Journal of Global Antimicrobial Resistance 30 (2022) 308–310

Contents lists available at ScienceDirect

# Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Letter to the Editor

## Characterization of a novel *cfr*(D)/*poxtA*-carrying plasmid in an oxazolidinone-resistant *Enterococcus casseliflavus* isolate from swine manure, Italy

Editor: Dr Francesco Santoro

Sir,

Oxazolidinones are bacteriostatic antibiotics primarily used as a last resort to treat severe infections due to multidrug-resistant Gram-positive pathogens. They inhibit bacterial protein synthesis by binding to the peptidyl transferase centre of the 50S ribosomal subunit.

However, over the past two decades, the onset of ribosomal mutations but even more the acquisition of transferable resistance genes (*cfr*/*cfr*-like, *optrA*, *poxtA* and its variant *poxtA2*) [1,2] have led to the spread of linezolid-resistant bacteria in healthcare, as well as in animal and environmental settings.

Recently, we reported on the occurrence of the *cfr*(D)/*poxtA2*-carrying plasmid in oxazolidinone-resistant *Enterococcus faecalis* and *Enterococcus casseliflavus* isolates from porcine manure [3].

There is concern about environmental pollution via livestock manure since phenicols and other antibiotic residues can generate a selective pressure able to promote the spread of linezolid resistance genes. The abundance of enterococci in animal faeces and their capacity to acquire new resistance traits pose a risk to human health [4].

Here we characterized an oxazolidinone-resistant *E. casseliflavus* (V378) isolated from the same pig manure lagoon already used before for sampling activities [3]. The enterococcal strain, isolated following the protocol previously described by Brenciani et al. [5] and identified by MALDI-TOF (Vitek-MS, bioMérieux), exhibited resistance to linezolid (MIC, 8 mg/L), tedizolid (MIC, 2 mg/L), florfenicol (MIC, 32 mg/L), chloramphenicol (32 mg/L), and vancomycin (MIC, 8 mg/L) and susceptibility to tetracycline (MIC 0.5 mg/L), according to clinical breakpoints (EUCAST version 11.0, 2021).

The molecular basis of oxazolidinone resistance was investigated by PCR assays for the presence of *cfr* and its variants, *optrA* and *poxtA* genes (Supplementary Table S1). *E. casseliflavus* V378 carried both *cfr*(D) and *poxtA* determinants, and Sanger sequencing showed that these genes were identical to the relevant wild-type sequences.

S1-PFGE/hybridization experiments revealed the presence of two plasmids of ~80 kb and ~100 kb and showed that *cfr*(D) and *poxtA* were co-located on the ~80-kb one; both genes also had a chromosomal location (Supplementary Figs. S1 and S2).

Conjugation and transformation assays, using 10 mg/L florfenicol for selection, failed to demonstrate the transferability of *cfr*(D) and *poxtA* to *E. faecalis* JH2-2 recipient.

In order to characterize the *cfr*(D)/*poxtA*-carrying plasmid, WGS of *E. casseliflavus* V378 was carried out by a hybrid process using both short-read Illumina MiSeq platform (MicrobesNG, Birmingham, UK) with a 2 × 250 approach and a long-read sequencing using MinION with a Rapid Sequencing Kit on a R9.4.1 flow cell and high-accuracy basecalling (Oxford Nanopore Technologies, Oxford, UK). Hybrid assembly was performed with Unicycler v. 0.4.8 (https://github.com/rrwick/Unicycler), and genome quality was assessed using BUSCO (https://busco.ezlab.org/). The WGS data of the *E. casseliflavus* V378 isolate is available under the BioProject ID PRJNA761559 (accession no: JAMQQH000000000). WGS analysis also ruled out the presence of ribosomal mutations using a LRE-finder tool (https://cge.food.dtu.dk/services/LRE-finder/).

Bioinformatic data revealed the *cfr*(D) and *poxtA* were co-located on a 79 407-bp mosaic plasmid (36% GC content) (GenBank accession no. OM621815), named pEc378 and belonging to the Rep3 replicon type. The main characteristics of ORFs are detailed in Supplementary Table S2.

Though hybridization experiments detected the *poxtA* and *cfr*(D) genes both in plasmid and chromosome, they have only been identified in pEc378 plasmid.

BLASTN analysis showed that part of the pEc378 (coverage 45%) was 99% identical to the plasmid unnamed1 of *Enterococcus* sp. FDAARGOS_553 from the United States (accession no. CP033739.1) (Fig. 1a); this identity region included the genes encoding a relaxase (*orf5*), a carbohydrate metabolism operon (*orf6*-*orf12*), a bacteriocin immunity protein (*orf14*), a prophage maintenance system killer protein (*orf16*), a D-lactate dehydrogenase (*orf42*), a plasmid partition protein A (*orf47*), and a truncated $\acute{\omega}$-$\varepsilon$-$\Delta\zeta$ toxin/antitoxin genetic cluster (*orf48*, *orf49*, and Δ*orf50*) (Supplementary Table S2).

Moreover, another region of pEc378 (coverage range from 24% to 31%) was 99% identical to four *poxtA*-carrying plasmids: p18-465_1 of *Enterococcus faecium* 18-465 (accession no. CP065753.1), p16-164_3 of *E. faecium* 16-164 (accession no. CP065776.1), both from France, pM18/0011 of *E. faecalis* M18/0011 from Ireland (accession no. MN831412.1), and pF179_3 of *E. faecium* F179 from Switzerland (accession no. CP072887.1) (Fig. 1a). The region carrying the phenicol exporter gene *fexB* and *poxtA* exhibited a 99% identity (coverage 37%) with part of Tn*6657* of *Staphylococcus aureus* AOUC 09-15 from Italy (accession no. MH746818.1); in pEc378, the Tn*6657* was rearranged and inserted within of the $\zeta$ toxin gene (*orf50*).

An in-depth analysis of the *cfr*(D)/*poxtA* genetic context revealed that *poxtA*, flanked by two IS*1216* with the same polarity, was 2258 bp away from *cfr*(D), which in turn was closely associated with a truncated *guaA* gene in 5′ due to IS*1216* insertion. The *poxtA* region showed a 99% DNA identity with the corresponding segment of pM18/0011 found in *E. faecalis* from Ireland (accession no. MN831412.1), whereas the region containing

https://doi.org/10.1016/j.jgar.2022.07.007
2213-7165/© 2022 The Author(s). Published by Elsevier Ltd on behalf of International Society for Antimicrobial Chemotherapy. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

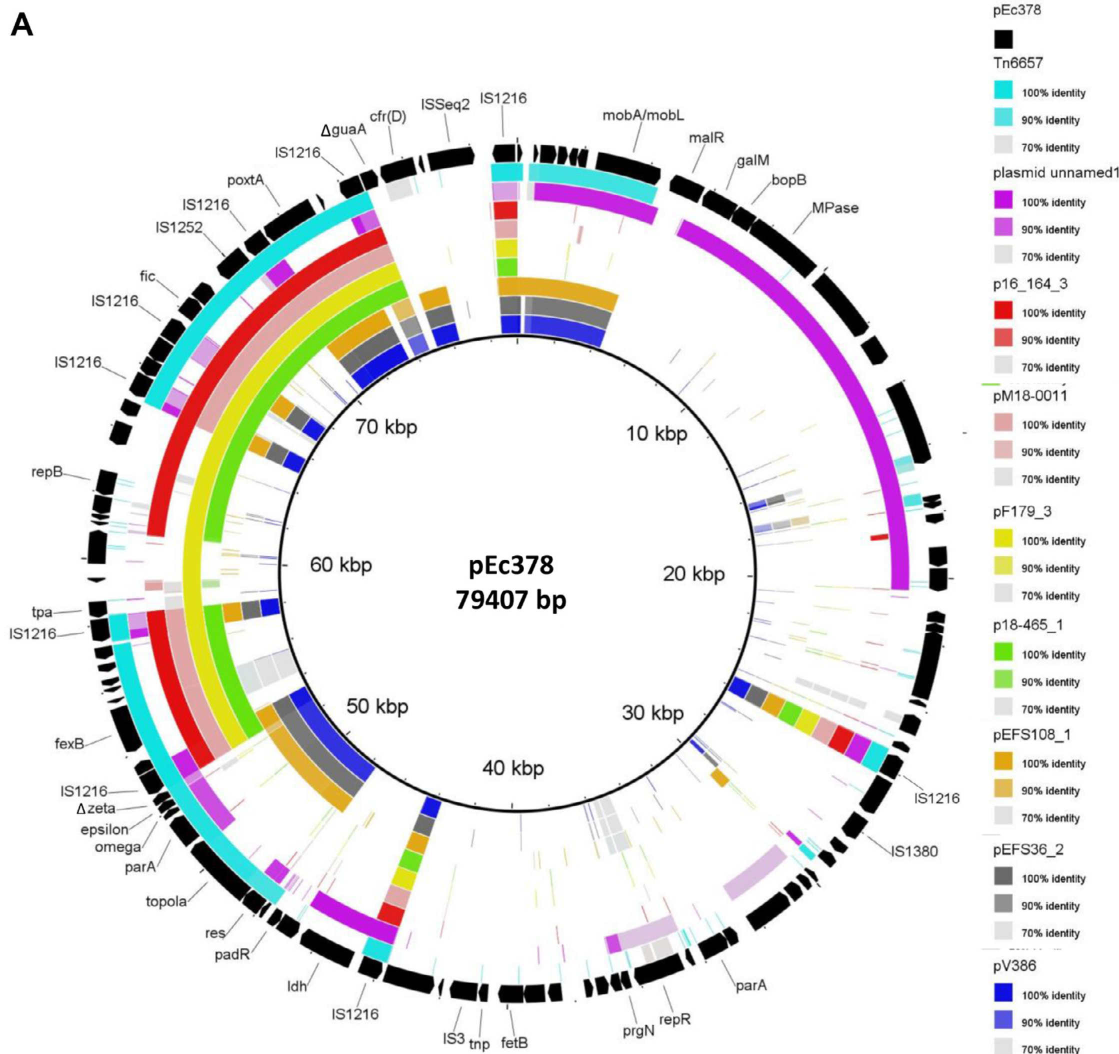


**Fig. 1.** (A) Circular map of the pEc378 plasmid in comparison with similar reported plasmids using BRIG software. Plasmids and transposon included in the analysis were as follows: (inner to outer circles) pV386 (MZ603802), pEFS36_2 (NZ_CP085293), pEFS108_1 (NZ_CP085295), p18-465_1 (CP065753.1), pF179_3 (CP072887.1), pM18/0011 (MN831412.1), p16-164_3 (CP065776.1), plasmid unnamed1 (CP033739.1), Tn*6657* (MH746818.1), and pEc378 (OM621815). Black arrows indicate the positions and orientations of genes; some antibiotic resistance determinants and relevant genes described in this study are shown. (B) Schematic representation and comparison between the *cfr*(D)-*poxtA* genetic context of pEc378 and the sequences with the highest degree of nucleotide similarity. The positions and transcriptional directions of the ORFs are represented by arrows. Insertion sequences, antibiotic resistance genes, and other plasmid ORFs are indicated as black, green, and white arrows, respectively. "Δ" symbol indicates truncated gene. Grey shading indicates regions with a high degree of nucleotide similarity (>90%).

the *cfr*(D) had 99% similarity with the relevant region of the plasmid 4 of *E. faecium* isolate E8014 from the Netherlands (accession no. LR135354.1) (Fig. 1b). The IS*1216* elements, with the same polarity, could play a role in the *poxtA*/*cfr*(D) genetic context insertion in pEc378, as previously described in enterococci [1].

To date, only three plasmids co-carrying *cfr*(D) and *poxtA2* genes [but no genetic linkage between *poxtA* and *cfr*(D)] have been identified: pV386 (accession no. MZ603802) detected in *E. faecalis* and *E. casseliflavus* strains from manure in Italy [3], and pEFS36_2 (accession no. NZ_CP085293) and pEFS108_1 (accession no. NZ_CP085295) found in *E. faecalis* isolates from food-producing animals and meat in South Korea [6]. Overall, therefore, these plasmids showed a 95%–97% DNA identity only with few regions of the pEc378 plasmid (coverage 25%) (Fig. 1a).

To the best of our knowledge, this is the first description of a novel *cfr*(D)/*poxtA*-carrying plasmid in *E. casseliflavus* from manure; this finding again emphasises the role of enterococci from animal slurry as a reservoir of oxazolidinone resistance genes for human bacteria.

## Funding

This work was supported by PRIN 2017 grant prot. 20177J5Y3P from MIUR Italy and by Ministry of Health RC003/2018.

## Competing interests

None to declare.

## Ethical approval

Not required.

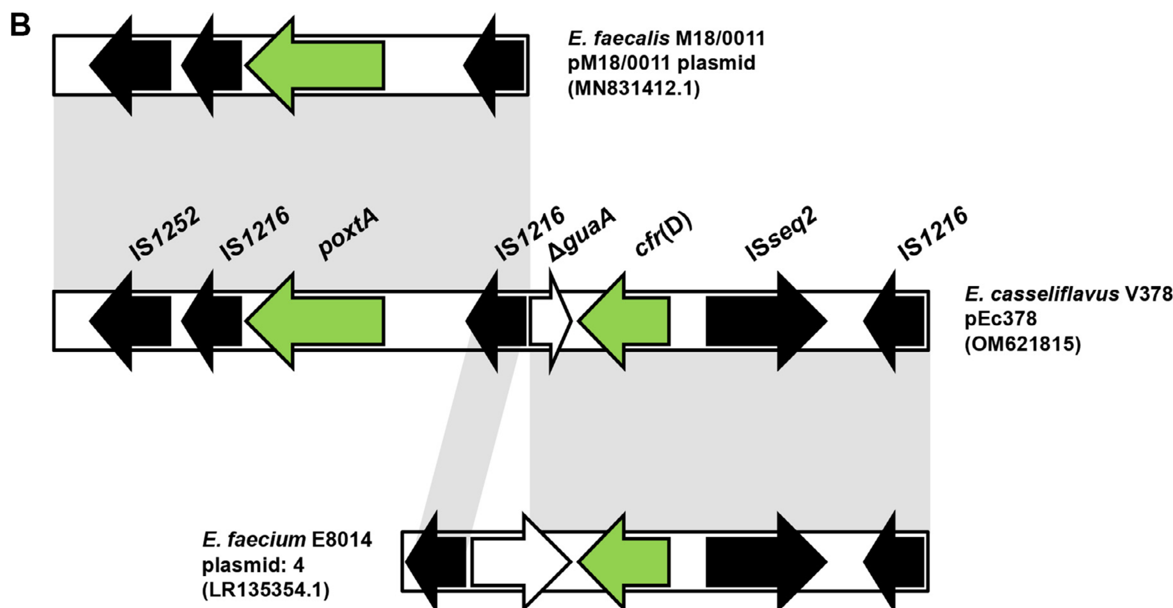


**Fig. 1.** Continued

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.07.007.

## References


[1] Schwarz SS, Zhang W, Du XD, Krüger H, Feßler AT, Ma S, et al. Mobile oxazolidinone resistance genes in Gram-positive and Gram-negative bacteria. Clin Microbiol Rev 2021;34:e00188 -20. doi:10.1128/CMR.00188-20.

[2] Baccani I, Antonelli A, Di Pilato V, Coppi M, Di Maggio T, Spinicci M, et al. Detection of *poxtA2*, a presumptive *poxtA* ancestor, in a plasmid from a linezolid-resistant *Enterococcus gallinarum* isolate. Antimicrob Agents Chemother 2021;65:e0069521. doi:10.1128/AAC.00695-21.

[3] Cinthi M, Coccitto SN, Fioriti S, Morroni G, Simoni S, Vignaroli C, et al. Occurrence of a plasmid co-carrying *cfr*(D) and *poxtA2* linezolid resistance genes in *Enterococcus faecalis* and *Enterococcus casseliflavus* from porcine manure, Italy. J Antimicrob Chemother 2022;77:598–603. doi:10.1093/jac/dkab456.

[4] Wang Y, Lia X, Fu Y, Chen Y, Wang Y, Ye D, et al. Association of florfenicol residues with the abundance of oxazolidinone resistance genes in livestock manures. J Hazard Mater 2020;399:123059. doi:10.1016/j.jhazmat.2020.123059.

[5] Brenciani A, Fioriti S, Morroni G, Cucco L, Morelli A, Pezzotti 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;74:817–18. doi:10.1093/jac/dky505.

[6] Kim E, Shin SW, Kwak HS, Cha MH, Yang SM, Gwak YS, et al. Prevalence and characteristics of phenicol-oxazolidinone resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* isolated from food-producing animals and meat in Korea. Int J Mol Sci 2021;22:11335. doi:10.3390/ijms222111335.



Marzia Cinthi
*Unit of Microbiology, Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy*

Sonia Nina Coccitto, Gloria D'Achille, Gianluca Morroni
*Unit of Microbiology, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche Medical School, Ancona, Italy*

Serena Simoni
*Unit of Microbiology, Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy*

Simona Fioriti
*Unit of Microbiology, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche Medical School, Ancona, Italy*

Chiara Francesca Magistrali
*Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche 'Togo Rosati', Perugia, Italy*

Andrea Brenciani*
*Unit of Microbiology, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche Medical School, Ancona, Italy*

Eleonora Giovanetti
*Unit of Microbiology, Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy*

*Corresponding author.
*E-mail address:* a.brenciani@univpm.it (A. Brenciani)


Revised 15 June 2022