Investigation of a multiresistance gene \textit{cfr} that fails to mediate resistance to phenicols and oxazolidinones in \textit{Enterococcus faecalis}

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Objectives: To investigate the basis of susceptibility to phenicols and oxazolidinones of the porcine \textit{Enterococcus faecalis} CPPF5 despite the presence of the multiresistance gene \textit{cfr}.

Methods: Southern blotting, conjugation and transformation analyses were conducted to confirm the plasmid location and transferability of \textit{cfr} in CPPF5. The genetic environment of \textit{cfr} was determined by sequence analysis. Transcription and translation of \textit{cfr} were examined by RT–PCR and western blotting, respectively, and modifications at A2503 within the 23S rRNA sequence were identified by primer extension.

Results: Electrottransformation and Southern blotting indicated that CPPF5 and its transformant SB2-3 contained two \textit{cfr}-carrying plasmids ~50 and ~12 kb in size. The complete 12270 bp sequence of the smaller plasmid, pCPPF5, was determined and shared 99.9% (12269/12270 bp) identity with the corresponding region of the \textit{cfr}-carrying plasmid pEF-01 in \textit{E. faecalis} of cattle origin. Moreover, the genetic environment of \textit{cfr} in the ~50 kb plasmid was the same as that in pCPPF5 according to sequencing results. Although \textit{cfr} mRNA, Cfr protein and a modification at A2503 site were detected, the \textit{cfr}-carrying transformant SB2-3 did not have elevated MICs of chloramphenicol, flarfenicol and linezolid, indicating that \textit{cfr} fails to mediate resistance to the respective antibiotics in \textit{E. faecalis}.

Conclusions: This is the first report of the \textit{cfr} gene failing to elevate MICs of the corresponding antibiotics. Although the genetic basis for the apparent ‘no resistance’ phenotype remains to be determined, this finding may have implications for surveillance studies that target the \textit{cfr} gene.

Keywords: pigs, linezolid, transcription, translation, RNA methylation

Introduction

The emergence and global spread of the multiresistance gene \textit{cfr} reduces the efficacy of linezolid in the control of Gram-positive bacteria. Linezolid, an oxazolidinone antibiotic, was introduced for clinical use in the United States in 2000 to treat serious infections caused by vancomycin-resistant enterococci (VRE), methicillin-resistant \textit{Staphylococcus aureus} (MRSA) and penicillin-resistant pneumococci.\cite{1,2} The \textit{cfr} gene encodes an RNA methyltransferase that methylates the C-8 position of nucleotide A2503 in the 23S rRNA,\cite{3,4} and confers resistance to five chemically unrelated antimicrobial classes, namely phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (PhLOPSa phenotype), and decreased susceptibility to the 16-membered macrolides spiramycin and josamycin.\cite{5,6} Since its first identification in a bovine \textit{Staphylococcus scouri} isolate in 2000, \textit{cfr} has been detected in several bacterial genera, including \textit{Enterococcus},\cite{7} derived from humans, pigs, cattle, horses and poultry.\cite{8} LaMarre et al.\cite{9} suggested that a low fitness cost is associated with the carriage of \textit{cfr}, which may contribute to the worldwide spread of \textit{cfr} among pathogens.

The \textit{cfr} and \textit{cfr}-like genes confer resistance to PhLOPSa antibiotics in both Gram-positive and Gram-negative bacteria.\cite{7,10–12} The possibility that unexpressed antibiotic resistance genes are present within bacterial genomes has seldom been investigated,\cite{13} possibly because of the low incidence (~1%), although examples of such genes have been reported.\cite{14} In this study, we present evidence of a \textit{cfr} gene carried on the plasmid pCPPF5 in \textit{Enterococcus faecalis} strain CPPF5 isolated from pig faeces, and in its transformant SB2-3, which does not display resistance to phenicols and oxazolidinones \textit{in vitro}. These findings are particularly important because \textit{E. faecalis} is implicated in the spread of the multiresistance gene \textit{cfr} among other pathogens in a clinical setting.
Materials and methods

Location and transferability of cfr in CPPF5

During a surveillance study of antimicrobial resistance in Enterococcus species isolated from animal farms in Shandong Province and Beijing, China, in 2009,5 a cfr-positive E. faecalis strain CPPF5 was isolated and typed by multilocus sequence typing (MLST).16 Conjugation and transformation experiments were performed following methods described previously,17 and both E. faecalis JH2-2 and FA2-2,18 respectively, served as the recipients. Transconjugants and transformants were further confirmed by Smal–PFGE, S1 nuclease PFGE assays, specific PCRs and Southern blotting as described previously.7 The strains, plasmids and primers used in this study are listed in Table 1.

Antimicrobial susceptibility testing

Susceptibility of the tested strains to antibiotics was assessed using the broth microdilution method as recommended by the CLSI documents M31-A3 (2008)19 and M100-S21 (2011).20 The following antimicrobial agents were tested with the test ranges in mg/L, given in parentheses: ampicillin (1–512), chloramphenicol (1–512), ciprofloxacin (0.125–64), erythromycin (0.125–256), florfenicol (1–512), gentamicin (1–512), linezolid (0.25–128), tetracycline (0.5–256) and vancomycin (0.5–256). E. faecalis ATCC 29212 served as a quality control strain.

Sequencing and analysis

The cfr-carrying plasmids from transformant SB2-3 were first sequenced by primer walking as described previously,17 then by shotgun sequencing using the Illumina HiSeq2000 system (San Diego, USA) to produce 100 bp paired-end reads. The latter approach was performed at the University of Virginia, USA. A draft assembly for the cfr plasmid was conducted using CLC Genomics Workbench 5 (CLC Bio, Aarhus, Denmark).

Functional cloning of the cfr gene

A 1426 bp DNA fragment containing the intact cfr gene plus the promoter region was amplified from SB2-3 by PCR using the primer pair g1-F and g1-R listed in Table 1. This PCR product was digested with EcoRI and HindIII and then cloned into pBluescript SK+ (pBSK+). Agilent Technologies, Santa Clara, CA, USA), and the recombinant plasmid vector was transformed into Escherichia coli TransSa (TransGen, Beijing, China) with subsequent selection on Luria–Bertani (LB) agar plates supplemented with 50 mg/L ampicillin, and further confirmed by PCR for the presence of the cfr gene. Subsequently, these transformants were investigated for the MICs of chloramphenicol and florfenicol.

RNA extraction, RT–PCR and real-time quantitative RT–PCR

Total bacterial RNA was isolated from transformant SB2-3 and the control strain JH2-2+pEF-01 using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Trace amounts of contaminating DNA in isolated RNA were removed using a TURBO DNA-free Kit (Ambion, Austin, TX, USA). Purified total RNA (1 μg) was subjected to reverse transcription with 10 U AMV reverse transcriptase (Takara, Dalian, China) using 10 pmol each of specific cfr downstream primers, according to the manufacturer’s instructions. A standard PCR was carried out sequentially by using cfr gene-specific primers and cDNA template.10 A control PCR experiment was also performed to detect the presence of residual DNA using the DNase-digested RNA preparations and the reaction conditions described above. RT–PCR products were separated on agarose gels stained with Goldview (SBS Genetech, Beijing, China) and visualized under UV illumination. In addition, the real-time quantitative RT–PCR was performed to accurately compare the amount of mRNA expression of cfr between SB2-3 and JH2-2+pEF-01 using a DNA Engine Chromo4 system (Bio-Rad, Hercules, CA, USA). The primer pairs RT–PCR-cfr-F/R for the cfr gene and 23S-F/R for 23S rRNA as housekeeping gene are described in Table 1. Amplification, detection and data analysis were performed twice and in duplicate with three different RNA samples. The value used for the comparison of gene expression was the number of PCR cycles required to reach the threshold cycle (CT). To relate the CT value back to the abundance of an mRNA species, CT was converted to an n-fold difference by comparing mRNA abundance in the SB2-3 with JH2-2+pEF-01. Statistical comparison of means was performed using ∆∆CT (ΔCT SB2-3-pEF-01 − ΔCT JH2-2). The n-fold difference was calculated by the formula 2ΔΔCT.

Production of polyclonal antibodies and western blot analysis

Because the Cfr protein of SB2-3 was identical to that of pEF-01, but differed from the pSCFS121 Cfr by only two amino acid substitutions (K88E and N123D), the cfr genes of SB2-3 and Enterococcus thailandicus W3, whose Cfr sequence is identical to that in pSCFS1, were cloned into the pET28a expression vector. The expression and purification of the recombinant proteins was performed as described previously.21 Briefly, the crude protein extracts after centrifugation were loaded on a 1 mL Ni-NTA agarose (Qiagen, Germany) column previously equilibrated with Buffer B (8 M urea, 100 mM NaH2PO4, and 100 mM Tris-HCl, pH 8.0). The column was washed with 10 volumes of Buffer C (8 M urea, 100 mM NaH2PO4, and 100 mM Tris-HCl, pH 6.3), and the Cfr protein was eluted with 5 volumes of Buffer E (8 M urea, 100 mM NaH2PO4, and 100 mM Tris-HCl, pH 4.5). The purified Cfr proteins from SB2-3 and W3 were separately used to raise polyclonal antibodies in BALB/c mice. The mice were first immunized with 100 μg of Cfr protein in complete Freund’s adjuvant by subcutaneous injection. After 4 weeks they were boosted three times using the same amounts of protein in incomplete Freund’s adjuvant at 2 week intervals, and serum was obtained after the last bleeding. The work of sampling from animals complied with the principles of the Beijing Municipality Review of Welfare and Ethics of Laboratory Animals (BAOLA 2005). The polyclonal antibodies were designated anti-Cfr (SB2-3) and anti-Cfr (W3). Total protein extractions and western blotting were carried out as described previously,22 using the antiserum and HRP-conjugated goat anti-mouse IgG (Dingguo, Beijing, China) as primary and secondary antibodies, respectively.

Premier extension analysis

Considering that E. faecalis contains four chromosomal copies of the RNA operon (rrn),24 the four different copies of the 23S rRNA domain V region were amplified and sequenced separately as described previously to confirm the presence of the A residue at position 2503.25 Methylation at A2503 was examined by primer extension analysis following a previously described method,17 and using a modified Cs5-labelled deoxyoligonucleotide primer (S’-GACGCCCACCGCCCTGG-3’) that is complementary to nucleotides 2540−2556 of the E. faecalis 23S rDNA sequence (E. coli numbering).

Results and discussion

Identification of the cfr-carrying isolate CPPF5

The E. faecalis CPPF5 isolate harbouring cfr exhibited resistance to chloramphenicol, erythromycin, tetracycline and ciprofloxacin, with elevated MICs against florfenicol and gentamicin, intermediate levels of resistance to linezolid and susceptibility to ampicillin and vancomycin (Table 2). MLST of CPPF5 indicated that it belongs to sequence type 21 (ST21), within the clonal complex 21 (CC21), which is the same as E. faecalis EF-01 isolated from
Southern blot analysis showed that Staphylococcus lentus strain CPPF5 showed 100% and 99% nucleotide identity to Staphylococcus aureus strain (accession number NC_014508) and S. aureus strain (accession number AJ549214), respectively.

Electrotransformation using the plasmids extracted from CPPF5 and E. faecalis JH2-2 as the recipient strain was not successful when 10 mg/L florfenicol was used as the selective antimicrobial agent. Considering that macrolide resistance genes often coexist with fexA-carrying plasmids, PCR screening to detect erythromycin resistance genes in E. faecalis JH2-2 and FA2-2, JH2-2 + pEF-01 and FA2-2 + pEF-01 was conducted with selection of transformants at 2 mg/L erythromycin. This transformant was resistant to CHL FFC LZD ERY AMP V AN TET GEN CIP.

Localization of the cfr gene, and antimicrobial susceptibility of CPPF5 and its transformant

Electrotransformation using the plasmids extracted from CPPF5 and E. faecalis JH2-2 as the recipient strain was not successful when 10 mg/L florfenicol was used as the selective antimicrobial agent, while PCR showed that all cfr-carrying transconjugants isolated on selective agar plates containing florfenicol (10 mg/L) also harboured the fexA-carrying plasmid. Considering that macrolide resistance genes often coexist with cfr,6,18,19,26 electrotransformation experiments were conducted with selection of transformants at 2 mg/L erythromycin to reduce the possibility of co-selection of the fexA-carrying plasmid. A cfr-positive and fexA-negative transformant, designated 5B2-3, was successfully obtained. Smal–PFGE analysis of the donor (CPPF5), the recipient (JH2-2) and the transformant (5B2-3) detected at least one visible extra fragment, ~12 kb in size, in the 5B2-3 Smal pattern compared with that of JH2-2 (Figure 1a). S1 nuclease–PFGE analysis exhibited at least four size, in the 5B2-3 Smal pattern compared with that of JH2-2 (Figure 1a). Southern blot analysis showed that the cfr probe hybridized to two bands (~50 and ~12 kb) in both CPPF5 and 5B2-3, while the fexA probe hybridized to a single 50 kb band in CPPF5, but showed no hybridization in 5B2-3 (Figure 1b). These results suggested that the transformant 5B2-3 harboured two cfr-carrying plasmids. Although the cfr-carrying plasmids in CPPF5 were transferable among enterococci, no cfr-positive and fexA-negative transformant could be isolated on agar plates containing florfenicol. This might be due to the fact that the cfr-carrying plasmids could not contribute to the florfenicol resistance phenotype in CPPF5.

PCR screening to detect erythromycin resistance genes in transformant 5B2-3 using previously described primers27,28 revealed the presence of erm(B) in this isolate. Moreover, another transformant was identified by electrotransformation assays using 2 mg/L erythromycin. This transformant was resistant to bovine faeces.7 However, Smal–PFGE analysis revealed a distinct genomic heterogeneity of <50% similarity between these two isolates (data not shown). The nucleotide sequences of the amplified cfr (518 bp) and phenicol exporter gene fexA (977 bp) regions in strain CPPF5 showed 100% and 99% nucleotide identity to cfr on the plasmid pEF-01 (accession number NC_014508) and fexA of Staphylococcus lentus (accession number AJ549214), respectively. Southern blot analysis showed that cfr and fexA were located on different plasmids.

### Table 1. Bacterial strains, plasmids and primers used in this study

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<th>Strain, plasmid or primer</th>
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<td>this study</td>
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<td>a modified Cy5-labelled deoxyoligonucleotide primer</td>
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### Table 2. Antimicrobial susceptibility profiles of cfr-positive E. faecalis CPPF5 and its transformants 5B2-3 and FA2-2 + CPPF5, E. faecalis strains JH2-2 and FA2-2, JH2-2 + pEF-01 and FA2-2 + pEF-01

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<th>VAN</th>
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CHL, chloramphenicol; FFC, florfenicol; LZD, linezolid; ERY, erythromycin; AMP, ampicillin; VAN, vancomycin; TET, tetracycline; GEN, gentamicin; CIP, ciprofloxacin.
Analysis of the regions flanking the cfr gene in pCPPF5

To find out whether the reason for the apparent failure of cfr-mediated resistance is due to mutational changes, the complete sequence of the 12,270 bp cfr-carrying plasmid, designated pCPPF5, was obtained by primer walking. The 5B2-3 plasmids were used as template for the primers P1 and P2 (Figure 2, accession number KC954773). The pCPPF5 sequence exhibited 99.9% nucleotide sequence identity to the part of plasmid pEF-01 that could be looped out by IS1216 recombination. However, it should be noted that pCPPF5 did not harbour a copy of IS1216. Only a single nucleotide exchange, G→C at position 24,816 in pEF-01, was observed, which caused an amino acid change at position 179 (Gly→Ala) of tnp in pEF-01. To determine the sequence of the larger cfr-carrying plasmid (~50 kb), deep sequencing of the 5B2-3 plasmids was performed and produced ~3.9 billion nucleotides, with an average G+C content of 37.4%. The 224 assembled contigs comprised 2.95 Mb of sequence and represented a complete 980-fold coverage. Sequence analysis revealed the presence of a single copy of the complete cfr gene on a 12,286-bp contig, which displayed >99.9% identity to plasmid pCPPF5. Together, these results suggested that the cfr genes contained by the two plasmids in 5B2-3 shared the same genetic environments. However, the possibility that integration of the small cfr-carrying plasmid pCPPF5 into a second plasmid led to the formation of the ~50 kb cfr-carrying plasmid in pCPPF5 cannot be excluded.

Sequence analysis showed that the 5B2-3 Cfr amino acid sequence had all amino acids predicted to be required for biochemical activity, including the conserved cysteine-rich CX2CX2C motif that is characteristic of radical S-adenosyl-L-methionine (SAM) enzymes. Moreover, the potential promoter sequence of cfr in pCPPF5 was identical to that in pEF-01. However, the MICs of chloramphenicol, florfenicol and linezolid for transformants 5B2-3 and JH2-2 were significantly different, showing susceptibility and resistance, respectively.

E. coli DHSx harbouring pBSK+ with the cloned cfr gene exhibited MICs of 16 mg/L to chloramphenicol and of 32 mg/L to florfenicol. Compared with E. coli DHSx and E. coli DHSx carrying pBSK+, these MICs corresponded to an increase of at least 4-fold and
suggest that the cloned cfr gene is functionally active in E. coli. Although the cfr gene in E. faecalis 5B2-3 did not exhibit cfr-mediated resistance, this observation strongly suggested that this cfr gene is functionally active after transfer to another bacterial genus. Such a situation has also been described for the ICE Pmu1-associated blaOXA-2 gene which was non-functional in Pasteurella multocida and Mannheimia haemolytica, but highly active after transfer of the integrative and conjugative element (ICE) to E. coli. Al-

Verifying transcription and translation of the cfr gene and RNA methylation at A2503 in the transformant 5B2-3

Because of the unexpected MIC results observed for transformant 5B2-3 carrying the multiresistance gene cfr, RT–PCR was performed to detect transcription of cfr in 5B2-3. JH2-2 + pEF-01 was used as a positive control because its cfr gene shares the same genetic environment as that of 5B2-3. Interestingly, cfr mRNA was detected in 5B2-3 at levels similar to those in JH2-2 + pEF-01 (Figure 3a). Moreover, based on results of real-time qRT–PCR, the x-fold change of expression of cfr gene between 5B2-3 and JH2-2 + pEF-01 was 1.37; this <1.5-fold change also suggested that no significant difference in mRNA expression of the cfr gene was observed between these two strains. Reports of unexpressed resistance genes in bacteria are rare, but can be divided into two major types. One type includes mecA in S. sciuri and cfiA in Bacteroides fragilis, both of which lack promoters that express the gene sufficiently to reduce the antibiotic susceptibility of the host. The second type includes adaA1, blaOXA-2, sul1 and tet(A) in E. coli, and the extended-spectrum β-lactamase (ESBL) genes in Klebsiella pneumoniae. The resistance genes in

Figure 3. Transcription, translation and methylation of cfr in 5B2-3. (a) RT–PCR was used to analyse the transcription of cfr in 5B2-3. Following reverse transcription, PCR was carried out using different templates: cDNA and RNA from JH2-2 + pEF-01 and 5B2-3. Lane +, chromosomal DNA from 5B2-3; lane –, water; lane M, Trans 2K. (b) Whole-cell proteins extracted from 5B2-3 with or without 1 mg/L florfenicol were analysed by western blot using anti-Cfr(W3) or anti-Cfr(5B2-3) antibodies. Lanes JH2-2 + pEF-01 were positive controls induced with 8 mg/L florfenicol, while lane JH2-2 shows the negative control. A minus symbol indicates that the isolates were cultured without any drug, while a plus symbol indicates pre-incubation with 8 or 1 mg/L florfenicol. (c) The nucleotide at position 2503 (E. coli numbering) in four different copies of the 23S rRNA sequence from isolate 5B2-3. (d) Primer extension analysis of modification of A2503 in 23S rRNA from 5B2-3. Lanes A, U, G and C refer to dideoxynucleotide sequencing generated using the E. faecalis RNA as template. The black arrow indicates the stop mediated by methylation modification by the Cfr protein.
These strains retain their original promoter sequences but cannot be transcribed as normal, in a process designated ‘antibiotic resistance gene silencing’, although the mechanism by which this occurs is unknown. Neither of these mechanisms described the situation observed with cfr in this study, as cfr in SB2-3 retained the intact promoter structure and could be transcribed just like JH2-2 + pEF-01.

To examine the expression of cfr in SB2-3, anti-Cfr antibody was produced. The expressed Cfr proteins were insoluble, with a size of 40 kDa. After four rounds of immunization, the polyclonal antibodies anti-Cfr(SB2-3) and anti-Cfr(W3) were obtained and used to detect the Cfr protein in SB2-3. Whole-cell proteins from the recipient strain JH2-2 alone and with plasmid pEF-01, which harbours a functionally active cfr, were included as controls. Strains SB2-3 and JH2-2 + pEF-01 were induced by florfenicol at a concentration of 1/8 MIC (1 or 8 mg/L). Although the polyclonal antibodies were produced by two slightly different Cfr proteins, the same results were observed. A single protein band reacting with the polyclonal antibodies was detected in SB2-3 and positive control JH2-2 + pEF-01, while the corresponding position of the negative control JH2-2 was blank (Figure 3b). Pre-incubation in the presence of florfenicol had no marked effect on the expression of the cfr gene by SB2-3 or JH2-2 + pEF-01.

Cfr methylates the C-8 position of 23S rRNA nucleotide A2503 at the ribosomal peptidyl transferase centre.3,6 Because the Cfr protein could be expressed in the transformant SB2-3, the modified site was tested by primer extension. No mutations were observed at position A2503 in four different copies of the 23S rRNA gene from SB2-3, suggesting the possibility of methylation of the Cfr protein in this site. The purified total RNA from SB2-3 was then subjected to primer extension using reverse transcriptase. A Cy5-labelled oligonucleotide complementary to nucleotides 2540–2556 of the 23S rRNA gene was annealed to a region of 23S rRNA sequence, downstream of A2503, and extended until stopped by modifications or secondary structures. Although dideoxynucleotide sequencing reactions did not produce clear results, the Cfr from SB2-3 mediated a strong stop at A2503, which was also observed for positive control JH2-2 + pEF-01. As expected, the negative control, JH2-2, did not cause any stop at the A2503 position (Figure 3d). The stop thus confirms that Cfr in SB2-3 modifies A2503 in the same way as Cfr in JH2-2 + pEF-01, although their MICs for chloramphenicol, florfenicol and linezolid were strikingly different.

Conclusions
These observations provide experimental evidence that the cfr gene in pCPPF5 fails to mediate resistance to the respective antibiotics, even though (i) the complete coding DNA sequence (CDS) and (ii) an intact promoter were present, (iii) cfr-specific mRNA was transcribed and translated, (iv) the Cfr protein was detected by western blotting and (v) the Cfr-specific methylation of A2503 could be shown. In contrast, when cloned and transferred to E. coli, the cfr gene confers the expected resistance phenotype. This finding strongly suggests that the failure to confer Cfr-mediated resistance in E. faecalis is a strain-specific issue rather than a cfr-gene-related issue. Moreover, the functionally active cfr gene in JH2-2 + pEF-01 suggested another gene product present on the remainder of pEF-01 may be responsible for elevating the MIC of phenicols and oxazolidinones in E. faecalis. In addition, the possibility that other plasmids from CPPF5 and SB2-3, that were not identified by S1-PFGE and PFGE, are involved in overcoming the effect of cfr-catalysed modification on antibiotic susceptibility cannot be excluded. This is a disturbing finding and deserves to be further studied, especially with regard to the underlying mechanism. The finding of an apparent failure of an intact cfr gene to mediate resistance is important for two reasons: (i) this observation indicates that there might be an unknown reservoir of ‘silent’ antibiotic resistance genes in pathogenic bacteria that would not be detected by diagnostic methods that rely only on the resistance phenotype; and (ii) surveillance studies that target the cfr gene by PCR or microarray approaches may yield false-positive results.

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Transparency declarations
None to declare.

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