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Transferable Multiresistance Plasmids Carrying *cfr* in *Enterococcus* spp. from Swine and Farm Environment

Yang Liu,[a] Yang Wang,[a] Stefan Schwarz,[b] Yun Li,[c] Zhangqi Shen,[d] Qijing Zhang,[d] Congming Wu,[a] Jianzhong Shen[a]

Key Laboratory of Development and Evaluation of Chemical and Herbal Drugs for Animal Use, College of Veterinary Medicine, China Agricultural University, Beijing, People's Republic of China[a]; Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany[b]; Shijiazhuang Livestock Products Quality Inspection and Supervision Center, Shijiazhuang, Hebei, People's Republic of China[c]; Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA[d]

Seventy-seven porcine *Enterococcus* isolates with florfenicol MICs of ≥16 μg of were/ml screened for the presence of the multiresistance gene *cfr*, its location on plasmids, and its genetic environment. Three isolates—*Enterococcus thailandicus* 3-38 (from a porcine rectal swab collected at a pig farm), *Enterococcus thailandicus* W3, and *Enterococcus faecalis* W9-2 (the latter two from sewage at a different farm), carried the *cfr* gene. The SmaI pulsed-field gel electrophoresis patterns of the three isolates differed distinctly. In addition, *E. faecalis* W9-2 was assigned to a new multilocus sequence type ST469. Mating experiments and Southern blot analysis indicated that *cfr* is located on conjugative plasmids pW3 (~75 kb) from *E. thailandicus* W3, p3-38 (~72 kb) from *E. thailandicus* 3-38, and pW9-2 (~55 kb) from *E. faecalis* W9-2; these plasmids differed in their sizes, additional resistance genes, and the analysis of the segments encompassing the *cfr* gene. Sequence analysis revealed that all plasmids harbored a 4,447-bp central region, in which *cfr* was bracketed by two copies of the novel insertion sequence IS*Enfa4* located in the same orientation. The sequences flanking the central regions of these plasmids, including the partial *tra* gene regions and a ω-ε-ζ toxin-antitoxin module, exhibited >95% nucleotide sequence identity to the conjugative plasmid pAMβ1 from *E. faecalis*. Conjugative plasmids carrying *cfr* appear to play an important role in the dissemination and maintenance of the multiresistance gene *cfr* among enterococcal isolates and possibly other species of Gram-positive bacteria.

Enterococci are Gram-positive bacteria that generally colonize the gastrointestinal tracts of animals. They are also found in food, water, and environmental samples. Enterococci were once considered harmless commensals of humans, but they have emerged as important nosocomial pathogens over the past 3 decades. *Enterococcus* spp., especially *Enterococcus faecalis* and *Enterococcus faecium*, are now the second major cause of surgical and urinary tract infections and the third major cause of bacteremia (1). The ability of *Enterococcus* spp. to acquire mobile genetic elements, such as plasmids and transposons carrying the determinants of antimicrobial resistance and virulence, has contributed to their emergence as often multiresistant pathogens. Antimicrobial multiresistance drastically limits the therapeutic options in the treatment of infections, and the emergence and rapid spread of glycopeptide-resistant enterococci has presented a particular challenge, since there are few remaining options for antimicrobial treatment (2). As donors and recipients of antimicrobial resistance genes, *Enterococcus* spp. play an important role in the dissemination of resistance genes by horizontal gene transfer. The transmission of the *vanA* gene cluster-carrying transposon Tn*1546* from vancomycin-resistant enterococci (VRE) to methicillin-resistant *Staphylococcus aureus* (MRSA), creating a vancomycin- and methicillin-resistant *S. aureus* strain in a hospitalized patient, represents a dramatic example of this phenomenon (3).

As the first oxazolidinone introduced for clinical use in the United States since 2000 and in China since 2007, linezolid is currently the most efficient antimicrobial against VRE, MRSA, and penicillin-resistant pneumococci (4). As a last-resort antimicrobial agent, linezolid not only exhibits strong antibacterial activity but also has properties that mitigate against the development of drug resistance in bacteria (5). No evidence of a significant increase in the percentage of linezolid-resistant bacteria has been reported in the LEADER Program, whose results are available on an annual basis since 2006 (6). The most recent results from the LEADER Program for the year 2009 reported an overall percentage of linezolid resistance of 0.34% (6). The most common mechanism of linezolid resistance involves mutations in the central loop of domain V of 23S rRNA, with G2576T (*Escherichia coli* numbering) being the most frequent mutation (7), followed by mutations in the genes for the ribosomal proteins L3 and L4 (8). Transferable linezolid resistance is due to the gene *cfr* encoding an rRNA methyltransferase which methylates position 8 of A2503 (9) and inhibits ribose methylation at nucleotide C2498 in the 23S rRNA (10). Thereby, Cfr confers resistance to five chemically unrelated antimicrobial classes including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (11) and decreased susceptibility to the 16-membered macrolides spiramycin and josamycin (12).

The observation that the gene *cfr* is often located on plasmids underlines the potential for the spread of this gene (11). Although it was initially discovered on the 17.1-kb multiresistance plasmid, pSCFS1, in a bovine *Staphylococcus sciuri* isolate (13), the gene *cfr* has been detected in seven bacterial genera, including *Staphylococcus*, *Bacillus*, *Enterococcus*, *Macrococcus*, *Jeotgalicoccus*, *Proteus*, and *Escherichia*. Most of the corresponding isolates were derived

Received 2 August 2012 **Returned for modification** 29 August 2012
Accepted 4 October 2012
Published ahead of print 15 October 2012
Address correspondence to Jianzhong Shen, sjz@cau.edu.cn.

doi:10.1128/AAC.01605-12

TABLE 1 Overviewof the so far identified *cfr*-carryingplasmids in different bacteria

Host bacteria	Plasmid	Size (kb)	Reference
Staphylococcus spp.	pSCFS1	17.1	24
	pSCFS3	35.7	16
	pSCFS6	~43	24
	pSCFS7	~45	25
	p004-737X	~55	26
	p426-3147L	~175	26
	pSS-01	~40	22
	pSS-02	~35.4	22
	pSS-03	7.1	22
	pERGB	~50	27
Bacillus spp.	pBS-01	16.5	28
	pBS-02	16.5	23
	pBS-03	7.4	19
Jeotgalicoccus and *Macrococcus* spp.	pJP1	~53	21
E. faecalis	pEF-01	32.4	17
	pHOU-cfr	~97	14
E. coli	pEC-01	~110	29

from domestic animals (mainly pigs) (14–23), and plasmids seemed to play an important role in the interspecies and intergenus transfer of the *cfr* gene. To date, 17 different *cfr*-carrying plasmids have been reported (Table 1). Among these plasmids, only the staphylococcal plasmids pSCFS7 and pERGB and the enterococcal plasmid pHOU-*cfr* were characterized as conjugative plasmids (14, 25, 27). In the present study, we describe three transferable multiresistance plasmids obtained from *Enterococcus* spp. from domestic pigs and farm environments which carry the *cfr* gene in new genetic environments.

MATERIALS AND METHODS

Bacterial isolates and detection of florfenicol resistance genes. A total of 77 *Enterococcus* isolates (*E. faecalis* [*n* = 49], *E. faecium* [*n* = 15], *E. hirae* [*n* = 6], *E. thailandicus* [*n* = 3], *E. durans* [*n* = 2], *E. asini* [*n* = 1], and *Enterococcus* sp. strain CIFRI D-TSB15 [*n* = 1]) with florfenicol MIC values of ≥16 μg of were/ml collected from individual pigs (*n* = 65; 57 from rectal swabs and 8 from nasal swabs), pig farm environments (*n* = 9; 8 from sewage and 1 from soil), and farm workers (*n* = 3; all from rectal swabs) in three pig farms and one slaughterhouse in Shandong province in 2011. Although no clinical breakpoints for florfenicol applicable to enterococci are currently available, isolates with an MIC of ≥16 μg of were/ml tentatively considered as florfenicol resistant. Whole-cell DNA from the *Enterococcus* isolates was extracted using a commercial kit (TianGen, Beijing, China) according to the manufacturer's instructions. The presence of the florfenicol resistance genes *cfr*, *fexA*, and *fexB* was investigated using previously described methods (28, 30).

Molecular typing. Pulsed-field gel electrophoresis (PFGE) was performed to investigate the clonality of the *cfr*-positive enterococcus isolates. DNA preparation and subsequent restriction analysis were conducted as described previously (31). Whole-cell DNA was incubated with SmaI (TaKaRa, Dalian, China) for 6 h at 30°C. The resulting restriction fragments were separated using a CHEF-DRIII system (Bio-Rad) with a clamped homogeneous electric field of 6 V/cm, using a 120° switch angle for 16 h at 14°C, with the pulse time linearly ramped from 1 s to 20 s. The *cfr*-positive bovine *E. faecalis* isolate EF-01, which was the first *Enterococcus* isolate of animal origin carrying the *cfr* gene (17), was included for comparison. In addition, typing of the *cfr*-positive *E. faecalis* isolates was performed by multilocus sequence typing (MLST), using an established set of MLST primers described previously (32).

Filter mating. Filter mating was performed with each of the three *cfr*-positive original isolates using *E. faecalis* JH2-2 (rifampin resistance) as the recipient, following the method described previously (33). The concentrations of antibiotics in brain heart infusion agar plates used for the selection of transconjugants were 25 μg/ml for rifampin and 10 μg/ml for florfenicol. Transconjugants were confirmed as *cfr* positive and *fexA* and *fexB* negative by PCR analysis; PFGE was also performed in all transconjugants using SmaI to confirm that they were derivatives of the recipient strain JH2-2.

Antimicrobial susceptibility testing. The MICs of all of the *cfr*-positive original *Enterococcus* isolates, transconjugants, and *E. faecalis* JH2-2 were determined using broth microdilution according to the recommendations of documents M31-A3 (34) and M100-S21 (35) of the Clinical and Laboratory Standards Institute. The reference strain *E. faecalis* ATCC 29212 served as a quality control.

Analysis of *cfr*-carrying plasmids. To estimate the size of the *cfr*-carrying plasmids, whole-cell DNA of the enterococcal isolates in agarose gel plugs were treated with S1 nuclease (TaKaRa) and then separated by PFGE as described previously (36, 37); gels were run for 14 h. Plasmid DNA was extracted using a plasmid extraction midi kit (Qiagen, Germany) using a modification described previously (17). The extracted plasmids and S1 nuclease-linearized PFGE-separated plasmid DNA fragments were transferred to Hybond N+ membranes (Amersham Biosciences, USA) and hybridized with digoxigenin (DIG)-labeled *cfr*-, *fexA*-, or *fexB*-specific probes. The DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Sciences, Germany) was used for detection. The regions flanking the *cfr* gene were sequenced by a modified random primer sequencing walking strategy, as previously described (38). To determine the stability of the IS*Enfa4* flanked segments in plasmids in both the original strains and in the transconjugants, inverse PCR was performed using the primers cfrIF and cfrIR (these are complementary to locations inside the *cfr* gene) (22).

Nucleotide sequence accession numbers. The nucleotide sequences of a 27,360-bp segment of plasmid pW3, a 21,116-bp fragment of p3-38, and a 25,761-bp segment of pW9-2 have been deposited in GenBank under accession numbers JQ911739 (pW3), JQ911740 (p3-38), and JQ911741 (pW9-2), respectively.

RESULTS

Identification of the florfenicol resistance genes *cfr*, *fexA*, and *fexB* in *Enterococcus* isolates. PCR analysis of whole-cell DNA with primers specific for the three florfenicol resistance genes—*cfr*, *fexA*, and *fexB*—thus far identified in *Enterococcus* demonstrated that these genes were present alone or in different combinations in most of the isolates included in the present study. Of the 77 enterococci studied, 68 isolates carried either *fexA* (*n* = 36), *fexB* (*n* = 14), or both genes (*n* = 18); only two isolates (*E. thailandicus* 3-38 and *E. faecalis* W9-2) carried both *cfr* and *fexA*, whereas only one isolate (*E. thailandicus* W3) carried both *cfr* and *fexB*. Interestingly, six isolates did not harbor any of these genes. Of the three *cfr*-positive isolates, *E. thailandicus* 3-38 was collected from a rectal swab of a pig, whereas *E. thailandicus* W3 and *E. faecalis* W9-2 were collected from sewage at a different pig farm. The nucleotide sequences of the amplified *cfr* genes in these three strains were 100% identical to the *cfr* gene of the *S. sciuri* plasmid pSCFS1 (accession number NC_005076). The 977-bp *fexA* amplicon from *E. faecalis* W9-2 was identical to the corresponding *fexA* sequence of the *S. aureus* plasmid pSCFS7 (FN995110), whereas a single nucleotide exchange (T→A) at position 551 was detected in the *fexA* amplicon of *E. thailandicus* 3-38R, which caused an amino acid change at position 71 of the phenicol exporter protein

TABLE 2 Antimicrobial susceptibility profiles of *cfr*-positive enterococci isolates W3, 3-38, and W9-2 and their transconjugants JHW3, JH3-38 and JHW9-2, for which *E. faecalis* JH2-2 served as recipient strain

Isolate	Organism	Origin	MIC(μg/ml)[a]							
			CHL	FFC	LIZ	ERY	TET	AMP	VAN	CIP
W3	*E. thailandicus*	Sewage	64	64	4	>128	128	≤1	≤1	>8
3-38	*E. thailandicus*	Swine	64	64	8	>128	128	4	≤1	>8
W9-2	*E. faecalis*	Sewage	64	128	4	>128	128	≤1	≤1	>8
JH2-2	*E. faecalis*	NA[b]	8	8	2	0.25	2	≤1	≤1	2
JHW3	*E. faecalis*	NA	32	32	8	8	2	≤1	≤1	2
JH3-38	*E. faecalis*	NA	32	32	8	8	2	≤1	≤1	2
JHW9-2	*E. faecalis*	NA	32	32	8	8	2	≤1	≤1	2

[a] CHL, chloramphenicol; FFC, florfenicol; LIZ, linezolid; ERY, erythromycin; TET, tetracycline; AMP, ampicillin; VAN, vancomycin; CIP, ciprofloxacin.
[b] NA, not applicable.

(Phe→Ile). Finally, the 786-bp *fexB* amplicon obtained from *E. thailandicus* W3 was identical to *fexB* from plasmid pEFM-1 (JN201336) of *E. faecium*.

Antimicrobial resistance, clonality, and plasmid profiles of the *cfr*-positive enterococci isolates. All three *cfr*-positive isolates exhibited resistance to chloramphenicol, erythromycin, tetracycline, ciprofloxacin, and rifampin and had elevated MICs against florfenicol (up to 64 to 128 μg/ml) but were susceptible to ampicillin and vancomycin. In addition, the MICs for linezolid were 8 μg/ml (*E. thailandicus* 3-38) and 4 μg/ml (*E. thailandicus* W3 and *E. faecalis* W9-2) (Table 2). PFGE analysis of the four *cfr*-positive *Enterococcus* isolates, including the previously described bovine *cfr*-carrying *E. faecalis* isolate EF-01, revealed marked genomic heterogeneity of less than 65% similarity in their SmaI patterns (Fig. 1A). MLST of *E. faecalis* EF-01 indicated that it belongs to ST21 within the clonal complex 21 (CC21) (39), whereas *E. faecalis* W9-2 represented the novel ST type ST469.

Conjugation proved successful using isolates W3, 3-38, and W9-2 as the donor strains and JH2-2 as the recipient strain; three transconjugants, designated JHW3, JH3-38 and JHW9-2, respectively, were obtained. The sizes of the plasmids present in the original strains and their transconjugants were determined by S1 nuclease-PFGE. Each of the original strains harbored at least three plasmids, but at least one visible plasmid band could be observed in each of the three transconjugants (Fig. 1B). Southern blot analysis showed that the *cfr* probe hybridized to a band of ~75 kb in both W3 and JHW3, a band of ~72 kb in both 3-38 and JH3-38, and a band of ~55 kb in both W9-2 and JHW9-2 (Fig. 1C); these three *cfr*-carrying plasmids were designated pW3, p3-38 and pW9-2, respectively. In addition, we confirmed that the florfenicol exporter gene *fexA* was not associated with any of the plasmids in *E. thailandicus* 3-28 and located on an ~40-kb plasmid in *E. faecalis* W9-2, while the *fexB* gene was located on an ~50-kb plasmid in *E. thailandicus* W3 (data not shown).

MIC testing revealed that all transconjugants exhibited at least 4-fold-elevated MICs for chloramphenicol, florfenicol, and linezolid compared to the recipient JH2-2 strain. No observable MIC changes were found for tetracycline and ciprofloxacin. Interestingly, all transconjugants showed 32-fold elevated MICs for erythromycin, compared to JH2-2 (Table 2). PCR screening to detect erythromycin resistance genes using previously described primers (40, 41) revealed the presence of the *erm*(B) gene in transconjugants JHW3 and JHW9-2 and their original strains, while the *erm*(A) gene was detected in both JH3-38 and 3-38. The *erm*(B) gene in pW3 and pW9-2 encode a 245-amino-acid (aa) rRNA methylase that shares 100% identity with Erm(B) from *Streptococcus suis* D12 (AER19841), and the Erm(B) protein differs by only four aa exchanges (S100N, H163Y, H217Y, and N222D) from that from pAMβ1 (ACY79534). The 571-bp *erm*(A) amplicon from

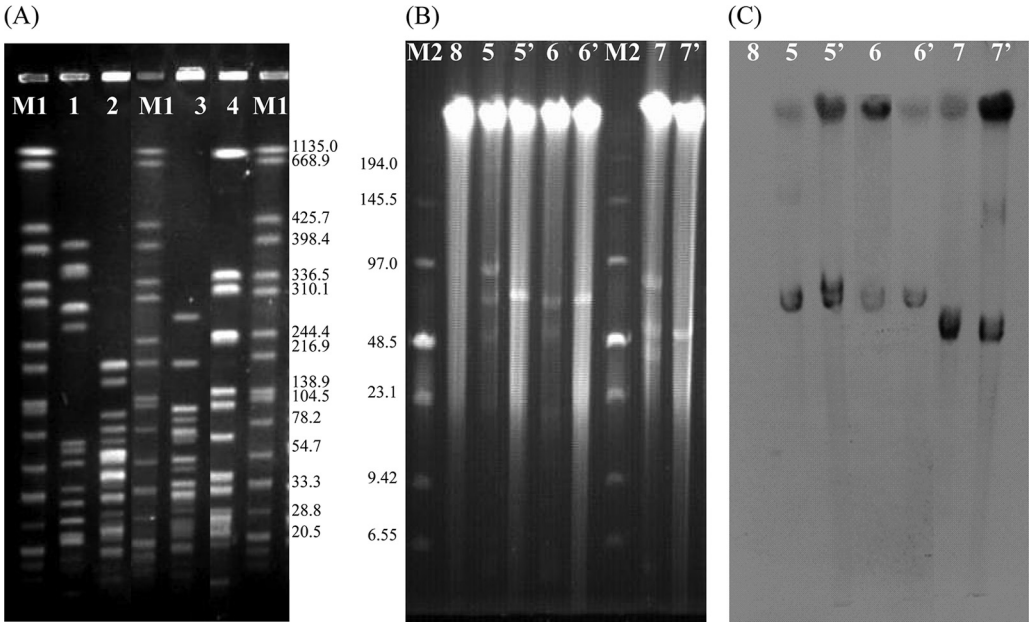

FIG 1 (A) SmaI-PFGE patterns from *E. faecalis* EF-01 and three *cfr*-positive isolates used in the present study. Lanes 1 to 4, *E. faecalis* EF-01, *E. thailandicus* W3, *E. thailandicus* 3-38, and *E. faecalis* W9-2, respectively; lanes M1 contain the XbaI pattern of *Salmonella braenderup* H9812 with the fragment sizes given in kilobases on the right-hand side. (B) Numbers and sizes of plasmids as determined by S1 nuclease PFGE. Lane M2, low-range PFG marker (NEB); lanes 5 and 5′, W3R and its transconjugant JHW3; lanes 6 and 6′, 3-38 and its transconjugant JH3-38; lanes 7 and 7′, W9-2 and its transconjugant JHW9-2; lane 8, the recipient *E. faecalis* JH2-2. (C) Southern hybridization with the *cfr* probe. The lane numbers correspond to those in panel B.

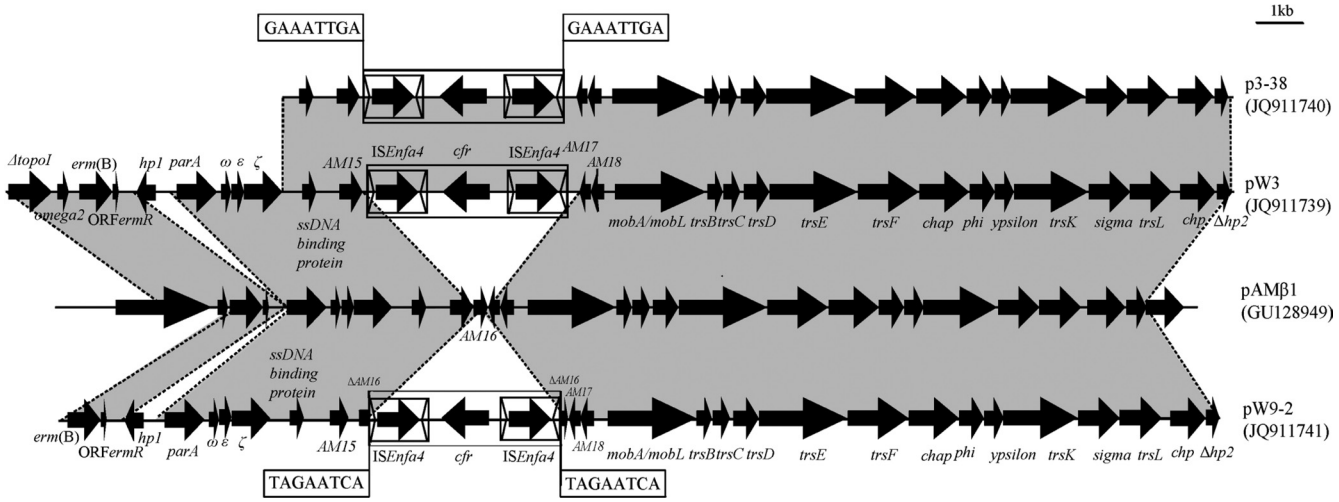

FIG 2 Genetic environment of *cfr* gene in the conjugative plasmids p3-38, pW3, and pW9-2 and structural comparison with the conjugative plasmid pAMβ1 from *E. faecalis* strain DS5. Regions of >95% homology are marked by gray shading.

both 3-38 and JH3-38 was identical to the corresponding *erm*(A) sequence of the *Streptococcus suis* isolate B1 (EU047809).

Analysis of the genetic environment of *cfr* in the conjugative plasmids pW3, p3-38, and pW9-2. To gain insight into the genetic environment of the *cfr* gene in the three newly identified conjugative plasmids, *cfr*-carrying segments of 27,360, 21,116, and 25,550 bp of the ~75-kb plasmid pW3, the ~72-kb plasmid p3-38, and the ~55-kb pW9-2 plasmid, respectively, were sequenced. The corresponding maps are shown in Fig. 2.

All three segments had an overall nucleotide sequence identity of >96% with respect to each other. The 4,447-bp central region, including *cfr* and the two 1,324-bp insertion sequences located in the same orientation, even displayed 100% nucleotide sequence identity. The further flanking sequences in pW3, p3-38, and pW9-2 exhibited a high nucleotide sequence identity (>95%) to the corresponding regions of the conjugative plasmid pAMβ1 (GU128949) from *E. faecalis* (Fig. 2).

In the *cfr* upstream region, the putative promoter and the partly overlapping reading frames for peptides of 59 aa (ORF1) and 44 aa (ORF2) were present. However, in comparison to pSCFS1, a 51-bp deletion had occurred in ORF1 which resulted in the loss of the first 17 aa of ORF1. This deletion was also present in the *cfr* upstream region of pEF-01 from bovine *E. faecalis* (17) but differed from the 35-bp deletion in the region between the stop codon of ORF2 and the start codon of *cfr* in the staphylococcal plasmid pSCFS3 (16). Two copies of a 1,324-bp insertion sequence harboring a 1,173-bp transposase gene and imperfect 26-bp terminal inverted repeats were found. These insertion sequences are most closely related to IS*1542* from *E. faecium* with an overall nucleotide sequence identity of 91.6% (1,213/1,324 bp) and 93.8% aa sequence identity (366/390 aa) in the transposase protein (42). This insertion sequence was submitted to the IS database (http://www-is.biotoul.fr/is.html) and received the designation IS*Enfa4*. Except for an 823-bp sequence, including the *orf1* located downstream of the *cfr* gene on pSS-01, the 4,447-bp central region shared >99% nucleotide identity with the corresponding region of plasmid pSS-01 (accession no. JQ041372) present in *S. cohnii* and *S. saprophyticus* (Fig. 3).

Immediately upstream of the righthand IS*Enfa4* element and downstream of the left-hand IS*Enfa4* element in the 4,447-bp central region, two different 8-bp direct repeats (DR) at the integration site were observed in the three conjugative plasmids. One DR (5′-TAGAATCA-3′), located in the open reading frame of *AM16* (which exhibited >99% nucleotide [304/306 bp] identity to that in pAMβ1), was found in the *E. faecalis* plasmid pW9-2. The other DR (5′-GAAATTGA-3′), located in a noncoding region between the genes *AM15* and *AM17*, was found in both *E. thailandicus* plasmids pW3-1 and p3-38. It should be noted that the *AM16* gene could not be detected in these two plasmids (Fig. 3). To determine the stability of the IS*Enfa4* flanked segment in the three conjugative plasmids, inverse PCR assays were performed using cfrIF and cfrIR primers. Amplicons 3,123 bp in size were obtained from each of the three parental strains and their transconjugants. Sequence analysis of these amplicons confirmed that they contained an intact *cfr* gene region and one complete IS*Enfa4* element.

DISCUSSION

Since only little a information is currently available about the presence of the multiresistance gene *cfr* among enterococci of animal origin (17), we screened 77 enterococcal isolates for the presence of this gene. The detection of the *cfr* gene in 3 of 77 (3.9%) florfenicol-resistant enterococcus strains of swine and farm environmental origin suggested that this multiresistance gene is relatively rare among enterococci from pig farms in Shandong Province, China. To the best of our knowledge, this is the first report of the *cfr* gene in the species *E. thailandicus*. *E. thailandicus* is a newly discovered enterococcal species, which was initially isolated from fermented sausage in Thailand (43). To date, no clinical *E. thailandicus* isolate has been reported; however, two strains of *Enterococcus sanguinicola*, which in the meantime have been reclassified as *E. thailandicus*, were recovered from clinically relevant human sources (44). The presence of the *cfr* gene in *E. thailandicus* from swine and sewage origins points toward the presence of this multiresistance gene in enterococci from animal settings and the surrounding environment.

The predominant resistance gene among florfenicol-resistant enterococci was *fexA*, which was detected in 72.7% (56/77) of the isolates tested in the present study; this finding was similar to the

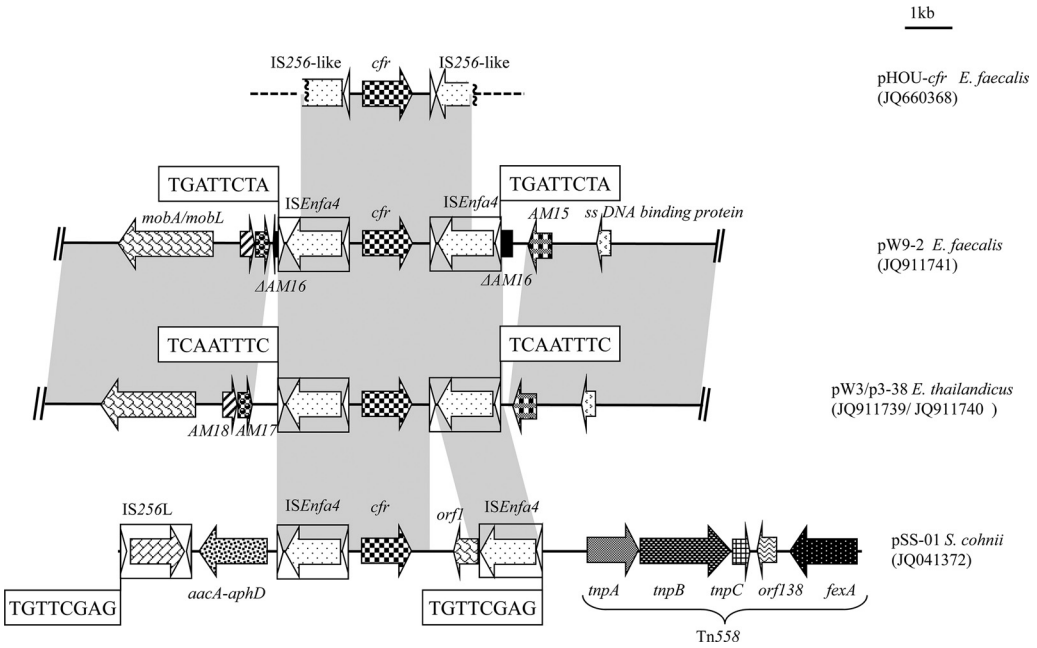

FIG 3 Schematic presentation of detailed genetic environment of the *cfr* gene in 4,447-bp center regions in conjugative plasmids pW9-2, pW3, and p3-38 and structure comparison with plasmid pHOU-*cfr* from *E. faecalis* 603-50427X of clinical human origin and pSS-01 from *S. cohnii* of swine origin. The arrows indicate the positions and direction of the transcription of the genes. The regions of >96% homology are marked using gray shading. The direct target site duplication is boxed. Δ, Truncated gene. A distance scale in kilobases is displayed in the upper right corner.

percentage of *fexA*-positive isolates among florfenicol-resistant staphylococci of swine origin (73.2%, 109/149) (22). In addition, the recently identified florfenicol exporter gene *fexB* was detected in 41.6% (32/77) of the isolates tested in the present study. The coexistence of *fexA* and *fexB* in the same isolate was also observed in 18 (23.4%) of the strains tested. In addition, 7.8% (6/77) of the florfenicol-resistant isolates examined here did not harbor any of the three genes *cfr*, *fexA*, or *fexB*, which suggests that potential nonenzymatic or enzymatic mechanisms conferred by other, as-yet-identified resistance genes might be involved in florfenicol resistance in enterococci.

The different SmaI PFGE patterns observed suggested that the three porcine and the single bovine *cfr*-positive enterococcal isolates are not related. Furthermore, MLST of two *cfr*-carrying *E. faecalis* strains originating from swine and cattle in China revealed that one of these isolates (EF-01) belongs to ST21, and the other (W9-2) represents the novel type ST469. The *E. faecalis* EF-01 isolate, which belonged to the clonal complex CC21, is mainly found in animals and in the community, while clinical isolates of this CC have rarely been detected (45). Concerning the W9-2 isolates, the ST469 belongs to a singleton, which did not have single or double locus variants by performing an eBURST analysis (data not shown).

In the present study, three different types of large *cfr*-carrying conjugative enterococcal plasmids—pW3, p3-38, and pW9-2—could be differentiated on the basis of their sizes, their additional erythromycin resistance genes, and the sequences surrounding the *cfr* gene. Despite these differences, high structural similarities between these three plasmids and plasmid pAMβ1 were observed. Plasmid pAMβ1, a member of a family of low-copy-number conjugative plasmids (46), was originally identified in a *E. faecalis* DS5 clinical isolate that harbored the MLS_B resistance gene *erm*(B) (47) and could be transferred to, and maintained in, a wide range of Gram-positive bacteria (e.g., enterococci, streptococci, *S. aureus*, *Bacillus subtilis*, and some *Lactobacillus casei* strains), indicating a broad host range (48). In the present study, the MLS_B resistance genes *erm*(A) and *erm*(B), the partial nucleotide sequence of the *tra* gene regions, and an ω-ε-ζ toxin-antitoxin module were observed in all three partially sequenced conjugative plasmids. This suggested that the 4,447-bp central region with the *cfr* gene and two IS*Enfa4* elements had integrated into a pAMβ1-like backbone. A closer look at the sequences identified two integration sites. We propose that the 4,447-bp central region in pW9-2 has integrated into the coding sequence of *AM16* via sample transposition; while in pW3 and p3-38, this element probably integrated into the region between the *AM15* and *AM17* genes, most likely by homologous recombination. The PCR-based stability tests also revealed that the *cfr* gene and one IS*Enfa4* copy can easily be excised, suggesting that this multiresistance gene may also be transferable via IS*Enfa4*-mediated recombination.

With the exception of the multiresistance gene *cfr*, the MLS_B genes *erm*(A) or *erm*(B) have also been found in three conjugative plasmids in our study. The coexistence of MLS_B genes and *cfr* in the same plasmid will allow for persistence and coselection of the *cfr* gene under the positive selective pressure imposed by the use of macrolides, such as tylosin and tilmicosin. The macrolides, as well as a number of other classes of antimicrobials, including ampicillin, florfenicol, trimethoprim-sulfamethoxazole, lincomycin, and tiamulin, have been used for curing or preventing bacterial infections in three of the pig farms investigated here, according to the antibiotic usage records of these farms. The persistence of the *cfr*-carrying plasmids among enterococci identified here may also be attributed to the coexistence of ω-ε-ζ toxin-antitoxin module, which is known to promote the persistence of plasmids by encod-

ing a system that kills or prevents the growth of plasmid-free cells (49). Therefore, the potential threat that the accumulation of multiple resistance genes including *cfr* on a plasmid with a toxin-antitoxin module poses is of great concern.

In conclusion, the data presented here described three conjugative plasmids carrying the multiresistance gene *cfr* in enterococci from pigs and their surrounding environments. The *cfr*-carrying center region flanked by the IS*Enfa4* elements in these conjugative plasmids harboring *tra* regions and toxin-antitoxin systems is a potential risk factor for dissemination of this multiresistance gene among enterococci isolates and possibly other species of Gram-positive bacteria, which many believe cause a threat to public health.

ACKNOWLEDGMENTS

This study was supported by grants from the National Natural Science Foundation of China (grants 31001087 and U1031004) and by the Special Fund for Agro-Scientific Research in the Public Interest (grant 201203040).

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