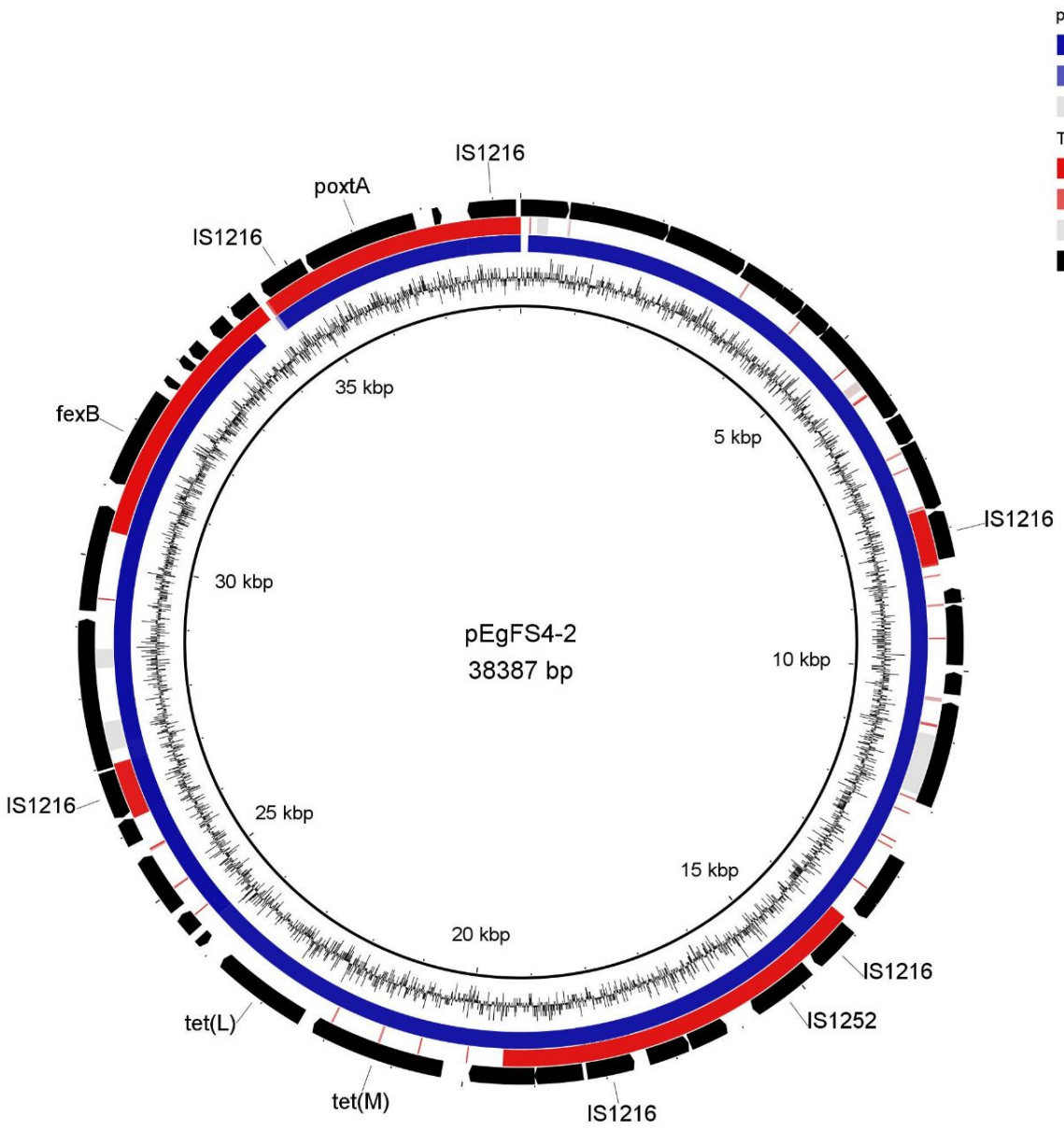


Figure 2. Circular map of the pEgFS4-2 plasmid in comparison with similar reported plasmids using BRIG software. Plasmids and transposons included in the analysis were as follows: (inner to outer circles) pSBC1 (CP038169) and Tn*6657* (MH746818.1). Black arrows indicate the positions and orientations of genes; some antibiotic resistance determinants and relevant genes described in this study are shown.



Supplementary materials

Table S1. Primer pairs used in this study.

Gene	Primer Designation	Sequence (5'-3')	Reference	Product size (bp)
cfr	cfr-FW[a]	TGAAGTATAAAGCAGGTTGGGAGTCA	1	746
	cfr-RV[a]	ACCATATAATTGACCACAAGCAGC		
optrA	optrA-FW[a]	TACTTGATGAACCTACTAACCA	1	422
	optrA-RV[a]	CCTTGAACTACTGATTCTCGG		
poxtA	poxtA-FW[a]	GAACGCTTGGAGTATTTCGACTTC	1	778
	poxtA-RV[a]	CTGGACTGAGAATACCCATC		
Detection of circular forms and analysis of the excision sites in pEgFS4-1				
orf10	orf10inv	CGAAAAACGGTTGGCACGGTA	This study	
orf30	orf30-fw	CGTTTATTGTGTATCCAGAA		
orf8	orf8-fw	GGGCAACCAGGGTCAGGGAAAA	This study	
orf32	orf32-inv	AAGAAAGAAAAAAGGAAGAAGA		
Detection of circular forms and analysis of the excision sites in pEgFS4-2				
orf12	orf12inv	AAACTGATTTTTTGTTGATTCG	This study	
orf29	orf29-fw	AAAGGCTGAAAGAGTAAAAGA		
orf9	orf9-fw	CCAAAGGAGCAGGACGGT	This study	
orf10	orf10-rv	TTCAGGGAAAATGGGTAAAT		
poxtA	poxtA-3	GACGAGCCGACCAACCACCT	2	
	poxtA-4	TTGGATTTTTGTCCGCCTGAA		

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

References:

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

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

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	2	625	207	Serine Recombinase	Recombinase family protein [*Enterococcus faecium*]	EGP4902831.1	99 (100)
orf2	625	2,769	714	DNA topoisomerase IA	DNA topoisomerase III [*Enterococcus faecium* Ef_aus0100]	HAQ1370444.1	99 (99)
orf3	2,895	3,128	77	Omega transcriptional repressor	Omega2 protein [*Enterococcus faecalis* HIP11704]	EEU72717.1	100 (100)
orf4	3,397	4,134	245	Ribosomal RNA adenine demethylase	23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(B) [*E. faecalis*]	EGO2511360.1	100 (100)
orf5	4,667	5,563	298	Plasmid-partitioning protein	ParA family protein [*Enterococcus gallinarum*]	WP_096711234.1	100 (100)
orf6	5,661	5,870	69	Omega Transcriptional Repressor	Omega Transcriptional Repressor family protein [*E. faecalis* 62]	ADX79061.1	100 (100)
orf7	5,888	6,160	90	Epsilon antitoxin	Bacterial epsilon antitoxin family protein [*E. faecalis* 62]	ADX79060.1	100 (100)
orf8	6,162	6,491	109	Zeta toxin	Zeta toxin family protein [*E. faecium*]	WP_172689917.1	94 (95)
orf9	7,207	6,521	228	IS6 family transposase	IS*1216* transposase [*E. faecalis*]	ARQ19110.1	100 (100)
orf10	7,929	7,255	224	AAA family ATPase	AAA family ATPase [*Staphylococcaceae*]	WP_119881052.1	99 (99)
orf11	9,379	7,922	482	Integrase core domain	DDE-type integrase/transposase/recombinase [*Staphylococcaceae*]	WP_119890407.1	100 (100)
orf12	10,017	9,403	204	Serine Recombinase	Recombinase family protein [*Staphylococcaceae*]	WP_032495683.1	100 (100)
orf13	11,810	10,752	352	23S rRNA methyltransferase	rRNA methylase [*Staphylococcus aureus*]	AFF18416.1	100 (100)
orf14	12,099	11,920	59		Hypothetical protein [*S. epidermidis*]	AJW29121.1	100 (100)
orf15	13,319	12,285	344	IS30 family transposase	IS30 family transposase [*Oceanobacillus* sp. AG]	WP_156858118.1	99 (99)
orf16	14,329	13,577	250	DNA replication protein DnaC	Integrase/resolvase B [*Staphylococcus saprophyticus*]	AEP69231.1	100 (100)
orf17	15,104	14,322	518	Integrase core domain	Integrase/resolvase A [*S. saprophyticus*]	AVE17230.1	100 (100)
orf18	16,577	16,173	134	Bleomycin binding protein	Bleomycin resistance protein [*S. saprophyticus*]	AVE17232.1	100 (100)
Δorf19	17,944	16,703	413	Truncated Tn3 transposase DDE domain	Tn3 family transposase, partial [*E. faecalis*]	EGO2516840.1	100 (100)
orf20	20,117	18,150	655	ABC-F type ribosomal protection protein OptrA	ABC-F type ribosomal protection protein OptrA [*Streptococcus suis*]	WP_050571857.1	100 (100)
orf21	20,815	21,735	306	Aminoglycoside 2"-phosphotransferase	Aminoglycoside 2'-phosphotransferase-IIIa [*E. gallinarum*]	3TDV_A	100 (100)
orf22	22,906	22,136	256	Aminoglycoside 4'-nucleotidyltransferase	Aminoglycoside O-nucleotidyltransferase ANT(4')-Ia [*S. aureus*]	WP_137075613.1	100 (100)
orf23	24,153	23,467	228	IS6 family transposase	IS*1216* transposase [*E. faecalis*]	ARQ19110.1	100 (100)
orf24	24,209	24,646	145	Plasmid replication protein	Replication protein Rep [*Lactobacillales*]	WP_011117198.1	98 (100)

496	*Δorf25*	24,897	24,970	24	Truncated relaxase domain of MobM	Plasmid recombination protein, partial [*Lactobacillus*]	WP_089143251.1	99 (99)
497	*orf26*	24,981	25,799	272	Aminoglycoside 9-nucleotidyltransferase	Aminoglycoside nucleotidyltransferase ANT(9) [*S. aureus*]	MVJ61453.1	100 (100)
498	*Δorf27*	26,298	25,889	97	Truncated 23S rRNA dimethyltransferase	rRNA adenine N-6-methyltransferase, partial [*S. aureus* M1150]	EWN25939.1	97 (97)
499	*Δorf25*	26,287	27,154	313	Truncated relaxase domain of MobM	Plasmid recombination protein, partial [*Lactobacillus*]	WP_089143251.1	99 (99)
500	*orf28*	27,946	27,461	161	Lincosamide nucleotidyltransferase	Lincosamide nucleotidyltransferase Lnu(A) [*E. faecalis*]	EGS7980767.1	100 (100)
501	*orf29*	28,337	28,501	54	Transcriptional repressor protein CopG	Ribbon-helix-helix protein, CopG family [*Aerococcus urinaeequi*]	WP_069286785.1	98 (100)
502	*Δorf30*	28,555	28,746	63	Truncated replication initiation protein	Replication protein, partial [*E. faecalis*]	EGS7980764.1	100 (100)
503	*orf31*	29,466	28,780	228	IS6 family transposase	IS*1216* transposase [*E. faecalis*]	ARQ19110.1	100 (100)
504	*orf32*	30,094	29,723	123		Hypothetical protein pEF-01_031 [*E. faecalis*]	ADN34780.1	100 (100)
505	*orf33*	31,007	30,096	303	Plasmid-partitioning protein	Chromosome partitioning protein ParA [*E. faecium*]	AWB15771.1	100 (100)
506	*orf34*	31,558	33,057	499	Primase C terminal 1 (PriCT-1)	Primase C-terminal domain-containing protein [*Enterococcus*]	WP_002333826.1	100 (100)
507	*orf35*	33,193	33,480	95	Replication control protein PrgN	PrgN protein [*E. faecalis*]	ADN34751.1	100 (100)
508	*Δorf36*	33,646	34,002	118	Truncated IS6 family transposase	Truncated IS1216 [*E. faecalis*]	ADN34752.1	98 (99)
509	*orf37*	33,986	34,156	56		Hypothetical protein [*E. faecium*]	HAZ0642166.1	100 (100)
510	*orf38*	34,503	34,673	56		Hypothetical protein [*E. faecalis*]	ADM24823.1	100 (100)

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512 [a]For each ORF, only the most significant identity detected is listed.
513 [b]Δ represented a truncated ORF.
514

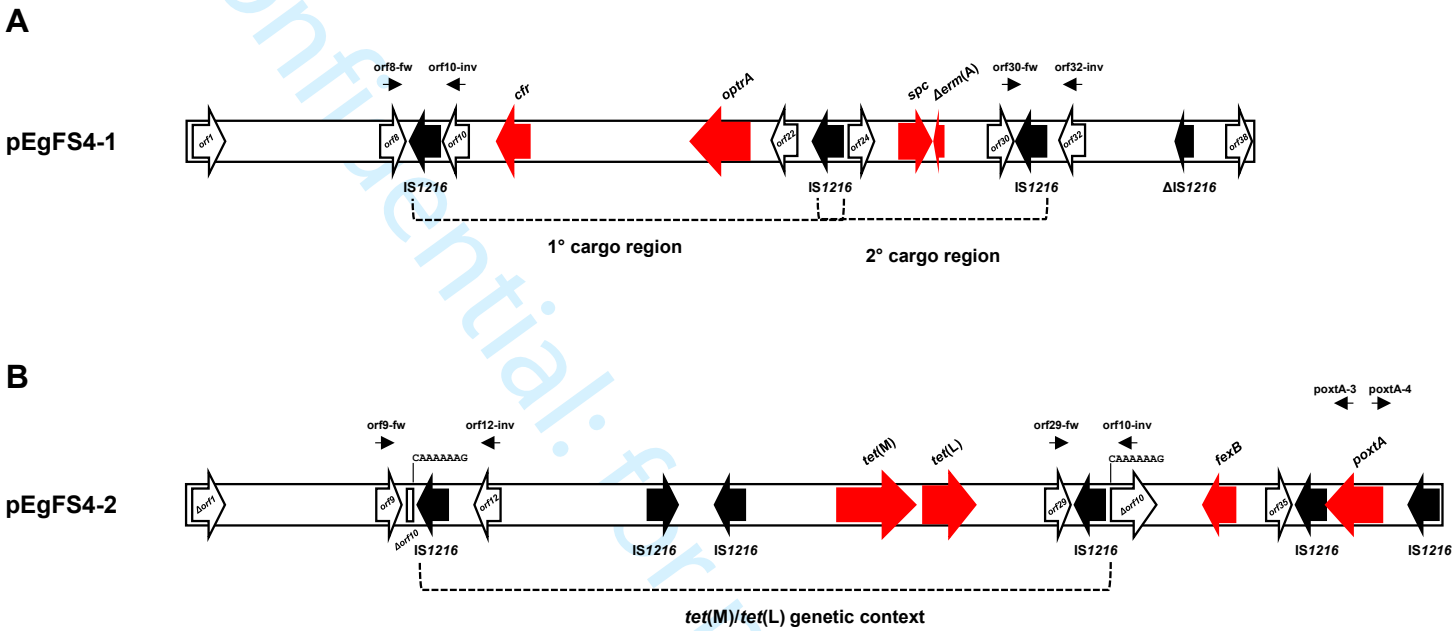
Table S3. Amino acid sequence identities/similarities of putative proteins encoded by the pEgFS4-2 (GenBank accession no. MZ291453).

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)
Δorf1	3	684	238	Truncated TrwB/TraG/TraD/VirD4 family of bacterial conjugation proteins	Conjugal transfer protein, partial [*Enterococcus faecium*]	HAP7631544.1	100 (100)
orf2	697	2,115	472	Protein secretion system	Conjugal transfer protein [*E. faecium*]	WP_159373493.1	100 (100)
orf3	2,117	3,286	389	Phage tail lysozyme	CHAP domain-containing protein [*E. faecium*]	WP_159373492.1	100 (100)
orf4	3,304	3,921	205		Hypothetical protein [*E. faecium*]	WP_159373491.1	100 (100)
orf5	3,899	4,282	127		Hypothetical protein [*Lactiplantibacillus plantarum*]	KZU18644.1	89 (96)
orf6	4,283	4,741	152		Conjugal transfer protein [*E. faecium*]	WP_159373490.1	100 (100)
orf7	4,738	6,264	508	Type IV secretory system Conjugative DNA transfer	Type IV secretory system conjugative DNA transfer family protein [*E. faecium*]	WP_159373489.1	100 (100)
orf8	6,283	6,699	138		Hypothetical protein [*E. faecium*]	WP_159373488.1	100 (100)
orf9	6,712	7,581	289		Conjugal transfer protein [*Enterococcus faecalis*]	KII46943.1	100 (100)
Δorf10	7,598	7,668	24	Truncated DNA topoisomerase IA	Type IA DNA topoisomerase, partial [*E. faecalis*]	WP_033922359.1	100 (100)
orf11	8,409	7,723	228	IS*6* family transposase	IS*6*-like element IS*1216* family transposase [*E. faecium*]	WP_002354485.1	100 (100)
orf12	9,048	8,836	70		Hypothetical protein [*E. faecium*]	WP_002324171.1	100 (100)
orf13	9,920	9,069	283		Hypothetical protein [*E. faecium* Aus0085]	AGS77122.1	100 (100)
orf14	10,338	10,021	105		PrgN [*E. faecium*]	ABB46244.1	99 (99)
orf15	11,945	10,452	497	Primase C terminal 1 (PriCT-1)	Primase C-terminal domain-containing protein [*Bacteria*]	WP_000947691.1	100 (100)
orf16	12,557	13,510	317	Cellulose biosynthesis protein BcsQ;	Putative PrgP protein [*E. faecium* Aus0085]	AGS77160.1	100 (100)
orf17	13,482	13,757	91		Hypothetical protein EFAU085_p3041 [*E. faecium* Aus0085]	AGS77159.1	100 (100)
orf18	13,979	14,665	228	IS*6* family transposase	IS*6*-like element IS*1216* family transposase [*E. faecium*]	WP_002354485.1	100 (100)
orf19	14,793	15,752	319	IS*30* family transposase	IS*30*-like element IS*1252* family transposase [*Enterococcus sp.*]	MBC9710599.1	100 (100)
orf20	16,757	16,185	190	Serine Recombinase (SR) family	Recombinase family protein [*Enterococcus* sp.]	MBC9710598.1	100 (100)
orf21	17,378	16,773	201	Fic/DOC family	Fic family protein [*E. faecium* Aus0085]	AGS77151.1	100 (100)
orf22	18,262	17,576	228	IS*6* family transposase	IS*6*-like element IS*1216* family transposase [*E. faecium*]	WP_002354485.1	100 (100)
orf23	18,318	19,001	227	NlpC/P60 family	Lipoprotein, NLP/P60 family [*E. faecium*]	QDL89967.1	100 (100)

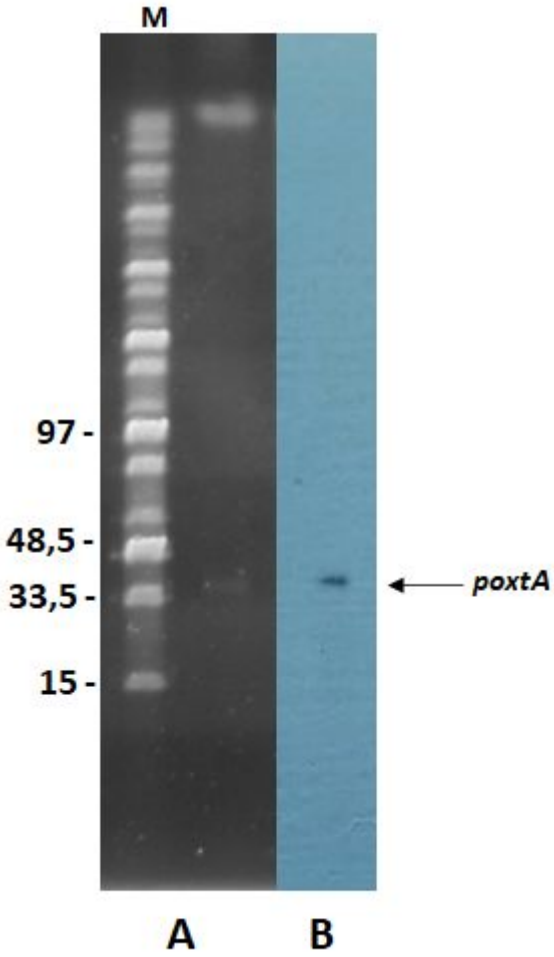
548	*orf24*	18,998	19,930	310	Conjugative transposon protein TcpC	Conjugal transfer protein [*Bacteria*]	WP_001224319.1	100 (100)
549	*orf25*	20,307	22,226	639	Tetracycline resistance, ribosomal protection Tet(M)	Tetracycline resistance ribosomal protection protein Tet(M) [*E. faecium* Ef_aus0098]	HAQ1362153.1	100 (100)
551	*orf26*	22,420	23,796	458	Tetracycline resistance, MFS efflux pump	Tetracycline efflux MFS transporter Tet(L) [*E. faecalis*]	EHA4049641.1	100 (100)
552	*Δorf27*	24,360	24,656	98	MobM_relaxase	Plasmid recombination enzyme, partial [*E. faecium*]	QDL89972.1	100 (100)
553	*Δorf28*	24,756	25,622	288	MobM_relaxase	Plasmid recombination protein, partial [*E. faecium*]	WP_192423965.1	100 (100)
554	*orf29*	25,846	26,217	123	Replication protein	Truncated replication protein [*E. faecium*]	QRN45573.1	100 (100)
555	*orf30*	26,937	26,251	228	IS*6* family transposase	IS*6*-like element IS*1216* family transposase [*E. faecium*]	WP_002354485.1	100 (100)
556	*Δorf10*	27,014	29,117	714	Truncated DNA topoisomerase IA	Type IA DNA topoisomerase, partial [*E. faecalis*]	WP_033922359.1	98 (98)
557	*orf31*	29,231	30,736	501		Hypothetical protein [*Enterococcus*]	WP_013330743.1	100 (100)
558	*orf32*	32,475	31,066	469	Chloramphenicol/florfenicol MFS transporter	Chloramphenicol/florfenicol exporter [*E. faecium*]	AKU20099.1	100 (100)
559	*orf33*	33,376	33,164	70		Hypothetical protein HMPREF1327_00680 [*E. faecalis* 599]	EJU93012.1	100 (100)
560	*orf34*	33,843	33,586	85		Putative transposase [*E. faecalis*]	ADN34760.1	100 (100)
561	*orf35*	34,357	33,980	125		Hypothetical conserved protein [*E. faecalis*]	ADN34761.1	100 (100)
562	*orf36*	35,200	34,514	228	IS*6* family transposase	IS*1216* transposase [*E. faecalis*]	ADN34762.1	100 (100)
563	*orf37*	36,876	35,248	542	Ribosomal protection protein	ARE-ABC-F family resistance factor PoxtA [*Staphylococcus aureus*]	AVI44920.1	100 (100)
564	*orf38*	38,319	37,633	228	IS*6* family transposase	IS*6*-like element IS*1216* family transposase [*E. faecalis*]	TKN59864.1	100 (100)

[a]For each ORF, only the most significant identity detected is listed.
[b]Δ represented a truncated ORF.

Figure S1. Schematic representation, not in scale, of the pEgFS4-1 (A) and pEgFS4-2 (B) plasmids from *E. gallinarum* FS4. IS*1216* elements are represented as black arrows, antibiotic resistance genes were indicated as red arrows and other plasmid ORFs are specified as white arrows. Thin arrows indicate the primer pairs used for the stability tests of each cargo region. In pEgFS4-2 the target site duplication (CAAAAAAG) of the *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 *poxtA* 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).
581



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