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Molecular and functional analysis of the novel *cfr*(D) linezolid resistance gene identified in *Enterococcus faecium*

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Objectives: To characterize the novel *cfr*(D) gene identified in an *Enterococcus faecium* clinical isolate (15-307.1) collected from France.

Methods: The genome of 15-307.1 was entirely sequenced using a hybrid approach combining short-read (MiSeq, Illumina) and long-read (GridION, Oxford Nanopore Technologies) technologies in order to analyse in detail the genetic support and environment of *cfr*(D). Transfer of linezolid resistance from 15-307.1 to *E. faecium* BM4107 was attempted by filter-mating experiments. The recombinant plasmid pAT29Ω*cfr*(D), containing *cfr*(D) and its own promoter, was transferred to *E. faecium* HM1070, *Enterococcus faecalis* JH2-2 and *Escherichia coli* AG100A.

Results: As previously reported, 15-307.1 belonged to ST17 and was phenotypically resistant to linezolid (MIC, 16 mg/L), vancomycin and teicoplanin. A hybrid sequencing approach confirmed the presence of several resistance genes including *vanA*, *optrA* and *cfr*(D). Located on a 103 kb plasmid, *cfr*(D) encoded a 357 amino acid protein, which shared 64%, 64%, 48% and 51% amino acid identity with Cfr, Cfr(B), Cfr(C) and Cfr(E), respectively. Both *optrA* and *cfr*(D) were successfully co-transferred to *E. faecium* BM4107. When expressed in *E. faecium* HM1070 and *E. faecalis* JH2-2, pAT29Ω*cfr*(D) did not confer any resistance, whereas it was responsible for an expected PhLOPS$_A$ resistance phenotype in *E. coli* AG100A. Analysis of the genetic environment of *cfr*(D) showed multiple IS*1216* elements, putatively involved in its mobilization.

Conclusions: Cfr(D) is a novel member of the family of 23S rRNA methyltransferases. While only conferring a PhLOPS$_A$ resistance phenotype when expressed in *E. coli*, enterococci could constitute an unknown reservoir of *cfr*(D).

Introduction

Enterococci are major opportunistic pathogens responsible for approximately 5%–10% of nosocomial infections and associated with numerous hospital outbreaks.[1] There is notably a worldwide spread of vancomycin-resistant *Enterococcus faecium* (VREF), for which therapeutic options are limited.[2] Linezolid, the first member of the oxazolidinone class, possesses potent activity against MDR Gram-positive pathogens, including VREF clinical isolates.[3] It exerts its antibacterial activity through inhibition of protein synthesis by binding to the central loop of domain V in the 23S rRNA.[3]

Although the overall prevalence of linezolid resistance among enterococcal clinical isolates remained low (<1%) in international surveillance programmes from 2008 to 2016, there is currently an emergence of linezolid-resistant enterococci (LRE) worldwide.[4–6] Linezolid resistance can be mediated either by chromosomal mutations in 23S rRNA (mainly G2576T mutation) and/or in L3, L4 and L22 ribosomal proteins or by acquisition of transferable resistance determinants (such as *cfr*-like, *optrA* and *poxtA* genes) that have recently emerged.[6,7]

The *cfr* gene encodes a 23S rRNA methyltransferase that modifies the C-8 position of residue A2503 and confers cross-resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics (the so-called PhLOPS$_A$ phenotype).[8] It was initially identified in a plasmid from a bovine *Staphylococcus sciuri* isolate in 1997 and then many studies have reported *cfr*

mainly on plasmids among staphylococci of animal and human origin and sporadically reported in a few clinical isolates of enterococci.[9–11] The *cfr*(B) gene, encoding a protein variant sharing 75% amino acid identity with Cfr and conferring a similar phenotype to that of Cfr, was identified in *E. faecium* and *Clostridioides difficile*.[12,13] Another *cfr*-like gene conferring linezolid resistance, denominated *cfr*(C), was identified in *C. difficile* from France and *Campylobacter coli* from China.[14,15] The *cfr*(D) gene was first identified in an *E. faecium* clinical isolate from France in 2015,[16] then in an *E. faecium* blood isolate recovered from an Australian patient in 2019.[17] Finally, a new determinant, termed *cfr*(E), was very recently discovered in a linezolid-resistant *C. difficile* clinical isolate collected in Mexico.[18]

Even though *cfr*(D) was designated according to the nomenclature for MLS resistance genes (http://faculty.washington.edu/marilynr/), only limited data are available about this novel resistance determinant. The aim of this study was to characterize the genetic support and environment of the *cfr*(D) gene and to study its functionality in different bacterial host species.

Materials and methods

Bacterial isolates and antimicrobial susceptibility testing

As previously reported, the *E. faecium* 15-307.1 strain was isolated in 2015 from a rectal swab of an 87-year-old male patient admitted to the Sud Francilien hospital (Corbeil-Essonnes, France) as a sanitary repatriation from India.[16] Strains used for conjugation and transformation experiments are described in Table S1 (available as Supplementary data at *JAC* Online).

Antibiotic susceptibility testing was performed by the disc diffusion method following EUCAST criteria (http://www.eucast.org/). MICs were determined by the broth microdilution reference method according to EUCAST guidelines (http://www.eucast.org/).

Conjugation

Transfer of linezolid resistance from strain 15-307.1 to *E. faecium* BM4107 was attempted by filter-mating experiments, as previously described.[19] Transconjugants were selected on brain heart infusion (BHI) agar plates containing rifampicin (60 mg/L), fusidic acid (50 mg/L) and linezolid (8 mg/L).

Cloning

From the total DNA of *E. faecium* 15-307.1, the fragment encompassing the *cfr*(D) gene and its promoter region was amplified by PCR using specific primers (Table S2). The PCR product was then digested with XbaI and cloned into the spectinomycin-resistant shuttle vector pAT29 after subcloning into pCR2.1-TPOPO in *Escherichia coli* TOP10 (Table S1). The resulting recombinant plasmid, pAT29Ω*cfr*(D), was transferred by electroporation to *E. faecium* HM1070, *Enterococcus faecalis* JH2-2 and *E. coli* AG100A (Table S1).

RNA manipulations

Total RNA was extracted using the Fungal/Bacterial RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). Residual chromosomal DNA was removed by treating samples with the TURBO DNA-*free* Kit (Life Technologies, Saint Aubin, France) and total RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). The absolute quantification of *cfr*(D) level expression was determined by reverse transcription quantitative PCR (RT–qPCR) in triplicate using specific primers and an external calibration curve (Table S2). The transcription start site (TSS) was determined using the 5′ RACE System (Life Technologies) using specific primers (Table S2).

WGS and bioinformatic analysis

Genomic DNA of *E. faecium* 15-307.1 was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). From the WGS, a hybrid approach combining short- and long-read sequencing technologies was employed. For short-read sequencing, DNA libraries were prepared using the Celero PCR Workflow with Enzymatic Fragmentation library preparation kit (NuGEN Technologies, Redwood City, CA, USA) and sequenced as paired-end reads (2×300 bp) using an Illumina MiSeq platform and the MiSeq Reagent Kit version 3. Long-read sequencing was performed using a GridION X5 device (Oxford Nanopore Technologies, Oxford, UK) and the Ligation Sequencing Kit 1D (R9.4) (CGFB, Bordeaux, France). From fast5 files generated from GridION sequencing, reads were base-called with Albacore 2.0.1 and long reads were assembled using Canu 1.8. Hybrid assembly was then performed using both Illumina and GridION reads with Unicycler (version 0.4.8) and a homemade pipeline. The annotation was done using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). MLST was performed using the MLST database (version 1.8; https://pubmlst.org/) and the nucleotide sequences were also submitted to ResFinder 3.2 and LRE-Finder 1.0 servers (https://cge.cbs.dtu.dk) for identification of acquired resistance genes and chromosomal mutations.

Nucleotide sequence accession numbers

Nucleotide sequences of the chromosome and plasmids of *E. faecium* 15-307.1 obtained by hybrid sequencing and assembly have been deposited in the GenBank database under accession numbers CP044316–CP044327.

Results

Characteristics of *E. faecium* 15-307.1

As previously shown,[16] the 15-307.1 strain was phenotypically resistant to ampicillin, gentamicin (high-level), norfloxacin, erythromycin, clindamycin, chloramphenicol, tetracycline, linezolid, vancomycin and teicoplanin, while it remained susceptible to daptomycin and tigecycline (Table 1). Notably, MICs of linezolid and tedizolid were elevated (16 and 4 mg/L, respectively).

WGS using a hybrid approach (short- and long-read sequencing) led to an assembly of 12 contigs, including the chromosome (2 856 007 bp) and 11 plasmids (with sizes from 2056 to 199 401 bp). It confirmed the presence of *vanA*, *erm*(A), *erm*(B), *tet*(L), *tet*(M), *optrA*, *aac(6′)-Ii*, *ant(6)-Ia*, *aph(3′)-III*, *aac(6′)-aph(2″)*, *msr*(C) and *dfrG* resistance genes as well as chromosomal mutations involved in ampicillin (PBP5) and fluoroquinolone (GyrA S83T; ParC S80I) resistance.[16] As expected, *E. faecium* 15-307.1 belonged to ST17.[16]

Transferability of linezolid resistance

Resistance to linezolid was successfully transferred from *E. faecium* 15-307.1 to *E. faecium* BM4107 at a low frequency (efficiency, $\sim 10^{-8}$ transconjugants per donor). The transconjugant was phenotypically resistant to linezolid (MIC, 16 mg/L) and was positive for both *optrA* and *cfr*(D) (Table 1). Co-transfer of *erm*(A) and *erm*(B) genes also occurred, but not the *vanA* cluster. The transconjugant was resistant to macrolides/lincosamides/streptogramin B antibiotics, while it remained entirely susceptible to glycopeptides (Table 1). Since the transconjugant harboured both

Table 1. Resistance genes and MICs for *E. faecium*, *E. faecalis* and *E. coli* strains

	Resistance gene						MIC (mg/L)																
	vanA	*erm*(A)	*erm*(B)	*tet*(L)	*tet*(M)	*optrA*	*cfr*(D)	LZD	TZD	AMP	GEN	LVX	CHL	ERY	CLI	TIA	DAL	QUI	Q/D	DAP	TET	TGC	VAN	TEC
E. faecium																								
15-307.1	+	+	+	+	+	+	+	16	4	≥256	≥512	256	64	≥256	≥256	128	≥128	32	4	1	128	0.25	≥256	64
BM4107	–	–	–	–	–	–	–	1	1	0.5	2	1	4	0.12	8	32	≥128	1	4	1	0.25	0.01	0.5	0.25
TC_15-307.1	–	+	+	–	–	+	+	**16**	**4**	0.5	2	1	**16**	**≥256**	**128**	**128**	**≥128**	**128**	4	1	0.5	0.03	1	0.25
HM1070	–	–	–	–	–	–	–	1	0.5	0.06	2	0.25	2	0.03	0.03	0.5	2	1	0.25	0.25	0.25	0.01	0.5	0.25
pAT29	–	–	–	–	–	–	–	1	0.25	0.06	2	0.25	1	0.03	0.03	0.25	1	2	0.25	0.25	0.25	0.03	0.5	0.25
pAT29Ω*cfr*(D)	–	–	–	–	–	–	+	1	0.25	0.06	2	0.25	2	0.03	0.03	0.5	2	1	0.25	0.25	0.25	0.03	0.25	0.25
E. faecalis																								
JH2-2	–	–	–	–	–	–	–	1	0.25	1	8	0.25	4	0.12	8	128	≥128	4	4	1	0.5	0.5	0.5	0.25
pAT29	–	–	–	–	–	–	–	1	0.25	1	8	0.25	4	0.12	8	128	≥128	4	4	0.5	0.25	0.5	0.5	0.25
pAT29Ω*cfr*(D)	–	–	–	–	–	–	+	1	0.25	1	8	0.25	4	0.12	8	128	≥128	4	4	0.5	0.5	0.5	0.5	0.25
E. coli																								
AG100A	–	–	–	–	–	–	–	4	2	1	<0.12	<0.12	2	4	4	16	16	≥128	16	ND	0.25	1	ND	ND
pAT29	–	–	–	–	–	–	–	4	2	1	<0.12	<0.12	2	4	4	16	16	≥128	16	ND	0.25	1	ND	ND
pAT29Ω*cfr*(D)	–	–	–	–	–	–	+	**16**	**4**	0.5	<0.12	<0.12	**8**	4	**≥256**	**128**	**256**	≥128	**128**	ND	0.25	1	ND	ND

LZD, linezolid; TZD, tedizolid; AMP, ampicillin; GEN, gentamicin; LVX, levofloxacin; CHL, chloramphenicol; ERY, erythromycin; CLI, clindamycin; TIA, tiamulin; DAL, dalfopristin; QUI, quinupristin; Q/D, quinupristin/dalfopristin; DAP, daptomycin; TET, tetracycline; TGC, tigecycline; VAN, vancomycin; TEC, teicoplanin; ND, not determined.
Values in bold indicate significant changes in MIC.

optrA and *cfr*(D) and the parental strain displayed an LS_A phenotype, it was not possible to determine whether *cfr*(D) conferred a $PhLOPS_A$ phenotype or not.

Cloning and expression of cfr(D)

With a length of 1074 bp (G + C content, 32.96%), *cfr*(D) encoded a protein of 357 amino acids (~41 kDa). This protein was related to members of the Cfr family (Figure S1) and shared 64%, 64%, 48% and 51% amino acid identity with Cfr, Cfr(B), Cfr(C) and Cfr(E), respectively. Notably, it contained the conserved cysteine-rich CX_3CX_2C motif that is characteristic of radical *S*-adenosyl-L-methionine (SAM) enzymes (Figure S2).

The TSS of the *cfr*(D) gene was experimentally determined 24 bp upstream of the start codon of *cfr*(D) and promoter elements (−35 and −10 boxes) were deduced (Figure 1). To test the functionality of *cfr*(D) and its involvement in linezolid resistance, the structural gene, along with its promoter, was cloned in the shuttle vector pAT29 and expressed in different bacterial hosts. In both *E. faecium* HM1070 and *E. faecalis* JH2-2, the introduction of the recombinant plasmid pAT29Ω*cfr*(D) did not confer any resistance (Table 1). By contrast, it was responsible for a $PhLOPS_A$ resistance phenotype in *E. coli* AG100A, with significant increases in MICs of chloramphenicol (4-fold), clindamycin (≥64-fold), linezolid (4-fold), tedizolid (2-fold), tiamulin (8-fold) and dalfopristin (16-fold) (Table 1). The absence of resistance in enterococci could not be explained by gene silencing since we confirmed the expression of *cfr*(D) by RT–qPCR in *E. faecium* 15-307.1 and HM1070 as well as in *E. faecalis* JH2-2, which was similar to that in *E. coli* AG100A (Figure S3).

Genetic support and environment of cfr(D)

Thanks to the hybrid sequencing approach, we identified that *cfr*(D) was part of a 103 kb conjugative plasmid. The analysis of flanking regions showed the presence of a *guaA* gene coding for a glutamine-hydrolysing guanosine monophosphate (GMP) synthase (truncated by an IS*1216* element) downstream of *cfr*(D) (Figure 1). Two other IS*1216* elements were also found in the close vicinity (Figure 1), but the implication of these elements in the mobilization of *cfr*(D) was not so clear since no duplication of the target site was evidenced after detailed genetic analysis.

Discussion

In this study, we describe a novel plasmid-mediated *cfr*-like determinant in an *E. faecium* clinical isolate. Cfr(D) is phylogenetically distant from other Cfr variants since it shares <65% amino acid identity with Cfr, Cfr(B), Cfr(C) and Cfr(E). However, it definitely belongs to the SAM enzyme superfamily since it contains the conserved cysteine-rich CX_3CX_2C motif. Similar to other Cfr-like proteins, Cfr(D) confers resistance to five classes of antibiotics, at least when expressed in *E. coli*. Notably, a 2-fold increase in the MIC of tedizolid was observed; this is different to Cfr, which apparently does not confer resistance to this new oxazolidinone.[20]

This lack of *cfr*-mediated resistance to phenicols and oxazolidinones has already been reported for *cfr* and *cfr*(B) in both *E. faecalis* and *E. faecium*.[13,21–23] Importantly, Liu *et al.*[21] conducted a thorough experimental investigation to understand the silent

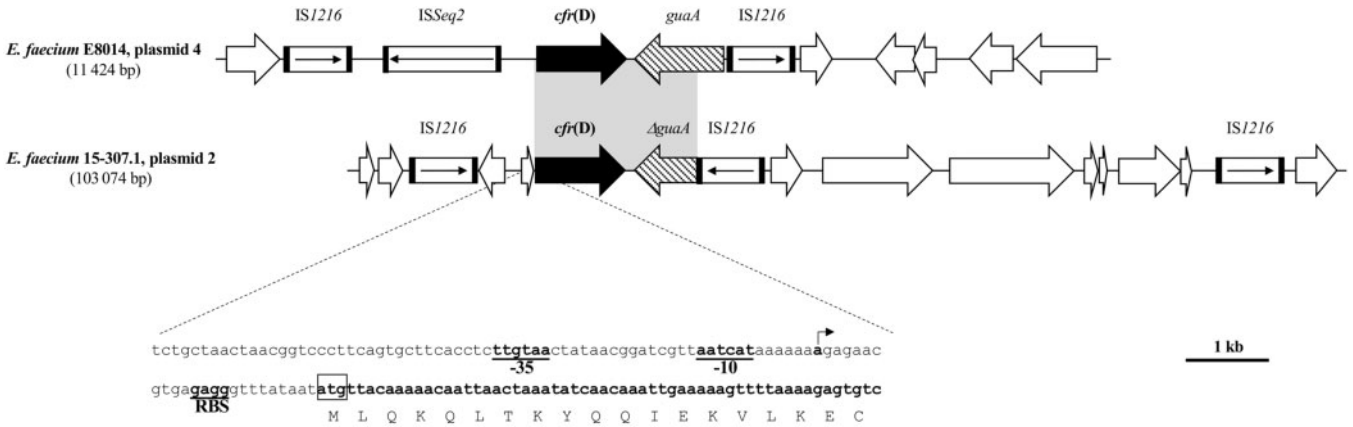

Figure 1. Schematic map of the genetic environment of *cfr*(D) located on plasmid 2 of *E. faecium* 15-307.1 and on plasmid 4 of *E. faecium* E8014 (GenBank accession no. LR135354). A region of >98% nucleotide sequence identity is shaded in grey. ORFs are shown as arrows indicating the orientation of their coding sequence. The gene *guaA* encodes a glutamine-hydrolysing GMP synthase. The nucleotide sequence corresponding to the upstream region of the *cfr*(D) gene is represented in detail. The −35 and −10 promoter boxes are underlined and the TSS is represented by an arrow. The start codon of *cfr*(D) and its putative ribosome-binding site (RBS) are also indicated.

phenotype of a plasmid-mediated *cfr* gene and found no modification in coding sequence and promoter regions, no alterations in *cfr* transcription, translation or production (Cfr was detected by western blot) and no lack of rRNA methylation of A2303. Although the authors were not able to explain this apparent 'no resistance' phenotype in *E. faecalis*, they observed that when cloned and transferred to *E. coli*, the *cfr* gene conferred the expected resistance phenotype,[21] as demonstrated here for *cfr*(D). These findings and ours strongly suggest that the failure to mediate Cfr-mediated resistance is a species-specific issue, probably related to the ribosome structure. Further investigations are needed to decipher the underlying mechanism.

As mentioned above, *cfr*(D) has been reported in a linezolid-resistant ST872 *E. faecium* clinical isolate collected from Australia in 2019.[17] This vancomycin-susceptible (but *vanA*-positive) strain also co-harboured *optrA* and *cfr*(D), but the genetic support and environment were not described. In the latter study, the functionality was not tested.

Even if the duplication of the target site was not evidenced in this study, ISs of this family are able to move without target site duplication.[24] Therefore, it is likely that IS*1216* elements may be involved in the mobilization of *cfr*(D) since multiple copies of this IS were identified in its close vicinity. The possible implication of these elements (having the same polarity) in the mobilization of *cfr*(D) was attempted by PCR experiments, but we failed to demonstrate this event (data not shown). Interestingly, the association of IS*1216* and other linezolid resistance genes has been previously reported in enterococci.[10,25] Finally, the origin of *cfr*-like genes remains to be elucidated, but enterococcal species could constitute a reservoir of these resistance determinants.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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