First Report of the Multidrug Resistance Gene cfr in Enterococcus faecalis of Animal Origin

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First Report of the Multidrug Resistance Gene cfr in Enterococcus faecalis of Animal Origin

Abstract
The multiresistance gene cfr was identified for the first time in an Enterococcus faecalis isolate of animal origin. The 32,388-bp plasmid pEF-01, which carried the cfr gene, was sequenced completely. Three copies of the insertion sequence IS1216 were identified in pEF-01, and the detection of a cfr- and IS1216-containing amplicon by inverse PCR suggests that IS1216 may play a role in the dissemination of cfr by a recombination process.

Comments

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The multiresistance gene \textit{cfr} was identified for the first time in an \textit{Enterococcus faecalis} isolate of animal origin. The 32,388-bp plasmid pEF-01, which carried the \textit{cfr} gene, was sequenced completely. Three copies of the insertion sequence \textit{IS1216} were identified in pEF-01, and the detection of a \textit{cfr}- and \textit{IS1216}-containing amplicon by inverse PCR suggests that IS1216 may play a role in the dissemination of \textit{cfr} by a recombination process.

The \textit{cfr} gene encodes a methyltransferase that modifies A2503 in bacterial 23S rRNA (12) and confers resistance to five chemically unrelated antimicrobial classes, including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (15), and decreased susceptibility to the 16-membered macrolides spiramycin and josamycin (26). Since its initial identification on the multiresistance plasmid pSCFS1 from \textit{Staphylococcus sciuri} in 2000 (21), several studies have reported the \textit{cfr} gene in staphylococcal isolates of animal and human origin. In most reports, the \textit{cfr} gene was located on different plasmids (9, 10, 16), while in a few cases, it was also detected in the chromosomal DNA (9, 14, 27). Recently, the first outbreak case of linezolid-resistant, \textit{cfr}-carrying \textit{Staphylococcus aureus} was reported in Spain (17), and the \textit{cfr} gene has also been identified in Panton-Valentine leukocidin-positive sequence type 8 (ST8) methicillin-resistant \textit{S. aureus} IVa (USA300) (23). These observations underline the increasing threat of this resistance determinant to public health. In recent studies, the \textit{cfr}-harboring plasmids pBS-01 and pBS-02 have also been identified in \textit{Bacillus} strains from swine feces (5, 29). In addition, a poster presented by Cercenado and coworkers described two human clinical isolates of \textit{cfr}-carrying enterococci, one \textit{Enterococcus faecalis} and one \textit{Enterococcus faecium} (2). To date, there has been no report of \textit{cfr} in \textit{Enterococcus} species of animal origin.

During a surveillance study on bacterial susceptibility to commonly used antibiotics on cattle farms in Sichuan province, China, in 2009, an enterococcal isolate from bovine feces exhibited elevated MICs of florfenicol and chloramphenicol, as determined by broth microdilution according to CLSI recommendations (3). This isolate, designated EF-01, was initially identified by Gram staining and \textit{Enterococcus}-specific PCR (6) and confirmed as \textit{E. faecalis} by the Rapid ID 32 Strept system (bioMérieux, Craponne, France).

Isolate EF-01 was screened for the genes \textit{cfr} and \textit{fexA} using previously described primers (5). A \textit{cfr}-specific PCR product was obtained and confirmed by sequence analysis. To determine the location of the \textit{cfr} gene, whole-cell DNA in agarose gel plugs from EF-01 was treated with S1 nuclease (TaKaRa, Shiga, Japan) and then separated by pulsed-field gel electrophoresis (PFGE) as described previously (1). Two plasmids were observed in EF-01, and their sizes were approximately 32 kb and 48 kb, as estimated by using the standard low-range PFGE markers (NEB, United Kingdom) (Fig. 1A). In a Southern blot analysis, a \textit{cfr}-specific digoxigenin-labeled probe hybridized to the ca. 32-kb plasmid, designated pEF-01 (Fig. 1B).

To investigate the transferability of plasmid pEF-01, conjugation and transformation assays were performed. Plasmid DNA was extracted by using the Qiagen plasmid extraction midi kit (Qiagen, Hilden, Germany) with the following modification: after the enterococci were suspended in buffer P1, lysozyme was added at a final concentration of 20 µg/ml, and the mixture was incubated for 2 h at 37°C before adding buffer P2. Transfer of the purified plasmid DNA was attempted with \textit{E. faecalis} JH2-2 and protoplasts of \textit{S. aureus} RN4220 by electrotransformation (4, 19). The transformants were selected on brain heart infusion (BHI) agar supplemented with 10 µg/ml florfenicol. Additionally, conjugative mating into \textit{E. faecalis} JH2-2 was attempted as described elsewhere (8). Although the conjugation was not successful, pEF-01 was successfully transferred into strains JH2-2 (JH2-2 + pEF-01) and RN4220 (RN4220 + pEF-01) by electrotransformation, as confirmed by a Southern blot analysis (Fig. 1A and B). Compared to the recipient strains, the transformants JH2-2 + pEF-01 and RN4220 + pEF-01 exhibited elevated MICs of phenicols, clindamycin, linezolid, and tiamulin (Table 1), which indicated the functionality of the \textit{cfr} gene in the new host bacteria.

The \textit{cfr} gene of pEF-01 encodes a 349-aa protein which differs from the Cfr proteins of pSCFS1 and pSCFS3 by only two
amino acid (aa) substitutions (K88E and N123D) (11, 21). To determine the genetic environment of the cfr gene, pEF-01 DNA purified from the transformant JH2-2+pEF-01 was sequenced by shotgun sequencing combined with primer walk- ing for gap closure, with both performed by the Beijing Genomics Institute (BGI; China). Sequences were annotated using the VectorNTI program (Invitrogen), and the predicted coding sequences (CDSs) were identified via Glimmer software (25). A smaller region (6,925 bp; from CDS 28 through part of CDS 6) of pEF-01 also showed 99.0% identity to the correspond- ing region of pVEF4 from an Enterococcus faecium strain derived from poultry in Norway (24). The common DNA segments in these four plasmids suggest recombination between plasmids and exchange among DNA segments in these four plasmids suggest recombination between plasmids and exchange among different origins. Second, multiple replication genes were identified in pEF-01 (13). Comparative analyses of the sequences of pEF-01 and the vanA-carrying, vancomycin-resistant plasmids pVEF1/pVEF2/pVEF4 from Enterococcus faecium revealed three interesting observ- ations. First, a region of 9,771 bp of pEF-01 spanning from CDS 28 to CDS 7 (Fig. 2; see also Table S1 in the supplemental material) showed 98.4% identity to the corresponding region of pVEF4 from an E. faecium strain derived from poultry in Norway (25). A smaller region (6,925 bp; from CDS 28 through part of CDS 6) of pEF-01 also showed 99.0% identity to the corresponding regions of pVEF1 and pVEF2 from E. faecium strains isolated from poultry and a poultry farmer in Norway (24). The common DNA segments in these four plasmids suggest recombination between plasmids and exchange among Enterococcus strains of different origins. Second, multiple replication genes were identified in these plasmids (pVEF1, pVEF2, pVEF4, and pEF-01). Three replication genes in pEF-01 showed similarities to those from three different bacteria. Specifically, CDS 1 is identical to the rep gene in pVEF1/pVEF2/pVEF4 from E. faecium, while the deduced aa sequences of CDS 23 and CDS 24 showed identities of 45% and

### TABLE 1: Impact of pEF-01 on antimicrobial susceptibility in E. faecalis and S. aureus

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (μg/ml) for E. faecalis</th>
<th>MIC (μg/ml) for S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EF-01</td>
<td>JH2-2</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>128</td>
<td>8</td>
</tr>
<tr>
<td>Linezolid</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* MIC = minimum inhibitory concentration.

**TABLE 2:** Impact of pEF-01 on antimicrobial susceptibility in E. faecalis and S. aureus.
87% to the RepB proteins of pWV05 from Lactococcus lactis and Leuconostoc mesenteroides subsp. cremoris, respectively (22). The presence of three different rep genes in pEF-01 suggests that this plasmid might have the ability to replicate in different bacteria—as also confirmed by its ability to replicate in S. aureus RN4220—and thus contribute to the dissemination of the multidrug-resistance gene cfr. Third, all of these plasmids have multiple copies of the insertion sequence (IS) IS1216 (three intact and one truncated copy in pEF-01) and the same resolvase (CDS 5 in pEF-01). The presence of IS elements, transposases, group II intron reverse transcriptase, integrase, and resolvases (Fig. 2; see also Table S1 in the supplemental material) in these plasmids might facilitate intra- or interplasmid recombination. Additionally, pEF-01 carries a bacterial epsilon antitoxin gene, suggesting that it may possess a toxin-antitoxin system to maintain its stability in bacterial hosts.

FIG 2 Genetic map of pEF-01. Coding regions larger than 50 amino acids are represented by arrows indicating the direction of transcription. All CDSs are colored according to their predicted functions. Truncated CDSs are indicated with a Greek delta symbol (e.g., ΔIS1216).

FIG 3 Genetic features flanking the cfr gene and detection of IS1216-mediated formation of a circular product by inverse PCR. (A) Comparison of plasmids pEF-01 (from E. faecalis EF-01) and pSCFS1 (from S. sciuri) in the regions flanking cfr. The positions and orientations of the genes are indicated by solid box arrows. The area of homology (1,626 bp) between the two plasmids is indicated by two dashed lines. Arrows P1, P2, P3, and P4 indicate the locations and orientation of the primers used for inverse PCR. (B) A circular product detected by inverse PCR and confirmed by sequence analysis. The locations and orientation of the primers (P1 to P4) used for inverse PCR are indicated by arrows. (C) Agarose gel analysis of the inverse PCR products amplified with primers P1 and P2 (lane 1) or primers P3 and P4 (lane 2). Lane M contains the Trans15K DNA marker.
Moreover, identical inverse PCR products were amplified using primers P1 (5′-TGAGTCTCGGTAGATCCG TGT-3′) and P2 (5′-TTTGCTCTCGTAAGACGTGAT-3′) and total DNA purified from EF-01 as the template. Indeed, a ca. 13-kb PCR product was obtained, and sequence analysis of the PCR product confirmed that it contained one copy of IS1216 and the intact region between the two direct repeat copies of IS1216, as shown in Fig. 3B and C. To further confirm the formation of a circular form, a second inverse PCR assay was conducted using primers P3 (5′-GACTGGCTTGATCTAAACCG-3′) and P4 (5′-GAGTCTCTAATGAACCCAATACAG-3′), which are located near the two direct repeat copies of IS1216 (Fig. 3A). This inverse PCR yielded a ca. 1.5-kb product that contained one intact copy of IS1216 and partial sequences of hpa2 and ΔrecF1 (Fig. 3B and C). Moreover, identical inverse PCR products were amplified using either plasmid or whole-cell DNA of the original strain EF-01 and its transformant JH2-2-tPEF-01. These results might suggest that intraplasmid recombination occurred between the two direct repeat copies of IS1216 in pEF-01 (Fig. 3B). Thus, the association of cfr with IS1216 may facilitate its dissemination; however, the definitive role of IS1216 in mobilizing cfr remains to be confirmed in future studies.

In conclusion, we report the first identification of a cfr-carrying plasmid in E. faecalis of animal origin and present evidence for the involvement of IS1216 in the mobility of the cfr gene. Given that enterococci are widely distributed in the environment and are known to be able to transmit antibiotic resistance determinants to other pathogens (18), a mobile cfr gene in this Gram-positive organism will likely increase the spread of this multiresistance determinant. Thus, an enhanced surveillance effort is needed to monitor the emergence and spread of cfr in enterococci and other pathogens in clinical settings.

Nucleotide sequence accession number. The sequence of a 32,388-bp sequence of the cfr-carrying plasmid pEF-01 has been deposited in the GenBank database under accession no. NC_014508.

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