Detection of the staphylococcal multiresistance gene cfr in *Macrococcus caseolyticus* and *Jeotgalicoccus pinnipedialis*

Yang Wang††, Yu Wang††, Stefan Schwarz², Zhangqi Shen³, Nan Zhou¹, Juchun Lin⁴, Congming Wu¹ and Jianzhong Shen¹*

¹Key Laboratory of Development and Evaluation of Chemical and Herbal Drugs for Animal Use, College of Veterinary Medicine, China Agricultural University, Beijing 100193, P. R. China; ²Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Höltystr. 10, 31535 Neustadt-Mariensee, Germany; ³Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA; ⁴College of Veterinary Medicine, Sichuan Agricultural University, Ya’an 625014, Sichuan Province, P. R. China

*Corresponding author. Tel: +(86-10)-62732803; Fax: +(86-10)-62731032; E-mail: sjz@cau.edu.cn
†These authors contributed equally to this study.

Received 17 January 2012; returned 28 February 2012; revised 30 March 2012; accepted 11 April 2012

**Objectives:** To investigate the presence and the genetic environment of the multiresistance gene *cfr* in *Jeotgalicoccus pinnipedialis* and *Macrococcus caseolyticus* from pigs.

**Methods:** A total of 391 bacterial isolates with florfenicol MICs ≥16 mg/L were obtained from nasal swabs of 557 individual pigs; of these, 75 Gram-positive isolates other than staphylococci and enterococci were screened by PCR for the presence of known florfenicol resistance genes. Species assignments of the *cfr*-carrying isolates were based on the results of biochemical profiling and 16S rDNA sequencing. The locations of the *cfr* gene were determined by Southern blotting. Regions flanking each *cfr* gene were sequenced by a modified random primer walking strategy, and the transferability of *cfr* was assessed by electrotransformation.

**Results:** Two *M. caseolyticus* isolates and one *J. pinnipedialis* isolate were *cfr* positive. The *cfr* gene was located either on a 7057 bp plasmid, pSS-03, which was widely distributed among staphylococci of pig origin, or on the ~53 kb plasmid pJP1. The region of pJP1 that included the *cfr* gene and the adjacent IS21-558, showed 99.7% identity to the corresponding region of plasmid pSCFS3. In addition, the genes *aadD*+*, aacA*-aphD*, ble* and *erm(C)*, coding for aminoglycoside, bleomycin and macrolide—lincosamide—streptogramin B resistance, respectively, were also identified on plasmid pJP1.

**Conclusions:** This study showed that plasmids carrying the multidrug resistance gene *cfr* are present in two new genera of commensal and environmental bacteria, *Macrococcus* and *Jeotgalicoccus*. This observation underlines the role of commensal and environmental flora in the dissemination of clinically important resistance genes, such as *cfr*.

**Keywords:** linezolid resistance, intergenus transfer, swine, food safety

### Introduction

The multidrug resistance gene *cfr* was originally identified as a chloramphenicol-florfenicol resistance determinant on the multiresistance plasmid pSCFS1 from bovine *Staphylococcus sciuri* in 2000.¹ Further studies indicated that Cfr methyltransferase catalyses methylation of A2503 in the 23S rRNA gene of the large ribosomal subunit. This configuration confers resistance to chemically unrelated classes of antimicrobial agents, including phenics, oxazolidinones, lincosamides, pleuromutilins and streptogramin A, and decreased susceptibility to the 16-membered macrolides spiramycin and josamycin.² The veterinary use of phenics (florfenicol), lincosamides (clindamycin, pirlimycin and lincomycin) and pleuromutilins (tiamulin and valnemulin) for treatment or prevention of bacterial diseases may provide the selective pressure for the spread of *cfr*-mediated multiresistance. Based on the frequency of detection, coagulase-negative staphylococci, which commonly colonize livestock, may constitute the main reservoir of *cfr* in veterinary medicine.³,⁴ However, it should be noted that *cfr*-positive *Staphylococcus aureus* strains have also been identified in both human and veterinary medicine.⁵ Recent studies from China showed that the...
presence of the cfr gene is not restricted to staphylococci as it has been found in porcine Bacillus spp.,7 bovine Enterococcus faecalis,8 and even in Proteus vulgaris9 and Escherichia coli10 of porcine origin.

In this study, we report for the first time the presence of plasmids harbouring the cfr gene in species from two genera of Gram-positive bacteria, Macrococcus caseolyticus and Jeotgalicoccus pinnipedialis, both of porcine origin.

Materials and methods

Sample collection, detection of florfenicol resistance genes and species assignment

In 2010, nasal swabs were collected from 557 individual animals during a survey for the presence of cfr in bacteria from pigs. Samples were collected from three pig farms in Shandong province, China. Samples were streaked on brain heart infusion (BHI) agar plates supplemented with 10 mg/L florfenicol. The resulting colonies were screened using PCR for the currently known florfenicol resistance genes, cfr, fexA, fexB and flor, using previously described primers.7,9,11 Species assignment of the cfr-carrying strains was performed by Gram’s staining, colony morphology analysis, 16S rDNA sequencing and the use of a commercially available biochemical test system (ID32 STAPH; bioMérieux, Craponne, France).

Susceptibility testing, transformation experiments and molecular analyses

MICs of the antimicrobial agents listed in Table 1 were determined by broth microdilution according to the recommendations for staphylococci given in document M100-S21 of the CLSI. S. aureus ATCC 29213 was used as a quality control strain.

Plasmid DNA was introduced into recipient strain S. aureus RN4220 by electroporation. Transformants were selected on BHI agar plates supplemented with 10 mg/L florfenicol, and presence of the cfr gene in transformants was further confirmed by PCR. Plasmids were extracted from the transformants, and their sizes were estimated by electrophoresis and compared with plasmid standards purified from E. coli V517. These transformants were subsequently assayed for MICs of the antimicrobial agents and combinations of antimicrobial agents listed in Table 1.

Genomic DNA was isolated from bacteria using a Wizard Genomic DNA Purification Kit (Promega, Beijing, China). Plasmids were extracted using a QIAGEN Midi plasmid extraction kit (Qiagen, Hilden, Germany). Southern blotting was used to determine the location of each cfr gene.9 The flanking regions of each cfr gene were sequenced by primer walking and a modified random primer walking sequencing strategy, as previously described.8 A 1466 bp amplicon, corresponding to the 16S rDNA gene, was obtained for each species using universal bacteria-specific primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GTCACCTTGTAGACTT-3′).9 Amplicons were sequenced by the Beijing Genomic Institution (BGI, Beijing, China). Further resistance genes, such as erm(C), erm(B) and aacA-aphD, present on previously described cfr-carrying plasmids, were detected by PCR.5,7

Results and discussion

Analysis of 75 Gram-positive non-staphylococcal and non-enterococcal isolates collected from 391 bacterial isolates with florfenicol MICs ≥16 mg/L from the 557 samples tested for the presence of florfenicol resistance genes revealed that three isolates, designated K3 (from farm 1), 102 (farm 1) and 207 (farm 2), carried the cfr gene. Sequence analysis confirmed that the nucleotide sequence of the cfr gene in each of these three isolates showed 100% identity to the cfr gene found on plasmid pSCFS1 from S. sciuri (accession number NC_005076).1 Gram’s staining, colony morphology, ID32 STAPH tests and 16S rDNA sequencing identified isolates K3 and 207 as M. caseolyticus, while isolate 102 was Gram-positive but could not be identified by the ID32 STAPH system. However, 16S rDNA sequencing of isolate 102 revealed >99% nucleotide sequence identity to the 16S rDNA sequence of J. pinnipedialis. To the best of our knowledge, this is the first report of the multiresistance gene cfr in M. caseolyticus and J. pinnipedialis. M. caseolyticus, a commensal bacterium of livestock, is typically isolated from animal skin and food products, such as milk and meat.12 J. pinnipedialis was originally isolated from a Southern elephant seal (Mirounga leonina).13 To date, little is known about this genus; however, some investigations have indicated that bacteria belonging to the genus Jeotgalicoccus are widely distributed in the environment.14 The existence of the cfr gene in these ubiquitous free-living bacteria implies a wide distribution of this multiresistance gene, both in veterinary settings and the surrounding environment.

All three isolates showed high MICs of chloramphenicol, florfenicol, clindamycin, erythromycin, tiamulin, valnemulin, kanamycin and gentamicin, but exhibited low MICs of amoxicillin/clavulanic acid, ceftazidime, cefotiofur, ciprofloxacin, trimethoprim and sulfamethoxazole/trimethoprim (Table 1). Plasmid analysis indicated that isolates K3, 207 and 102 contained plasmids of different sizes (data not shown). Electrotansformation identified that a ~7 kb plasmid from M. caseolyticus K3 and a ~53 kb plasmid from both M. caseolyticus 207 and J. pinnipedialis 102 were associated with phenicol resistance. Interestingly, the ~7 kb plasmid isolated from K3 presented an EcoRI restriction pattern that was indistinguishable from that of plasmid pSS-03, originally identified in coagulase-negative staphylococci.15 Sequence analysis revealed that the 7057 bp plasmid is indeed pSS-03. Interestingly, the pSS-03-carrying M. caseolyticus K3 was isolated from pig farm 1, where plasmid pSS-03 had also been detected in two S. sciuri, two Staphylococcus cohnii and one Staphylococcus saprophyticus.5 This observation suggested an intergenus transfer of this small plasmid between staphylococci and M. caseolyticus. In addition, the ~53 kb plasmids from transformants of M. caseolyticus 207 and J. pinnipedialis 102, designated pJP1, showed indistinguishable HindIII and XbaI restriction patterns, and the corresponding S. aureus RN4220 transformants exhibited the same MICs of all tested antimicrobial agents (Table 1). Southern blot analysis confirmed the presence of cfr on both ~53 kb plasmids in the original strains and on that of the transformants (data not shown). The existence of plasmid pJP1 in strains M. caseolyticus 207 and J. pinnipedialis 102 suggests that this cfr-carrying plasmid can replicate in, and be disseminated between, different genera of Gram-positive bacteria. Susceptibility testing of transformants RN4220+pJP1 and RN4220+pSS-03 showed that both plasmids conferred increased MICs of chloramphenicol, florfenicol, clindamycin, tiamulin, valnemulin, linezolid and erythromycin (Table 1). In addition, RN4220+pJP1 showed elevated MICs of aminoglycosides, including gentamicin (64 mg/L), kanamycin (64 mg/L) and neomycin (4 mg/L).

The sequenced 8896 bp part of plasmid pJP1 (accession number JQ320084) revealed a segment of 4698 bp that
comprised the insertion sequence IS21-558 and the cfr gene and showed 99.7% (4685/4698 bp) identity to the corresponding segment of the S. aureus plasmid pSCFS3 (accession number AM086211) (Figure 1).14 This IS21-558 element has been reported to play an important role in the mobility of cfr.14 The 1612 bp up-stream region of IS21-558, which contained the kanamycin/neo-mycin resistance gene aadD and the bleomycin resistance gene ble, showed 100% identity to the S. aureus plasmid pGO1 (acces-sion number FM207042).14 The tnp gene downstream of the cfr gene in pJP1 presented 76% amino acid identity to a putative transposase in Bacillus cereus AH820 plasmid pAH820_272 (acces-sion number AM159501). PCR screening for macrolide and gentamicin resistance genes in S. aureus RN4220 revealed the presence of the genes erm(C) and aacA-aphD. The coexistence of the cfr gene and the aminoglycoside resistance genes aadD and aacA-aphD in pJP1 as well as the macrolide–lincosa-mide–streptogramin B resistance gene erm(C) in pSS-03 and pJP1 will allow persistence and co-selection of the cfr gene under selective pressure of aminoglycosides or macrolides.

In summary, this study extends the known host range of the staphylococcal multiresistance gene cfr. Although there are no reports of M. caseolyticus or J. pinnipedialis being isolated from humans, the presence of cfr-carrying plasmids in these genera may allow horizontal transfer to other commensal or pathogenic bacteria, such as staphylococci or enterococci, in the agricultural environment. It may also be possible for these plasmids to find their way through the food chain to commensal or pathogenic bacteria of humans, which then may result in a non-negligible threat to public health.

Funding
This work was supported by the National Natural Science Foundation of China (31001087 and U1031004) and the Program for Chang Jiang Scholars and the Innovative Research Team at the University of China (IRT0866).

### Table 1. MICs for cfr-carrying M. caseolyticus and J. pinnipedialis strains, as well as for S. aureus RN4220 and S. aureus RN4220 transformants carrying plasmids pSS-03 and pJP1

<table>
<thead>
<tr>
<th>Strain</th>
<th>FFC</th>
<th>CHL</th>
<th>CLI</th>
<th>ERY</th>
<th>TIA</th>
<th>VAL</th>
<th>LZD</th>
<th>GEN</th>
<th>KAN</th>
<th>NEO</th>
<th>AMC</th>
<th>CAZ</th>
<th>CEF</th>
<th>CIP</th>
<th>SXT</th>
<th>TMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. caseolyticus K3</td>
<td>128</td>
<td>32</td>
<td>32</td>
<td>&gt;64</td>
<td>128</td>
<td>64</td>
<td>8</td>
<td>16</td>
<td>128</td>
<td>1</td>
<td>1/0.5</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>9.5/0.5</td>
<td>1</td>
</tr>
<tr>
<td>M. caseolyticus 207</td>
<td>128</td>
<td>32</td>
<td>16</td>
<td>&gt;64</td>
<td>64</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>64</td>
<td>4</td>
<td>0.25/0.125</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.25</td>
<td>&lt;0.125</td>
<td>4.75/0.25</td>
</tr>
<tr>
<td>J. pinnipedialis 102</td>
<td>32</td>
<td>32</td>
<td>16</td>
<td>&gt;64</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>64</td>
<td>2</td>
<td>0.25/0.125</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.25</td>
<td>&lt;0.125</td>
<td>4.75/0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>S. aureus RN4220</td>
<td>4</td>
<td>4</td>
<td>≤0.06</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td>0.25</td>
<td>0.5/0.25</td>
<td>8</td>
<td>&lt;0.25</td>
<td>0.25</td>
<td>19/1</td>
<td>2</td>
</tr>
<tr>
<td>RN4220+pSS-03</td>
<td>256</td>
<td>32</td>
<td>32</td>
<td>&gt;64</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
<td>0.5/0.25</td>
<td>8</td>
<td>0.5</td>
<td>0.25</td>
<td>19/1</td>
<td>2</td>
</tr>
<tr>
<td>RN4220+pJP1</td>
<td>128</td>
<td>32</td>
<td>32</td>
<td>&gt;64</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>64</td>
<td>4</td>
<td>0.5/0.25</td>
<td>8</td>
<td>0.5</td>
<td>0.25</td>
<td>19/1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

FCC, florfenicol (1–256 mg/L); CHL, chloramphenicol (0.5–128 mg/L); CLI, clindamycin (0.06–64 mg/L); ERY, erythromycin (0.25–64 mg/L); TIA, tiamulin (0.125–128 mg/L); VAL, valnemulin (0.125–128 mg/L); LZD, linezolid (0.5–64 mg/L); GEN, gentamicin (0.25–64 mg/L); KAN, kanamycin (0.5–256 mg/L); NEO, neomycin (0.25–128 mg/L); AMC, amoxicillin/clavulanic acid (0.25/0.125–128/64 mg/L); CAZ, ceftazidime (0.125–128 mg/L); CEF, cefotaxime (0.25–128 mg/L); CIP, ciprofloxacin (0.125–64 mg/L); SXT, sulfamethoxazole/trimethoprim (2.375/0.125–1216/64 mg/L); TMP, trimethoprim (0.5–128 mg/L). The test ranges for the different antimicrobial agents are presented in parentheses.

*pJP1-carrying transformants obtained from both M. caseolyticus 207 and J. pinnipedialis 102 showed the same MICs.

Figure 1. Genetic environment of the cfr gene in plasmid pJP1 from M. caseolyticus 207 and J. pinnipedialis 102, and structural comparison with plasmids pSCFS3 and pGO1 from S. aureus. The arrows indicate the positions and directions of transcription for each gene. Regions with >98% identity are indicated by grey shading.
Transparency declarations
None to declare.

References