Molecular analysis of florfenicol-resistant *Escherichia coli* isolates from pigs

Maren Blickwede and Stefan Schwarz*

Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL), Höiltystrasse 10, 31535 Neustadt-Mariensee, Germany

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Objectives: The aim of this study was to analyse florfenicol-resistant *Escherichia coli* isolates from pigs for the genetic basis of florfenicol resistance, and to compare these data with those previously determined for *E. coli* isolates from cattle and poultry.

Methods: Fourteen porcine *E. coli* isolates were included in this study and subjected to serotyping, plasmid profiling and macrorestriction analysis. MICs of florfenicol were determined by broth microdilution. The presence of the gene *floR* was confirmed by hybridization and PCR analysis. Transformation experiments were conducted to isolate florfenicol resistance plasmids. The *floR* region of a florfenicol resistance plasmid was cloned and sequenced.

Results: All florfenicol-resistant *E. coli* isolates exhibited MICs of florfenicol >128 mg/L and carried the *floR* gene. A single isolate had a *floR*-carrying plasmid of ~35 kb, designated pMBSF1. Sequence analysis identified the *floR* gene flanked by truncated transposase genes. Moreover, a truncated copy of Tn5393 with complete streptomycin resistance genes strA and strB was found upstream of the *floR* gene of pMBSF1. Chromosomally resistant *E. coli* isolates, which shared the same *BlnI* macrorestriction pattern, differed in their *floRh*ybridization patterns.

Conclusion: The plasmid pMBSF1 is the smallest *floR*-carrying plasmid reported to date. Its *floR* region differed from those previously found in *E. coli* isolates from cattle. Variations in the RFLPs of chromosomal EcoRI fragments carrying *floR* in isolates that had the same macrorestriction pattern might suggest variable chromosomal integration sites.

Keywords: florfenicol resistance, *floR* gene, transposon Tn5393, transposon Tn1721, recombination

Introduction

The fluorinated chloramphenicol derivative florfenicol was licensed in Europe for the control of bacterial respiratory tract infections in cattle and pigs in 1995 and 2000, respectively. A recent study showed that since its introduction into clinical veterinary use, no resistance to florfenicol has developed in the major target bacteria from cattle, e.g. *Pasteurella multocida* and *Mannheimia haemolytica*, or in those from pigs, e.g. *P. multocida*, *Actinobacillus pleuropneumoniae* and *Streptococcus suis*. However, during the past 5 years, florfenicol resistance has been detected in a wide variety of enteric bacteria, including various *Salmonella enterica* serovars, such as Typhimurium, Agona, Albany, Paratyphi B and Newport, *Klebsiella pneumoniae*, *Vibrio cholerae*, and *Escherichia coli*. In all of those cases, the gene *floR*, coding for an efflux protein of 12 transmembrane segments, was responsible for florfenicol resistance. In the *Salmonella* serovars Typhimurium, Agona, Albany and Paratyphi B, as well as in *V. cholerae*, this gene was detected in the chromosomal DNA as part of resistance gene clusters, whereas plasmid-borne *floR* gene variants have been described in *K. pneumoniae* and *S. Newport*. In *E. coli*, both plasmid and chromosomal locations of this gene are known.

While all these data on florfenicol resistance in *E. coli* were based on strains from cattle or poultry, little is known about the situation in *E. coli* from pigs. Therefore, we investigated 14 florfenicol-resistant *E. coli* isolates from pigs for the genetic basis of florfenicol resistance, and compared the data obtained with those from *E. coli* strains of other animal sources.

*Corresponding author. Tel: +49-5034-871-241; Fax: +49-5034-871-246; E-mail: stefan.schwarz@fal.de*
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Materials and methods

Bacterial isolates, serotyping and antimicrobial susceptibility testing

Fourteen florfenicol-resistant porcine E. coli isolates were included in this study. All isolates were obtained from faecal samples of individual animals suffering from diarrhoea. The samples were collected in 2001 and the animals belonged to the same herd. Biochemical confirmation of the isolates as E. coli followed the specifications given by Koneman et al. Serotyping was performed at the Federal Institute for Risk Assessment, Dessau, Germany.

In vitro susceptibility testing was performed by agar disc diffusion on Mueller–Hinton agar (Oxoid, Wesel, Germany) with discs containing ampicillin (10 μg), carbenicillin (100 μg), chloramphenicol (30 μg), florfenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), minocycline (30 μg), neomycin (30 μg), streptomycin (10 μg), sulfamethoxazole (25 μg), tetracycline (30 μg) or trimethoprim (5 μg). The zones of growth inhibition were evaluated according to NCCLS standards.18 In addition, MICS of florfenicol were determined by the broth microdilution method.18

DNA preparation, PCR analysis, hybridization and PFGE experiments

Plasmid DNA for hybridization studies was prepared according to a previously described modification of the alkaline lysis procedure, which proved to be particularly suitable for the recovery of large enterobacterial plasmids.16 In addition, plasmid DNA for cloning experiments and sequence analysis was prepared by alkaline denaturation and subsequent purification by affinity chromatography on Qiagen columns (Qiagen, Hilden, Germany). The plasmids of E. coli V517 and S. Typhimurium LT2 and the K. pneumoniae plasmid R55 served as size standards. A previously described method for the isolation of whole-cell DNA from salmonellae served for the preparation of whole-cell DNA of the 14 E. coli isolates for Southern blot hybridization experiments.15 Preparation of whole-cell DNA for macrorestriction analysis followed a previously described protocol.20

The gene floR was detected by PCR with primers flo3 and flo4 (flo3, 5′-GCACTCGTAACGAGCAGCCGCT-3′; flo4, 5′-GCTGTT-GGTCTGTACGTAAGCCG-3′). Cycling conditions included an initial denaturation step of 2 min at 94°C followed by 31 cycles, each consisting of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C, followed by a final extension step of 7 min at 72°C. The resulting 1031 bp amplicon comprised almost the entire floR reading frame. The floR gene from a Salmonella enterica serovar Typhimurium DT104 isolate (GenBank accession no. AJ251806) served as a positive control in the PCR experiments. One such amplicon was cloned into PCR Blunt II TOPO (Invitrogen, Groningen, The Netherlands), sequenced and used as a specific gene probe in hybridization experiments. Transfer of uncut plasmid DNA or EcoRI-digested whole-cell DNA from agarose gels to nitrocellulose membranes (Hybond N, Amersham-Buchler, Braunschweig, Germany) was achieved by the capillary blot procedure. The amplicons were labelled either by the non-radioactive enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Freiburg, Germany) or by the DIG-High Prime DNA labelling and detection system (Roche Diagnostics GmbH, Mannheim, Germany). Hybridization and signal detection were carried out strictly according to the manufacturer’s recommendations. Detection of the streptomycin resistance gene strA by PCR followed a previously described protocol.21

Plasmid or chromosomal location of the floR gene was confirmed as described previously. PFGE was performed using the enzymes BlnI and I-CeuI. The pulse times for BlnI digests were increased from 7 to 12 s for 11 h, and from 20 to 65 s for the next 13 h, whereas those for I-CeuI were increased from 2.2 to 63.8 s for 10.5 h.16 Electrophoresis was carried out at 14°C, 5.5 V/cm and 0.15 A in a Bio-Rad CHEF-DR III system (Bio-Rad, Munich, Germany) with 0.5× TBE as running buffer. The Smal fragments of Staphylococcus aureus 832521 served as size markers.

Cloning and sequence analysis of the floR region of plasmid pMBSF1

Transformation of plasmids into E. coli strain JM109 was achieved using the CaCl2 method and transformants were selected on Luria–Bertani agar (Oxoid) supplemented with 20 mg/L florfenicol. E. coli JM109 transformants that grew on the selective medium were analysed for their plasmid content and the presence of the floR gene as described. The floR-carrying plasmid pMBSF1 was subjected to restriction mapping and initially, a 5.4 kb BamHI–EcoRI fragment was cloned into pBluescript II SK+ (Stratagene, Amsterdam, The Netherlands). Further on, two SphI fragments of 4.5 kb (one harbouring the EcoRI site, the other harbouring the BamHI site), as well as a 1.1 kb SphI fragment, were cloned into pCR Blunt II TOPO (Invitrogen). To complete the sequence of the streptomycin resistance genes upstream of the floR gene, an Real fragment of 1.8 kb was cloned. Sequence analysis was performed using an automated sequencer (ALF DNA Analysis System; Amersham Pharmacia Biotech). For this, the commercially available standard primers M13 universal and M13 reverse (Stratagene) were used first. Twelve further 16–20mer oligonucleotide primers, derived from the sequence obtained with the standard primers, were designed to complete the sequence analysis. The sequence of a 10910 bp segment of plasmid pMBSF1 has been deposited with the GenBank database under the accession number AJ518835. Sequence comparisons were carried out using the BLAST program, available at http://www.ncbi.nlm.nih.gov/BLAST/.

Results

Serotyping, in vitro susceptibility testing and plasmid profiling

Biochemical analysis confirmed that all 14 isolates belonged to the species E. coli. Serotyping showed that 13 of the 14 isolates were positive for the O antigen 108, but non-typeable by K antigens (O108:K-). The remaining isolate exhibited the serotype O108:K-. The remaining isolate showed an extended resistance pattern, including resistances to members of six different classes of antimicrobials (Table 1). MICS of florfenicol were >128 mg/L for all 14 E. coli isolates. Two to five plasmids in the size range between 4 and 95 kb were identified in each of the 14 E. coli isolates (Table 1).

Genotyping and detection of the floR gene

While all isolates were indistinguishable by their I-CeuI macrorestriction patterns, a total of eight different BlnI macrorestriction patterns were observed (Figure 1). The isolates of serotype O108:K- exhibited seven closely related patterns that differed by one to five bands. Among them, a dominant pattern was detected, which was represented by seven isolates. Each of the other six patterns was represented by a single isolate (Table 1). The remaining BlnI pattern of the O141:K85ac isolate differed distinctly from the other patterns.

All E. coli isolates carried the floR gene as confirmed by PCR. Hybridization and transformation experiments identified only the single isolate of serotype O148:K85ac to carry its floR gene on a plasmid of ~35 kb. The remaining 13 isolates carried one or two copies of the floR gene in their chromosomal DNA. A total of six different hybridization patterns were seen when using EcoRI-digested whole-cell DNA as target for the floR-specific gene probe
Table 1. Characteristics of the 14 florfenicol-resistant porcine E. coli isolates

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Serotype</th>
<th>Resistance phenotype</th>
<th>BlmI macrorestriction profile</th>
<th>Sizes of the floR-carrying EcoRI fragment (kb)</th>
<th>Approximate plasmid sizes (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A1</td>
<td>18</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>2</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A2</td>
<td>18</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>3</td>
<td>O141:K85ac</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, KAN, FF, CHL</td>
<td>B</td>
<td>&gt;23</td>
<td>35, 95</td>
</tr>
<tr>
<td>4</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A3</td>
<td>12</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>5</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A1</td>
<td>16</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>6</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A3</td>
<td>&gt;23, 12</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>7</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A4</td>
<td>23</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>8</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A2</td>
<td>16</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>9</td>
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<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A4</td>
<td>16</td>
<td>4, 50, 80</td>
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<tr>
<td>10</td>
<td>O108:K-</td>
<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A2</td>
<td>23</td>
<td>4, 50, 80</td>
</tr>
<tr>
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<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A5</td>
<td>12</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
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<td>AMP, CAR, TET, MIN, TMP, SUL, STR, FF, CHL</td>
<td>A1</td>
<td>18</td>
<td>4, 50, 53, 80</td>
</tr>
<tr>
<td>13</td>
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<td>A6</td>
<td>18</td>
<td>4, 50, 80</td>
</tr>
<tr>
<td>14</td>
<td>O108:K-</td>
<td>AMP, TET, TMP, SUL, STR, MIN, CAR, FF, CHL</td>
<td>A7</td>
<td>23</td>
<td>50, 53, 80</td>
</tr>
</tbody>
</table>

AMP, ampicillin; CAR, carbenicillin; CHL, chloramphenicol; FF, florfenicol; KAN, kanamycin; MIN, minocycline; STR, streptomycin; SUL, sulphamethoxazole; TET, tetracycline; TMP, trimethoprim.

*Based on agarose gel analysis of plasmid profiles, a distinctly thicker band suggests the presence of two plasmids of almost the same size.

The floR gene was found to be flanked by truncated copies of transposase genes (∆tnp). A stretch of −3.75 kb (positions 2468–6221 in the pMBSF1 sequence) including the ∆tnp gene upstream of floR, the floR gene itself and −0.45 kb of a non-coding region downstream of floR, showed striking homology (99% identity) to the corresponding regions previously found on the floR plasmid from bovine E. coli 10660 (GenBank accession no. AF231986), as well as in the chromosomal SXT element of V. cholerae (accession no. AB114188). The ∆tnp gene downstream of floR and the region immediately upstream of it (positions 6210–8891 in the pMBSF1 sequence) were highly homologous (99% identity) to the corresponding region of the tetracycline resistance transposon Tn1721 (GenBank accession no. X61367). This region represented the right end of Tn1721 including its 38 bp terminal inverted repeat.

The pMBSF1 sequences upstream of the ∆Tn5393 element (positions 1–320) and downstream of the Tn1721-homologous part (positions 8892–10636) did not show significant homology to any sequences deposited in the databases. Moreover, only small reading frames of up to 231 amino acids were detected in these two regions. However, their deduced protein sequences also did not reveal significant homology to proteins with known functions deposited in the databases. Finally, a stretch of 258 bp (positions 10637–10894 in the pMBSF1 sequence) revealed close homology (95% identity) to the oriT region of the E. coli plasmid R388 (GenBank accession no. X51505).

Discussion

Similar to the situation of florfenicol resistance in E. coli from bovine and avian sources, florfenicol resistance in porcine E. coli isolates was also mediated by the gene floR, and this gene was located either in the chromosomal DNA or on a plasmid. While previous data on chromosomally florfenicol-resistant isolates were from epidemic-
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![BlnI macrorestriction patterns of the 14 florfenicol-resistant porcine *E. coli* isolates. Numbering of the lanes refers to the numbering of the isolates as listed in the Table 1. Lanes M represent the size standard (*Sma*I-digested DNA of *S. aureus* 8325).](image)

logically unrelated *E. coli* isolates, the porcine isolates investigated in this study were from the same farm. Therefore, it was not a surprise to see that all 13 chromosomally florfenicol-resistant isolates were closely related, as confirmed by serotyping, *in vitro* susceptibility testing, plasmid profiling and PFGE (Table 1). However, the detection of six different *floR* hybridization patterns among these 13 isolates was an interesting observation, and might point towards the presence of a yet unidentified mobile *floR*-carrying element which integrated at different sites within the chromosomal DNA. Two previous studies on chromosomally florfenicol-resistant bovine *E. coli* isolates, which revealed either structural variations in the chromosomal regions flanking the *floR* gene or different chromosomal integration sites, might support this hypothesis.

Two types of mobile elements, both of which are widespread among Enterobacteriaceae, may be considered to carry the *floR* gene: gene cassettes/integrons or transposons. Although the *floR* gene is located between two integron structures in the chromosomal multi-resistance gene clusters present in several *Salmonella* serovars, analysis of the sequences flanking *floR* in all *floR*-associated database entries did not reveal any sequences that suggested direct involvement of gene cassettes/integrons in the spread of *floR*. Therefore, the location of this gene on a transposable element appeared to be a likely explanation for its various positions on plasmids or in the chromosomal DNA. In this regard, there are parallels between *floR* and the tetracycline resistance genes tet(A), tet(B) and tet(H), which have been identified as transposon-borne genes. Previous studies on these tet genes in Enterobacteriaceae and Pasteurellaceae showed that chromosomal integrates of the corresponding transposons, Tn1721 [tet(A)], Tn10 [tet(B)] and Tn5706 [tet(H)], were truncated in the vast majority of the cases. In contrast, analysis of plasmids carrying the genes tet(A) or tet(H) led to the identification of complete copies of the transposons Tn1721 and Tn5706.

Based on these experiences, our main interest with regard to the identification of a mobile genetic element harbouring *floR* focused on the *floR* gene and its flanking regions located on plasmid pMBSF1, to date the smallest known *floR*-carrying plasmid in Enterobacteriaceae.

The only other detailed report of the *floR* flanking regions on plasmids in *E. coli* showed that the *floR* gene located on the 110 kb plasmid from the bovine *E. coli* isolate 10 660 was preceded by a truncated copy and followed by a complete copy of a putative transposase gene. In the case of plasmid pMBSF1 from porcine *E. coli*, only the part immediately upstream of *floR* including the ∆tnp gene was virtually identical to the corresponding region of the *floR* plasmid from *E. coli* 10 660. However, the complete copy downstream of *floR* in the plasmid from *E. coli* 10 660 was replaced by a ∆tnp gene from Tn1721 in the case of plasmid pMBSF1. Another novel observation was the presence of a Tn5393 relic with complete streptomycin resistance genes strA and strB further upstream of *floR* in
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Thus, the floR gene area of plasmid pMBSF1 consisted of three parts that exhibited homology to Tn5393, the floR plasmid from E. coli 10 660, or Tn1721 (Figure 3). Analysis of these three different parts and their junctions suggested that recombination events obviously played an important role in the development of this region on plasmid pMBSF1.

Although we did not find a novel transposon carrying floR on plasmid pMBSF1, the data presented in this study enlarged the current knowledge of the diversity of floR gene areas in Gram-negative bacteria. Since the sequence of the floR region of plasmid pMBSF1 is only in part similar to the corresponding regions found either on other floR-carrying plasmids from E. coli,14 K. pneumoniae,10 or Pasteurella piscicida (now reclassified as Photobacterium damselae subsp. piscicida),32 or on floR-carrying chromosomal multiresistance gene clusters,2,8,11,12 both interplasmid recombination and recombination between floR-carrying plasmids and the chromosomal DNA must be considered when speculating about the spread of floR. Such recombination events may also play an important role in the development of novel floR-carrying plasmids, which might carry additional resistance genes, such as strA in the present case. Since many of the so far known floR-carrying plasmids from E. coli also mediate resistances to antimicrobials other than florfenicol,14 the spread of these plasmids by co-selection in the presence of the respective antimicrobials might be facilitated. In this regard, detailed information on the floR plasmids and their additional resistance markers are necessary to understand how and under what conditions florfenicol resistance spreads among members of the same and/or different genera of the family Enterobacteriaceae.

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Figure 2. Southern blot hybridization patterns of EcoRI-digested whole-cell DNA obtained with the floR gene probe. The sizes of the DNA marker (lane M, λ DNA, HindIII digested; Invitrogen) are indicated on the left-hand side. The numbering of the hybridization patterns corresponds to the numbering of the respective chromosomally florfenicol-resistant porcine E. coli isolates 1, 2 and 4–7 as listed in the Table 1.

Figure 3. Organization of the floR gene area on plasmid pMBSF1. The reading frames for strA, strB, floR and Δtnp are shown as arrows. A distance scale in kilobases is indicated below the map. Restriction sites are abbreviated as follows: B, BamHI; E, EcoRI; EV, EcoRV; K, KpnI; Hp, HpaI; P, PstI; Pv, PvuII; and Sp, SspI. The potential recombination site between the Tn1721-homologous part and the region similar to that of the floR plasmid from E. coli 10 660 is shown as a box below the map of the floR gene area. The numbering refers to the GenBank database accession no. AJ518835 of the pMBSF1 sequence.
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Figure 4. Comparative analysis between the original Tn5393 and the Tn5393 relic located on plasmid pMBSF1. The reading frames for *strA, strB, mprA*, and *mprR* are shown as arrows, the insertion element IS1133 as a box. The terminal inverted repeats of Tn5393 are displayed as black boxes and those of IS1133 are shown as horizontal stripes. A distance scale in kilobases is indicated below both maps. The two potential recombination sites A and B that might explain the development of the truncated Tn5393 element on plasmid pMBSF1 are shown as boxes below the map of the *floR* gene area. The numbering of the sequences refers to the respective positions in the database entries of pMBSF1 (GenBank accession no. AJ518835) and Tn5393 (accession no. M95402).

**References**


