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Short Communication

Detection of *poxtA*- and *optrA*-carrying *E. faecium* isolates in air samples of a Spanish swine farm

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ABSTRACT

Objective: Two linezolid-resistant *Enterococcus faecium* isolates, C10004 and C10009, were recovered from air samples of a Spanish swine farm and comprehensively characterized.
Methods: Detection of linezolid resistance mechanisms (mutations and acquisition of resistance genes) was performed by PCR/sequencing. Isolates were characterized by multilocus sequence typing (MLST), antimicrobial susceptibility testing, detection of antimicrobial resistance and virulence genes, and analysis of the genetic environment of the linezolid resistance genes. The characterization of isolate C10009 was performed by Whole-Genome-Sequencing and of isolate C10004 by PCR and amplicon sequencing, where applicable. Conjugation experiments to assess the transferability of the *optrA* and *poxtA* genes implicated in linezolid resistance were performed.
Results: The linezolid-resistant *E. faecium* isolates C10004 and C10009, assigned to ST128 and ST437, respectively, harbored the *optrA* and *poxtA* genes. Neither mutations in the 23S rRNA nor in the genes for the ribosomal proteins L3, L4 and L22 were detected. C10004 and C10009 carried fourteen and thirteen antimicrobial resistance genes, respectively. The sequence alignment indicated that the genetic environment of the *poxtA* gene was identical in both isolates, with a downstream-located *fexB* gene. The *poxtA* gene was transferred by conjugation together with the *fexB* gene, and also with *tet*(M) and *tet*(L) in the case of isolate C10004. The *optrA* gene could not be transferred.
Conclusions: This is the first report of the *poxtA* gene in Spain. The presence of *poxtA*- and *optrA*-carrying *E. faecium* isolates in air samples represents a public health concern, indicating an involvement of swine farms in the spread of linezolid-resistant bacteria.

1. Introduction

The oxazolidinone linezolid is a highly effective antimicrobial agent to treat serious infections caused by multidrug-resistant Gram-positive bacteria in humans, including vancomycin-resistant enterococci (VRE) [1]. This antimicrobial is not approved for use in veterinary medicine worldwide, however, active surveillance of oxazolidinone-resistant enterococci, and the detection of transferable oxazolidinone resistance genes in the livestock-setting have increased in recent years [2–5].

Since its introduction into clinical use in 2000, several mechanisms of linezolid resistance have been described related either to mutations in the 23S rRNA, to amino acid changes in the ribosomal proteins L3, L4, and L22, or to the acquisition of resistance genes [1]. Recently, two novel transferable genes coding for members of the ABC-F family of ATP-binding cassette proteins were described, *optrA* and *poxtA* [2,6]. The *optrA* gene, which confers resistance to oxazolidinones and phenicols, was first described in *Enterococcus faecium* and *Enterococcus faecalis* of human and animal origin [2], and thereafter in other *Enterococcus* spp., *Staphylococcus* spp., and *Streptococcus suis* worldwide [3,7,8]. The *optrA* gene is commonly located on plasmids or in the chromosomal DNA [9], but has recently also been detected as part of integrative and conjugative elements, a prophage, and a novel transposon of the Tn*554* family [8,10]. The *poxtA* gene, mediates decreased susceptibility to oxazolidinones, phenicols and tetracyclines. It was first reported in a clinical MRSA isolate [6], but has meanwhile also been detected in different Gram-positive bacteria, primarily of animal origin [3–5,11].

Although more than 99% of the Gram-positive pathogens are still linezolid-susceptible [12], linezolid resistance represents a

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serious public health concern as oxazolidinones are considered as last-resort antimicrobial agents in human medicine. Therefore, the objective of this work was to characterize two *poxtA*- and *optrA*-positive *E. faecium* isolates recovered from environmental samples of a Spanish swine farm, as, to our knowledge, this is the first report of the *poxtA* gene in Spain.

2. Materials and methods

In a previous study [13], two linezolid-resistant *E. faecium* isolates, C10004 and C10009, were recovered from air samples of a Spanish swine farm. *E. faecium* C10004 was isolated from the air inside the farm, and *E. faecium* C10009 at a distance of 150 meters outside the farm. Slanetz-Bartley agar (SB; Scharlau, Barcelona, Spain) was used for the recovery of enterococci, and the species identification was performed using MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) (Bruker Daltonik GmbH, Bremen, Germany) [13].

The susceptibility to the following eighteen antimicrobial agents was determined by the MicroScan® system (Beckmann Coulter, Nyon, Switzerland): penicillin, ampicillin, oxacillin, erythromycin, clindamycin, quinupristin-dalfopristin, gentamicin, tobramycin, tetracycline, ciprofloxacin, levofloxacin, vancomycin, teicoplanin, daptomycin, mupirocin, fosfomycin, fusidic acid, and trimethoprim-sulfamethoxazole [14,15]. Moreover, the minimum inhibitory concentration (MIC) to linezolid was determined by E-test® (bioMérieux, Durham, USA) according to the manufacturer's instructions, and to florfenicol, chloramphenicol and rifampicin by broth macrodilution [14].

Isolates were PCR-screened for the presence of the linezolid resistance genes *poxtA*, *optrA*, and *cfr*. Mutations within the domain V of the 23S rRNA and in the genes for ribosomal proteins L3 (*rplC*), L4 (*rplD*), and L22 (*rplV*) were investigated by PCR and amplicon sequencing (the primers used in this study are listed in the Supplementary Table S1 and S2). The corresponding sequences of *E. faecium* strain Aus0004 (GenBank accession number CP003351) were used as references.

The molecular characterization of isolate *E. faecium* C10009 was performed by Whole-Genome-Sequencing (WGS). Genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) and the libraries for WGS were prepared using the Nextera XT library preparation kit (Illumina Inc., San Diego, USA) according to the manufacturer's recommendations. The DNA was sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, USA) (2 × 300 bp paired-end sequencing in 40-fold multiplexes). The draft genomes were *de novo* assembled using the software MIRA 4.0 (Biomatters, Auckland, New Zealand) and annotated using the RAST annotation server [16]. The sequences were analyzed using Geneious v 2019.0.4 (Biomatters, Auckland, New Zealand), and the online tools ResFinder, VirulenceFinder, and MLST from the Center for Genomic Epidemiology website (www.genomicepidemiology.org). Nucleotide alignments were performed using Geneious with default settings, and amino acid alignments with the BLOSUM62 cost matrix.

E. faecium C10004 was characterized by PCR and amplicon sequencing. Multilocus sequence typing (MLST) was performed as recommended (www.pubmlst.com). Besides linezolid resistance genes, the presence of the antimicrobial resistance genes *erm*(A), *erm*(B), *erm*(C), *msr*(A), *msr*(C), *lnu*(A), *lnu*(B), *lsa*(B), *lsa*(E), *aac*(6')-Ii, *aac*(6')-Ie-*aph*(2")-Ia, *aph*(3')-III, *ant*(6)-Ia, *str*, *ant*(4')-Ia, *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *vat*(D), *vat*(E), *fexA*, *fexB*, cat_{pC194}, cat_{pC221}, cat_{pC223} and *catA* was studied (Supplementary Table S1). Moreover, mutations leading to amino acid changes in the GyrA and ParC proteins related to reduced susceptibility to fluoroquinolones were investigated, using the corresponding sequences of *E. faecium* strain DO (GenBank accession number CP003583) as references. The virulence genes investigated in isolate C10004 were the following: *esp*, *hyl*, *ace*, *agg*, *acm* and *gelE* (Supplementary Table S1). The primers designed to determine the genetic environment of the *poxtA* gene are listed in the Supplementary Table S2.

Conjugation experiments to assess the transferability of the *optrA* and *poxtA* genes were performed by filter-mating experiments [17]. The strain *E. faecalis* JH2-2 (rifampicin-resistant) was used as recipient strain, and the transconjugants were selected on Brain Heart Infusion agar (BHI; Difco Laboratories, Detroit, USA) containing florfenicol (10 mg/L) and rifampicin (100 mg/L). The obtained transconjugants were identified using MALDI-TOF MS and their antimicrobial resistance genotype was determined by PCR (Supplementary Table S1). The MICs to tetracycline, chloramphenicol, florfenicol, linezolid and rifampicin were determined by broth macrodilution [14].

3. Results and Discussion

3.1. Characterization of E. faecium isolates

The two *E. faecium* isolates harbored the resistance genes *optrA* and *poxtA*, while they were negative for the multiresistance gene *cfr*. So far, the *poxtA* gene has been reported in enterococci and staphylococci from clinical, livestock-associated, pet-associated, and animal-derived food samples [3–6,11,18], but, to the best of our knowledge, this is the first report of this gene in environmental samples. Moreover, as far as we know, this is also the first description of the *poxtA* gene in Spain. The *optrA* gene has been previously described in environmental samples, such as in urban waste waters [19]. Both *poxtA* and *optrA* genes are disseminated among enterococci of livestock origin in countries such as China, Tunisia and Italy [2–5,9,11,18,19], possibly due to co-selection because of the extended use of florfenicol and tetracyclines in livestock-farming [2–5,8–10,18,19].

Mutations in the domain V of the 23S rRNA are supposed to be the most common mechanism of linezolid resistance among enterococci, but no mutational changes were detected in our case, neither in the 23S rRNA nor in the genes coding for the ribosomal proteins L3, L4 and L22 of the two investigated *E. faecium* isolates. Such mutational changes are often associated with the use of linezolid in human infections [19], but the isolates investigated in this study were not supposed to be under the selective pressure of linezolid as it is not approved for use in livestock.

The isolates *E. faecium* C10004 and C10009 were typed as ST128 and ST437, respectively. While *E. faecium* of these specific STs have not been identified before co-harboring *poxtA* and *optrA*, other *E. faecium* STs have been reported carrying those genes [4]. This fact suggests that a clear association between the ST and the detection of these two genes does not exist.

Both *E. faecium* isolates displayed a multiresistance phenotype (resistance to three or more classes of antimicrobial agents), but differed in their susceptibility to fluoroquinolones, fusidic acid and trimethoprim-sulfamethoxazole (Table 1). They showed a linezolid MIC of 8 mg/L, and were also resistant to chloramphenicol (MICs 32 mg/L) and florfenicol (MICs 64 mg/L), and showed susceptibility to fosfomycin, vancomycin and daptomycin, among others. Besides the concomitant presence of the *optrA* and *poxtA* genes, both isolates harbored the phenicol resistance gene *fexB*, and isolate *E. faecium* C10004 also *fexA*. A large number of antimicrobial resistance genes were detected in the two isolates, many shared by both isolates (Table 1). Thirteen amino acid changes in the penicillin binding protein 5 (PBP5) were detected in isolate C10009 which showed high-level resistance to β-lactams (Table 1), compared with the reference strain *E. faecium* BM4107 (GenBank accession number AF364092). Amino acid changes in the PBP5 in

Table 1
Molecular characterization of the two *poxtA*- and *optrA*-positive *E. faecium* isolates.

Isolate	Origin	MLST	Antimicrobial resistance phenotype[a]	Antimicrobial resistance genotype	Virulence genes
C10004	Air inside the swine farm	ST128	PEN-AMP-ERY-CLI-QDA-GEN-TOB- TET-CIP[I]-FFN-CHL-LZD-FUS	*erm*(B), *msr*(C), *lnu*(B), *lsa*(E), *aac*(6')-Ie-*aph*(2")-Ia, *ant*(6)-Ia, *aac*(6')-Ii, *aph*(3')-III, *tet*(L), *tet*(M), *fexA*, *fexB*, *optrA*, *poxtA*	*esp*
C10009	Air outside the swine farm (150 m)	ST437	PEN[b]-AMP[b]-ERY-CLI-QDA-GEN-TOB-TET-CIP-LEV[I]-FFN-CHL-LZD-SXT	*erm*(B), *msr*(C), *lnu*(B), *lsa*(E), *aac*(6')-Ie-*aph*(2")-Ia, *ant*(6)-Ia, *aac*(6')-Ii, *aph*(3')-III, *tet*(L), *fexB*, *optrA*, *poxtA*, *dfrG*	*efaAfm*, *acm*

[a] PEN, penicillin; AMP, ampicillin; ERY, erythromycin; CLI, clindamycin; QDA, quinupristin-dalfopristin; GEN, gentamicin; TOB, tobramycin; TET, tetracycline; CIP, ciprofloxacin; LEV, levofloxacin; FFN, florfenicol; CHL, chloramphenicol; LZD, linezolid; FUS, fusidic acid; SXT, trimethoprim-sulfamethoxazole; I, intermediate.
[b] Amino acid changes detected in the penicillin binding protein 5 (PBP5): V24A, S27G, R34Q, G66E, E100Q, K144Q, T127A, L177I, A216S, T324A, N496K, A499I and E525D.

isolate C10004 were not analyzed. The *E. faecium* C10009 was resistant to fluoroquinolones, whereas the isolate *E. faecium* C10004 showed an intermediate phenotype, but no amino acid changes were detected in the deduced sequences of the GyrA and ParC proteins in either isolates. Multiresistance among *E. faecium* harboring *optrA* and/or *poxtA* genes has been extensively observed, with high resistance rates to macrolides and lincosamides, aminoglycosides, and tetracycline [3,5].

Regarding virulence factors, isolate *E. faecium* C10004 carried the *esp* gene, while *E. faecium* C10009 harbored the virulence genes coding for the collagen-binding adhesin (Acm) and the adhesion-associated protein (EfaA) (Table 1). The enterococcal surface protein (Esp) is one of the most important virulence factors among enterococci. It is strongly associated with clinical strains, although, like the Acm protein, has also been described among *Enterococcus* spp. of environmental and animal origin [3]. Concerning EfaA, it has been postulated that this virulence factor is widely distributed among *E. faecium* isolates regardless of their origin [20].

3.2. Genetic environment of the optrA and poxtA genes and conjugation assays

Sequence analysis revealed that *E. faecium* C10009 harbored the wild type *poxtA* gene with 100% nucleotide sequence identity to that of *S. aureus* strain AUOC-0915 (GenBank accession number MF095097) [6], whereas *E. faecium* C10004 carried a PoxtA variant with the amino acid change I219L. The analysis and alignment of the obtained nucleotide sequences showed an identical genetic environment of the *poxtA* gene in both isolates, with the insertion sequence IS*1216*, two small reading frames for proteins with unknown functions and the *fexB* gene located downstream of *poxtA* (Fig. 1). The nucleotide sequence alignment of the genetic environment of our two isolates, and the corresponding sequence of the *E. faecium* P36 strain (GenBank accession number KP834591) showed the presence of a second copy of IS*1216* downstream of *fexB* in *E. faecium* P36, which was absent in *E. faecium* C10009 (GenBank accession number MN661250) (Fig. 1). In isolate C10004, the nucleotide sequence of the amplicon was not large enough to identify the presence or absence of the second copy of IS*1216*. In the reference *E. faecium* P36, the two copies of IS elements flank the composite Tn*6246*-like transposon which contains the *fexB* resistance gene. As the genetic environment of the *poxtA* gene in our isolates could only be partially revealed because of the nucleotide sequence length, we cannot confirm if the second copy of the insertion sequence is missing. In case that the second copy of IS*1216* is absent, the intervening DNA segment could not be mobilized as a composite transposon.

Regarding the *optrA* gene, the alignment of the amino acid sequences revealed that both *E. faecium* isolates harbored the wild type OptrA of *E. faecalis* E394 strain (GenBank accession number KP399637) [2]. In the genetic context of the *optrA* gene in isolate C10009, due to the short sequence size, we could only determine the presence of a reading frame for one protein with unknown function located upstream.

The transferability of *optrA* by conjugation failed for both isolates, but transconjugants of the isolates C10004 (TC-C10004) and C10009 (TC-C10009) harboring the *poxtA* and *fexB* resistance genes, were obtained. Moreover, the PCR screening showed the presence of *tet*(L) and *tet*(M) genes encoding an efflux protein and a ribosomal protection protein implicated in tetracycline resistance, respectively, in the isolate TC-C10004 (Table 2). An increase by two to three and four dilution steps, respectively, was observed in the MICs of chloramphenicol and florfenicol in both transconjugants after acquisition of *poxtA* and *fexB* genes. In relation to linezolid, the increase in the MICs was lower (one dilution step). For tetracycline, the MIC of the TC-C10009 isolate increased by one dilution step compared to the recipient strain, whereas in the case of the TC-C10004, which also harbored the *tet*(M) and *tet*(L) genes, the MIC was identical to that of the donor strain (Table 2). The MICs of the transconjugants are consistent with the resistance genotype

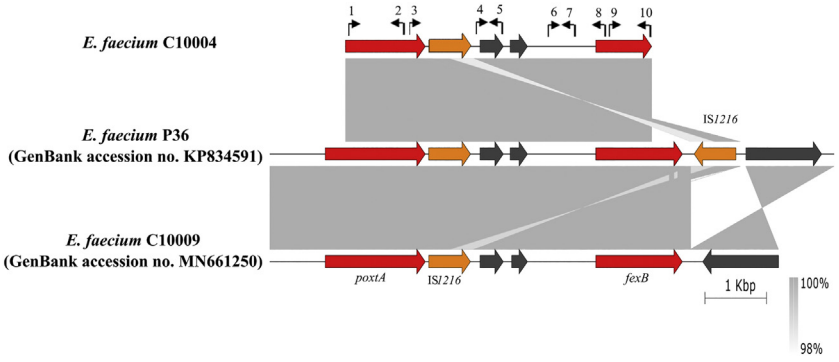

Fig. 1. Genetic environment of the *poxtA* gene in the isolates *E. faecium* C10004 and C10009 and the previously described genetic structure of the *Enterococcus* P36 strain (GenBank accession number KP834591). Coding Sequences (CDS) are colored according to their function: red, antimicrobial resistance; orange, transposition; black, unknown function. The direction and approximate position of the primers used to determine the genetic environment of the *poxtA* gene in the isolate C10004 are indicated by arrows (1, poxtA-F; 2, poxtA-R; 3, poxtA-inv; 4, poxtA-reg1; 5, poxtA-reg2; 6, poxtA-reg3; 7, poxtA-reg4; 8, poxtA-reg5; 9, fexB-F; 10, fexB-R) (Supplementary Table S2).

Table 2
Minimum inhibitory concentrations (MICs) of the recipient *E. faecalis* JH2-2, the C10004 and C10009 donor strains and the two transconjugants (TC).

Strain	MIC (mg/L)[a]				
	TET	CHL	FFN	LZD	RIF
E. faecium C10004 (donor)	256	32	64	8	1
E. faecium C10009 (donor)	256	32	64	8	1
TC-C10004 [*poxtA*, *fexB*, *tet*(L), *tet*(M)]	256	16	64	4	256
TC-C10009 (*poxtA*, *fexB*)	0.25	32	64	4	256
E. faecalis JH2-2 (recipient)	0.125	4	4	2	256

[a] TET, tetracycline; CHL, chloramphenicol; FFN, florfenicol; LZD, linezolid; RIF, rifampicin.

observed. However, in the case of linezolid, the increase of the transconjugants' MICs by one dilution step should be interpreted with caution, since variations of +/- one dilution step are typical variances seen in antimicrobial susceptibility testing. The transferability of the *poxtA* gene together with other antimicrobial resistance genes, including *fexB*, *tet*(L), and *tet*(M), has been previously reported due to their location on the same plasmid or transposon [4,5,11,18]. This co-location could contribute to the co-selection and dissemination of the *poxtA* gene. Although the exact location of the *poxtA* gene in isolate C10009 could not be determined, the analysis of whole genome sequence identified a contig containing one *rep* gene and other genes involved in plasmid replication. This contig was identical to part of the *Enterococcus faecium* strain 27 plasmid pC25-1 which also carries the *poxtA* and *fexB* genes (GenBank accession number MH784601) [5].

In summary, we present the first report of the *poxtA* gene in Spain and the first detection of this gene in environmental samples worldwide. The results of this study suggest that swine farms may play a role in the spread of linezolid-resistant bacteria to the environment. The presence of *poxtA*- and *optrA*-carrying *E. faecium* in air samples represents a public health concern, especially because one of the isolates was detected at 150 meters distance outside the swine farm, suggesting their dissemination within and outside the farm.

Ethical approval

Not required.

Competing interests

None declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.12.012.

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