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

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Running title:

Linezolid resistance genes in seawater enterococci

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30 **Abstract**

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

36 In this study we investigated the occurrence of linezolid-resistant enterococci in
37 marine samples from two coastal areas in Italy. Isolates grown on florfenicol-
38 supplemented Slanetz-Bartley were investigated for their carriage of *optrA*, *poxA*
39 and *cfr* genes: *optrA* was found in one *E. faecalis*, *poxA* in three *E. faecium* and
40 two *E. hirae* and *cfr* was not found. Two of the three *poxA*-carrying *E. faecium*
41 and the two *E. hirae* showed related PFGE profiles. Two *E. faecium* belonged to
42 the new ST1710, which clustered in the clonal complex CC94, encompassing
43 nosocomial strains. S1-PFGE/hybridization assays showed a double (chromosome
44 and plasmid) location of *poxA* and plasmid location of *optrA*. WGS revealed that
45 *poxA* was contained in a Tn6657-like element carried by two plasmids (pEfm-EF3
46 and pEh-GE2) of similar size, found in different species, and that *poxA* were
47 flanked by two copies of *IS1216* in both plasmids. In mating experiments all but
48 one (*E. faecalis* EN3) strains were able to transfer the *poxA* gene to *E. faecium*
49 64/3.

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

54

55 **Importance**

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

67

68

69 **Introduction**

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

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

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

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

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

94 mutations in 23S rRNA and/or in L3, L4, and L22 ribosomal proteins (6, 8),
95 linezolid resistance can develop following acquisition of the resistance genes *cfr*
96 and its variants, *optrA* and *poxxA*. Cfr and Cfr-like methylases confer resistance to
97 five classes of antimicrobial agents including phenicols, lincosamides,
98 oxazolidinones, pleuromutilins and streptogramin A (PhLOPS_A phenotype) by a
99 post-transcriptional methylation of the 23S rRNA (9, 10-13). The ABC-F proteins
100 OptrA and PoxxA leads to a decreased susceptibility to phenicols, oxazolidinones
101 (including tedizolid) and tetracyclines (PoxxA protein only) by a ribosomal
102 protection mechanism (14-16).
103 In enterococci, linezolid resistance genes are often carried by mobile genetic
104 elements and are easily transferred between bacteria by HGT (14, 17-20).
105 Enterococci spread in many natural habitats and, besides the occurrence of
106 linezolid-resistant enterococci in hospitals, their detection in other reservoirs is of
107 special concern (21).
108 The purpose of this study was to investigate the occurrence of linezolid resistance
109 genes in enterococci isolated from marine samples collected at two coastal urban
110 areas in Italy.
111 To our knowledge, this is the first report of linezolid resistance genes in
112 enterococci from the marine environment.

113

114 **Results and discussion**

115

116 **Detection of oxazolidinone resistance genes in florfenicol-resistant enterococci** 117 **and antimicrobial susceptibility profiles**

118 Out of 77 total samples (seawater=33, sediment=33 and zooplankton=11) only ten
119 sediment and one zooplankton samples from six sampling sites (Figure 1) were
120 positive for the presence of florfenicol-resistant enterococci. Thirty-five isolates
121 were found positive for *poxA* or *optrA*, however only six different pulsotypes (one
122 by site) were detected by SmaI-PFGE assay. The six isolates – 1 *Enterococcus*
123 *faecalis*, 3 *E. faecium* and 2 *Enterococcus hirae* - were then characterized (Table
124 1). The *optrA* gene was only detected in the *E. faecalis* isolate, *poxA* was
125 identified in the 3 *E. faecium* and the 2 *E. hirae*, whereas *cfr* was not found (Table
126 1).

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

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

140 The six enterococcal isolates were all resistant to florfenicol (MIC range, 32-128
141 mg/L), chloramphenicol (MIC range, 16-128 mg/L), and tetracycline (MIC range,
142 128->128 mg/L)) and either susceptible or resistant to linezolid (MIC range, 2–8
143 mg/L) and tedizolid (MIC range, 2-4 mg/L). All tested strains were susceptible to
144 vancomycin (MIC range, 0.5-1 mg/L) (Table 1).

145

146 **Typing assays**

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

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

158

159 **Location of oxazolidinones resistance genes and detection of circular forms**

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

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

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

171 Since the *optrA* is located on plasmids of different size (~20 kb and ~140 kb), and
172 WGS revealed a single *optrA* genetic context with no evidences of circularisation,
173 it is reasonable to assume that recombination events between plasmids occurred
174 (38).

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

178

179 **Transferability of oxazolidinones resistance genes**

180 Five of six isolates successfully transferred the linezolid resistance genes in intra-
181 and interspecific mating experiments with frequencies ranging from 6.5×10^{-1} to 3
182 $\times 10^{-6}$ CFU per recipient. MICs and genotypes for both donors and selected
183 transconjugants, and transfer frequencies are indicated in Table 2. The higher
184 frequencies were observed in intraspecific transfer of *poxA* from *E. faecium* ES2
185 and *E. faecium* TF3 donors to *E. faecium* 64/3 recipient. Conversely, *E. hirae* GE2
186 and GE5 successfully transferred *poxA* to the *E. faecium* recipient.

187 In both *E. faecium* and *E. hirae* transconjugants, *poxA* gene was located on
188 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 *IS1216* 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 *rep9*-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).

215 Since the hybridization assays suggested that a *poxA*-carrying plasmid of ~30 kb
 216 was shared by *E. faecium* isolates, we decided to proceed with its assembly.
 217 In *E. faecium* EF3, *poxA* gene was located on a 27,703-bp plasmid designed pEfm-
 218 EF3 (G+C content, 35.0%) (accession no. MT683615). The genetic context of
 219 *poxA* (4,003 bp), flanked by two IS1216 in the same orientation, was in turn
 220 inserted in a Tn6657-like transposon also containing *fexB* as originally described
 221 in the MRSA strain AOUC-0915 (accession no. MF095097) (20). Upstream the
 222 Tn6657-like transposon a tetracycline resistance region containing *tet*(L) and
 223 *tet*(M) genes arranged in tandem was found; downstream the Tn6657-like four
 224 genes (*orf28-orf31*) involved in plasmid partitioning and replication were detected
 225 (Figure 3). pEfm-EF3 exhibited 99% DNA identity (cover 100%) with regions of
 226 pC25-1 and pC27-2, two broad-host-range Inc18 plasmids from a CC17 *E. faecium*
 227 of pig origin from China (accession numbers MH784601 and MH784602,
 228 respectively) (44).
 229 In *E. faecium* ES2 and *E. faecium* TF3, *poxA*-carrying plasmids identical to pEfm-
 230 EF3 were found. It is noteworthy that the three *E. faecium* isolates have been
 231 collected from different sampling sites (Table 1). Furthermore, the closely related
 232 *E. faecium* EF3 and *E. faecium* ES2 belonged to ST1710, while *E. faecium* TF3
 233 was assigned to ST1711 suggesting a spread of pEfm-EF3 by HGT may occur
 234 among isolates with different backgrounds.
 235 WGS analysis of *E. hirae* GE2 revealed that the *poxA* gene was located on a
 236 24,793-bp plasmid, named pEh-GE2 (G+C content, 38.0%) (accession no.
 237 MT683616). BLASTN analysis displayed that in pEh-GE2 two regions exhibited a
 238 high DNA identity with different genetic elements. The 12.8-kb region containing
 239 the *poxA* genetic context (*orf1* to *orf18*) showed high DNA identity (99%) with a
 240 Tn6657-like transposon (20). As observed in pEfm-EN3, the *poxA* genetic context

241 was bracketed by *IS1216* elements in the same orientation (Figure 4). The pEh-
242 GE2 region spanning from *orf19* to *orf31* (14.7 kb) and carrying the *Tn916*
243 conjugation region (including the *rep* gene) showed 99% DNA identity with
244 plasmid 3 of *E. faecium* E4457 (accession no. LR135260) (Figure 4).
245 WGS analysis of *E. hirae* GE5 displayed a *poxxA*-carrying plasmid with a complete
246 synteny to the pEh-GE2, despite the two strains come from different sampling sites
247 and samples (sediment and zooplankton, respectively) (Table 1).
248 Interestingly, the *poxxA*-carrying plasmids of *E. hirae* and *E. faecium* isolates
249 shared only the *Tn6657*-like region (cover 55%, DNA identity 99%) suggesting the
250 widespread of this element in enterococci. The pEh-GE2 resulted to belong to the
251 Rep_trans family which includes small size plasmids largely spread among
252 enterococcal populations (42).
253 Hybridization analysis also showed the presence of an *optrA*-carrying plasmid
254 (~140 kb) in *E. faecalis* EN3 and a *poxxA* plasmid (~100 kb) in *E. hirae* GE2 and
255 GE5 isolates that were not assembled.
256 WGS analysis also ruled out the presence of *cfr*(B), *cfr*(C), *cfr*(D) and *cfr*(E)
257 genes. No mutations were detected in the genes encoding the 23S rRNA or
258 ribosomal proteins.

259

260 **Conclusions**

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

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

271 **Materials and methods**

272 273 **Sampling sites, sample processing and bacterial isolation**

274 Sampling activities were carried out at 11 sites in two areas located on the
275 Western and Eastern coast of Italy (in Ligurian and Adriatic Sea), in a framework
276 of a research project aimed at the detection of antibiotic-resistant bacteria from
277 the marine environment (unpublished results). Sampling sites located in Ligurian
278 Sea (n=3) were in front of the harbor and the hospital of Genoa city (GEN, GES,
279 GEF), whereas sampling sites in the Adriatic Sea (n=8) were in front of an urban
280 area close to the river Esino estuary and to an oil refinery (ESN, ESS, ESF), and in
281 front of the hospital (TN, TS, TF) and the harbor of Ancona city (PN, PS) (Figure
282 1).

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

285 All 77 samples were incubated overnight at 37°C in Azide broth (Oxoid,
286 Basingstoke, UK) for the selective enrichment of enterococci. Sediment (5g)
287 samples were immediately added to the enrichment broth whereas seawater and
288 zooplankton samples were processed as follows. Seawater (400 ml) were filtered
289 through 0,22 µm filter membranes (Merk Life Science, Milano, Italy) and filters
290 incubated in 30 ml Azide broth. Zooplankton (50 ml aliquots) organisms were
291 collected by dragging the water horizontally (~1m depth) with a 200 µm mesh
292 plankton net. Aliquots (50 ml) of the collected material were centrifuged 10 min at
293 15000xg; pellets were resuspended in 5 ml artificial sterile seawater and added to
294 40 ml of Azide broth. Each enrichment culture (100 µl) was spread on Slanetz

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

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

299

300 **Genotypic and phenotypic characterization**

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

304 Isolates carrying linezolid resistance genes were identified by MALDI-TOF
305 (Vitek-MS, bioMérieux) and tested for their susceptibility to florfenicol,
306 chloramphenicol, linezolid, tetracycline and vancomycin (Sigma Aldrich, St.
307 Louis, MI) by standard broth microdilution assay, and to tedizolid using Etest
308 strips (Liofilchem, Roseto degli Abruzzi, Italy). Susceptibility tests were
309 interpreted according to clinical EUCAST (version 10.0, 2020.

310 <http://www.eucast.org>) or CLSI breakpoints

311 (<https://clsi.org/standards/products/free-resources/access-our-free-resources/>). *E.*

312 *faecalis* ATCC 29212 was used as quality control (EUCAST QC tables v 10.0,

313 2020. <http://www.eucast.org>).

314

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

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

317 Genomic DNA embedded in agarose gel plugs was digested with S1 nuclease

318 (Thermo Fisher Scientific, Milan, Italy) and chromosome and plasmids separated

319 by PFGE as previously described (46). After S1-PFGE, total DNA was blotted onto

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

322

323 **Detection of circular forms**

324 To investigate the excision of genetic contexts carrying linezolid resistance genes,
325 PCR assays were performed using outward-directed primer pairs targeting the
326 linezolid resistance genes: (i) *poxA*div-FW GACGAGCCGACCAACCACCT and
327 *poxA*div-RV TTCAGGCGGACAAAAATCCAA; (ii) *optrA*div-FW
328 GAAAAATAACACAGTAAAAGGC and *optrA*div-RV
329 TTTTCCACATCCATTTCTACC.

330 Briefly, 5 µl of genomic DNA was added in a final volume of 25 µl of mastermix
331 containing 0.2 µM of each primer, 500 mM dNTP mix, 7 mM MgCl₂, and 2 U
332 Dream Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA). PCR
333 conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C for 1 min, 58 °C
334 for 1 min, and 72 °C for 5 min; and 72 °C for 5 min. PCR was performed in a
335 GeneAmp PCR System 9700 (Applied Biosystems System 9700 GeneAmp PCR
336 Thermal Cycler). PCR products were resolved by electrophoresis on 1.0% agarose
337 gel.

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

341

342 **Conjugation experiments**

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

346 Transconjugants were selected on brain heart infusion agar (Oxoid, Basingstoke,
347 UK) containing florfenicol (10 mg/L), fusidic acid (25 mg/L) and rifampicin (25
348 mg/L), grown colonies were tested for the presence of linezolid resistance genes
349 by PCR and for their susceptibility to florfenicol and linezolid.
350 SmaI-PFGE was carried out and patterns analysed to confirm the genetic
351 background of transconjugants. Conjugation frequencies were expressed as ratio of
352 cell number (CFU/ml) of transconjugants to recipient.

353

354 **WGS and sequence analysis**

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

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

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

374

375 **Data availability**

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

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384 and natural sources”.

385

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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 *poxA*-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 *poxA*-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	
				<i>optrA</i>	<i>cfr</i>	<i>poxA</i>	FFC ^a	CHL	LZD	TZD	TE	VAN		SmaI-PFGE	MLST	<i>optrA</i>	<i>poxA</i>
EN3	<i>E. faecalis</i>	EN	sediment	+	-	-	128	128	4	4	128	1		-	ST585	20 ^b ,140	-
EF3	<i>E. faecium</i>	EF	sediment	-	-	+	64	16	8	2	128	1		C	ST1710	-	30, c ^c
ES2	<i>E. faecium</i>	ES	sediment	-	-	+	32	16	8	2	128	1		C ₁	ST1710	-	15, 30, c
TF3	<i>E. faecium</i>	TF	sediment	-	-	+	64	32	2	2	>128	0.5		B	ST1711	-	30, 50, 80, c
GE5	<i>E. hirae</i>	GEN	sediment	-	-	+	64	64	4	2	128	0.5		A ₁	-	-	25, 100
GE2	<i>E. hirae</i>	GES	zooplankton	-	-	+	64	64	8	3	128	0.5		A	-	-	25, 100

610

611

612 ^aFFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TDZ, tedizolid; TE, tetracycline; VAN, vancomycin; ^bEstimated plasmid size (in kb)

613 ^cc, 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.

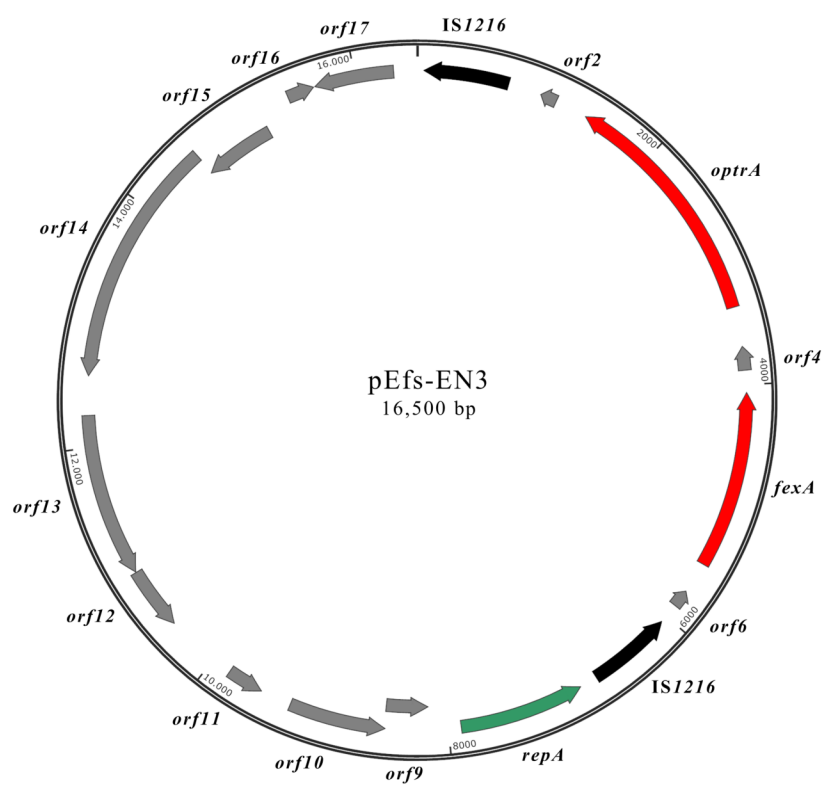
Table 2. Florfenicol and linezolid MICs, resistance genotypes and genes location for relevant transconjugants.

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		
<i>E. faecalis</i> EN3	128	4	<i>optrA</i>	<i>E. faecium</i> 64/3 ^b	ND ^c	-	-	-	-
<i>E. faecium</i> EF3	64	8	<i>poxA</i>	<i>E. faecium</i> 64/3	5 x 10 ⁻⁵	64	4	<i>poxA</i>	30 ^d , c ^e
<i>E. faecium</i> ES2	32	8	<i>poxA</i>	<i>E. faecium</i> 64/3	6.5 x 10 ⁻¹	64	4	<i>poxA</i>	30, c
<i>E. faecium</i> TF3	64	2	<i>poxA</i>	<i>E. faecium</i> 64/3	1.1 x 10 ⁻¹	32	2	<i>poxA</i>	30, c
<i>E. hirae</i> GE5	64	4	<i>poxA</i>	<i>E. faecium</i> 64/3	7.5 x 10 ⁻⁵	64	4	<i>poxA</i>	25, c
<i>E. hirae</i> GE2	64	8	<i>poxA</i>	<i>E. faecium</i> 64/3	3 x 10 ⁻⁶	64	4	<i>poxA</i>	25, c

^aFFC, florfenicol; LZD, linezolid. ^bThe MICs of FFC and LZD for *E. faecium* 64/3 were 4 mg/L and 1 mg/L, respectively.

^cND, not detectable; ^dEstimated plasmid size (in kb); ^ec, chromosome





- Antibiotic resistance genes
- rep* genes
- Insertion sequences
- Hypothetical protein and other ORFs

