AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Characterization of a *cfr*-Carrying Plasmid from Porcine *Escherichia coli* That Closely Resembles Plasmid pEA3 from the Plant Pathogen *Erwinia amylovora*

Rongmin Zhang,[a] Bin Sun,[b] Yang Wang,[a] Lei Lei,[a] Stefan Schwarz,[a,c] Congming Wu[a]

Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety, College of Veterinary Medicine, China Agricultural University, Beijing, People's Republic of China[a]; Animal Science and Veterinary Medicine College of Heilongjiang Bayi Agriculture University, Daqing, Heilongjiang, People's Republic of China[b]; Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany[c]

The multiresistance gene *cfr* was found in two porcine *Escherichia coli* isolates, one harboring it on the conjugative 33,885-bp plasmid pFSEC-01, the other harboring it in the chromosomal DNA. Sequence analysis of pFSEC-01 revealed that a 6,769-bp fragment containing the *cfr* gene bracketed by two IS*26* elements was inserted into a plasmid closely related to pEA3 from the plant pathogen *Erwinia amylovora*, suggesting that pFSEC-01 may be transferred between different bacterial genera of both animal and plant origin.

The multiresistance gene *cfr* confers resistance to phenicols, lincosamides, pleuromutilins, streptogramin, and oxazolidinones and decreased susceptibility to the 16-membered macrolides spiramycin and josamycin (1–3). While the *cfr* gene is found mainly in Gram-positive bacteria (4), it has also been detected in two species of Gram-negative bacteria, *Proteus vulgaris* and *Escherichia coli*, during recent years (5–10). Here, we report IS*26*-flanked *cfr* genes from porcine *E. coli* are located either on a plasmid that most likely originated from the plant pathogen *Erwinia amylovora* or in the chromosomal DNA.

During July and August 2013, a total of 396 samples were collected from the lungs of diseased animals (pigs, $n = 334$; chickens, $n = 40$; ducks, $n = 22$) at an animal diagnostic laboratory at Foshan University, Foshan City, Guangdong, China. A total of 64 *E. coli* isolates grew on MacConkey agar plates supplemented with 10 mg/liter florfenicol. Of these, only two isolates, FSEC-01 and FSEC-02, which were obtained from swine suffering from pneumonia and sepsis, respectively, that originated from two different pig farms were positive for the *cfr* gene by PCR as described previously (11, 12).

In vitro susceptibility testing (13, 14) showed that isolates FSEC-01 and FSEC-02 were resistant to chloramphenicol, ampicillin, tetracycline, gentamicin, and streptomycin and also had high MIC values of florfenicol (128 μg/ml). Isolate FSEC-02 also exhibited resistance to amoxicillin-clavulanic acid and sulfamethoxazole. In addition to harboring the *cfr* gene, both isolates harbored the phenicol exporter gene *floR*, the β-lactam resistance gene bla_{TEM-1}, and the tetracycline resistance gene *tet*(A). Isolate FSEC-01 also carried the aminoglycoside resistance gene *aacC2* and the β-lactam resistance gene $bla_{CTX-M-65}$, whereas isolate FSEC-02 also harbored the aminoglycoside resistance genes *aac(3)-VI*, *aadA2*, *aacC3*, *strA*, and *strB*, the sulfonamide resistance gene *sul2*, and the β-lactam resistance gene bla_{OXA-30} (Table 1).

S1 nuclease pulsed-field gel electrophoresis (PFGE) and Southern blot analysis (15) showed that the *cfr* gene was located on an ~34-kb plasmid, designated pFSEC-01, in isolate FSEC-01 and in the chromosomal DNA of isolate FSEC-02 (see Fig. S1 in the supplemental material). Conjugation experiments by filter mating (15) using isolate FSEC-01 as the donor and *E. coli* C600 as the recipient proved to be successful. The transconjugant, designated EC-600-FS-01, which harbored only the *cfr*-carrying plasmid pFSEC-01, was negative for *floR* and exhibited resistance to both chloramphenicol and florfenicol (Table 1), suggesting that *cfr* was functionally active.

The complete DNA sequence of the *cfr*-carrying plasmid pFSEC-01, extracted from a transconjugant, and the regions flanking the *cfr* gene in the chromosome of FSEC-02 were obtained by whole-plasmid and genomic-DNA sequencing, respectively, using the Illumina HiSeq 2500 system, which produced 125-bp paired-end reads (Berry Genomics Company, Beijing, China). The draft assembly of the sequences was generated using Genomics Workbench 5 (CLC Bio, Aarhus, Denmark). The gap closure was performed by a modified random-primer walking strategy (15), using the primers listed in Table S1 in the supplemental material. The plasmid pFSEC-01 had a size of 33,885 bp, assembled from two contigs (4,390 bp and 27,928 bp), with a coverage of >5,000-fold and subsequent gap closure. The average GC content was 44.8%, and 27 predicted open reading frames coding for proteins of ≥100 amino acids were detected. The plasmid contained two main regions (region A and region B), which differed in GC content (Fig. 1). The 5,990-bp fragment located in 6,769-bp region A, containing the *cfr* gene flanked by two copies of IS*26* located in the same orientation, had a distinctly low GC content of 38.9% and showed 99.5% nucleotide sequence identity to the corresponding region of plasmid pSD6 (GenBank accession

Received 31 August 2015 **Returned for modification** 28 September 2015 **Accepted** 26 October 2015

Accepted manuscript posted online 2 November 2015

Citation Zhang R, Sun B, Wang Y, Lei L, Schwarz S, Wu C. 2016. Characterization of a *cfr*-carrying plasmid from porcine *Escherichia coli* that closely resembles plasmid pEA3 from the plant pathogen *Erwinia amylovora*. Antimicrob Agents Chemother 60:658–661. doi:10.1128/AAC.02114-15.

Address correspondence to Congming Wu, wucm@cau.edu.cn, or Stefan Schwarz, stefan.schwarz@fli.bund.de.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02114-15.

TABLE 1 Antimicrobial susceptibility profiles of *E. coli* FSEC-01, FSEC-02, and EC-600 and the transconjugant EC-600-FS-01, which contains plasmid pFSEC-01

Bacterial isolate	Resistance gene(s)	MIC (mg/liter) of[a]:								
		CHL	FFC	AMP	AMC	TET	SMZ	GEN	STR	AMK
FSEC-01	*cfr*, *floR*, *aacC2*, bla_{TEM-1}, $bla_{CTX-M-65}$, *tet*(A)	512	128	512	8/16	256	32	64	512	4
FSEC-02	*cfr*, *floR*, *aac(3)-IV*, *aadA2*, *strA*, *strB*, *aacC3*, *sul2*, bla_{OXA-30}, bla_{TEM-1}, *tet*(A)	512	128	512	32/64	256	>512	128	512	16
EC-600-FS-01	*cfr*	128	64	1	2/4	0.5	16	1	2	1
EC-600		2	8	1	2/4	0.5	16	1	2	1

[a] The antimicrobial agents are abbreviated as follows: CHL, chloramphenicol; FFC, florfenicol; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; TET, tetracycline; SMZ, sulfamethoxazole; GEN, gentamicin; STR, streptomycin; and AMK, amikacin.

number NG_041755.1) from porcine *E. coli* 8GZ12D (Fig. 1 and 2) (8). Fragments of 1,700 bp and 1,492 bp in region A, comprising mainly the *rep* gene and the *cfr* gene, respectively, shared >99.9% nucleotide identity to the corresponding regions of plasmid pSA8589 (GenBank accession no. KC561137.1) from *Staphylococcus aureus* isolated from a medical institution in Ohio, USA (16) (Fig. 2). No direct repeats were found immediately upstream and downstream of these IS*26* elements in region A (Fig. 2). Moreover, no *cfr*-carrying intermediate circular forms were detected by inverse PCR in either FSEC-01 or the transconjugant EC-600-FS-01. The entire 5,990-bp region is the internal part of a 6,769-bp segment that was inserted into the pEA3 backbone. The insertion of this segment was accompanied by the loss of a 1,412-bp segment in pEA3 which harbored mainly genes for hypothetical proteins 5 to 8 (Fig. 2).

Region B of pFSEC-01 had a size of 27,116 bp and a high GC content (46.1%) (Fig. 1). It displayed 98.2% nucleotide sequence identity to the corresponding region in plasmid pEA3. Plasmid

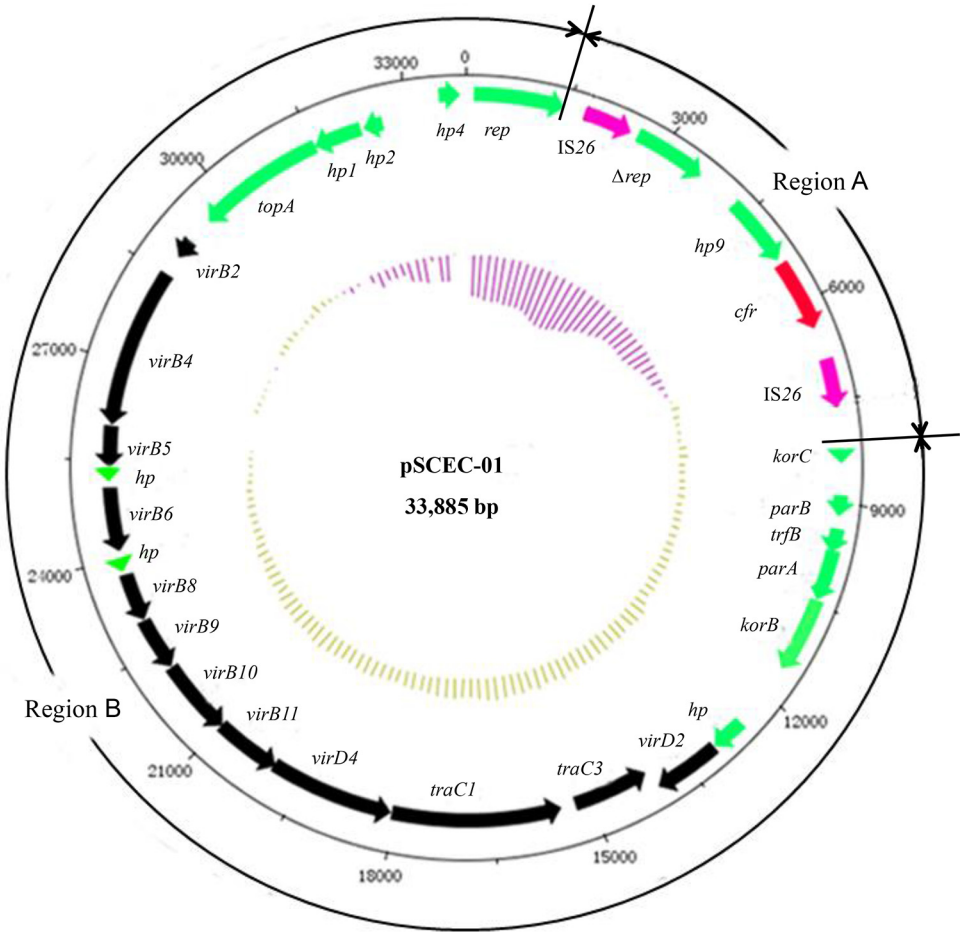

FIG 1 Circular representation of plasmid pFSEC-01. The circles display (from outside to inside) (i) sizes in base pairs, (ii) the positions of predicted coding sequences, with arrows indicating the positions and directions of transcription of the genes, and (iii) the GC contents plotted against 44.8%, with pink indicating <44.8% and yellow indicating >44.8% GC. Genes are color coded, depending on functional annotations, as follows: pink, transposition; black, type IV secretory components; red, antimicrobial resistance; and green, transposition/recombination/replication and other putative functions/hypothetical proteins.

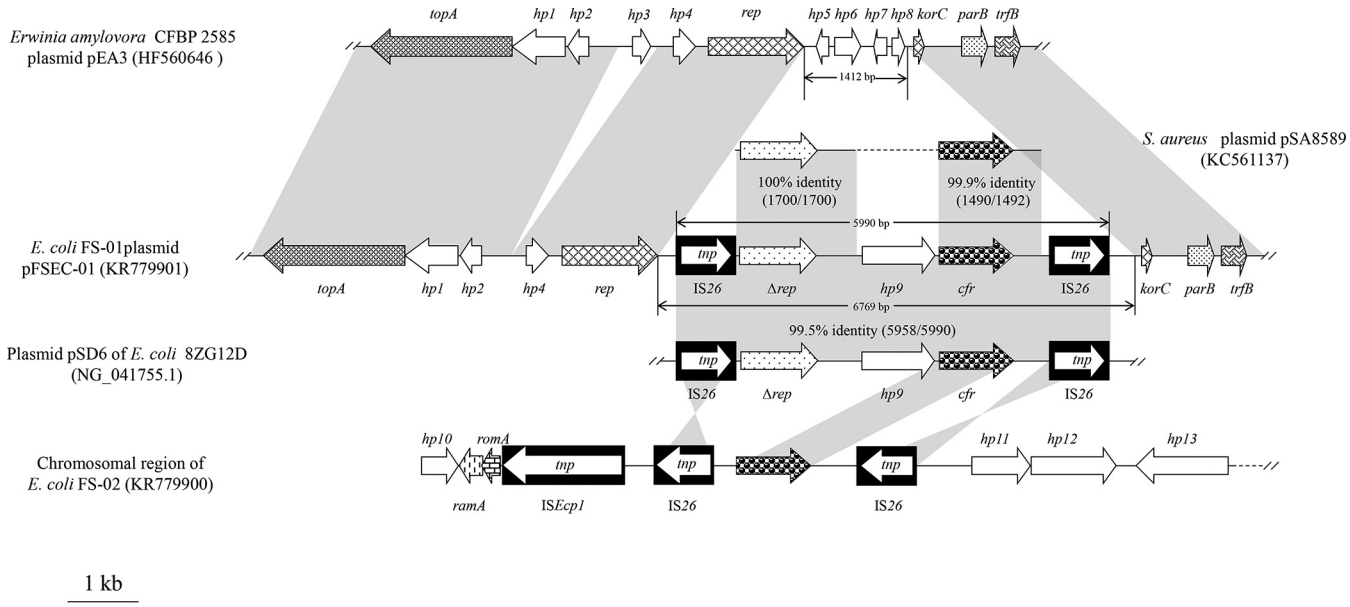

FIG 2 Genetic environment of the *cfr* gene in plasmid pFSEC-01 and in the chromosome of FSEC-02. A structural comparison was made with plasmid pEA3 from *E. amylovora* CFBP 2585 (plant origin), plasmid pSA8589 from *S. aureus*, and plasmid pSD6 from *E. coli* 8ZG12D. The arrows indicate the positions and directions of transcription of the genes. Different genes are indicated by different types of shading. Regions of ≥97% nucleotide sequence identity are marked by gray shading.

pEA3 has a size of 29,585 bp and was originally identified in the plant pathogen *Erwinia amylovora* CFBP 2585 (GenBank accession number HF560646.1), which causes fire blight, a devastating disease that threatens a wide range of plants, including apple, pear, cotoneaster, and hawthorn shrubs and trees (17, 18). Region B also harbored the genes of a type IV secretion system (T4SS) gene cluster (Fig. 1). This T4SS might play a role in the conjugative transfer of plasmids, and its presence might explain how a basic pEA3 plasmid might have been transferred between the plant pathogen *E. amylovora* and *E. coli* of swine origin. However, it is unknown whether the plasmids pFSEC-01 and pEA3 were directly transferred between *E. amylovora* and *E. coli* or whether other intermediate-host bacteria have also been involved in the dissemination of these plasmids. It is also not known where and when the insertion of the IS*26*-flanked *cfr* segment into the pEA3 replicon occurred.

In the chromosomal DNA of FSEC-02, a fragment of 18,482 bp comprising the *cfr* gene was assembled from three contigs (2,945, 2,062, and 11,492 bp) with a >500-fold coverage and subsequent gap closure (see Fig. S2 in the supplemental material). The primers used for confirmation of the fragment order are listed in Table S2. Similarly to the situation in pFSEC-01, the *cfr* gene in the chromosomal DNA of FSEC-02 was bracketed by two IS*26* elements in the same orientation. However, the IS*26* elements in isolate FCEC-02 were in the orientation opposite to that of the *cfr* gene (Fig. 2). A 2,130-bp amplicon containing the *cfr* gene and one intact IS*26* was obtained from FSEC-02 by inverse PCR, suggesting that this *cfr*-carrying segment might be looped out via IS*26*-mediated recombination. To date, 18 *cfr*-carrying Gram-negative bacterial isolates, including the two *E. coli* isolates from this study, have been reported, and in 17 of them, the *cfr* gene was bracketed by two copies of IS*26* (5, 6, 8–10). This is further evidence that IS*26* plays a role in the dissemination of the *cfr* gene in Gram-negative bacteria, not only for insertion into different types of plasmids but also for integration into the chromosomal DNA.

Nucleotide sequence accession numbers. The *cfr*-carrying region in FSEC-02 and the complete sequence of plasmid pFSEC-01 have been deposited in GenBank under accession numbers KR779900 and KR779901, respectively.

ACKNOWLEDGMENTS

We thank Zongji Lv of the Animal Diagnostic Laboratory at Foshan University for sample collection.

FUNDING INFORMATION

Special Fund for Agro-Scientific Research in the Public Interest provided funding to and Congming Wu under grant number 201203040. National Natural Science Foundation of China (NSFC) provided funding to and Yang Wang under grant number 31370046. German Federal Ministry of Education and Research (BMBF) through the German Aerospace Center (DLR) provided funding to and Stefan Schwarz under grant numbers 01KI1301D and 01KI1313D.

REFERENCES

1. **Kehrenberg C, Schwarz S, Jacobsen L, Hansen LH, Vester B.** 2005. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. Mol Microbiol **57:** 1064–1073. http://dx.doi.org/10.1111/j.1365-2958.2005.04754.x.
2. **Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S, Vester B.** 2006. The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. Antimicrob Agents Chemother **50:**2500–2505. http://dx.doi.org/10.1128/AAC.00131-06.
3. **Smith LK, Mankin AS.** 2008. Transcriptional and translational control of the *mlr* operon, which confers resistance to seven classes of protein synthesis inhibitors. Antimicrob Agents Chemother **52:**1703–1712. http://dx.doi.org/10.1128/AAC.01583-07.
4. **Shen J, Wang Y, Schwarz S.** 2013. Presence and dissemination of the multiresistance gene *cfr* in Gram-positive and Gram-negative bacteria. J Antimicrob Chemother **68:**1697–1706. http://dx.doi.org/10.1093/jac/dkt092.
5. **Wang Y, He T, Schwarz S, Zhou D, Shen Z, Wu C, Wang Y, Ma L, Zhang Q, Shen J.** 2012. Detection of the staphylococcal multiresistance

gene *cfr* in *Escherichia coli* of domestic-animal origin. J Antimicrob Chemother **67:**1094–1098. http://dx.doi.org/10.1093/jac/dks020.

6. **Wang Y, Wang Y, Wu CM, Schwarz S, Shen Z, Zhang W, Zhang Q, Shen JZ.** 2011. Detection of the staphylococcal multiresistance gene *cfr* in *Proteus vulgaris* of food animal origin. J Antimicrob Chemother **66:**2521–2526. http://dx.doi.org/10.1093/jac/dkr322.
7. **Zhang WJ, Xu XR, Schwarz S, Wang XM, Dai L, Zheng HJ, Liu S.** 2014. Characterization of the IncA/C plasmid pSCEC2 from *Escherichia coli* of swine origin that harbours the multiresistance gene *cfr*. J Antimicrob Chemother **69:**385–389. http://dx.doi.org/10.1093/jac/dkt355.
8. **Deng H, Sun J, Ma J, Li L, Fang LX, Zhang Q, Liu YH, Liao XP.** 2014. Identification of the multi-resistance gene *cfr* in *Escherichia coli* isolates of animal origin. PLoS One **18:**e102378. http://dx.doi.org/10.1371/journal.pone.0102378
9. **Sun J, Deng H, Li L, Chen MY, Fang LX, Yang QE, Liu YH, Liao XP.** 2015. Complete nucleotide sequence of *cfr*-carrying IncX4 plasmid, pSD11, from *Escherichia coli*. Antimicrob Agents Chemother **59:**738–741. http://dx.doi.org/10.1128/AAC.04388-14.
10. **Zhang WJ, Wang XM, Dai L, Hua X, Dong Z, Schwarz S, Liu S.** 2015. Novel conjugative plasmid from *Escherichia coli* of swine origin that coharbors the multiresistance gene *cfr* and the extended-spectrum-beta-lactamase gene $bla_{CTX-M-14b}$. Antimicrob Agents Chemother **59:**1337–1340. http://dx.doi.org/10.1128/AAC.04631-14.
11. **Kim TW, Kim YH, Kim SE, Lee JH, Park CS, Kim HY.** 2010. Identification and distribution of *Bacillus* species in Doenjang by whole-cell protein patterns and 16S rRNA gene sequence analysis. J Microbiol Biotech **20:**1210–1214. http://dx.doi.org/10.4014/jmb.1002.02008.
12. **Zhang WJ, Wu CM, Wang Y, Shen ZQ, Dai L, Han J, Foley SL, Shen JZ, Zhang Q.** 2011. The new genetic environment of *cfr* on plasmid pBS-02 in a *Bacillus* strain. J Antimicrob Chemother **66:**1174–1175. http://dx.doi.org/10.1093/jac/dkr037.
13. **Clinical and Laboratory Standards Institute.** 2015. Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. CLSI document M100-S25. CLSI, Wayne, PA.
14. **Clinical and Laboratory Standards Institute.** 2015. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, 3rd ed. CLSI supplement VET01S. CLSI, Wayne, PA.
15. **Wang Y, Wu C, Zhang Q, Qi J, Liu H, He T, Ma L, Lai J, Shen Z, Liu Y, Shen J.** 2012. Identification of New Delhi metallo-beta-lactamase 1 in *Acinetobacter lwoffii* of food animal origin. PLoS One **7:**e37152. http://dx.doi.org/10.1371/journal.pone.0037152.
16. **Mendes RE, Deshpande LM, Bonilla HF, Schwarz S, Huband MD, Jones RN, Quinn JP.** 2013. Dissemination of a pSCFS3-like *cfr*-carrying plasmid in *Staphylococcus aureus* and *Staphylococcus epidermidis* clinical isolates recovered from hospitals in Ohio. Antimicrob Agents Chemother **57:**2923–2928. http://dx.doi.org/10.1128/AAC.00071-13.
17. **Malnoy M, Martens S, Norelli JL, Barny MA, Sundin GW, Smits TH, Duffy B.** 2012. Fire blight: applied genomic insights of the pathogen and host. Annu Rev Phytopathol **50:**475–494. http://dx.doi.org/10.1146/annurev-phyto-081211-172931.
18. **Mann RA, Smits TH, Buhlmann A, Blom J, Goesmann A, Frey JE, Plummer KM, Beer SV, Luck J, Duffy B, Rodoni B.** 2013. Comparative genomics of 12 strains of *Erwinia amylovora* identifies a pan-genome with a large conserved core. PLoS One **8:**e55644. http://dx.doi.org/10.1371/journal.pone.0055644.