Linezolid resistance genes in enterococci isolated from sediment and zooplankton in two Italian coastal areas

Original Research

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Running title:

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Abstract

Linezolid is a last resort antibiotic for the treatment of severe infections caused by multi-resistant Gram-positives; although linezolid resistance remains uncommon, the number of linezolid-resistant enterococci has increased during recent years due to worldwide spread of acquired resistance genes (*cfr*, *optrA* and *poxtA*) in clinical, animal and environmental setting.

In this study we investigated the occurrence of linezolid-resistant enterococci in marine samples from two coastal areas in Italy. Isolates grown on florfenicol-supplemented Slanetz-Bartley were investigated for their carriage of *optrA*, *poxtA* and *cfr* genes: *optrA* was found in one *E. faecalis*, *poxtA* in three *E. faecium* and two *E. hirae* and *cfr* was not found. Two of the three *poxtA*-carrying *E. faecium* and the two *E. hirae* showed related PFGE profiles. Two *E. faecium* belonged to the new ST1710, which clustered in the clonal complex CC94, encompassing nosocomial strains. S1-PFGE/hybridization assays showed a double (chromosome and plasmid) location of *poxtA* and plasmid location of *optrA*. WGS revealed that *poxtA* was contained in a Tn*6657*-like element carried by two plasmids (pEfm-EF3 and pEh-GE2) of similar size, found in different species, and that *poxtA* were flanked by two copies of IS*1216* in both plasmids. In mating experiments all but one (*E. faecalis* EN3) strains were able to transfer the *poxtA* gene to *E. faecium* 64/3.

The occurrence of linezolid resistance genes in enterococci from marine samples is of great concern and highlights the need to improve practices aimed at limiting the transmission of linezolid resistant strains to humans from the environmental reservoirs.

Importance

Linezolid is one of the few antimicrobials available to treat severe infections due to drug-resistant Gram-positive bacteria, thus the emergence of linezolid-resistant enterococci carrying transferable resistance determinants is of great concern for public health. Linezolid resistance genes (*cfr*, *optrA* and *poxtA*), often plasmid located, can be transmitted via horizontal gene transfer and have the potential to spread globally. This study highlights the first detection of enterococci carrying linezolid resistance genes from sediment and zooplankton samples in two coastal urban areas in Italy. The presence of clinically relevant resistant bacteria, such as linezolid-resistant enterococci, in marine environment could reflect their spillover from human and/or animal reservoirs and could indicate that also coastal seawaters could represent a source of these resistance genes.

Introduction

The anthropogenic release of antibiotics into environment, due to their intensive use in human and veterinary medicine and in agriculture, has raised global public health concerns.

The complex microbial community of aquatic environment also include transient bacteria from different sources, such as hospital, domestic and animal breeding effluents (1, 2). Antibiotic pollution imposes a selective pressure on bacterial populations which can facilitate the development and spread of antibiotic resistances through horizontal gene transfer (HGT). The evidence for horizontal dissemination of antibiotic resistances between environmental bacteria and human pathogens demonstrates the importance of environmental resistomes.

Enterococci are members of gut microbiota of humans and animals. They are released in large amounts into the environment with feces and therefore can be found in different niches including soil, foods of animal origin, vegetables, and water. Fecal indicator *Enterococcus* spp. has been well established for routine monitoring of water quality, and this principle has been extended to foods (3). More recently, enterococci have been also proposed for monitoring antibiotic resistance in food animals (4).

Although regarded as commensals, *Enterococcus* spp. are the leading causes of nosocomial infections worldwide (5). Acquired resistances are growing and considerably limit the therapeutic options and oxazolidinones are among the few available last-resort antibiotics recommended to treat severe infections caused by VRE and MDR enterococci (6).

Oxazolidinones – linezolid and tedizolid – bind in the V domain of the 23S rRNA of the 50S ribosomal subunit and inhibit protein synthesis (7). Besides the

mutations in 23S rRNA and/or in L3, L4, and L22 ribosomal proteins (6, 8), linezolid resistance can develop following acquisition of the resistance genes *cfr* and its variants, *optrA* and *poxtA*. Cfr and Cfr-like methylases confer resistance to five classes of antimicrobial agents including phenicols, lincosamides, oxazolidinones, pleuromutilines and streptogramin A ($PhLOPS_A$ phenotype) by a post-transcriptional methylation of the 23S rRNA (9, 10-13). The ABC-F proteins OptrA and PoxtA leads to a decreased susceptibility to phenicols, oxazolidinones (including tedizolid) and tetracyclines (PoxtA protein only) by a ribosomal protection mechanism (14-16).

In enterococci, linezolid resistance genes are often carried by mobile genetic elements and are easily transferred between bacteria by HGT (14, 17-20). Enterococci spread in many natural habitats and, besides the occurrence of linezolid-resistant enterococci in hospitals, their detection in other reservoirs is of special concern (21).

The purpose of this study was to investigate the occurrence of linezolid resistance genes in enterococci isolated from marine samples collected at two coastal urban areas in Italy.

To our knowledge, this is the first report of linezolid resistance genes in enterococci from the marine environment.

Results and discussion

Detection of oxazolidinone resistance genes in florfenicol-resistant enterococci and antimicrobial susceptibility profiles

Out of 77 total samples (seawater=33, sediment=33 and zooplankton=11) only ten sediment and one zooplankton samples from six sampling sites (Figure 1) were positive for the presence of florfenicol-resistant enterococci. Thirty-five isolates were found positive for *poxtA* or *optrA*, however only six different pulsotypes (one by site) were detected by SmaI-PFGE assay. The six isolates – 1 *Enterococcus faecalis*, 3 *E. faecium* and 2 *Enterococcus hirae* - were then characterized (Table 1). The *optrA* gene was only detected in the *E. faecalis* isolate, *poxtA* was identified in the 3 *E. faecium* and the 2 *E. hirae*, whereas *cfr* was not found (Table 1).

The *poxtA* gene, first described in a MRSA from a patient with cystic fibrosis (15), was shortly after reported in enterococci isolated from many different non-human sources, e.g. pigs and chicken (22-24), retail meat and food-producing animals (25), as well as from air samples of swine farm (26). Through metagenomic approach, this gene was recently detected in livestock manures (27), and even in microbiome of drinking water in environmental and clinical settings (28). The wide spread of *poxtA* in non-human enterococci, mainly *E. faecium* species, suggested that selection of this gene could occurred in the animal setting owing to extensive use of phenicols and doxycycline in veterinary medicine (29). *poxtA*-carrying strains can then reach water bodies, including coastal waters, through manure contamination and runoff from husbandry and agriculture activities. On the

other hand, *poxtA* has also been increasingly reported on clinical isolates (30, 31), confirming its diffusion also in human settings.

The six enterococcal isolates were all resistant to florfenicol (MIC range, 32-128 mg/L), chloramphenicol (MIC range, 16-128 mg/L), and tetracycline (MIC range, 128->128 mg/L)) and either susceptible or resistant to linezolid (MIC range, 2–8 mg/L) and tedizolid (MIC range, 2-4 mg/L). All tested strains were susceptible to vancomycin (MIC range, 0.5-1 mg/L) (Table 1).

Typing assays

Enterococcal isolates belonged to 3 different SmaI-PFGE types (A to C), and two subtypes (A1 and C1) (Table 1). *E. faecium* EF3 and ES2 were found to be closely related (C and C1, respectively), as well as *E. hirae* GE5 (from marine sediment) and *E. hirae* GE2 (from zooplankton) (A1 and A, respectively).

E. faecalis EN3 belonged to ST585 which has been associated with human enterococci (32-36). *E. faecium* EF3 and ES2 belonged to the same ST (ST1710), while *E. faecium* TF3 to the ST1711. Although both STs have never been described before, ST1710 clustered in the clonal complex CC94, encompassing human intestinal enterococci, recovered from both community and hospitalized hosts (37). The proximity of our sampling sites to the hospital and urban areas, could suggest the spread in the environment of human strains carrying linezolid resistance genes.

Location of oxazolidinones resistance genes and detection of circular forms

In *E. faecalis* EN3, *optrA* gene was located on two plasmids of ~20 kb and ~140 kb plasmids, while in the three *poxtA*-carrying *E. faecium* isolates hybridisation occurred on both chromosome and plasmids. The *poxtA* gene was located on plasmids of different sizes: ~30 kb in *E. faecium* EF3, ~15 and ~30 kb in *E.*

faecium ES2 and ~30, ~50 and ~80 kb in *E. faecium* TF3. In the closely related *poxtA*-carrying *E. hirae* GE5 and *E. hirae* GE2 only a plasmid localization of *poxtA* gene was detected. In both isolates the *poxtA* probe hybridized on two plasmids of ~25 and ~100 kb in size (Table 1).

Inverse PCR experiments and sequencing showed that no circular form of *optrA* genetic context was detectable. Conversely, minicircles were obtained from all the *poxtA* genetic contexts.

Since the *optrA* is located on plasmids of different size (~20 kb and ~140 kb), and WGS revealed a single *optrA* genetic context with no evidences of circularisation, it is reasonable to assume that recombination events between plasmids occurred (38).

As regards *poxtA*, its location on plasmids of different sizes and even on the chromosome, suggests an intracellular mobility of the *poxtA*-carrying element due to IS-mediated recombination events.

Transferability of oxazolidinones resistance genes

Five of six isolates successfully transferred the linezolid resistance genes in intra- and interspecific mating experiments with frequencies ranging from 6.5×10^{-1} to 3×10^{-6} CFU per recipient. MICs and genotypes for both donors and selected transconjugants, and transfer frequencies are indicated in Table 2. The higher frequencies were observed in intraspecific transfer of *poxtA* from *E. faecium* ES2 and *E. faecium* TF3 donors to *E. faecium* 64/3 recipient. Conversely, *E. hirae* GE2 and GE5 successfully transferred *poxtA* to the *E. faecium* recipient.

In both *E. faecium* and *E. hirae* transconjugants, *poxtA* gene was located on plasmids of ~30 kb and ~25 kb, respectively and on the chromosome (Table 2).

189 Despite several attempts *E. faecalis* EN3 was not able to transfer *optrA* gene to *E.*
190 *faecium* 64/3 recipient. The interspecific transfer of the resistance genes from *E.*
191 *hirae* to *E. faecium* is worrisome since the former species is more common in
192 animals where phenicols and tetracyclines are widely used and therefore could be a
193 reservoir of linezolid resistance genes for more pathogenic species such as *E.*
194 *faecium*.
195
196 **WGS analysis**
197 All six test strains were subjected to WGS analysis. The maps of the plasmids are
198 shown in Figures 2-4.
199 Bioinformatics analysis of the draft genome of *E. faecalis* EN3, coupled with PCR
200 mapping and Sanger sequencing experiments, revealed that the *optrA* gene was part
201 of a 16,500 bp plasmid, named pEfs-EN3 (G+C content, 33.0%) (accession no.
202 MT683614). According to the nomenclature of *optrA* variants reported by Morroni
203 *et al.* (39). *E. faecalis* EN3 showed the *optrA* DP variant which has been described
204 in different *E. faecalis* clones from human and pigs (40). The *optrA* genetic
205 environment (6,810 bp), bounded by two IS*1216* insertion sequences arranged in
206 the same orientation, also contained the *fexA* gene located 687 bp upstream *optrA*
207 (Figure 2). A similar organization has been previously described in plasmids of *E.*
208 *faecalis* isolates from dogs in China (41). The *repA*, *parA* and *prgN* genes (*orf8*,
209 *orf10*, and *orf11*, respectively) responsible for the plasmid replication and
210 partitioning were also detected. The plasmid pEfs-EN3 belonged to the RepA_N
211 family and showed a rep_9-type, which are both typical features of *E. faecalis* sex
212 pheromone-responsive plasmids (42). Interestingly, pheromone-responsive
213 conjugative *optrA*-carrying plasmids have been identified in *E. faecalis* of swine
214 origin (43).

Since the hybridization assays suggested that a *poxtA*-carrying plasmid of ~30 kb was shared by *E. faecium* isolates, we decided to proceed with its assembly.

In *E. faecium* EF3, *poxtA* gene was located on a 27,703-bp plasmid designed pEfm-EF3 (G+C content, 35.0%) (accession no. MT683615). The genetic context of *poxtA* (4,003 bp), flanked by two IS*1216* in the same orientation, was in turn inserted in a Tn*6657*-like transposon also containing *fexB* as originally described in the MRSA strain AOUC-0915 (accession no. MF095097) (20). Upstream the Tn*6657*-like transposon a tetracycline resistance region containing *tet*(L) and *tet*(M) genes arranged in tandem was found; downstream the Tn*6657*-like four genes (*orf28*-*orf31*) involved in plasmid partitioning and replication were detected (Figure 3). pEfm-EF3 exhibited 99% DNA identity (cover 100%) with regions of pC25-1 and pC27-2, two broad-host-range Inc18 plasmids from a CC17 *E. faecium* of pig origin from China (accession numbers MH784601 and MH784602, respectively) (44).

In *E. faecium* ES2 and *E. faecium* TF3, *poxtA*-carrying plasmids identical to pEfm-EF3 were found. It is noteworthy that the three *E. faecium* isolates have been collected from different sampling sites (Table 1). Furthermore, the closely related *E. faecium* EF3 and *E. faecium* ES2 belonged to ST1710, while *E. faecium* TF3 was assigned to ST1711 suggesting a spread of pEfm-EF3 by HGT may occur among isolates with different backgrounds.

WGS analysis of *E. hirae* GE2 revealed that the *poxtA* gene was located on a 24,793-bp plasmid, named pEh-GE2 (G+C content, 38.0%) (accession no. MT683616). BLASTN analysis displayed that in pEh-GE2 two regions exhibited a high DNA identity with different genetic elements. The 12.8-kb region containing the *poxtA* genetic context (*orf1* to *orf18*) showed high DNA identity (99%) with a Tn*6657*-like transposon (20). As observed in pEfm-EN3, the *poxtA* genetic contest

was bracketed by IS*1216* elements in the same orientation (Figure 4). The pEh-GE2 region spanning from *orf19* to *orf31* (14.7 kb) and carrying the Tn*916* conjugation region (including the *rep* gene) showed 99% DNA identity with plasmid 3 of *E. faecium* E4457 (accession no. LR135260) (Figure 4).

WGS analysis of *E. hirae* GE5 displayed a *poxtA*-carrying plasmid with a complete synteny to the pEh-GE2, despite the two strains come from different sampling sites and samples (sediment and zooplankton, respectively) (Table 1).

Interestingly, the *poxtA*-carrying plasmids of *E. hirae* and *E. faecium* isolates shared only the Tn*6657*-like region (cover 55%, DNA identity 99%) suggesting the widespread of this element in enterococci. The pEh-GE2 resulted to belong to the Rep_trans family which includes small size plasmids largely spread among enterococcal populations (42).

Hybridization analysis also showed the presence of an *optrA*-carrying plasmid (~140 kb) in *E. faecalis* EN3 and a *poxtA* plasmid (~100 kb) in *E. hirae* GE2 and GE5 isolates that were not assembled.

WGS analysis also ruled out the presence of *cfr*(B), *cfr*(C), *cfr*(D) and *cfr*(E) genes. No mutations were detected in the genes encoding the 23S rRNA or ribosomal proteins.

Conclusions

The emergence of linezolid-resistant enterococci due to transferable resistance determinants is a matter of concern worldwide. This is – to the best of our knowledge – the first detection of enterococci carrying linezolid resistance genes in marine sediment and zooplankton. The evidence that also the coastal seawaters could serve as a reservoir of oxazolidinones resistance genes is of great concern for public health. Further surveillance and control efforts are needed to counteract

the spread of linezolid-resistant bacteria in human and animal settings to prevent the formation of environmental reservoirs of resistance genes transmissible to humans via different routes including bathing, aquaculture and seafood consumption.

Materials and methods

Sampling sites, sample processing and bacterial isolation

Sampling activities were carried out at 11 sites in two areas located on the Western and Eastern coast of Italy (in Ligurian and Adriatic Sea), in a framework of a research project aimed at the detection of antibiotic-resistant bacteria from the marine environment (unpublished results). Sampling sites located in Ligurian Sea (n=3) were in front of the harbor and the hospital of Genoa city (GEN, GES, GEF), whereas sampling sites in the Adriatic Sea (n=8) were in front of an urban area close to the river Esino estuary and to an oil refinery (ESN, ESS, ESF), and in front of the hospital (TN, TS, TF) and the harbor of Ancona city (PN, PS) (Figure 1).

At each site, seven samples (seawater n=3, sediment n=3, and zooplankton n=1) were collected in July 2019.

All 77 samples were incubated overnight at 37°C in Azide broth (Oxoid, Basingstoke, UK) for the selective enrichment of enterococci. Sediment (5g) samples were immediately added to the enrichment broth whereas seawater and zooplankton samples were processed as follows. Seawater (400 ml) were filtered through 0,22 μm filter membranes (Merk Life Science, Milano, Italy) and filters incubated in 30 ml Azide broth. Zooplankton (50 ml aliquots) organisms were collected by dragging the water horizontally (~1m depth) with a 200 μm mesh plankton net. Aliquots (50 ml) of the collected material were centrifuged 10 min at 15000xg; pellets were resuspended in 5 ml artificial sterile seawater and added to 40 ml of Azide broth. Each enrichment culture (100 μl) was spread on Slanetz

Bartley agar plates supplemented with florfenicol (10 mg/L) for the selection of resistant enterococcal isolates.

From each selective agar plate eight presumptive resistant enterococcal colonies were randomly picked for further analysis.

Genotypic and phenotypic characterization

Selected florfenicol-resistant enterococci were screened by PCR for the presence of *cfr*, *optrA* and *poxtA* genes using primer pairs previously described (22). The PCR products were subjected to Sanger sequencing.

Isolates carrying linezolid resistance genes were identified by MALDI-TOF (Vitek-MS, bioMérieux) and tested for their susceptibility to florfenicol, chloramphenicol, linezolid, tetracycline and vancomycin (Sigma Aldrich, St. Louis, MI) by standard broth microdilution assay, and to tedizolid using Etest strips (Liofilchem, Roseto degli Abruzzi, Italy). Susceptibility tests were interpreted according to clinical EUCAST (version 10.0, 2020. http://www.eucast.org) or CLSI breakpoints (https://clsi.org/standards/products/free-resources/access-our-free-resources/). *E. faecalis* ATCC 29212 was used as quality control (EUCAST QC tables v 10.0, 2020. http://www.eucast.org).

SmaI-PFGE, S1-PFGE, southern blotting and hybridisation assays

Typing was performed by SmaI-PFGE as previously described (45).

Genomic DNA embedded in agarose gel plugs was digested with S1 nuclease (Thermo Fisher Scientific, Milan, Italy) and chromosome and plasmids separated by PFGE as previously described (46). After S1-PFGE, total DNA was blotted onto

positively charged nylon membranes (Ambion-Celbio, Milan, Italy) and hybridised with biotin-labelled *cfr*, *optrA* and *poxtA* DNA probes as described elsewhere (47).

Detection of circular forms

To investigate the excision of genetic contexts carrying linezolid resistance genes, PCR assays were performed using outward-directed primer pairs targeting the linezolid resistance genes: (i) poxtAdiv-FW GACGAGCCGACCAACCACCT and poxtAdiv-RV TTCAGGCGGACAAAAATCCAA; (ii) optrAdiv-FW GAAAAATAACACAGTAAAAGGC and optrAdiv-RV TTTTTCCACATCCATTTCTACC.

Briefly, 5 µl of genomic DNA was added in a final volume of 25 µl of mastermix containing 0.2 µM of each primer, 500 mM dNTP mix, 7 mM $MgCl_2$, and 2 U Dream Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA). PCR conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 5 min; and 72 °C for 5 min. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems System 9700 GeneAmp PCR Thermal Cycler). PCR products were resolved by electrophoresis on 1.0% agarose gel.

The *cfr*-, *poxtA*-carrying *S. aureus* AOUC-0915 (48) and the *cfr*-, *optrA*-carrying *E. faecium* E35048 (49) isolates were used as positive controls in PCR experiments.

Conjugation experiments

Conjugal transfer was performed on a membrane filter as described previously (47). In mating experiments, all isolates carrying linezolid resistance genes were used as donors, and *Enterococcus faecium* 64/3 was used as a recipient (50).

Transconjugants were selected on brain heart infusion agar (Oxoid, Basingstoke, UK) containing florfenicol (10 mg/L), fusidic acid (25 mg/L) and rifampicin (25 mg/L), grown colonies were tested for the presence of linezolid resistance genes by PCR and for their susceptibility to florfenicol and linezolid.

SmaI-PFGE was carried out and patterns analysed to confirm the genetic background of transconjugants. Conjugation frequencies were expressed as ratio of cell number (CFU/ml) of transconjugants to recipient.

WGS and sequence analysis

Genomic DNA was extracted using a commercial kit (Sigma-Aldrich, St Louis, MO, USA). Next-generation sequencing (NGS) was carried out using the Illumina MiSeq platform (MicrobesNG, Birmingham, UK) by using a 2 x 250 paired end approach. De novo assembly was performed with SPAdes v 3.11.1 (http://cab.spbu.ru/software/spades/), and ORFs (minimum length, 50 amino acids) were annotated with the RAST Annotation server (http://rast.nmpdr.org) and ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). The quality of the final contigs was improved with Burrows–Wheeler Aligner. The gaps between the plasmid contigs were closed by PCR mapping using primers targeting unique DNA regions and Sanger sequencing of the resulting amplicons, after purification with a GenElute PCR Cleanup kit (Sigma-Aldrich).

The presence of mutations in genes encoding all copies of the 23S rRNA and ribosomal proteins L3 and L4 were investigated by WGS analysis, comparing the sequences to those from linezolid-susceptible *E. faecalis* ATCC 29212 (accession no: ALOD01000000). The nucleotide sequences were compared with sequences in the GenBank database using BLASTN (http://blast.ncbi.nlm.nih.gov/blast). The ST was determined through the Center for Genomic Epidemiology

(https://cge.cbs.dtu.dk/services/MLST/) and MLST database (https://pubmlst.org/general.shtml).

Data availability

The whole genomes of six isolates are available under the BioProject ID PRJNA679166. The sequence of plasmids characterized in this study were submitted to GenBank and assigned to accession numbers: MT683614, MT683615 and MT683616.

Funding

This work was supported by PRIN 2017 grant prot. 20177J5Y3P from MIUR-Italy and by Progetto Strategico di Ateneo 2017 – Polytechnic University of Marche, "In the hunt of new antibiotics: active compounds from both chemical synthesis and natural sources".

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583

584 **Figure legends**

585

586 **Figure 1.** Maps of two sampling areas located in Ligurian and Adriatic Sea. The
587 yellow pins indicate sites where florfenicol-resistant enterococci were isolated; the
588 white pins indicate sites where no florfenicol-resistant strains were recovered.
589 Geographic coordinates and depth of sampling sites: GEN (44°23'25.26"N/
590 8°56'40.56"E – 13,4 m); GES (44°23'22.06"N/ 8°56'44.59"E – 16.1 m); GEF
591 (44°23'21.48"N/ 8°56'39.77"E – 16,2 m); EN (43°38'51.06"N/13°22'6.66"E – 4 m);
592 EF (43°38'41.16"N/13°22'22.74"E – 3 m); ES (43°38'37.20"N/13°22'41.46"E – 3.4
593 m); TF (43°36'45.96"N/13°27'12.36"E – 3 m); PN (43°37'21.30"N/13°29'2.10"E –
594 8,8 m); PS (43°37'22.78"N/13°29'26.16"E – 7 m); TN
595 (43°37'17.94"N/13°27'26.64"E – 7,6 m); TS (43°36'40.02"N/13°27'22.08"E – 2,4
596 m).

597

598 **Figure 2.** Schematic representation of the *optrA*-carrying pEfs-EN3 plasmid
599 (16,500 bp) from *E. faecalis* EN3 (accession no. MT683614).
600 Arrows indicate the positions and directions of transcription of the different genes.

601

602 **Figure 3.** Schematic representation of the *poxtA*-carrying pEfm-EF3 plasmid
603 (27,703 bp) from *E. faecium* EF3 (accession no. MT683615).
604 Arrows indicate the positions and directions of transcription of the different genes.

605

606 **Figure 4.** Schematic representation of the *poxtA*-carrying pEh-GE2 plasmid
607 (24,793 bp) from *E. hirae* GE2 (accession no. MT683616).
608 Arrows indicate the positions and directions of transcription of the different genes.

609 **Table 1.** Linezolid resistance genes, antimicrobial susceptibility profiles, typing data and genes location.

Strain	Species	Sampling site	Sample	Oxazolidinone resistance genes				MIC (mg/L)							Typing		S1-PFGE and hybridization	
				optrA	*cfr*	*poxtA*		FFC[a]	CHL	LZD	TZD	TE	VAN		SmaI-PFGE	MLST	*optrA*	*poxtA*
EN3	*E. faecalis*	EN	sediment	+	-	-		128	128	4	4	128	1		-	ST585	20[b],140	-
EF3	*E. faecium*	EF	sediment	-	-	+		64	16	8	2	128	1		C	ST1710	-	30, c[c]
ES2	*E. faecium*	ES	sediment	-	-	+		32	16	8	2	128	1		C_1	ST1710	-	15, 30, c
TF3	*E. faecium*	TF	sediment	-	-	+		64	32	2	2	>128	0.5		B	ST1711	-	30, 50, 80, c
GE5	*E. hirae*	GEN	sediment	-	-	+		64	64	4	2	128	0.5		A_1	-	-	25, 100
GE2	*E. hirae*	GES	zooplankton	-	-	+		64	64	8	3	128	0.5		A	-	-	25, 100

610

611

612 [a]FFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TDZ, tedizolid; TE, tetracycline; VAN, vancomycin; [b]Estimated plasmid size (in kb)

613 [c]c, chromosome. MIC resistance breakpoints (EUCAST or CLSI): FFC, not applicable; CHL, R≥32mg/L; LZD, R>4 mg/L; TDZ, S≤0.5 mg/L

614 (only for *E. faecalis*); TE, R≥16 mg/L; VAN, R>4 mg/L.

Table 2. Florfenicol and linezolid MICs, resistance genotypes and genes location for relevant transconjugants.

Donor				Recipient	Transfer frequency	Transconjugant			
	MIC (mg/L)		LZD resistance genotype			MIC (mg/L)		LZD resistance genotype	S1-PFGE and hybridization
	FFC[a]	LZD				FFC	LZD		
E. faecalis EN3	128	4	*optrA*	*E. faecium* 64/3[b]	ND[c]	-	-	-	-
E. faecium EF3	64	8	*poxtA*	*E. faecium* 64/3	5×10^{-5}	64	4	*poxtA*	30[d], c[e]
E. faecium ES2	32	8	*poxtA*	*E. faecium* 64/3	6.5×10^{-1}	64	4	*poxtA*	30, c
E. faecium TF3	64	2	*poxtA*	*E. faecium* 64/3	1.1×10^{-1}	32	2	*poxtA*	30, c
E. hirae GE5	64	4	*poxtA*	*E. faecium* 64/3	7.5×10^{-5}	64	4	*poxtA*	25, c
E. hirae GE2	64	8	*poxtA*	*E. faecium* 64/3	3×10^{-6}	64	4	*poxtA*	25, c

[a]FFC, florfenicol; LZD, linezolid. [b]The MICs of FFC and LZD for *E. faecium* 64/3 were 4 mg/L and 1 mg/L, respectively.

[c]ND, not detectable; [d]Estimated plasmid size (in kb); [e]c, chromosome


Switzerland
Slovenia
Croatia
Italy
Albania
Genoa
Ancona
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H
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Marina di Rocca Priora
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Fiumesino
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TF
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TS
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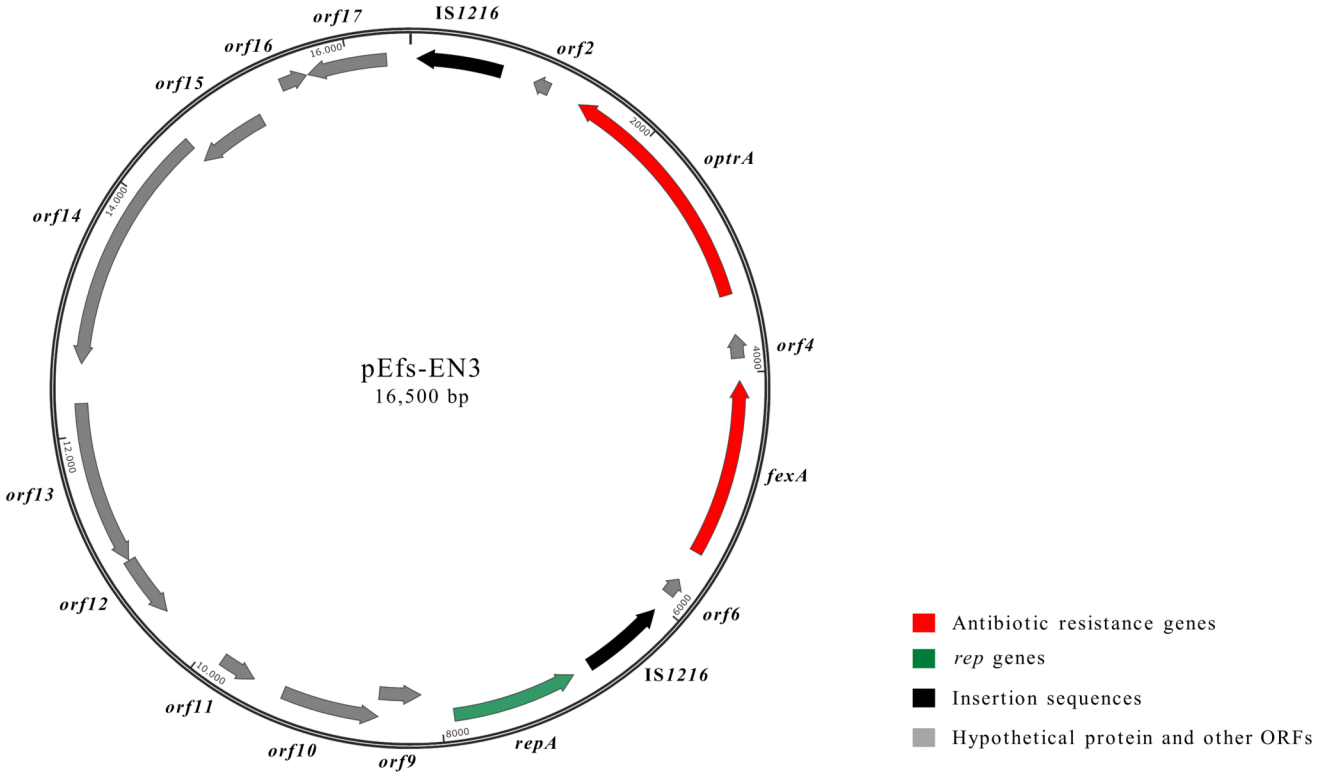
Oil refinery
Hospital
PN
PS
US Dept of State Geographer


pEfs-EN3
16,500 bp
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orf2
optrA
orf4
fexA
orf6
IS1216
repA
orf9
orf10
orf11
orf12
orf13
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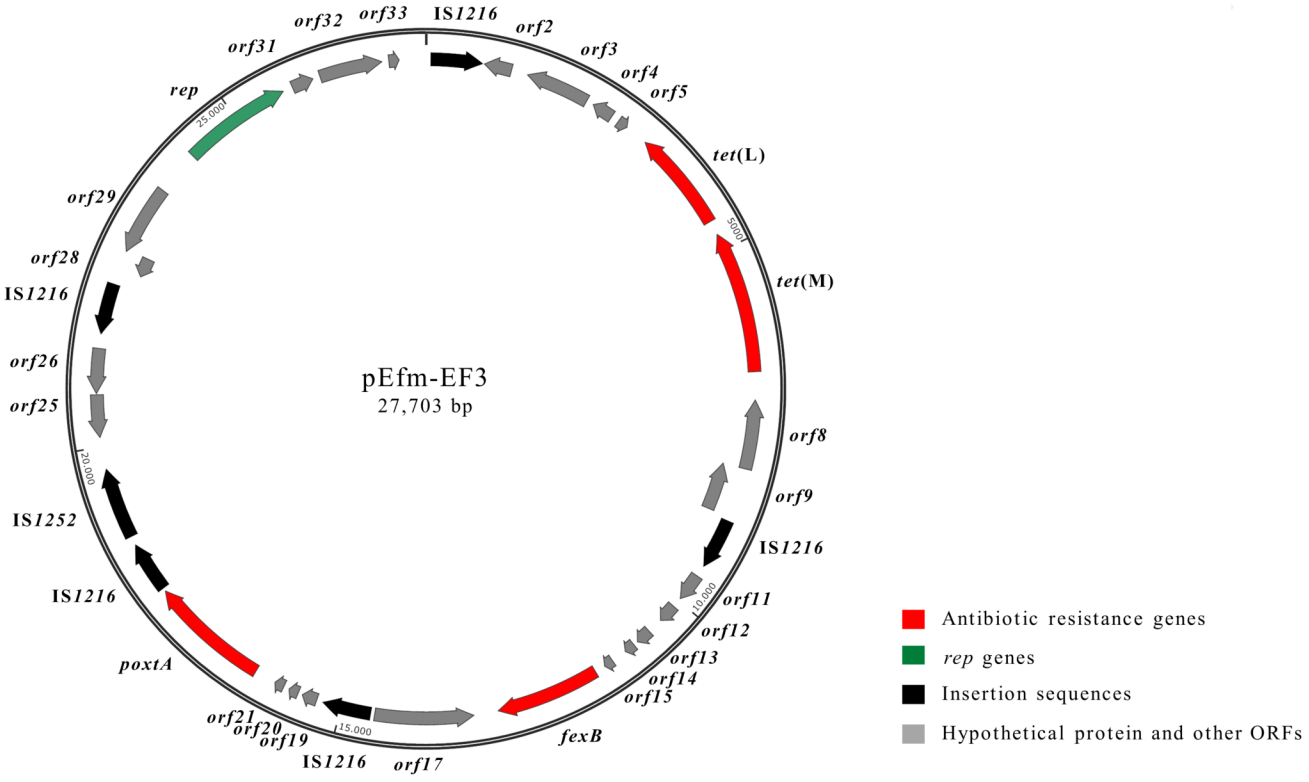
Antibiotic resistance genes
rep genes
Insertion sequences
Hypothetical protein and other ORFs


pEfm-EF3
27,703 bp
IS1216
orf2
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fexB
orf17
IS1216
orf19
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orf21
poxtA
IS1216
IS1252
orf25
orf26
IS1216
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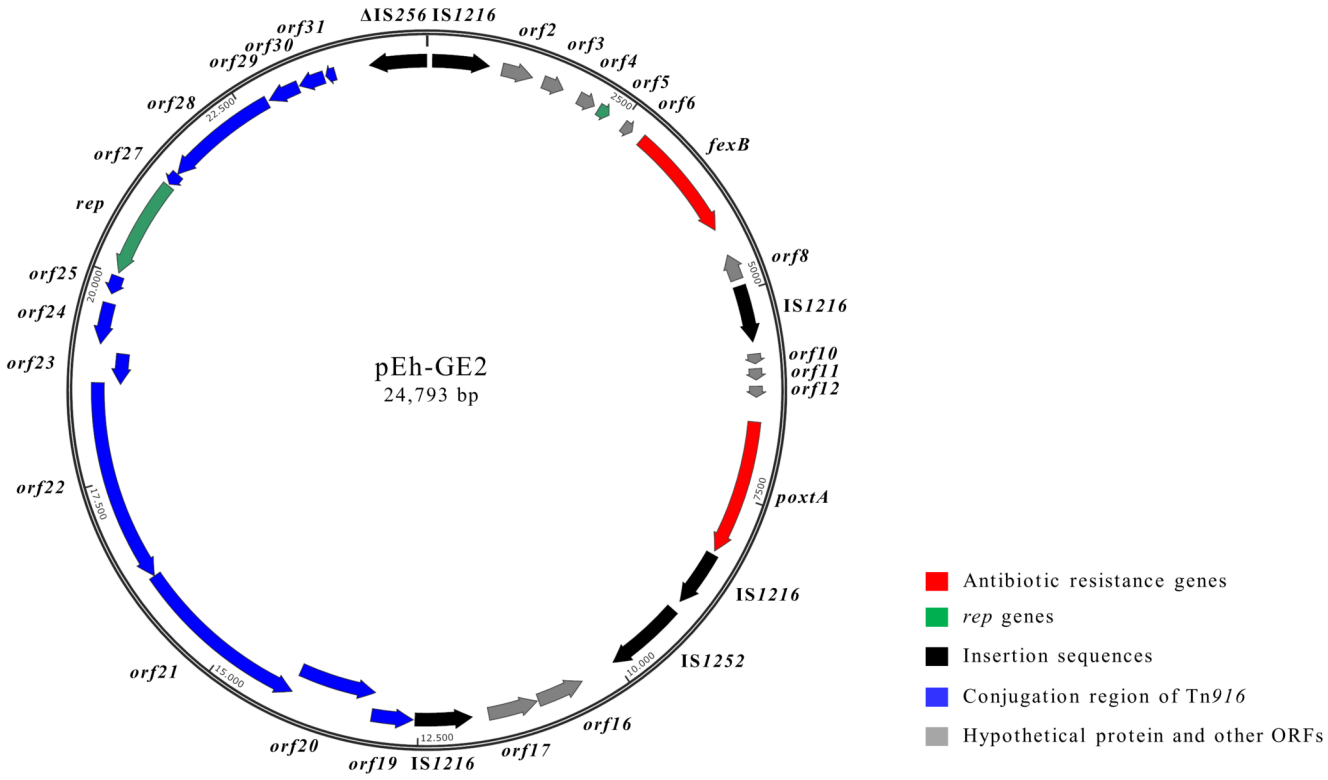
Antibiotic resistance genes
rep genes
Insertion sequences
Hypothetical protein and other ORFs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orf22
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orf24
orf25
rep
orf27
orf28
orf29
orf30
orf31
pEh-GE2
24,793 bp
Antibiotic resistance genes
rep genes
Insertion sequences
Conjugation region of Tn916
Hypothetical protein and other ORFs