Molecular analysis of florfenicol-resistant *Pasteurella multocida* isolates in Germany

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**Objectives:** Three florfenicol-resistant *Pasteurella multocida* isolates from Germany, two from swine and one from a calf, were investigated for the genetics and transferability of florfenicol resistance.  

**Methods:** The isolates were investigated for susceptibility to antimicrobial agents and plasmid content. Florfenicol resistance plasmids carrying the gene *floR* were identified by transformation and PCR. Plasmids were mapped, and a novel plasmid type was sequenced completely. PFGE served to determine the clonality of the isolates.  

**Results:** In one porcine and the bovine *P. multocida* isolate, florfenicol resistance was associated with the plasmid pCCK381 previously described in a bovine *P. multocida* isolate from the UK. The remaining porcine isolate harboured a new type of *floR*-carrying plasmid, the 10 226 bp plasmid pCCK1900. Complete sequence analysis identified an RSF1010-like plasmid backbone with the mobilization genes *mobA*, *mobB* and *mobC*, the plasmid replication genes *repA*, *repB* and *repC*, the sulphonamide resistance gene *sul2* and the streptomycin resistance genes *strA* and *strB*. The *floR* gene area was integrated into a region downstream of *strB*, which exhibited homology to the *floR* flanking regions found in various bacteria. PFGE revealed that the *floR*-carrying *P. multocida* strains from Germany were unrelated and also different from the UK strain.

**Conclusions:** After the UK and France, *floR*-mediated florfenicol resistance has now also been identified in target bacteria from Germany. PFGE data and the analysis of plasmids strongly suggested that the spread of florfenicol resistance is due to the horizontal transfer of plasmids rather than the clonal dissemination of a resistant *P. multocida* isolate.

Keywords: *floR* gene, respiratory tract pathogens, antimicrobial resistance, gene transfer

**Introduction**

The fluorinated thiamphenicol derivative florfenicol is one of the few antimicrobial agents that are exclusively approved for veterinary use.¹ For the control of respiratory tract pathogens from cattle and pigs, it has been licensed in Europe in 1995 and 2000, respectively.¹ Since then, continuous monitoring programmes have been conducted to determine the MICs of florfenicol for bovine and porcine respiratory tract pathogens. In Germany, MICs of florfenicol have been determined in the national monitoring programme GERM-Vet conducted by the Federal Office of Consumer Protection and Food Safety (BVL) as well as in a product-specific monitoring study conducted by the former Federal Research Centre for Agriculture, which has recently become part of the Friedrich-Loeffler-Institute (FLI). The results of the latter study indicated that no resistant target bacteria obtained from cattle (*Pasteurella multocida* and *Mannheimia haemolytica*) and pigs (*P. multocida* and *Actinobacillus pleuropneumoniae*) were detected up to now.² In contrast, single florfenicol-resistant *P. multocida* isolates were identified in the GERM-Vet studies 2002–03 and 2005–06.³,⁴ A thorough analysis of these isolates for their species assignment and re-checking of the MICs of florfenicol identified two porcine *P. multocida* isolates with MICs of 8 and 16 mg/L as resistant to florfenicol based on the veterinary-specific breakpoints available in the document M31-A3 of the CLSI.⁵ In addition, various diagnostic laboratories in Germany were asked to send us presumably florfenicol-resistant *P. multocida* isolates. A single bovine *P. multocida* isolate with an MIC of 32 mg/L was provided by a diagnostic laboratory in 2007.
These three *P. multocida* isolates represent the first and so-far only confirmed cases of florfenicol resistance in target bacteria in Germany. Florfenicol-resistant bovine *P. multocida* from the UK and bovine *Pasteurella multocida* (meanwhile reclassified as *Bibersteinia trehalosi*) from France, but also porcine *Bordetella bronchiseptica* isolates from Germany, have been reported previously. The aim of this study was to investigate these three *P. multocida* isolates for the genetic basis of florfenicol resistance and the location of the resistance genes. Moreover, PFGE was applied to determine the genetic relatedness of the three German *P. multocida* isolates and to compare them with the previously described UK isolate.

Materials and methods

**Bacterial strains and antimicrobial susceptibility testing**

The two porcine *P. multocida* isolates 1387-03 and 1900-03 were obtained from cases of respiratory tract infections of swine in 2003. The single bovine *P. multocida* isolate R77-07 originated from a fatal case of respiratory disease in a calf in 2007. *In vitro* susceptibility testing was performed by MIC determination via broth microdilution, according to the CLSI document M31-A3.5

**DNA techniques**

Capsular typing as well as PCR analysis and hybridization experiments for the detection of the chloramphenicol–florfenicol resistance gene floR were conducted, as described previously.6 Plasmid preparation by alkaline lysis and transformation experiments into the recipient strains *E. coli* HB101 and JM101 or *P. multocida* P4000 were performed, as described previously.7 Plasmid DNA obtained from the transformants was subjected to restriction mapping with the restriction endonucleases known to have cleavage sites in the so-far known two *floR*-carrying Pasteurellaceae plasmids pCCK381 and pCCK13698.6.7 Overlapping *SacI* fragments of ca. 2.1 and 8.1 kb as well as *EcoRV* fragments of ca. 4.5 and 5.7 kb obtained from the plasmid pCCK1900 were cloned into either pBluescript II SK+ (Stratagene, Amsterdam, The Netherlands) or pCR-Blunt II-TOPO (Invitrogen, Groningen, The Netherlands) and transformed into *E. coli* recipient strains JM109 or TOP10, respectively. Sequence analyses were started with the M13 reverse and universal primers and completed with primers derived from sequences obtained with the aforementioned standard primers (Eurofins MWG Operon, Ebersberg, Germany). Sequence comparisons were performed with the BLAST programs blastn and blastp (http://www.ncbi.nlm.nih.gov/BLAST; 11 July 2008, date last accessed) and with the ORF finder program (http://www.ncbi.nlm.nih.gov/Orf2html; 11 July 2008, date last accessed). The nucleotide sequence of the plasmid pCCK1900 has been deposited in the European Molecular Biology Laboratory (EMBL) database under accession no. FM179941.

Whole-cell DNA for PFGE was prepared as described previously.8 The separation of the *SmaI* fragments was conducted in a CHEF DR III system (Bio-Rad) at 15 V/cm, with 0.5 × Tris-borate-EDTA as the running buffer. To achieve a suitable separation of the multiple fragments in the low molecular weight range, the pulse times were increased from 2 to 5 s over 24 h.9 The *SmaI* fragments of *S. aureus* 8325 served as a size standard.

**Results and discussion**

**Molecular basis of florfenicol resistance in the three *P. multocida* isolates**

All three *P. multocida* isolates were positive in the PCR for the florfenicol resistance gene *floR*. The complete resistance patterns of the three *P. multocida* isolates are shown in Table 1. All isolates were susceptible to other antimicrobial agents used for the control of respiratory tract infections in cattle and swine, including ampicillin (MICs: 0.12 mg/L), amoxicillin/clavulanic acid (MICs: 0.12/0.06 mg/L), ceftriaxone (MICs: ≤0.03 mg/L), cefquinome (MICs: 0.03 mg/L), enrofloxacin (MICs: ≤0.008–0.015 mg/L), trimethoprim/sulfamethoxazole (MICs: ≤0.015/0.3–0.12/2.8 mg/L) and tulathromycin (MICs: 1–4 mg/L). Isolate 1900-03 was resistant to tetracycline with an MIC of 32 mg/L, whereas the other two isolates were susceptible with MICs of ≤0.12 mg/L (isolate R77-07) or 0.5 mg/L (isolate 1387-03).

Plasmids of 10.2 kb (isolate 1900-03) or 10.8 kb (isolates R77-07 and 1387-03) were identified by transformation experiments and subsequent PCR analysis and hybridization as carriers of the *floR* gene. Restriction analysis with nine restriction endonucleases showed that the 10.8 kb plasmids were indistinguishable from the plasmid pCCK381 on the basis of their single and double digestion patterns. Plasmid pCCK381 was identified in 2005 from a bovine *P. multocida* isolate in the UK. However, the 10.2 kb plasmid from the *P. multocida* isolate 1900-03,

### Table 1. Characteristics of the three German *P. multocida* isolates

<table>
<thead>
<tr>
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<th>R77-07</th>
<th>1387-03</th>
<th>1900-03</th>
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<tbody>
<tr>
<td>Animal origin</td>
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<td>swine</td>
<td>swine</td>
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<tr>
<td>Geographic origin</td>
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<td>Lower Saxony</td>
<td>Saxony-Anhalt</td>
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<td>Month/year of isolation</td>
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<td>02/2003</td>
<td>05/2003</td>
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<tr>
<td>Capsular type</td>
<td>non-typeable</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>Resistance pattern of the original isolate</td>
<td>CHL, FFC</td>
<td>CHL, FFC, SUL</td>
<td>CHL, FFC, STR, SUL, TET</td>
</tr>
<tr>
<td><em>floR</em> plasmid type</td>
<td>pCCK381</td>
<td>pCCK381</td>
<td>pCCK1900</td>
</tr>
<tr>
<td><em>floR</em> plasmid-associated resistance pattern</td>
<td>CHL/FFC</td>
<td>CHL/FFC</td>
<td>CHL/FFC, STR, SUL</td>
</tr>
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CHL, chloramphenicol; FFC, florfenicol; STR, streptomycin; SUL, sulphonamides; TET, tetracycline.
designated pCCK1900, differed distinctly in its restriction patterns from the so-far known florfenicol resistance plasmids of Pasteurellaceae and also conferred resistance to sulphonamides and streptomycin in addition to phenicicol resistance (Table 1). As plasmid pCCK381 has already been described in detail, further analyses focused on the novel plasmid type pCCK1900.

**Structural analysis of plasmid pCCK1900**

Complete sequencing of plasmid pCCK1900 revealed a size of 10 226 bp. A map of this plasmid in comparison with in-part related plasmids is shown in Figure 1. In general, plasmid pCCK1900 appeared to represent an RSF1010 derivative into which a floR gene region has been integrated.

The initial 3215 bp of plasmid pCCK1900 resembled closely (99.8% nucleotide sequence identity) the corresponding region of the 8684 bp broad host range plasmid RSF1010.10 This part contains the sulphonamide resistance gene *su*2 coding for a type II dihydropteroate synthase and the streptomycin resistance genes *strA* and *strB* coding for different types of streptomycin phosphotransferases. Although only two base pair substitutions and five additional base pairs were noted in this entire segment in comparison with RSF1010, four of these additional base pairs, which resulted in frame shifts, were detected within the *su*2 reading frame. As a consequence, the deduced amino acid sequence of the pCCK1900-associated 271 amino acid Sul2 protein differed distinctly from the 262 amino acid Sul2 protein of plasmid RSF1010. The analysis of the *E. coli* JM101 transformants carrying pCCK1900 showed a 128-fold increase in the MIC of sulfamethoxazole (when compared with the empty JM101 recipient strain). This observation suggested that this Sul2 protein is functionally active.

Downstream of the *su*2 gene, the slightly overlapping reading frames of the streptomycin resistance genes *strA* and *strB* were detected. The *strA* gene, which coded for a 267 amino acid protein, exhibited two base pair substitutions in comparison with that of RSF1010, both of which also resulted in amino acid substitutions at positions 156 and 157. In contrast, the *strB* gene, which coded for a 278 amino acid protein, was indistinguishable from the *strB* gene of RSF1010. It should be noted that *sul2* and *strA* genes indistinguishable from or closely related to those of plasmid pCCK1900 have been identified in a large number of Gram-negative bacteria including various members of the family Pasteurellaceae, whereas *strB* genes have often been found to be largely truncated in Pasteurellaceae.11

Plasmid RSF1010 carries a truncated ISCR2 element of 524 bp downstream of the *strB* reading frame. Such ISCR2 elements have been assumed to play a role in the mobility of the chloramphenicol–florfenicol resistance gene *floR.*12 A complete element and a truncated ISCR2 element, both encompassing this 524 bp segment, have recently been described to bracket the *floR* gene on the multiresistance plasmid pAB559 from *Aeromonas bestiarum.*13 In plasmid pCCK1900, a 2325 bp region was detected downstream of *strB*, which included the *floR* gene and differed by only 5 bp from the respective region in the *A. bestiarum* plasmid pAB559. This gene codes for a 404 amino acid phenicol-specific exporter of the Major Facilitator Superfamily,1 which differed by two amino acids from the unpublished FloR proteins of *Salmonella* Dublin (accession no. YP_001552094) and *Vibrio cholerae* (accession no. AAV84883) and by three amino acids from the FloR proteins of pAB559 from *A. bestiarum,* *B. bronchiseptica* and *E. coli.*14,15 Immediately downstream of the *floR* gene, a short reading frame for a 101 amino acid putative transcriptional regulator protein of the LysR family was detected. This reading frame was indistinguishable from those previously found downstream of the *floR* genes in *A. bestiarum,* *E. coli* and *B. thailand.*7,13–15 Based on the structural comparison between the RSF1010 and the pAB559 sequences, it is likely that the 524 bp sequence has served for the integration of the *floR* gene area into the RSF1010-related plasmid pCCK1900, possibly by homologous recombination.

The remaining part of the plasmid pCCK1900 again closely resembles plasmid RSF1010 (99.7% nucleotide sequence identity) and includes three mobilization genes *mobA, mobB* and *mobC* as well as three plasmid replication genes *repA, repB* and *repC* (Figure 1). The deduced sequence of the 94 amino acid

![Figure 1. Schematic representation of plasmid pCCK1900 (accession no. FM179941) in comparison with plasmid RSF1010 (accession no. M28829) and a part of plasmid pAB559 (accession no. EF495198). The reading frames are shown as arrows with the arrowhead indicating the direction of transcription (repA, repB and repC: plasmid replication; mobA, mobB and mobC: mobilization; sul2: sulphonamide resistance; floR: chloramphenicol–florfenicol resistance; strA and strB: streptomycin resistance). The *lysR* gene downstream of *floR* is indicated as a black arrow. The ISCR2 elements are shown as boxes with the inner arrow indicating the reading frame for the transposase gene. The Δ symbol indicates a truncated ISCR2 element. A distance scale in kilobase pairs is shown below each map. The grey-shaded areas mark the areas of >99% sequence identity between the three plasmids.](https://academic.oup.com/jac/article-abstract/62/5/951/729741)
MobC protein differed by a single amino acid substitution at position 55 from the corresponding protein of RSF1010, whereas those of the 709 amino acid MobA protein and the 137 amino acid MobB protein were indistinguishable from the RSF1010-associated MobA and MobB proteins. The deduced protein sequences of the 279 amino acid RepA protein and the 283 amino acid RepC protein differed by one or three amino acids, respectively, from RepA and RepC of RSF1010, whereas that of the 323 amino acid RepB protein was indistinguishable from the RSF1010-associated RepB protein.

Genomic relationships between the florfenicol-resistant
P. multocida isolates

As three of the four so-far confirmed florfenicol-resistant P. multocida isolates harboured the floR-carrying plasmid pCCK381, it was necessary to investigate whether this observation is due to the spread of a single clone across country and host animal borders or due to the horizontal transfer of the plasmid between different P. multocida isolates.

Capsular typing of the two German porcine P. multocida isolates identified isolate 1387-03 as type A and isolate 1900-03 as type D. The bovine isolate R77-07 did not produce an amplicon with the multiplex-PCR applied. The bovine UK isolate 381 had been assigned to capsular type A.\textsuperscript{5} Pulsed-field gel electrophoretic separation of the Smal macrorestriction patterns clearly showed that all four florfenicol-resistant P. multocida isolates were non-related (Figure 2). Hence, a horizontal dissemination of plasmid pCCK381 is the most likely explanation for the occurrence of this plasmid in two bovine and one porcine P. multocida from the UK and Germany. This assumption is also backed up by the finding that plasmid pCCK381 carried a replication/mobilization segment virtually identical to that of the mobilizable broad host range plasmid pDN1 from Dichelobacter nodosus.\textsuperscript{16} Moreover, it has been shown previously that plasmid pDN1 as well as plasmid pCCK381 can replicate in different Gram-negative hosts.\textsuperscript{5,16} The novel plasmid type pCCK1900 described in this study also carries a replication/mobilization part closely related to that of another broad host range plasmid, namely RSF1010. Further monitoring of florfenicol susceptibility in the target pathogens will show whether any of these plasmids will be further disseminated among bovine and porcine respiratory tract pathogens. The high susceptibility of these floR-carrying P. multocida isolates to a wide range of antimicrobial agents commonly used in veterinary medicine might explain why such isolates have not yet emerged.

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Transparency declarations

None to declare.

References

Florfenicol-resistant *P. multocida*


