

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

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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.

The *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 *Staphylococcus sciuri* in 2000 (21), several studies have reported the *cfr* gene in staphylococcal isolates of animal and human origin. In most reports, the *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, *cfr*-carrying *Staphylococcus aureus* was reported in Spain (17), and the *cfr* gene has also been identified in Pantone-Valentine leukocidin-positive sequence type 8 (ST8) methicillin-resistant *S. aureus* IVa (USA300) (23). These observations underline the increasing threat of this resistance determinant to public health. In recent studies, the *cfr*-harboring plasmids pBS-01 and pBS-02 have also been identified in *Bacillus* strains from swine feces (5, 29). In addition, a poster presented by Cercenado and coworkers described two human clinical isolates of *cfr*-carrying enterococci, one *Enterococcus faecalis* and one *Enterococcus faecium* (2). To date, there has been no report of *cfr* in *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 *Enterococcus*-specific PCR (6) and confirmed as *E. faecalis* by the Rapid ID 32 Strep system (bioMérieux, Craponne, France).

Isolate EF-01 was screened for the genes *cfr* and *fexA* using previously described primers (5). A *cfr*-specific PCR product was obtained and confirmed by sequence analysis. To determine the location of the *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 PFG markers (NEB, United Kingdom) (Fig. 1A). In a Southern blot analysis, a *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 μ g/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 *E. faecalis* JH2-2 and protoplasts of *S. aureus* RN4220 by electrotransformation (4, 19). The transformants were selected on brain heart infusion (BHI) agar supplemented with 10 μ g/ml florfenicol. Additionally, conjugative mating into *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 *cfr* gene in the new host bacteria.

The *cfr* gene of pEF-01 encodes a 349-aa protein which differs from the Cfr proteins of pSCFS1 and pSCFS3 by only two

Received 8 November 2011 Returned for modification 4 December 2011

Accepted 18 December 2011

Published ahead of print 27 December 2011

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Supplemental material for this article may be found at <http://aac.asm.org/>.

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doi:10.1128/AAC.06091-11

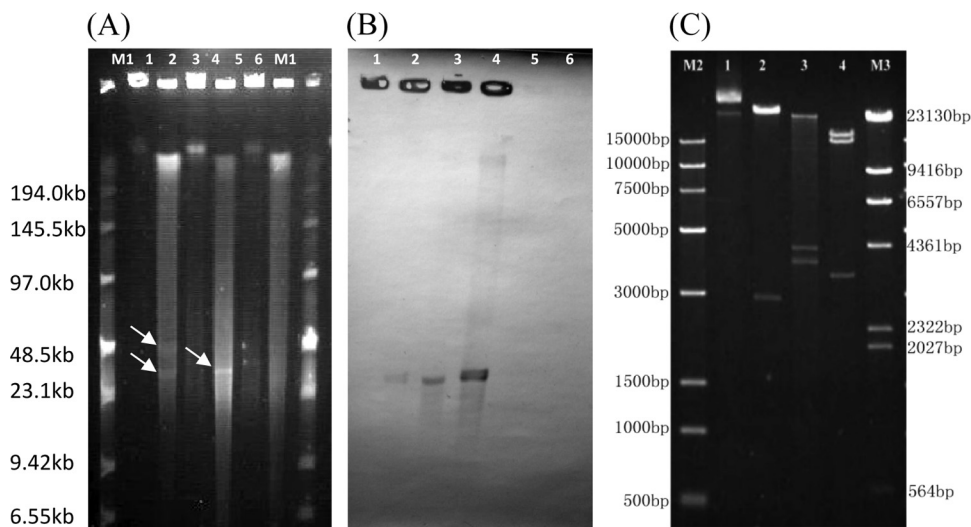


FIG 1 Identification and characterization of *cfr*-carrying pEF-01. (A) Plasmid size determination by S1 nuclease treatment coupled with pulsed-field gel electrophoresis. Lane M1, low-range PFG marker (NEB); lanes 1 and 2, EF-01; lanes 3 and 4, the transformant JH2-2+pEF-01; lanes 5 and 6, *E. faecalis* JH2-2 (control for lanes 3 and 4). The samples (gel plug) in lanes 1, 3, and 5 were not treated with S1 nuclease, while the samples in lanes 2, 4, and 6 were incubated with S1 nuclease prior to electrophoresis. Arrows indicate the locations of plasmids. (B) Southern blot hybridization with the *cfr* probe. The lane numbers correspond to those in panel A. (C) Restriction digestion analysis of plasmid pEF-01, extracted from *E. faecalis* JH2-2+pEF-01. Lane 1 contains the nondigested plasmid pEF-01, while lanes 2, 3, and 4 contain the plasmid DNA digested with StuI, EcoRI, and XbaI, respectively. Lanes M2 and M3 contain the *Trans15K* (TransGen) and λ -HindIII digest (TaKaRa) DNA markers, respectively.

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 walking 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 and manually by correlation scores of the open reading frames (ORFs) with ≥ 50 amino acids. Sequence comparison was performed using the BLAST system (<http://www.ncbi.nlm.nih.gov/BLAST/>). The final assembly of the whole plasmid was verified by two methods. Based on the assembled plasmid sequence, we designed multiple pairs of PCR primers, and by using these primers, we obtained amplicons of the expected sizes from JH2-2+pEF-01. The amplicons were sequenced, and they confirmed the assembled plasmid sequence. Second, we conducted restriction analyses of the plasmid using EcoRI, HindIII, MluI, NheI, NdeI, StuI, XbaI, and XhoI. All obtained fragment pat-

terns were consistent with the assembled plasmid sequence. Some of the restriction patterns are shown in Fig. 1C.

Plasmid pEF-01 consists of 32,388 bp and contains 30 potential coding sequences (CDSs) for proteins of ≥ 50 aa. The putative functions of 28 CDSs were predicted on the basis of their sequence homology to previously characterized proteins (see Table S1 in the supplemental material). The G+C content of pEF-01 is 35.3%, which is similar to that of *E. faecalis* genomic DNA (13). The G+C contents of individual CDSs ranged from 27.7% to 47.3%, suggesting that pEF-01 is composed of DNA regions which originated from multiple sources. A circular genetic map of pEF-01 is shown in Fig. 2. In addition to *cfr*, the phenicol exporter gene *fecB* (CDS10), recently described in *E. faecium* and *Enterococcus hirae*, was 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 observations. 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*

Antimicrobial agent	MIC ($\mu\text{g/ml}$) for ^a :				
	<i>E. faecalis</i> strain			<i>S. aureus</i> strain	
	EF-01	JH2-2	JH2-2+pEF-01	RN4220	RN4220+pEF-01
Chloramphenicol	64	8	32	4	64
Florfenicol	128	8	64	4	128
Linezolid	4	2	8	2	8
Tiamulin	–	–	–	≤ 0.5	32
Clindamycin	–	–	–	≤ 0.0625	8

^a –, MIC was not measured.

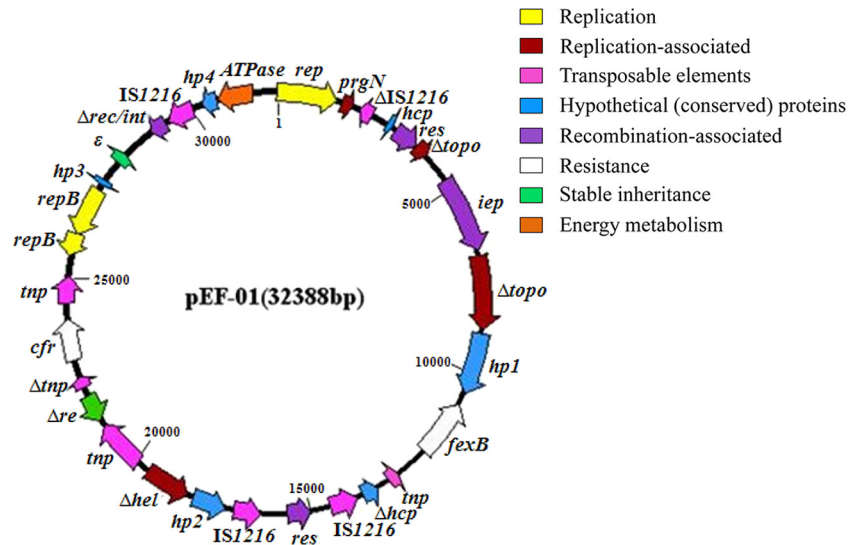


FIG 2 Genetic map of pEF-01. Coding regions larger than 50 amino acids are represented by arrows indicating the direction of transcription. All CDSs are colored according to their predicted functions. Truncated CDSs are indicated with a Greek delta symbol (e.g., Δ IS1216).

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 multi-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.

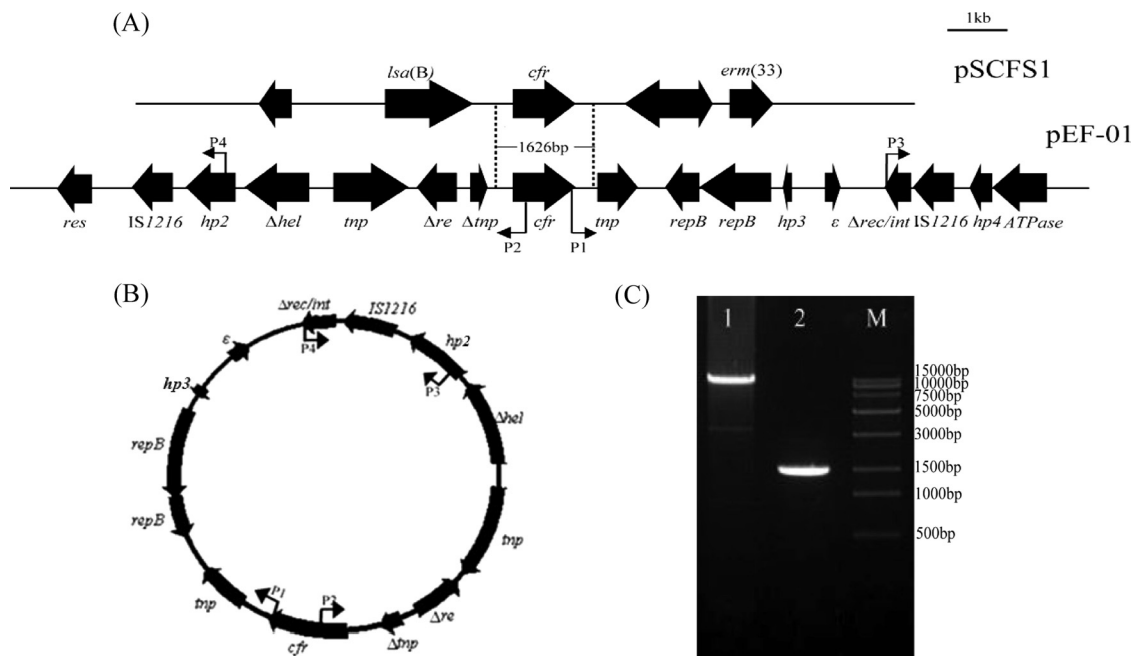


FIG 3 Genetic features flanking the *cfr* gene and detection of IS1216-mediated formation of a circular product by inverse PCR. (A) Comparison of plasmids pEF-01 (from *E. faecalis* EF-01) and pSCFS1 (from *S. sciuri*) in the regions flanking *cfr*. The positions and orientations of the genes are indicated 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.

In pEF-01, two direct repeat copies of the *IS1216* element flanked a 12.4-kb segment that contains the *cfr* gene (Fig. 3A). It has been reported that *IS1216* plays an important role in the dissemination of antimicrobial resistance determinants (28) and in plasmid recombination (20). Since IS-mediated transpositions involve a simple cut-and-paste mechanism and the formation of circular forms (7, 9), we performed inverse PCR to detect whether *IS1216* in pEF-01 mediated the formation of a *cfr*-carrying circular form. The inverse PCR assay was first performed using primers P1 (5'-TGAGTCTGCTGGTATCCA TGT-3') and P2 (5'-TTTGCTCTGCTAAGAGCTTGAT-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'-GAGTCCTAATGAACCAATACAG-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 *hp2* and $\Delta rec/int$ (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 + pEF-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.

ACKNOWLEDGMENTS

We thank Don B. Clewell (University of Michigan) for providing *Enterococcus faecalis* reference strain JH2-2. We also thank Anne-Kathrin Schink (Friedrich-Loeffler-Institut) for expert assistance in the S1 nuclease PFGE assay.

This work was supported by grants from the National Natural Science Foundation of China (no. 31001087 and no. U1031004), the Program for Chang Jiang Scholars, and the Innovative Research Team at the University of China (no. IRT0866).

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