Transferable Plasmid-Mediated Resistance to Linezolid Due to *cfr* in a Human Clinical Isolate of *Enterococcus faecalis*

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Nonmutational resistance to linezolid is due to the presence of *cfr*, which encodes a methyltransferase responsible for methylation of A2503 in the 23S rRNA. The *cfr* gene was first described in animal isolates of staphylococci, and more recently, it has been identified in *Staphylococcus aureus* from human clinical infections, including in an outbreak of methicillin-resistant *S. aureus*. In enterococci, *cfr* has been described in an animal isolate of *Enterococcus faecalis* from China. Here, we report an isolate of linezolid-resistant *E. faecalis* (603-50427X) recovered from a patient in Thailand who received prolonged therapy with the antibiotic for the treatment of atypical mycobacterial disease. The isolate lacked mutations in the genes coding for 23S rRNA and L3 and L4 ribosomal proteins and belonged to the multilocus sequence type (MLST) 16 (ST16), which is commonly found in enterococcal isolates from animal sources. Resistance to linezolid was associated with the presence of *cfr* on an ~97-kb transferable plasmid. The *cfr* gene environment exhibited DNA sequences similar to those of other *cfr*-carrying plasmids previously identified in staphylococci (nucleotide identity, 99 to 100%). The *cfr*-carrying plasmid was transferable by conjugation to a laboratory strain of *E. faecalis* (OG1RF) but not to *Enterococcus faecium* or *S. aureus*. The *cfr* gene was flanked by IS*256*-like sequences both upstream and downstream. This is the first characterization of the potential horizontal transferability of the *cfr* gene from a human linezolid-resistant isolate of *E. faecalis*.

L inezolid was the first oxazolidinone introduced to clinical use (in 2000), and since then, it has been widely prescribed to treat infections caused by Gram-positive organisms and, in many instances, mycobacterial infections. Linezolid is currently approved by the Food and Drug Administration (FDA) for the treatment of complicated skin and skin structure infections and nosocomial pneumonia caused by susceptible organisms. Linezolid has an FDA indication for the treatment of vancomycin-resistant *Enterococcus faecium* (VRE) infections (including bacteremia) (61). Linezolid alters protein synthesis by binding to the 50S ribosomal subunit, with recent data suggesting that the oxazolidinone binds to the A site of the peptidyl-transferase center (PTC) of the bacterial ribosome, interfering with the positioning of aminoacyl-tRNA; as a result, protein synthesis is inhibited (71). Although the prevalence of linezolid resistance among Gram-positive organisms is still low (18, 56), the mechanisms of linezolid resistance have been extensively characterized. Indeed, the most common mechanism involves mutations in domain V of 23S rRNA (20, 67) with the G2576T (*Escherichia coli* numbering) substitution being the most frequently reported (18, 67). Additional mutations observed in resistant isolates include T2500A (44) C2192T (22), G2447T (34, 64), A2503G (34), T2504C (35), G2505A (50), G2766T (34), and C2461T (17). Additionally, mutations in the ribosomal proteins L3 and L4 have also been associated with linezolid resistance (17, 36–39). The above-mentioned mechanisms of linezolid resistance have been shown not to be transferable and are associated with previous exposure to linezolid.

In 2005, a Colombian patient developed nosocomial pneumonia with a linezolid-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) isolate (3, 65). Characterization of the mechanism of resistance in the isolate indicated that *cfr* (for chloramphenicol-florfenicol resistance gene), encoding a methyltransferase that catalyzes the posttranscriptional methylation of nucleotide A2503 in

the 23S rRNA, was responsible for the resistance phenotype (65). This gene had been previously described in a 17,108-bp transferable plasmid (pSCFS1) from an animal isolate of *Staphylococcus sciuri* (60) and had also been detected in several other staphylococci of animal origin in Europe (2, 26), which exhibited resistance to phenicols (florphenicol and chloramphenicol). Interestingly, Cfr methylation also affects susceptibility to other antimicrobial compounds, such as lincosamides, pleuromutilins, streptogramin A, and 16-member ring macrolides, which, like linezolid, all bind to the PTC on the 50S ribosomal subunit (63). This phenotype has been designated PhLOPS$_A$ (for phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics) (41). Linezolid resistance due to *cfr* in human clinical isolates of staphylococci has now been reported in several countries (5, 45–48), including a recent report of an outbreak of *cfr*-containing MRSA in a critical care unit in Spain (48). Dissemination of the *cfr* gene among staphylococci is of great concern, since evidence of transferability of the gene has been provided both *in vitro* and *in vivo* (45, 57). The initial report of *cfr* in a human isolate of MRSA suggested that a possible source of the gene may have been enterococci. Indeed, *cfr* has been detected in human clinical isolates of enterococci (7, 8), although a complete characterization of these isolates has not been published. In this work, we present the characterization of a human clinical isolate of linezolid-resis-

TABLE 1 Antimicrobial susceptibilities of *E. faecalis* 603-50427X and OG1RF and transconjugants

	MIC (µg/ml)[a]			
	E. faecalis			
Antibiotic	603-50427X	OG1RF	Tc6	Tc11
Linezolid	32	1	8	8
Linezolid[b]	24	2	8	6
Ampicillin	2	4	1	1
Vancomycin	1	2	2	2
Teicoplanin	≤1	≤1	≤1	≤1
Daptomycin	1	2	1	1
Ciprofloxacin	>4	2	1	1
Levofloxacin	>4	2	1	1
Chloramphenicol	64	4	64	64
Tigecycline	0.06	0.12	0.12	0.12
Quinupristin/dalfopristin	4	16	>16	>16
Tiamulin	64	>64	>64	>64
Clindamycin	>128	32	>128	>128
Fusidic acid	8	>128	>128	>128
Rifampin	0.25	>128	>128	>128

[a] MICs were determined by broth microdilution according to Clinical and Laboratory Standards Institute guidelines (10, 11).

[b] MICs were determined by Etest.

tant *Enterococcus faecalis* carrying the *cfr* gene on a transferable plasmid.

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MATERIALS AND METHODS

Bacterial isolates, species identification, and molecular typing. A linezolid-resistant *E. faecalis* strain (603-50427X) was isolated in July 2010 from the skin of a 72-year-old diabetic woman in Bangkok, Thailand, who presented with multiple skin abscesses due to *Mycobacterium abscessus* and pulmonary tuberculosis. The patient received meropenem, amikacin, moxifloxacin, azithromycin, isoniazid, rifampin, ethambutol, metronidazole, and linezolid for at least 3 months prior to the isolation of the linezolid-resistant organism. The susceptibilities of the *E. faecalis* isolate are shown in Table 1. *E. faecalis* OG1RF (14, 51), rifampin- and fusidic acid-resistant derivatives of *E. faecium* GE1 (16), and *S. aureus* RN4220 (30) (RN4220-RF) were used as recipients for conjugation experiments (see below). Species identification of the enterococcal isolate and determination of the presence of vancomycin resistance genes were performed using a PCR assay as described previously (15). Typing of the organism was performed by multilocus sequence typing (MLST) using the established set of *E. faecalis* MLST primers (targeting *aroE*, *gdh*, *gki*, *gyd*, *pstS*, *xpt*, and *yqiL*) (1, http://efaecalis.mlst.net). DNA sequencing was performed in both strands using the dideoxynucleotide chain termination method (59) with fluorescent cycle sequencing with dye-labeled terminators (Applied Biosystems [Foster City, CA] BigDye Terminator V3.1 cycle sequencing kit) on an ABI Prism 3730xl DNA analyzer.

Susceptibility testing and detection of linezolid resistance. MICs were determined by a broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI) guidelines (10, 11). Additional susceptibilities to linezolid were determined using Etest (BioMerieux, Marcy l'Étoile, France) on Mueller-Hinton agar (Oxoid Basingstoke, Hampshire, United Kingdom) following the recommendations of the manufacturer. The presence of mutations in genes encoding all copies of the 23S rRNA and ribosomal proteins L3 and L4 were investigated by PCR and sequencing. The 23S rRNA-encoding genes were PCR amplified used previously published primers (43), while the L3- and L4-encoding genes

were amplified using specifically designed primers, as follows: ESP rplC-F, ATGACCAAAGGAATCTTAGGG; ESP rplC-R, CACAGCTGATTTGAT WGTGATT; ESP rplD-F, GCCGAATGTAGCATTATTCAA; and ESP rplD-R, CAAGCACCTCCTCAATTTGAGT. The amplicons were sequenced on both strands, and the amino acid sequences were compared with those from wild-type linezolid-susceptible *E. faecalis* ATCC 29212. The resulting DNA sequences were compared with all *E. faecalis* isolates whose genomes have been sequenced (http://www.ncbi.nlm.nih.gov /genomes/geblast.cgi?taxid=1351). Detection of the presence of the *cfr* gene was performed by PCR using primers previously reported (27).

Bacterial mating and S1 nuclease assays. Conjugative transfer of *cfr* was conducted by filter mating (66) using *E. faecalis* 603-50427X as the donor and *E. faecalis* OG1RF, *E. faecium* GE1, and *S. aureus* RN4220-RF as recipients. Selection of transconjugants was performed using brain heart infusion (BHI) agar plates supplemented with chloramphenicol (20 µg/ ml) and fusidic acid (25 µg/ml). Purified single colonies of transconjugants were also plated on BHI agar containing chloramphenicol (20 µg/ ml) and rifampin (100 µg/ml). Pulsed-field gel electrophoresis (PFGE) was performed in all transconjugants using the restriction enzyme SmaI (49) in order to confirm that they were derivatives of the recipient strain. Total DNA from *E. faecalis* transconjugants was subjected to S1 nuclease assays coupled with PFGE in order to determine the presence of transferred plasmids, as described previously (4). S1 nuclease-treated linearized plasmids were transferred to a nylon membrane and hybridized using a *cfr* probe (745 bp) (62).

Sequencing of *cfr* and its genetic environment. Total DNA was extracted by standard methodology using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), following the manufacturer's recommendations, with a modification consisting of adding lysozyme (20 mg/ml) to the lysis buffer, followed by incubation for 30 min at 37°C. In order to sequence the *cfr* gene, primers Cfr-F (5′-TGTATGTTTTGACTTTCGGCACCGG-3′) and Cfr-R (5′-ATTATCTTCCACCCAGTAGTCC-3′), which are located 138 bp upstream and 133 bp downstream of *cfr*, respectively, in *S. aureus* CM-05 (accession number JN849634), were used for PCR amplifications. The PCR amplicon was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA), sequenced by fluorescent cycle sequencing with dye-labeled terminators, assembled using CodonCode Aligner (version 3.7.1; CodonCode Corp., Dedham, MA), and compared to reference sequences. The DNA sequence upstream and downstream of *cfr* in *E. faecalis* 603-50427X was obtained by inverse PCR methodology (53). Briefly, total DNA from the bacterial isolate was extracted and digested with EcoRI. The digested DNA was purified and religated using T4 ligase. The inverse primers 5′-TGCTCTGAATTTTGCTCTGCTAAGA-3′ (targeting nucleotides 203 to 227 of *cfr*) and 5′-GGAGAAGCAAACGAAGGGCAGG-3′ (nucleotides 907 to 928) were used for the PCR assay. The resulting amplicon was purified and cloned into the pGEM-T Easy Vector for sequencing with universal M13 primers.

Nucleotide sequence accession number. The sequence of 1,274 bp upstream and and 1,170 bp downstream of the *cfr* gene has been deposited in GenBank under accession number JQ660368.

RESULTS AND DISCUSSION

Linezolid resistance in *E. faecalis* 603-50427X is associated with the presence of *cfr* on a plasmid. *E. faecalis* 603-50427X exhibited resistance to linezolid with MICs of 32 µg/ml by broth microdilution and 24 µg/ml by Etest (Table 1). In order to determine the mechanism of resistance, we initially investigated the presence of mutations in the genes encoding domain V of the 23S rRNA (the most common mechanism found in clinical isolates) and in ribosomal proteins L3 and L4. Sequencing of the above-mentioned genes revealed no mutations when comparing the sequences with those from wild-type *E. faecalis* genomes available in the NCBI database (http://www.ncbi.nlm.nih.gov/genomes/geblast.cgi?taxid =1351). Instead, we were able to obtain a PCR product of the

expected size (745 bp) when using primers previously used to amplify *cfr* from staphylococci. The amplicon was cloned and sequenced in its entirety on both strands, revealing a nucleotide sequence that was 100% identical to that of *cfr* genes from several human and animal staphylococcal isolates, including the original isolate of *S. sciuri* (25) and the first MRSA of human origin (65). The *cfr* gene from *E. faecalis* 603-50427X exhibited two nucleotide differences (G262→A and A367→G) from a *cfr* gene previously characterized from an animal isolate of *E. faecalis* (EF-01) (33), although the nucleotide changes caused an amino acid difference only in position 88 of the predicted enzyme (Glu→Lys). The putative Cfr amino acid sequence from *E. faecalis* 603-50427X had all the essential amino acids predicted to be required for biochemical activity, including the conserved CxxxCxxC motif characteristic of radical *S*-adenosyl-L-methionine (SAM) enzymes (24).

In several organisms, *cfr* has been described on plasmids, present as a monocistronic unit and associated with transposons, suggesting that the gene is likely to be mobilizable (40). Indeed, the *cfr* gene was initially described on a 17.1-kb plasmid from a multiresistant *S. sciuri* animal isolate and was designated pSCFS1, which also carries the rRNA methylase gene *erm*(33), the aminocyclitol phosphotransferase gene *spc* and the ABC transporter gene *lsa*(B), conferring resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, spectinomycin, and lincosamides, respectively (25). In order to determine the location of the *cfr* gene within the genome of *E. faecalis* 603-50427X, we performed S1 digestion, PFGE, and Southern hybridization with a *cfr* probe. This methodology has been used previously (4) to detect and estimate the sizes of enterococcal plasmids, since large plasmids are linearized on a PFGE gel. Figure 1 shows that *cfr* was indeed located on an *E. faecalis* plasmid of ca. 97 kb.

Conjugative transfer of linezolid resistance to *E. faecalis* OG1RF via a *cfr*-carrying plasmid. The transferability of the *cfr* gene was evaluated by performing filter-mating experiments, as described previously (66). As the recipients of the mating experiments, we used *E. faecalis* OG1RF (14, 51), *E. faecium* GE1 (16), and *S. aureus* RN4220-RF (30). We were able to successfully transfer *cfr* to *E. faecalis* OG1RF (efficiency, 2.6×10^{-5} transconjugants per donor), but not to *E. faecium* or *S. aureus*, suggesting that the *cfr*-carrying plasmid was not able to replicate in a heterologous host. Two purified single colonies from the *E. faecalis* OG1RF transconjugants, designated Tc6 and Tc11, were characterized by PCR (targeting *cfr*), PFGE, S1 nuclease digestion, and hybridization in order to compare DNA banding and plasmid profiles between the donor and the corresponding transconjugants. Figure 1 confirms that the *cfr*-carrying plasmid was transferred to the recipient *E. faecalis* strain via conjugation. Of note, several other plasmids were also transferred during the mating experiments, indicating that several mobilizable plasmids are present in the clinical strain *E. faecalis* 603-50427X (Fig. 1). The lack of transfer to an *S. aureus* recipient was not surprising, since it has been shown that some enterococcal plasmids may be unstable in staphylococcal backgrounds. Indeed, it has been shown that enterococcal plasmids containing the *vanA* gene cluster involved in vancomycin resistance are unstable in *S. aureus* (54). Moreover, the efficient transfer of DNA between bacteria of different species may be limited by one or more restriction/modification systems (23). In fact, at least three types of restriction systems that drastically reduce the frequency of horizontal gene transfer have been described in *S. aureus* (12). The lack of plasmid transfer to *E. faecium*
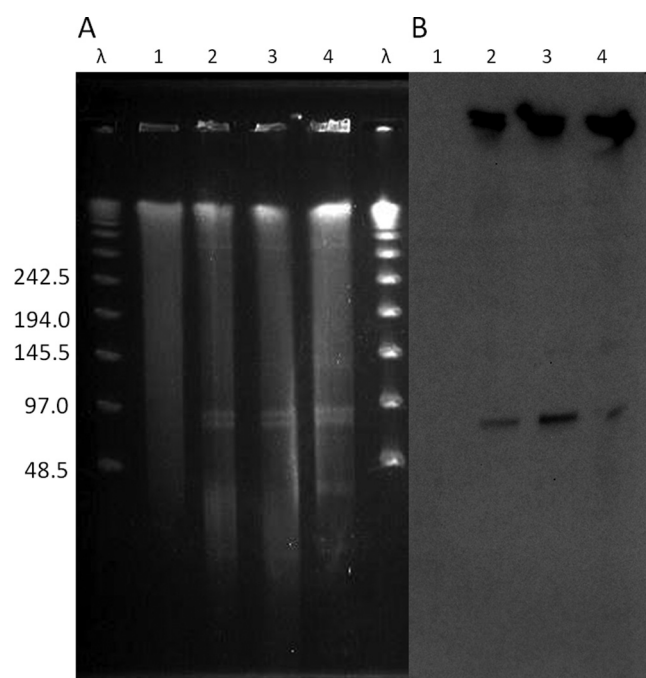

FIG 1 Localization and transfer of the *cfr* gene determined by S1-PFGE and Southern blot hybridization. S1 digestion of total DNA of *E. faecalis* strains was followed by PFGE (A) and Southern blot analysis with a *cfr* probe (B). λ denotes the lambda ladder (molecular sizes in kilobases are shown on the left). Lane 1, *E. faecalis* OG1RF; lanes 2 and 3, *E. faecalis* transconjugants Tc6 and Tc11, respectively; lane 4, *E. faecalis* 603-50427X. The figure is a composite of gels and hybridizations from different experiments.

was also not surprising, since most *E. faecalis* plasmids may not have the ability to replicate in *E. faecium* hosts. As an example, pheromone-responsive plasmids that are commonly found in *E. faecalis* isolates are rarely described in *E. faecium* (14).

The transfer of the *cfr* plasmid was associated with a 3- to 4-fold increase in the linezolid MIC (2 μg/ml to 6 μg/ml and 8 μg/ml in the transconjugants Tc11 and Tc6, respectively) (Table 1). Moreover, increases in the MICs of chloramphenicol, clindamycin, and quinopristin/dalfopristin (part of PhLOPS_A) were also observed in the transconjugant strains, supporting the notion that *cfr* was an important contributor to the resistance phenotype in the parental strain (Table 1). The increase of linezolid MICs observed in the transconjugants did not reach the level observed in *E. faecalis* 603-50427X, suggesting that other factors may influence susceptibility to linezolid in the original clinical strain. This phenomenon has also been observed in staphylococci when *cfr* has been expressed on plasmids in heterologous hosts (65).

Sequence type and genetic environment of the *cfr* gene in *E. faecalis* 603-50427X. MLST typing of *E. faecalis* 603-50427X indicated that it belonged to sequence type 16 (ST16). Unlike the majority of clinical isolates of *E. faecalis* belonging to clonal complex 2 (CC2) and CC9 (31, 58), isolates belonging to ST16 have been associated with both animal and human origins (9, 19, 52, 55). This finding is of particular interest, since the *cfr* gene has been characterized mainly in isolates from animal origin, and it suggests that *E. faecalis* 603-50427X may have been transmitted by direct contact with an animal source. In order to determine the genetic environment surrounding the *cfr* gene on the plasmid, we obtained the sequences 1,274 bp upstream and 1,170 bp down-
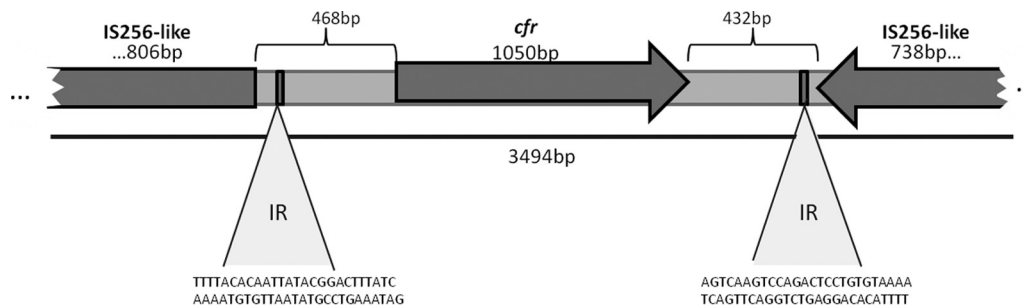
FIG 2 Genetic environment of *cfr* in *E. faecalis* 603-50427X. The *cfr* gene is flanked in both sites by IS*256*-like sequences. The 26-bp IR sequences are shown.

stream of the *cfr* gene (JQ660368). BLASTn analysis of these sequences showed a high degree of nucleotide identity (99%) to IS*256*-like sequences present in plasmid pSS-01, which was originally described in a *Staphylococcus cohnii* strain recovered from a porcine nasal swab in Shandong Province, China (69) (Fig. 2). Our findings indicate that *cfr* in *E. faecalis* 603-50427X is flanked by the same mobile transposable elements, a phenomenon that has been described in other *cfr*-carrying organisms (5, 26, 64, 68). IS*256* belongs to the mutator transposase family and is widespread in the genomes of several multiresistant enterococci and staphylococci (6). IS*256* consists of an open reading frame encoding a transposase protein flanked by noncoding regions harboring imperfect inverted repeats (IRs) (21). As shown in Fig. 2, we found two imperfect IRs flanking *cfr* in *E. faecalis* 603-50427X, suggesting that the gene may also be transferable via IS*256*-mediated transposition. Of note, this insertion sequence has been associated with aminoglycoside resistance (29, 42) and with the *ica* operon in biofilm producers of *Staphylococcus epidermidis* (28, 29). Since a high prevalence of transposons with IS*256* sequences in enterococcal isolates has been reported from Japan and Thailand (70, 32) and many *cfr*-carrying strains have been detected in the last 2 years in China (13, 33, 68, 69, 72), it is tempting to speculate that *cfr* has an important potential for dissemination from animals to humans in Southeast Asia.

In summary, we report the first characterization of a transferable *cfr*-carrying plasmid of human origin in an *E. faecalis* isolate (ST16), conferring resistance to linezolid. The genetic environment suggests high potential for dissemination of this gene, which confers multidrug resistance.

ACKNOWLEDGMENTS

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