Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente,
Ciclo XXXVIII

Isolation and molecular characterization of linezolid-resistant enterococci of human, animal, and environmental origin

Cinthi Marzia

DiSVA, Laboratorio di Microbiologia *Tutor: Prof. Eleonora Giovanetti*

INTRODUCTION

Enterococci are members of gut microbiota of human and animals. Although regarded as commensals, they also are an increasing cause of nosocomial infections worldwide. Due to their abundance in animal feces and persistence in the environment, enterococci spread in many habitats including soil, water, food of animal origin, sewage and plants. The treatment of infections due to MDR and VRE is limited to quinupristin/dalfopristin, oxazolidinones and daptomycin.[1] Oxazolidinones, including linezolid (LZD) and tedizolid (TZD), are synthetic molecules that bind the V domain of the 23S rRNA of the 50S ribosomal subunit and inhibit the protein synthesis.[2] Resistance to LZD can arise via mutations in 23S rRNA and in ribosomal proteins L3 and L4,[4] but also through the acquisition of transferable resistance genes commonly located on conjugative plasmids: *cfr* and its variants, *poxtA* and *optrA*.[2] The aim of my study has been: (i) to investigate the occurrence of LZD resistance genes in enterococci of animal and environmental origin, and (ii) to evaluate their transferability to human pathogens.

MATERIALS AND METHODS

Bacterial strains. In collaboration with the "Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche", sampling activities were carried out in order to isolate FFC-resistant enterococci of animal and environmental origin as previously described.[3] Furthermore, LZD-resistant enterococci were collected from different wards of the Ancona regional hospital. Species identification was performed by MALDI-TOF (*Biomerieux*).

Genotypic and phenotypic characterization. Enterococci were screened by PCR for the presence of *cfr, cfrD, optrA, poxtA* genes.[5]Isolates were tested for their susceptibility to several antibiotics by standard broth microdilution assay.

WGS and sequence analysis. The genomic DNA was extracted by the QIAcube automated extractor (*Qiagen*). Extracted DNA was subjected to WGS.

Mating experiments. Enterococci carrying LZD resistance genes were used as donors in filter mating experiments or in aquaria microcosm assays. *E. faecium* 64/3, *E. faecalis* JH2-2 or *E. faecium* Ef1 were used as recipients.

Table 1. Antimicrobial susceptibility profiles, ST, location of oxazolidinone resistance genes and transfer frequency for the enterococcal isolates. LZD, linezolid; TDZ, tedizolid; FFC, florfenicol; p, plasmid; chr, chromosome; ND, not detectable; [a], filter mating assay; [b], aquaria microcosm assays.

STRAIN	SPECIES	ORIGIN	ST	GENOTYPE				MIC µg/ml			GENES LOCATION	RECIPIENT	TRANSFER FREQUENCY (UFC/mL)
				poxtA	*optrA*	*cfr*	*cfrD*	LZD	TZD	FFC			
M1	*E. faecium*	Freshwater river	ST1036	+	-	-	-	4	0.5	64	pEfM1	*E. faecium* 64/3 *E. faecium* Ef1	5.8×10^{-2} [a] 6.3×10^{-3} [b] 5×10^{-3} [a]
909961	*E. faecalis*	Human	ST476	-	+	-	-	8	1	64	chr (Tn*6674*)	*E. faecalis* JH2-2	ND
30488	*E. faecalis*	Raptor	ST476	-	+	-	-	8	0.5	64	chr (Tn*6674*-like)	*E. faecium* 64/3	ND
249031-C	*E. faecium*	Bovine	ST22	+	+	-	-	4	2	128	p249031-S / p1818-c	*E. faecium* 64/3	2.9×10^{-3}

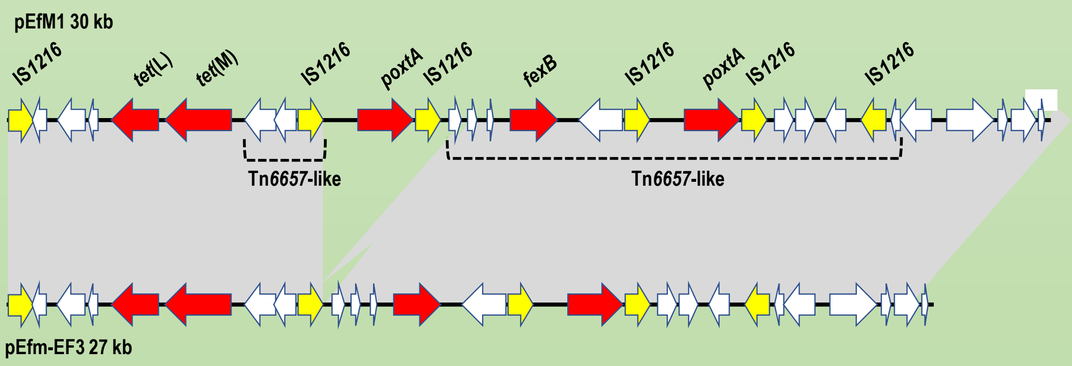

Figure 1. Schematic representation and comparison between pEfM1 plasmid from *E. faecium* M1 and pEfm-EF3 plasmid from *E. faecium* EF3.

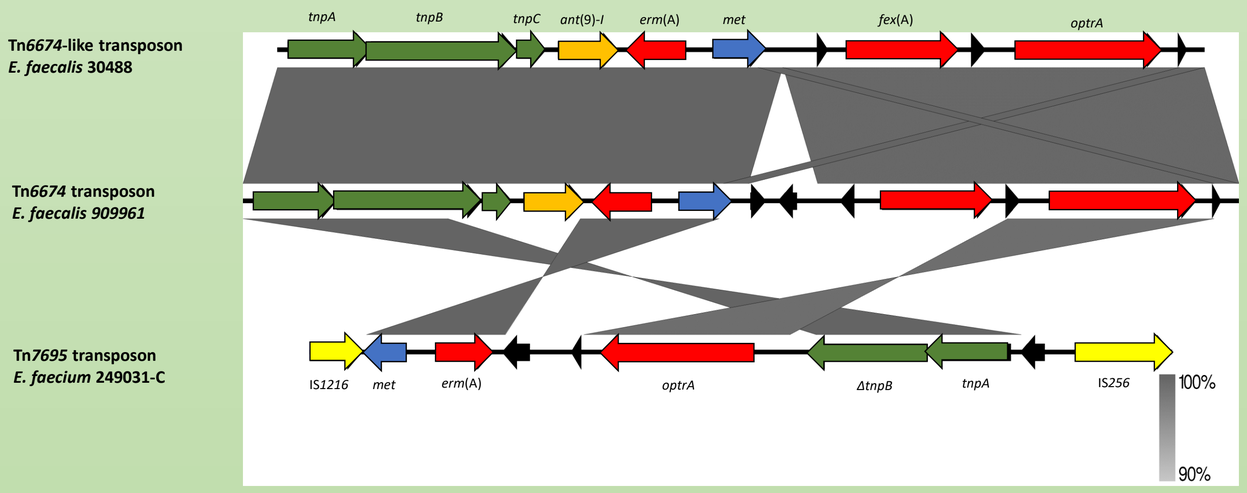

Figure 2. Schematic representation and comparison between Tn*6674*-like of *E. faecalis* 30488, Tn*6674* wild type of *E. faecalis* 909961 and Tn*7695* of *E. faecium* 249031-C

RESULTS

PCR experiments. Enterococci positive for at least one oxazolidinone resistance gene are shown in the Table 1.

Antimicrobial susceptibility profile. All strains were resistant to FFC, susceptible or resistant to LZD and TZD (Table1).

WGS analysis. LZD resistance genes showed chromosomal or plasmid location (Table 1). *E. faecium* M1 was characterized by a double copy of *poxtA* gene on a 30,877-bp plasmid (named pEfM1) with high identity to the enterococcal 27-kb plasmid pEfm-Ef3 detected in an *E. faecium* strain from sediment collected in an Italian coastal area.[4] The *poxtA* genetic context, flanked by two IS*1216*, was located in a Tn*6657*-like transposon also containing *fexB* and *tet*(L) and *tet*(M) genes in tandem (Figure 1). The second copy of *poxtA* was inserted within the Tn*6657*-like, maybe thanks to IS*1216* mediated recombination phenomena. The strain belonged to ST1036 which has been associated with human enterococci (PubMLST).

E. faecium 249031-C belonged to ST22 closely related to the CC17 clonal complex. Enterococci belonging to this clonal lineage, despite recovered from both environmental and animal samples, have adapted to the hospital setting causing numerous epidemic outbreaks. The isolate harbored two plasmids: the *optrA*-carrying p249031-S (179,049 bp) and the *poxtA*-carrying p1818-c (23,864 bp) . p249031-S showed a high degree of identity (100%) and coverage (73%) to the plasmid pF88_1 of *E. faecium* F88 isolated from river water in Switzerland [5] and it was characterized by a new *optrA*-carrying Tn*7695* transposon (11,319-bp), flanked by IS*1216* and IS*256* with the same polarity (Figure 2). The 24-kb plasmid carrying the *poxtA* gene was identical to the p1818-c, previously detected in *E. faecium* 1818 from a healthy human in Switzerland.[6]

The LZD-resistant and *optrA*-carrying *E. faecalis* 909961 (human) and 30488 (from raptor) both belonged to ST476, a clonal lineage detected in multiple countries from human, animal and environmental sources. In *E. faecalis* 909961 and 30488 the *optrA* gene was located respectively on the wild type Tn*6674* and on a Tn*6674*-like which was characterized by a 900-bp deletion in the intergenic region between *fexA* and *erm*(A) genes (Figure 2).

Mating experiments. *E. faecium* 249031-C was able to transfer the resistance to the recipient in filter mating assays (Table1). The *optrA* transferability from *E. faecium* M1 donor was performed both in filter mating experiments and in aquaria microcosm assays. M1 was able to transfer the double copy of *poxtA* gene to *E. faecium* 64/3 and Ef1 in filter mating experiments and only to *E. faecium* 64/3 in acquaria microcosm assays (Table 1).

DISCUSSION

FFC is extensively used in zootechnics and veterinary medicine; the selective pressure exerted by this antibiotic may have led to widespread of LZD resistance genes both in animal and environmental setting. The currently worrisome situation in humans may be the tip of the iceberg of a more widespread phenomenon. Antibiotic resistance is a complex and multifactorial phenomenon that requires a ONE HEALTH approach that connects the health of humans and animals with the environment in which they live. The emergence of LZD-resistant enterococci due to transferable resistance determinants is a matter of concern worldwide. This research will help to clarify the role of animal and environmental enterococci as a source of LZD resistance genes transferable to major human pathogens by horizontal gene transfer.

REFERENCES

1. Bender J et al. 2018. Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: towards a common nomenclature. Drug Resist Updat 40:25–39.
2. Brenciani A et al. 2022. Oxazolidinones: mechanisms of resistance and mobile genetic elements involved. J Antimicrob Chemot
3. Cinthi M et al. 2022. Occurrence of a plasmid cocarrying cfr(D) and poxtA2 linezolid resistance genes in Enterococcus faecalis and Enterococcus casseliflavus from porcine manure, Italy. J Antimicrob Chemother. 77: 598–603
4. Fioriti, S. et al. Linezolid resistance genes in enterococci isolated from sediment and zooplankton in two Italian coastal areas. Appl. Environ. Microbiol. **2021**, 87, e02958-20.
5. Biggel M, et al. Genetic context of optrA and poxtA in florfenicol-resistant enterococci isolated from flowing surface water in Switzerland. Antimicrob Agents Chemother 2021;65(10):e0108321; doi: 10.1128/AAC.01083-21
6. Nu¨esch-Inderbinen M et al. Faecal carriage of enterococci harbouring oxazolidinone resistance genes among healthy humans in the community in Switzerland. J Antim Chemother 2022;77(10):2779–2783; doi: 10.1093/jac/dkac260