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Linezolid-resistant *Enterococcus casseliflavus* strains of surface water and coexistence of *cfr* and *optrA* genes on an Inc18-type plasmid

Editor by: Stefania Stefani

Letter to the editor,

Enterococci are clinically important pathogens, and the detection of multidrug (MDR)- and linezolid-resistant enterococci has been increasing, which is worrying. Linezolid is one of the most important antimicrobial agents used to treat infections caused mainly by MDR Gram-positive bacteria, including vancomycin-resistant enterococci. On the other hand, plasmid-mediated linezolid resistance genes (e.g., *cfr, optrA*, and *poxtA*) have been identified at the human-animal-environment interface [1–3]. To date, *Enterococcus casseliflavus* strains co-carrying *optrA* + *poxtA* or *optrA* + *cfr* + *poxtA* were only reported in swine of Italy and China [4,5]. Here, we report MDR and linezolid-resistant *E. casseliflavus* strains in aquatic ecosystems and the genomic characterization of a strain co-carrying *cfr* and *optrA* genes, raising an alert of the dissemination of linezolid resistance in the environment.

Between July 2021 and January 2022, a surveillance study was conducted to monitor the occurrence of linezolid-resistant enterococci. For this, water samples were collected from 51 cities in the state of São Paulo, Brazil (Supplementary Fig. S1), and strains were obtained using plates containing Kanamycin Esculin Azide agar (HiMedia, India) supplemented with 4 mg/L of linezolid. Antimicrobial susceptibility testing was carried out using BrCast/EUCAST (https://brcast.org.br/) or CLSI (https://clsi.org/) guidelines. Bacterial identification and detection of antimicrobial resistance genes (ARGs), virulence genes, and plasmid replicons were performed using conventional polymerase chain reactions followed by Sanger sequencing (Supplementary Table S1).

Twelve linezolid-resistant *E. casseliflavus* strains with minimum inhibitory concentrations (MICs) of 8 and 16 mg/L were obtained. These strains were also resistant to other antimicrobials, especially vancomycin (MICs of 8 and > 64 mg/L) in three strains (EW1584, EW1687 and EW1689), which may be related to the intrinsic *vanC2*/3 gene. Furthermore, high-level gentamicin resistance (MIC of > 500 mg/L) was identified in EW1611 strain. Overall, 66.6% (n = 8) of the strains were classified as MDR, and 50% (n = 6; EW1529, EW1584, EW1611, EW1659, EW1689, and EW1690) were positive for the *optrA* gene; however, only EW1611 strain was identified co-carrying *cfr* and *optrA* genes. Furthermore, these strains also harbored different ARGs, virulence genes, and plasmid replicons (Supplementary Table S2).

In this context, EW1161 strain was selected for whole-genome sequencing. Genomic DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, USA) and sequencing was performed using the Illumina MiSeq platform (Illumina Inc., USA). The draft genome was *de novo* assembly by SPAdes v.3.15.2 (https://cab.spbu.ru/files/release3.15.2/manual.html) and annotated using RAST (https://rast.nmpdr.org/rast.cgi). Resistome, virulome, and plasmid replicons were identified using tools with default parameters available at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). Plasmid contigs were predicted using RFPlasmid (http://klif.uu.nl/rfplasmid/) and the plasmid assembly was carried out using combined strategies in BLASTn and Geneious Prime® v.2022.2.2 (Biomatters Ltd.). Insertion sequence elements were analyzed using ISfinder (https://www-is.biotoul.fr/index.php).

In addition to *cfr* and *optrA* genes, EW1611 strain also carried resistance genes to aminoglycosides [*ant(4′)-Ia, aph(2′′)-Ic*, and *ant(9)-Ia*], bleomycin (*bleO*), macrolide-lincosamide-streptogramin B [*erm(B)* and Δ*erm(A)*], lincosamide [*lnu(A)*], tetracyclines (*tetM*), and glycopeptides (*vanC2XY*), the latter a species-specific gene. The *optrA* gene presented 99.7% identity when compared to the reference sequence (GenBank accession number KP399637), representing the $OptrA_{EYD}$ variant previously reported in *Enterococcus faecalis, Enterococcus avium*, and *Enterococcus gallinarum* from swine in Italy [2,3], but not yet described in *E. casseliflavus*, in the environment, or in the Americas.

E. casseliflavus EW1611 harbored a plasmid, named pEW1611, co-carrying *cfr* and *optrA* genes, as well as *erm(B), ant(4′)-Ia, aph(2′′)-Ic, ant(9)-Ia*, Δ*erm(A)*, and *lnu(A)*. The pEW1611 plasmid belonged to the Inc18-type (rep_1) and was 35,541 bp in length, containing 36.1% GC content. The sequence length of the pEW1611 plasmid supported the plasmid size (~ 36 kb) visualized after plasmid extraction using QIAGEN® Plasmid Midi Kit (QIAGEN, Germany). BLASTn analysis revelated that pEW1611 was most related (97% query coverage and 99.9% nucleotide identity) only to pEgFS4-1 plasmid (GenBank accession no. MZ291452) from an *E. gallinarium* strain of swine origin in Italy [3].

Comparative analysis between pEW1611 and pEgFS4-1 showed a conserved structural and two cargo regions bounded by Δ*IS1216* isoforms (Fig. 1A). The cargo region of *cfr* and *optrA* genes in pEW1611 and pEgFS4-1 showed high-level of nucleotide identity, but with insertion of *ant(4′)-Ia* and *IS6-like*, corresponding to *IS1216* transposase (GenBank accession no. MT723957) of two strains also isolated of swine origin in Italy (Fig. 1B) [2]. Plasmid transfer by conjugation was unsuccessful, but EW1611 strain maintained the pEW1611 plasmid, as well as the linezolid resistance phenotype for 30 days in antimicrobial-free culture medium, corroborating with Coccitto et al. [3].

In conclusion, to the best of our knowledge, this is the first report of an Inc18-type plasmid co-harboring *cfr* and *optrA* in *E. casseliflavus*. Plasmid-mediated linezolid resistance genes in aquatic ecosystems represent a threat to environmental safety and support that the environment is a hotspot for antimicrobial resistance. Therefore, continued monitoring of enterococci in the environment remains necessary for mapping mainly MDR- and linezolid-resistant strains.

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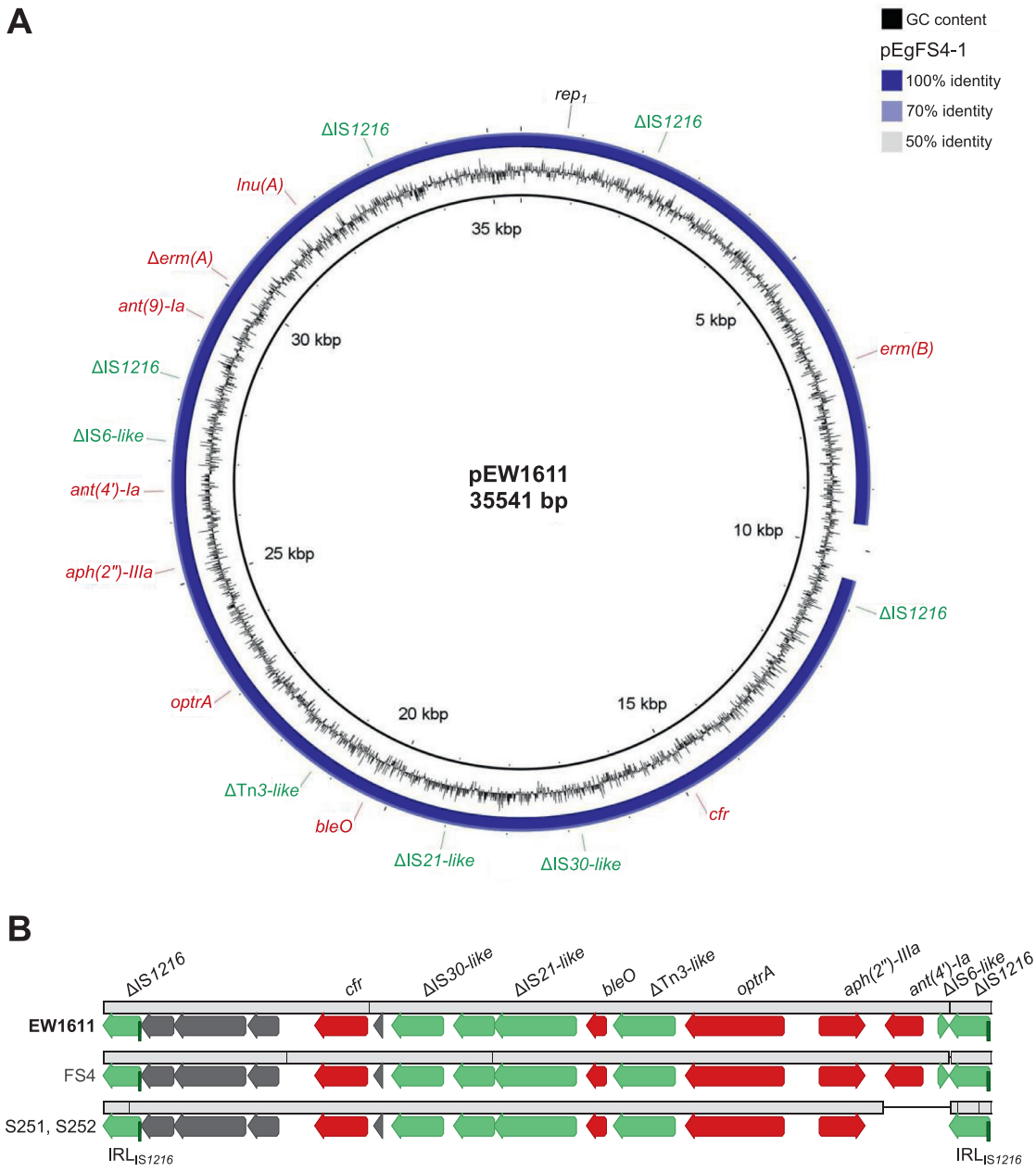

Fig. 1. Inc18-type plasmid co-harboring *cfr* and *optrA* genes in a strain isolated from the environment. A) Circular map of the pEW1611 plasmid in comparison with the pEgFS4-1 plasmid (GenBank accession no. MZ291452) using BRIG software (https://brig.sourceforge.net). Antimicrobial resistance genes, insertion sequences, and other genes are indicated in red, green, and black, respectively. B) Comparison between the genetic environments of *cfr* and *optrA* genes in *Enterococcus casseliflavus* str. EW1611 from surface water in Brazil (this study; in bold) and *Enterococcus gallinarum* str. FS4, *E. faecalis* str. S251, and *Enterococcus avium* str. S252 from swine in Italy (GenBank accession no. MZ291452 and MT723957). Colored arrows indicate the positions and orientations of genes. The vertical bars represent left inverted repeats (IRL). The gray shading denotes shared regions of homology.

Nucleotide sequence accession numbers: Nucleotide sequences of *E. casseliflavus* EW1611 have been deposited at GenBank under accession numbers: JAPKIH000000000 (whole-genome shotgun sequencing project), and OP852500 (pEW1611 plasmid).

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Ethical approval

Not required

Declaration of competing interest

None declared

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.02.008.

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Lucas David Rodrigues dos Santos, João Pedro Rueda Furlan, Rafael da Silva Rosa, Micaela Santana Ramos
Department of Clinical Analyses, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil

Eduardo Angelino Savazzi
Environmental Company of the State of São Paulo, Ribeirão Preto, São Paulo, Brazil

Eliana Guedes Stehling*
Department of Clinical Analyses, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil

*Corresponding author. Av. do Café, S/N, Monte Alegre, Ribeirão Preto, 14040-903, Brazil
E-mail address: elianags@usp.br (E.G. Stehling)
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