Molecular analysis of chromosomally florfenicol-resistant *Escherichia coli* isolates from France and Germany

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The aim of this study was to analyse chromosomally florfenicol-resistant *Escherichia coli* isolates for their genetic relatedness, and also for the presence of the *floR* gene and its adjacent regions, in order to compare these regions with those associated with a *floR* gene located on a conjugative plasmid from *E. coli*. Twenty-two bovine *E. coli* from France and Germany were examined. Florfenicol resistance was determined by MIC determination. The presence of the *floR* gene was confirmed by hybridization and PCR analysis. The *E. coli* isolates were investigated by macrorestriction analysis. The 22 florfenicol-resistant *E. coli* (MICs 64–128 mg/L) differed in their *BlnI* macrorestriction patterns. Single or double copies of the *floR* gene were detected by hybridization on different-sized chromosomal *EcoRI*, *BamHI* and *BglII* fragments. The *floR*-flanking regions also proved to be variable as confirmed by hybridization experiments. The detection of chromosomal *floR* gene copies in unrelated *E. coli* isolates supplements the observations of *floR* genes on plasmids in *E. coli* and confirms their potential to integrate into the chromosome. The RFLPs of *floR* gene-carrying restriction fragments might suggest variable chromosomal integration sites.

**Introduction**

Florfenicol is a fluorinated chloramphenicol derivative that was licensed in Europe in 1995 for the control of bacterial infections of the respiratory tract of cattle. Plasmid-mediated resistance to florfenicol by efflux of the drug was first encountered in the fish pathogen *Photobacterium damselae* subsp. *piscicida*, formerly known as *Pasteurella piscicida*\(^1\). The gene responsible for florfenicol resistance is referred to as *floR*, although other gene designations such as *pp-flo*, *cmlA*-like, *flo*\(_St\) or *floR* have been used\(^1\)–\(^7\). This gene was determined to be part of a chromosomal multi-resistance cluster in *Salmonella enterica* serovar Typhimurium definitive phage type (DT)104\(^4\)–\(^8\) and *Salmonella enterica* serovar Agona.\(^7\) Recently, the same gene has been detected on conjugative and non-conjugative plasmids in *Escherichia coli*.\(^5\)–\(^10\) From one such plasmid, a 6522 bp *EcoRI–BamHI* fragment including the *floR* gene was cloned and sequenced.\(^9\) Analysis of the sequences upstream and downstream of the *floR* gene revealed the presence of two reading frames that showed considerable homology to those of transposase proteins.\(^9\)

To date, very little is known about *E. coli* isolates carrying *floR* genes in chromosomal DNA.\(^10\) We therefore investigated 22 chromosomally florfenicol-resistant *E. coli* isolates from two countries for their clonal relationships and the genetic environment of the *floR* genes.

**Material and methods**

*Bacterial isolates, antimicrobial susceptibility testing and serotyping* A total of 22 florfenicol-resistant bovine *E. coli* isolates, 12 from France and 10 from Germany, were included. All were from faecal samples of individual animals of unrelated herds. The French isolates BN10871, BN11282 and BN11285 were obtained from animals suffering from res-
piratory diseases whereas the remaining 19 isolates were from cases of diseases of the digestive tract. Isolates were collected between 1997 and 2000. Biochemical confirmation of the species assignment followed the specifications given by Koneman et al.11

Resistance patterns were determined by the agar disc diffusion method on diagnostic sensitivity test agar (DST agar; Oxoid, Wesel, Germany), with discs containing ampicillin 10 µg, chloramphenicol 30 µg, florfenicol 30 µg, gentamicin 10 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.12 In addition, MICs of florfenicol and chloramphenicol were determined by broth micro- and macrodilution methods, respectively, as described previously.9

Serotyping was performed as a slide agglutination test with commercially available monovalent test sera directed against the antigens K99, K88, 987p and F107 (BgVV, Dessau, Germany).

DNA preparation, hybridization experiments and PCR analysis

Plasmid DNA was prepared according to a modification13 of the method of Kado & Liu,14 which is particularly suitable for the recovery of large enterobacterial plasmids. The 150 kb plasmid R55 served as a control to ensure identification of the presence of large plasmids.15 In addition, plasmids were also prepared by alkaline denaturation, and subsequently purified by affinity chromatography on Qiagen columns (Qiagen, Hilden, Germany). The preparation of whole-cell DNA of the 22 E. coli isolates followed a method described previously for the isolation of whole-cell DNA from salmonellae.13 Transformation of plasmids into E. coli strain JM107 or JM109 with subsequent selection on its CaCl2 method.13 Conjugation experiments with E. coli strain BM14 were performed as described.9,15 PCR-directed detection of the floR gene was achieved with primers flO1 and flO2 (flO1, 5’-GCATCTGAAACACGACGCCGCT-3’; flO2, 5’-CGACACCAGCCTGCAATTGCCG-3’), which results in the amplification of a 1031 bp amplicon that comprises almost the entire floR reading frame. For Southern blot hybridization the 1031 bp floR segment (GenBank accession no. AJ251806), amplified from the floR gene of S. enterica serovar Typhimurium DT104, served as specific probe. Chromosomal location of the floR gene was confirmed by: (i) positive floR-specific PCR results using whole-cell DNA; (ii) positive floR-specific hybridization results using whole-cell DNA; (iii) negative results of repeated plasmid transformation/conjugation experiments; (iv) negative results of repeated floR-specific hybridization experiments using plasmid profiles as target DNA; and (v) the location of the floR gene on a I-CeuI fragment of chromosomal DNA. For this latter approach, whole-cell DNA was digested with the homing restriction endonuclease I-CeuI, which is known to have its cleavage sites exclusively within the rnr operons of the E. coli genome.16 The fragment patterns were separated by pulsed-field gel electrophoresis. The pulse times were increased from 2.2 to 63.8 s for 10.5 h. The seven fragments were cut from the gel and slices of these gel pieces served as targets for the PCR-directed detection of the floR gene. In this particular case, PCR was performed for 45–60 cycles in a total volume of 80 µL.

To compare the chromosomal regions flanking the floR gene in the E. coli isolates, Southern blotting was performed using the 6522 bp segment of the floR-carrying plasmid pEF03 (GenBank accession no. AF231986) as a probe for hybridization with BglI-digested, whole-cell DNA. Labelling of the two probes and hybridization conditions were as reported previously.9,17

Macrorestriction analysis

Macrorestriction analysis using the enzyme BlnI18 was carried out to determine the genomic relationships of the 22 E. coli isolates. This enzyme has proved to be superior in its discriminatory power to other enzymes commonly used for E. coli, such as XbaI or SpeI (S. Schwarz, unpublished data). Preparation of the whole-cell DNA for macrorestriction analysis followed a protocol described previously.18 The pulse times were increased from 7 to 12 s for 11 h and from 20 to 65 s for the next 13 h. Electrophoresis was carried out at 14°C, 5.5 V/cm and 0.15 A in a Bio-Rad CHEFDR III system (Bio-Rad, Munich, Germany) using 0.5 × TBE as running buffer. The SmalI fragments of Staphylococcus aureus 832519 served as size markers. Cluster analysis using the unweighted pair group average (UPGMA) method was performed with the GelCompar software.

Results

Antimicrobial resistance testing and genotyping

Biochemical analysis confirmed the assignment of all isolates to the species E. coli. Serotyping identified four E. coli isolates (BN10678, BN11157, 9 and 29) as positive for the antigen K99. All E. coli proved to be multiresistant. In addition to florfenicol and chloramphenicol resistance the isolates exhibited in vitro resistance to between four and seven additional antimicrobial agents (Table). The MICs of chloramphenicol varied between 128 and >256 mg/L, and those of florfenicol between 64 and >128 mg/L.

Macrorestriction analysis identified two isolates from Germany (isolates 4 and 5) as indistinguishable by their BlnI macrorestriction patterns, as were three isolates from France (isolates BN10420, BN10656 and BN10935) (Figure 1a). The DNA of another isolate from France (BN11286) degraded rapidly during the preparation process. This isolate could therefore not be typed by macrorestriction
Table. Characteristics of the *E. coli* strains

<table>
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<tr>
<th>Strain</th>
<th>Origin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antibiotic resistance profile&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MIC&lt;sub&gt;Cm&lt;/sub&gt; (mg/L)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MIC&lt;sub&gt;Ff&lt;/sub&gt; (mg/L)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>floR hybridization approx. fragment size (kb)</th>
<th>Presence of conserved pEF03 BglI fragments&lt;sup&gt;d&lt;/sup&gt;</th>
<th>pEF03 BglI profile&lt;sup&gt;e&lt;/sup&gt;</th>
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<sup>a</sup>F, France; G, Germany.

<sup>b</sup>Amp, ampicillin; Cm, chloramphenicol; Ff, florfenicol; Gm, gentamicin; Nm, neomycin; Sm, streptomycin; Sul, sulfamethoxazole; Tc, tetracycline; Tmp, trimethoprim.

<sup>c</sup>MIC<sub>Cm</sub> and MIC<sub>Ff</sub>, MICs of chloramphenicol and florfenicol, respectively.

<sup>d</sup>As expected from the pEF03 insert nucleotide sequence: a, 1402 bp; b, 1976 bp; c, 906 bp (Figure 2a).

<sup>e</sup>With respect to Figure 2(b).
analysis in repeated attempts. Cluster analysis identified two major groups of isolates, one of which included mainly the isolates from France, the other those from Germany (Figure 1b). Only two of the German isolates, numbers 25 and 26, clustered with the French isolates; the two French isolates BN11282 and BN10657 proved to be more closely related to the majority of the strains from Germany (Figure 1b). Similarity between the two clusters was <20%.

The floR gene and its genetic environment

The floR gene was detected in all isolates using a specific PCR assay in addition to Southern blot hybridization with a specific gene probe. The latter approach identified single copies of the floR gene in 21 of the 22 E. coli, whereas two copies were found in a single French isolate (Table). The assumption that the floR gene is located in the chromosomal DNA of the E. coli isolates was confirmed by: (i) the presence of the 1031 bp amplicon when using whole-cell DNA for PCR; (ii) different-sized hybridizing bands using EcoRI-, BamHI- and BglII-digested whole-cell DNA; (iii) repeated negative hybridization results using plasmid DNA of the E. coli isolates; (iv) repeated negative transformation/conjugation results using the plasmids of the E. coli isolates; and (v) the PCR-directed detection of the floR gene on I-CeuI fragments of chromosomal DNA. This latter approach identified the floR gene in most cases on one of the two largest I-CeuI fragments. The French isolate BN10657 harboured its two floR gene copies on different I-CeuI fragments. To assess the genetic environment of the floR gene in the chromosomal DNA of the E. coli isolates, another set of hybridization experiments was performed using BglII-digested DNA and the 6522 bp EcoRI–BamHI insert of plasmid pEF03 as a probe. This insert contains in addition to the floR gene approximately 3.3 kb of upstream and 1.8 kb of downstream sequences containing reading frames for putative transposase proteins (orfA' and orfA). The insert exhibited four BglII sites accounting for three BglII fragments in the floR gene area of 1402, 1976 and 906 bp (Figure 2a). Hybridization of the 22 E. coli isolates revealed a total of nine BglII restriction patterns (Table and Figure 2b). The 1976 bp fragment that included the floR gene and the adjacent 906 bp fragment were seen in 17 of the 22 isolates. The 1402 bp fragment representing the floR upstream region including part of orfA' was detected in only two E. coli isolates. Additional hybridization experiments using the floR gene probe and EcoRI- or BamHI-digested whole-cell DNA yielded fragments of 9.3–23.1 kb or 7.0–21.8 kb, respectively. These hybridizing fragments were larger than the corresponding fragments expected from plasmid pEF03.

Discussion

The observation that the floR gene was found either on plasmids1,8–10 or in the chromosomal DNA3–7 of different bacterial pathogens raised the question of the genetic basis of mobility of this resistance gene. A first approach was made by identifying plasmids that carried the floR gene.8,9 Restriction analysis and hybridization with a floR-specific gene probe identified at least five different types of floR-carrying conjugative plasmid of 110–125 kb among European E. coli isolates,9 whereas the corresponding plasmids seen in E. coli from the USA8,10 were of approximately 225 kb. Cloning and sequencing of the upstream and downstream regions of the floR gene, located on a large multi-resistance plasmid from the French E. coli isolate BN10660,9...
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identified reading frames that showed homology to reading frames of transposase proteins. Although the functional activity of these potential transposases could not be confirmed, this observation suggests the involvement of a transposable element in the spread of the *floR* gene. The occurrence of the *floR* gene in the chromosome of *E. coli* has rarely been observed, and compared with the *S. enterica* serovars Typhimurium and Agona, very little is known about chromosomal *floR* genes in *E. coli*. In this respect two major questions had to be solved: (i) do the chromosomally *floR*-carrying *E. coli* isolates represent one clone and (ii) is the genetic environment of the chromosomal *floR* genes similar or identical to that of plasmid pEF03? To answer the first question, all *E. coli* isolates were subjected to macrorestriction analysis using *BlnI*. Two of the German and three of the French isolates proved to be indistinguishable based upon their *BlnI* fragment patterns, while the majority of the other isolates were distinctly different based on their macrorestriction patterns (Figure 1a). Two clusters that reflected mainly the geographical sources of the isolates were identified (Figure 1b) and confirmed that *floR*-carrying bovine *E. coli* from France and Germany represent independent populations. This was in accordance with the previous observation of strikingly different restriction patterns of *floR*-carrying plasmids of *E. coli* from these two countries. Based on the macrorestriction patterns and the resulting cluster analysis, it can be concluded that the chromosomally *floR*-carrying *E. coli* represented a wide variety of different genotypes.

To analyse the chromosomal regions adjacent to the *floR* genes, whole-cell DNA was digested with *BglI* and hybridized with the sequenced 6.522 kb *EcoRI–BamHI* fragment of plasmid pEF03. Of the three conserved *BglI* fragments, the 2.0 kb fragment carrying the *floR* gene and the 0.9 kb fragment harbouring part of the downstream-located *orfA* were seen in all of the German isolates, but only in seven of the 12 French isolates (Table and Figure 2). The 1.4 kb fragment located upstream of the *floR* gene in pEF03 was detected in only two isolates, one from France and one from Germany (Table). Among the remaining five French isolates, two completely different hybridization patterns were observed. In total, nine different *BglI* hybridization patterns were identified. This confirmed the genetic diversity in the chromosomal regions immediately upstream and downstream of the *floR* gene. Further hybridization experiments with the *floR* gene probe identified the *floR* gene on chromosomal *EcoRI* or *BamHI* fragments ranging in size from 7.0 to >23.1 kb.

Assuming that the *floR* gene is part of a transposable element, different chromosomal integration sites can explain the differences in the hybridization profiles. The detection of two *floR* gene copies on different I-*CeuI* fragments of the chromosome of strain BN10657 also supports the hypothesis of different chromosomal integration sites. In addition, there have been a number of structurally different *floR*-carrying plasmids identified in *E. coli*. However, the *floR*-flanking area of only one of them is known. Thus, integration of different *floR*-carrying plasmids—in part or *in toto*—in the chromosomal DNA of *E. coli* isolates might represent another possible explanation for the observed structural variations in the chromosomal *floR* gene area.

**Acknowledgements**

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**References**


Figure 2. (a) Organization of the *floR* locus on the 6522 bp *EcoRI–BamHI* insert of plasmid pEF03. The sizes and locations of the three *BglI* fragments are indicated. The reading frames for *orfA*, *floR* and *orfA* are shown as arrows. The PCR amplicon of 1031 bp is presented as a double-headed arrow. A distance scale in bp is indicated below the map. Restriction sites are abbreviated as follows: B, *BglI*; Ba, *BamHI*; E, *EcoRI*. (b) Presentation of the nine different patterns obtained from hybridization of *BglI*-digested whole-cell DNA with the 6522 bp *EcoRI–BamHI* fragment of plasmid pEF03.


