Detection of the staphylococcal multiresistance gene cfr in Proteus vulgaris of food animal origin

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Objectives: To investigate the presence and the genetic environment of the multiresistance gene cfr in naturally occurring Gram-negative bacteria of pigs.

Methods: A total of 391 bacterial isolates with florfenicol MICs of ≥16 mg/L, obtained from 557 nasal swabs of individual pigs, were screened by PCR for the known florfenicol resistance genes. The species assignment of the cfr-carrying isolate was based on the results of Gram’s staining, colony morphology, 16S rDNA sequencing and biochemical profiling. The location of the cfr and floR genes was determined by Southern blotting and the regions flanking the cfr gene were sequenced by a modified random primer walking strategy.

Results: A single Proteus vulgaris isolate, which carried the genes floR and cfr, was detected in this study. A cfr-carrying segment of 7 kb with homology to a staphylococcal plasmid was found to be inserted into the chromosomal fimD gene of P. vulgaris. This segment was flanked by two IS26 elements located in the same orientation, which are believed to have played a role in this integration process. Stability testing via inverse PCR approaches showed that this integrate is not entirely stable, but the cfr-carrying centre region plus one IS26 copy can be looped out via IS26-mediated recombination.

Conclusions: To the best of our knowledge, this is the first report of the cfr gene in a naturally occurring Gram-negative bacterium. Surveillance and monitoring of the cfr gene in Gram-negative bacteria are warranted with respect to food safety and consumer protection.

Keywords: linezolid resistance, inter-genus transfer, reserve antibiotics, food safety

Introduction

Linezolid, the first oxazolidinone used in clinical practice, has demonstrated potent antimicrobial activity against Gram-positive organisms, including methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant enterococci and Streptococcus spp. Since its introduction into clinical use in 2000, the emergence of resistant strains has remained relatively rare. Resistance is usually mediated by mutations in one or more alleles of the 23S rRNA, with G2576T being the most frequently observed mutation. Recently the gene cfr has been shown to mediate transferable resistance to linezolid. This gene encodes a methyltransferase that targets A2503 in the 23S rRNA and thereby confers resistance not only to linezolid, but also to members of five other chemically unrelated classes of antimicrobial agents, including phenicols, lincosamides, pleuromutilins, streptogramin A antibiotics and the 16-membered macrolides spiramycin and josamycin. Initially the gene cfr was identified in coagulase-negative staphylococci of animal origin, but it has also been detected in staphylococcal isolates from humans, including the first cfr-positive MRSA isolate, which was identified in a strain from Colombia, as well as the emergence of this multidrug resistance gene in Italy, the USA, Mexico and Spain. During recent years, the cfr gene has also been sporadically identified in other Gram-positive bacteria, such as Bacillus spp. Plasmids seem to play an important role in the interspecies and inter-genus transfer of this multiresistance gene.

Although cloning experiments have shown that a staphylococcal cfr gene is functionally active in an enterobacterial host, such as Escherichia coli, no information is available about the presence of the cfr gene in naturally occurring Gram-negative bacteria. In this report, we describe the first identification of a...
cfr gene in the chromosomal DNA of a *Proteus vulgaris* isolate of porcine origin.

**Materials and methods**

**Sample collection and detection of florfenicol resistance genes**

In 2010 a survey of the presence of the cfr gene in bacteria of food producing animal origin was performed at three pig farms in Shandong Province, China. For this, 557 nasal swabs were obtained from individual animals and were streaked on brain-heart-infusion agar plates supplemented with 10 mg/L florfenicol. Bacteria that grew on these selective plates were investigated for the currently known florfenicol resistance genes *floR*, *fexA* and *cfr* by PCR using previously described primers.\(^1^{13}\)

**Species assignment**

The species assignment of the cfr-carrying strains was performed by Gram's staining, colony morphology, 16S rDNA sequencing and the use of commercially available biochemical test systems, such as the API 20E system (bioMérieux, Craponne, France).

**Susceptibility testing**

MIC values were determined by broth microdilution according to the recommendations of the document M100-S21 of the CLSI.\(^1^{14}\) The reference strains *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 served as quality controls.

**Molecular analyses**

The genomic DNA was isolated from bacteria using a commercial DNA isolation kit (TianGen, Beijing, China). For this, 557 nasal swabs were obtained from individual animals and were streaked on brain-heart-infusion agar plates supplemented with 10 mg/L florfenicol. Bacteria that grew on these selective plates were investigated for the currently known florfenicol resistance genes *floR*, *fexA* and *cfr* by PCR using previously described primers.\(^1^{13}\)

### Functional analysis

A DNA fragment containing the cfr gene plus 1228 bp of the upstream sequence, including the truncated genes rep and trpB, was amplified by PCR using the primer pair E-F (5'-GCTCTAGAAGCGATTTAGACC AACTCAAT-3') and E-R (5'-GCTCTAGACTATTTTGATTTTAA TACC-3') (see Figure 2). This PCR product was digested with XbaI and then cloned into the XbaI site of the chloramphenicol- and tetracycline-resistant *E. coli-Enterococcus faecalis* shuttle vector pAM401 (ATCC 37429), which also replicates in *S. aureus*, to construct the recombinant plasmid pAME1. The cloned insert was confirmed by sequence analysis. The recombinant plasmid was introduced into *E. coli DH10B* and *S. aureus* RN4220 by electrottransformation.\(^1^{17}\) Transformants were selected on LB plates containing 10 mg/L chloramphenicol and further confirmed by PCR of the cfr gene. Subsequently these transformants were investigated for their MICs of linezolid, florfenicol, tiamulin and clindamycin.

### Results

Analysis of 391 bacterial colonies, obtained from florfenicol-supplemented media, for the presence of florfenicol resistance genes revealed the presence of a single Gram-negative isolate, designated PV-01, which carried the genes *floR* and *cfr* as confirmed by sequence analysis. The nucleotide sequence of the cfr gene in PV-01 showed 100% identity with the cfr gene of the *Staphylococcus sciuri* plasmid pSCFS1 (accession number NC_005076).\(^5\) Moreover, the 959 bp PCR product of the *floR* gene also was indistinguishable from the *floR* gene on plasmid 10660-1 from *E. coli* (accession number AF231986).\(^1^{13}\) Growth characteristics already suggested that this isolate might be a *P. vulgaris*, which was further confirmed by biochemical profiling with the API 20E system (bioMérieux, France). Moreover, the results of 16S rDNA sequencing showed 99% identity to the 16S rDNA sequence of *P. vulgaris*.

Isolate PV-01 exhibited high MIC values for gentamicin (≥128 mg/L) and sulfamethoxazole (≥512 mg/L), but showed low MIC values for ciprofloxacin (1 mg/L), amoxicillin/clavulanic acid (≤0.5/0.25 mg/L), ceftiofur (≤0.5 mg/L), ceftazidime (≤1 mg/L) and amikacin (≤1 mg/L). In addition, this strain exhibited chloramphenicol and florfenicol MICs of >256 mg/L (Table 1). Southern blot analysis with probes specific for *cfr* and *floR* identified the *floR* gene on a plasmid of ~6 kb, whereas the cfr probe hybridized only to an ~4.5 kb EcoRI fragment of the chromosomal DNA (Figure 1). The genetic environment

### Table 1. Antimicrobial susceptibility profiles of *P. vulgaris* PV-01, *S. aureus* RN4220, *E. coli* DH10B and their transformants with plasmids pAM401 and pAME1

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th><em>P. vulgaris</em></th>
<th><em>S. aureus</em></th>
<th><em>S. aureus</em></th>
<th><em>S. aureus</em></th>
<th><em>E. coli</em></th>
<th><em>E. coli</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PV-01</td>
<td>RN4220</td>
<td>RN4220+pAM401</td>
<td>RN4220+pAME1</td>
<td>DH10B</td>
<td>DH10B+pAM401</td>
<td>DH10B+pAME1</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>&gt;256</td>
<td>4</td>
<td>4</td>
<td>64</td>
<td>1</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Linezolid</td>
<td>ND</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>ND</td>
<td>0.12</td>
<td>0.12</td>
<td>32</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>ND</td>
<td>0.03</td>
<td>0.03</td>
<td>32</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined because of known intrinsic resistance (clindamycin)\(^1^{16}\) or naturally high MICs (tiamulin/linezolid).\(^2^{17}\)
of the cfr gene was sequenced by a modified random primer walking strategy, starting from each end of the cfr gene. In total, the sequence of a chromosomal segment of 11.2 kb containing the cfr gene was determined (Figure 2; GenBank accession number JF969273). Within this chromosomal fragment, an 8.6 kb element was detected. This element had a central region, which carried the cfr gene, whereas the flanking regions showed partial homology to plasmid pKKS825 from MRSA ST398. The central region was bracketed by two IS26 insertion sequences that were located in the same orientation. Immediately upstream of the left IS26 and downstream of the right IS26, 8 bp direct target site duplications (5'-GTTGATAC-3') were found (Figure 2). This entire IS26-flanked segment was integrated into a fimD gene encoding a putative fimbrial biogenesis usher protein.

To determine the stability of this IS26-flanked segment in PV-01, an inverse PCR was performed using the primers cfrIF (5'-TGAAGTCTGCTGGTATCCATGT-3') and cfrIR (5'-TTTGCTCTGTAAGACCTTGAT-3'), which were located inside the cfr gene. A 7791 bp amplicon was obtained and sequence analysis confirmed that it contained an intact cfr-carrying centre region and one intact IS26 (Figure 3a). Using the same DNA preparation, a PCR assay was carried out with primers PVFR6 (5'-CCGGTGTATTGATCAACAAGC-3') and PVRR5 (5'-CGTCGCAATTCCGATGTCGAT-3'), which were located in the fimD gene left and right of the IS26-flanked segment. Two PCR amplicons were obtained, one of which was 9201 bp and the other 1410 bp in size (Figure 3a). Sequence analysis indicated that the 1.4 kb amplicon contained only one complete IS26 and the flanking fimD sequences, while the 9.2 kb amplicon comprised the intact aforementioned IS26-flanked segment and the flanking fimD sequences.

![Figure 1](image1.png) **Figure 1.** Localization of the cfr gene in the chromosomal DNA of *P. vulgaris* PV-01. (a) Plasmid and chromosomal DNA extracted from PV-01 and (b) Southern blot hybridization of the plasmid and chromosomal DNA with the cfr-specific probe. Lane M1, plasmid standards purified from *E. coli* V517; lane M2, λ-DNA HindIII digested; lane 1, plasmids purified from PV-01; lane 2, undigested chromosomal DNA extracted from PV-01; lane 3, PV-01 chromosomal DNA digested by EcoRI.

![Figure 2](image2.png) **Figure 2.** Genetic environment of the cfr gene in chromosomal DNA of *P. vulgaris* PV-01 and structural comparison with plasmid pKKS825 from MRSA ST398 of swine origin and pSCFS1 from *S. sciuri* of bovine origin. The arrows indicate the positions and directions of transcription of the genes. Different genes are displayed using different shadings. The IS26 elements are shown as light grey boxes, with the white arrows indicating the transposase (tnp) genes and dark grey arrowhead indicating inverted repeats of IS elements. The 8 bp direct repeats up- and downstream of the IS26 elements are shown in boxes. The regions of >95% homology are marked using different grey shadings. Black arrowheads indicate the primers used for amplifying fragments to construct pAME1.
Figure 3. (a) The stability of the IS26-flanked segment in PV-01 was determined by an inverse PCR assay using the primers cfrIF and cfrIR, and by a regular PCR assay using the primers PVRR5 and PVFR6. The locations and orientations of these primers are shown in (b). Lane M1, Trans15K DNA Marker (TianGen, China); lane M2, 1 kb ladder marker (TianGen, China); lane 1, 7064 bp PCR product amplified by primers cfrIF and cfrIR; lane 2, 9201 bp and 1410 bp PCR products amplified by primers PVRR5 and PVFR6. (b) Schematic presentation of the simple transposition, co-integrate formation and resolution events of the cfr-containing region. The 8 bp direct repeats adjacent to each IS26 element and generated by the IS26-mediated simple transposition and co-integrate formation are shown in boxes.
Determination of MICs of linezolid, tiamulin, clindamycin and/or florfenicol for *S. aureus* RN4220 and *E. coli* DH1O8 carrying the recombinant plasmid pAME1, respectively, confirmed the functionality of the *cfr* gene of *P. vulgaris* PV-01. MICs of chloramphenicol have not been determined, since the cloning vector pAM401 carried a *cat* gene for chloramphenicol but not florfenicol resistance. *E. coli* DH1O8 and *S. aureus* RN4220 harbouring pAME1 exhibited at least 4-fold elevated MICs of the tested antibiotics as compared with the *cfr*-negative recipient strains (Table 1).

Discussion

The results of this study provided an example of a mainly plasmid-borne resistance gene of Gram-positive origin that was transferred into the chromosomal DNA of a Gram-negative bacterium. In this transfer, IS26 elements seemed to have played an important role. In a previous study, another IS element was identified in close proximity to *cfr* gene and has been assumed to play a role in the mobility of *cfr*. Based on the known transposition modes of IS26, a model was developed that explains how the *cfr* gene might have been integrated into the chromosomal DNA of *P. vulgaris* PV-01 (Figure 3b). In a first step, an IS26 element is assumed to have integrated into a replicon containing the *cfr* gene via simple transposition. Integration of IS26 into the *rep* gene disrupted this gene and resulted in the 8 bp direct repeats (5′-CTTAGAT-3′). A similar transposition event is believed to have played a role in the IS26 integration into the chromosomal *fimD* gene of *P. vulgaris* PV-01. The typical 8 bp direct repeats (5′-GGTGTACAT-3′) immediately upstream and downstream of this IS26 copy were also present. In a next step, a recombination between the IS26 copy on the *cfr*-harbouring replicon and the IS26 copy in the chromosomal *fimD* gene is assumed to have occurred. As a consequence, the *cfr*-harbouring replicon was bracketed by two copies of IS26 present in the same orientation. This recombination model is supported by the 8 bp repeats found upstream and downstream of each of the two IS26 copies (Figure 3b).

Furthermore, PCR-based stability tests revealed that this chromosomal integratge was not entirely stable. Instead, recombination between the two IS26 copies may occur, resulting in looping out of the *cfr*-carrying segment, including one IS26 copy. Such a circular form is not able to replicate as a plasmid due to the functional disruption of the *rep* gene by the IS26 copy. However, it may be able to re-integrate into the same position, as well as integrate into another IS26-carrying vector molecule present in the same cell. IS26 is widespread among plasmids of Gram-negative bacteria, but also shares significant homology with the staphylococcal insertion sequence IS431, present on various plasmids as well as the SCCmec cassettes of MRSA strains. Thus the *cfr* gene may not only be disseminated within the Gram-negative gene pool via IS26-mediated recombination events, but also may be re-introduced into the Gram-positive gene pool.

Antibiotic usage records for these swine farms indicated that the phenicol florfenicol, the lincosamide lincomycin and the pleuromutilin tiamulin have been used for curing disease, preventing disease and growth promotion, respectively. The selective pressure imposed by the use of these antibiotics in swine farms might have paved the way for an efficient spread and maintenance of the *cfr* gene among different porcine bacteria. Thus guidelines for the prudent use of antibiotics in livestock, as well as measures to enforce adherence to these guidelines, are needed in the future in China. Although the *cfr* gene has not yet been found in a clinical strain of human origin in China, it is likely that the *cfr* gene—once present in bacteria of food animal origin—might find its way through the food chain to commensal or pathogenic bacteria of humans and then may cause a serious threat to public health. Without overemphasizing the finding of this single *cfr*-carrying porcine *P. vulgaris* isolate, surveillance and monitoring of the prevalence of the *cfr* gene in Gram-negative bacteria are warranted to better trace the dissemination of this multiresistance gene and evaluate potential hazards with regard to food safety and consumer protection.

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Transparency declarations

None to declare.

References


