Efflux-mediated resistance to florfenicol and/or chloramphenicol in *Bordetella bronchiseptica*: identification of a novel chloramphenicol exporter

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Objectives: Twenty florfenicol- and/or chloramphenicol-resistant *Bordetella bronchiseptica* isolates of porcine and feline origin were investigated for the presence of floR and cml genes and their location on plasmids.

Methods: The *B. bronchiseptica* isolates were investigated for their susceptibility to antimicrobial agents by broth micro- or macrodilution and for their plasmid content. Hybridization experiments and PCR assays were conducted to identify resistance genes. Transformation and conjugation studies were performed to show their transferability. Representatives of both types of genes including their flanking regions were sequenced. Moreover, inhibitor studies with the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN) were performed.

Results: The gene floR was found in the chromosomal DNA of 9 of the 18 florfenicol/chloramphenicol-resistant isolates. Sequence analysis revealed that the deduced FloR protein sequence differed by a single amino acid exchange from FloR of *Vibrio cholerae*. A chloramphenicol-resistant, but florfenicol-susceptible isolate carried a novel plasmid-borne cml gene, designated cmlB1. The CmlB1 protein revealed only 73.8–76.5% identity to known CmlA proteins. The gene cmlB1 was not part of a gene cassette. The results of inhibitor studies with PAβN suggested that a so-far unidentified efflux system might play a role in phenicol resistance of the remaining florfenicol- and/or chloramphenicol-resistant isolates.

Conclusions: This is to the best of our knowledge the first report of a floR gene in *B. bronchiseptica* isolates. The identification of the first member of a new subclass of cml genes, cmlB1 from *B. bronchiseptica*, extends our knowledge on specific chloramphenicol exporters.

Keywords: cmlB1 gene, floR gene, efflux pump inhibitors, respiratory tract pathogen

Introduction

*Bordetella bronchiseptica* is frequently involved in respiratory tract infections of food-producing animals and companion animals. Antimicrobial agents are commonly used to treat these infections in animals. Initial studies of antimicrobial resistance in *B. bronchiseptica* from pigs revealed a decreased susceptibility to most of the antimicrobial agents currently approved for the treatment of respiratory tract infections, such as tilmicosin and ceftiofur, with MIC₉₀ values of 16 mg/L and 32 mg/L, respectively. For other antimicrobial agents, such as florfenicol, a fluorinated chloramphenicol derivative, the corresponding MIC values were distinctly lower. Following the European Union’s ban of chloramphenicol use in food-producing animals in 1994, florfenicol has been approved for the treatment of respiratory tract infections in cattle (1995) and in pigs (2000). By contrast, chloramphenicol is still approved for use in dogs, cats and other non food-producing animals, and based on its favourable susceptibility situation it is used for the control of a wide variety of infections in these animals.

Although florfenicol-resistant *B. bronchiseptica* isolates from respiratory tract infections in pigs have been detected in recent years, the genetic basis for this resistance in *B. bronchiseptica* had not been elucidated. In the present study, we analysed isolates classified as florfenicol/chloramphenicol-resistant or only chloramphenicol-resistant for the presence of known florfenicol and/or chloramphenicol resistance genes. In addition, an efflux pump inhibitor was used to assess whether efflux may play a role in phenicol resistance of *B. bronchiseptica*.

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Materials and methods

Bacterial isolates and susceptibility testing

A total of 496 *B. bronchiseptica* isolates from animals suffering from respiratory tract infections in Germany, including 349 isolates from pigs collected between 2000 and 2003, as well as 105 isolates from cats and 34 from dogs, all collected between 2004 and 2006, were investigated for their susceptibility to florfenicol and chloramphenicol. Biochemical species identification was confirmed by genus- and species-specific PCR analysis.\(^6\) Inhibition of efflux mechanisms

For the detection of the chloramphenicol resistance genes, PCR assays for the genes conferring combined resistance to florfenicol and chloramphenicol, namely *floR*, *fexA* and *cfr*, but also for the most common chloramphenicol resistance genes *catA1*, *catA2*, and *catA3* were performed according to previously described protocols. For the detection of the chloramphenicol resistance gene *cmlA*, previously described primers were used at an annealing temperature of 60°C. In addition, all isolates were investigated for class 1 integrons and their associated catB gene cassettes. For the gene *floR*, additional new primers were designed to amplify the entire *floR* gene and used with the annealing temperature of 50°C. The forward primer (5'-AGGGTTGATTCGTC-3') contained the start codon and the reverse primer (5'-CGGTAGACGA CTGGCAGC-3') the stop codon of the *floR* gene. To detect circular forms of the *floR*-carrying transposon *Tn*florR*, the primers *floRcirc1* and *floRcirc2* were used. In addition, PCR assays were conducted for the *opxAB* operon which has recently been described to mediate the efflux of chloramphenicol in addition to that of olequindox.

**Plasmid profiling, transfer experiments and Southern blot hybridization**

Plasmid profiles were prepared by alkaline lysis as described. \(^6\) Conjugation into *E. coli* HK225 was performed. \(^6\) Electrotransformation into *B. bronchiseptica* B543 and into *E. coli* HB101 was carried out as described previously for *Pasteurella*\(^1\) with the Gene Pulser II electroporation system (Bio-Rad, Munich, Germany). Transfer was confirmed by MIC determination and by plasmid isolation with subsequent restriction analysis and PCR assays. Southern blot hybridization was performed with a PCR-generated *floR* gene probe\(^6\) using either *EcoRI* or *SacI*-digested whole cell DNA or uncult plasmid profiles as target DNA. Probe labelling was achieved with the DIG-High Prime DNA labelling and detection system. Hybridization and signal detection followed the recommendations given by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

**Sequencing of resistance gene regions**

For sequence analysis, the *floR* and *cmlA* PCR amplicons were cloned into the vector pCR-blunt (Invitrogen, Groningen, The Netherlands) as described previously.\(^6\) To sequence the flanking regions of *floR*, chromosomal DNA was first digested with the restriction enzyme *AgeI* and the fragments re-ligated with *T4* DNA ligase. Subsequently, inverse PCR with the *floRcirc* primers was conducted. The resulting amplicon was cloned into pCR-blunt and sequenced. To sequence the flanking regions of the plasmid-borne *cmlA*-like gene, the plasmid was digested with *EcORI* and *KpnI* and the fragments cloned into pBluescript II SK+ (Stratagene, Amsterdam, The Netherlands). The clones were confirmed by plasmid profiling, restriction analysis, PCR-directed determination of the *cmlA*-like gene, and expression of chloramphenicol resistance. Sequences were deposited in the EMBL database under accession nos. AM296480 (*floR*) and AM296481 (*cmlB1*).

Results and discussion

**Susceptibility testing**

Of all the isolates analysed, only 18 *B. bronchiseptica* isolates, 17 from pigs and one from a cat, were classified as florfenicol-resistant by CLSI criteria with MICs of florfenicol of ≥8 mg/L.\(^7\) All florfenicol-resistant isolates (nos. 1–18) exhibited MICs of chloramphenicol of ≥16 mg/L (Table 1). In addition, two isolates (nos. 19 and 20) were chloramphenicol-resistant, but not florfenicol-resistant, and had MICs for chloramphenicol of 128 mg/L and 32 mg/L, respectively. The high MIC for chloramphenicol of 128 mg/L for isolate no. 19 suggested the presence of a specific chloramphenicol resistance gene. The remaining isolates (nos. 21–23) were used for control purposes and showed low MIC values of 4 mg/L for chloramphenicol and florfenicol (Table 1).

**FloR-mediated florfenicol/chloramphenicol resistance**

In isolates 3–11 (Table 1), the gene *floR* was detected by PCR. Hybridization studies confirmed that this gene was located in the chromosomal DNA in all nine cases. Plasmid profiling revealed that the *floR*-carrying strains were plasmid-free. Since all these *B. bronchiseptica* isolates shared indistinguishable or closely related XbaI macrorestriction patterns, one of these isolates, no. 5, was chosen for further analysis. Analysis of a 1638 bp region including the *floR* gene and 66 bp in its upstream and 356 bp in its downstream flanking regions revealed a single base-pair exchange as compared with the corresponding sequence of *Vibrio cholerae* (accession no. AY822603). This base-pair exchange resulted in an amino acid exchange of His-202 in *V. cholerae* versus Arg-202 in *V. cholerae*. Compared with FloR proteins so far found in other respiratory tract pathogens,
FloR from *B. bronchiseptica* differed by four amino acid exchanges each from FloR of *Pasteurella multocida* (Leu-178, His-202, Pro-207 and Phe-228 in *B. bronchiseptica* versus Arg-178, Arg-202, Ala-207 and Tyr-228 in *P. multocida*). Although the determined upstream and downstream flanking regions of floR were identical to the sequence of Tn<sup>floR</sup>, a circular intermediate (which would have confirmed the mobility of floR) could not be detected in any of the nine floR-carrying isolates.

In the remaining nine florfenicol-resistant isolates, the floR gene was not detectable by PCR or by specific hybridization. In addition, none of the other two so-far known florfenicol resistance genes, cfr and fexA, was detectable. PCR assays for genes conferring resistance to chloramphenicol only did not yield amplicons in any of the 18 florfenicol-resistant isolates, thus confirming that no additional chloramphenicol resistance gene is present in these isolates.

### CmlB1-mediated chloramphenicol resistance

Solely in isolate no. 19, which had an MIC for chloramphenicol of 128 mg/L, was a cmlA-like gene detected. Plasmid profiling as well as conjugation and transformation experiments revealed its localization on a non-conjugative plasmid of approx. 50 kb. Sequencing of the entire gene and analysis of the deduced amino acid sequence showed that the corresponding gene product differed distinctly from the amino acid sequences of all so-far known CmlA proteins. Based on a multi-sequence alignment with all CmlA amino acid sequences currently deposited in the databases, the 421 amino acid protein from *B. bronchiseptica* showed identities of only 73.7 to 76.5% to the different CmlA proteins, with least identity to the CmlA4 protein of *Salmonella enterica* serovar Agona<sup>21</sup> and the highest identity to CmlA5 from *Acinetobacter baumannii*.<sup>22</sup> Based on this level of identity, the chloramphenicol exporter from *B. bronchiseptica* was considered as the first representative of a novel class of CmlA-like proteins but different from the CmlA proteins. Therefore, it was designated CmlB1. A phylogenetic tree (Figure 1) confirmed the evolutionary distance of the CmlB1 protein from the different CmlA variants. In this regard, it should be noted that the CmlA protein sequences as deposited in the databases varied in size between 390 and 437 amino acids with most of the CmlA proteins having a size of 419 amino acids. The cmlA genes coding for proteins of 418 and 419 amino acids have been reported to start with GTG start codon.<sup>21</sup>

### Table 1. MICs for the *B. bronchiseptica* isolates of florfenicol (FFC), chloramphenicol (CHL) and nalidixic acid (NAL) determined in the absence (−) or presence (+) of the efflux pump inhibitor PAβN, PFGE patterns and the phenicol resistance genes detected in the isolates

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Year</th>
<th>MIC (mg/L)</th>
<th>PFGE pattern&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phenicol resistance gene present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CHL - PAβN</td>
<td>CHL + PAβN</td>
<td>FFC - PAβN</td>
</tr>
<tr>
<td>1</td>
<td>2003</td>
<td>128</td>
<td>4</td>
<td>128</td>
</tr>
<tr>
<td>2</td>
<td>2003</td>
<td>128</td>
<td>8</td>
<td>128</td>
</tr>
<tr>
<td>3</td>
<td>2000</td>
<td>256</td>
<td>128</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>2006</td>
<td>256</td>
<td>128</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>2003</td>
<td>256</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
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<td>ND</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>2002</td>
<td>256</td>
<td>ND</td>
<td>16</td>
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<td>8</td>
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<td>128</td>
<td>ND</td>
<td>32</td>
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<td>9</td>
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<td>128</td>
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<tr>
<td>16</td>
<td>2006</td>
<td>32</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>17</td>
<td>2006</td>
<td>32</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2006</td>
<td>16</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>19</td>
<td>2001</td>
<td>128</td>
<td>16</td>
<td>4</td>
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<tr>
<td>20</td>
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<td>21</td>
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<tr>
<td>23</td>
<td>2006</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

ND, not determined.

<sup>a</sup>A new letter was given if the pattern differed by three or more bands. Patterns indicated as, e.g. A1 or A2, differed by only one or two bands from pattern A.

<sup>b</sup>Isolate no. 18 was from a cat suffering from an upper respiratory tract infection, while all other isolates were from pigs.

FloR from *B. bronchiseptica* differed by four amino acid exchanges each from FloR of *Pasteurella multocida* (Leu-178, His-202, Pro-207 and Phe-228 in *B. bronchiseptica* versus Arg-178, Arg-202, Ala-207 and Tyr-228 in *P. multocida*) and from FloR of *Pasteurella trehalosi* (Ile-32, Met-147, His-202 and Met-225 in *B. bronchiseptica* versus Met-32, Ile-147, Arg-202 and Ile-225 in *P. trehalosi*).<sup>19,20</sup> Although the determined upstream and downstream flanking regions of floR were identical to the sequence of Tn<sup>floR</sup>, a circular intermediate (which would have confirmed the mobility of floR) could not be detected in any of the nine floR-carrying isolates.

In the remaining nine florfenicol-resistant isolates, the floR gene was not detectable by PCR or by specific hybridization. In addition, none of the other two so-far known florfenicol resistance genes, cfr and fexA, was detectable. PCR assays for genes conferring resistance to chloramphenicol only did not yield amplicons in any of the 18 florfenicol-resistant isolates, thus confirming that no additional chloramphenicol resistance gene is present in these isolates.
This unusual start codon has also been identified in the novel *cmlB1* gene. A closer look at the reading frames for the 390 amino acid proteins (accession nos. AAY43147, AAY43150, ABH07981, ABB71444, CAD31707) strongly suggested that these *cmlA* genes also have the GTG start codon rather than the proposed ATG start codon and thus code for a protein of 419 amino acids as well. A wrong annotation of the start codon (ATC at positions 43516–43518) in the nucleotide sequence of the *cmlA5* gene of *A. baumannii* resulted in the uncommon size of 437 amino acids of the respective gene product (CAJ77046). Most likely, the *cmlA5* gene of *A. baumannii* also starts with GTG (at positions 43570–43572) and codes for a 419 amino acid protein.

The analysis of a 2291 bp region encompassing the *cmlB1* gene revealed a 582 bp region in the upstream part which differed only by 1 bp from the respective part in the whole genome sequence of *B. bronchiseptica* strain RB50 (BX640441). Immediately downstream of the *cmlB1* gene, an incomplete reading frame was detected which resembled the N-terminus of a transposase from *Marinobacter aquaeolei* VT8 (ZP_00818190). Although many *cmlA* genes are part of gene cassettes located in class 1 integrons, no structures resembling the 5’- and 3’-conserved segments of integrons were detectable upstream and downstream of the *cmlB1* gene. Moreover, no 59-base element was detectable downstream of the translational termination codon of *cmlB1*.

In the area immediately upstream of the *cmlB1* gene, a putative regulatory region comprising a small reading frame for a 9 amino acid peptide and two pairs of imperfect inverted repeated (IR) sequences of 12 and 10 bp, respectively, were detected. Such an arrangement has also been described for the *cmlA1* gene of Tn1696 and is assumed to play a role in the chloramphenicol-inducible expression of the *cmlA1* gene by attenuated translation. The IR1 sequence in the *cmlB1* upstream region was detected immediately after the translational stop codon, whereas the IR4 sequence comprised the start of the *cmlB1* gene. Calculation of the mRNA stabilities suggested that IR1 : IR2 ($\Delta G = 90.3$ kJ/mol) and IR3 : IR4 ($\Delta G = 79.4$ kJ/mol), but also IR2 : IR3 ($\Delta G = 74.4$ kJ/mol) may be able to form stable mRNA secondary structures. In addition, the small reading frame also contained a ribosome stall sequence 5’-AACAAAGCAGAC-3’ which was indistinguishable from that in the small reading frame upstream of the inducibly expressed chloramphenicol resistance gene of the staphylococcal plasmid pC194. All these sequence features may support the assumption that *cmlB1* expression is also regulated by translational attenuation. MIC
Phenicol resistance in *Bordetella bronchiseptica*

Table 2. MICs of chloramphenicol (CHL) and florfenicol (FFC) for the *cmlB1*-carrying isolates determined with and without induction by CHL or FFC

<table>
<thead>
<tr>
<th>Isolates</th>
<th>CHL MIC (mg/L)</th>
<th>FFC MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>not induced</td>
<td>induced with</td>
</tr>
<tr>
<td>Bordetella bronchiseptica B1115</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>B. bronchiseptica B543</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>B. bronchiseptica B543::pKBB1115</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>Escherichia coli HB101</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>E. coli HB101::pKBB1115</td>
<td>8</td>
<td>32</td>
</tr>
</tbody>
</table>

*The test strains comprise the original *cmlB1*-carrying *B. bronchiseptica* isolate B1115, but also the recipient strains *B. bronchiseptica* B543 and *E. coli* HB101 and their transformants harbouring the *cmlB1*-carrying plasmid pKBB115.

determination for the original *cmlB1*-carrying *B. bronchiseptica* isolate and its *B. bronchiseptica* B543 and *E. coli* HB101 transformants revealed up to 16-fold increase in the MICs of chloramphenicol and up to 8-fold increase in the MICs of florfenicol after pre-incubation in subinhibitory concentrations of chloramphenicol or florfenicol (Table 2).

Inhibition of efflux-mediated phenicol resistance

To investigate efflux inhibition, we used three of the nine *floR*-carrying isolates, all nine florfenicol-resistant but *floR*-negative isolates, the two chloramphenicol-resistant isolates and, as controls, three isolates with lower MICs for chloramphenicol and florfenicol of 4 mg/L. The MIC values of the antimicrobial agents in the absence and in the presence of the efflux inhibitor PAβN are shown in Table 1. In isolates carrying *floR*, a 2- to 4-fold decrease in the MICs of both phenicols was seen in the presence of PAβN. In contrast, *floR*-negative florfenicol-resistant isolates showed a distinctly more pronounced susceptibility to both phenicols in the presence of PAβN, as illustrated by an 8- to 32-fold decrease in the corresponding MICs. A very similar situation was seen with the MICs for the *B. bronchiseptica* isolates classified as intermediately susceptible to florfenicol (MIC 4 mg/L) (Table 1).

Since PAβN interferes with multi-drug efflux systems of the resistance-nodulation-division (RND) family, it may be possible that one or more such systems, which are widespread among Gram-negative bacteria, are also present in *B. bronchiseptica* and may play a role in phenicol resistance. In other bacteria, such as *Salmonella enterica*, it has been shown that the MIC of florfenicol dropped distinctly in the presence of the efflux pump inhibitor PAβN. Efflux systems of the RND family, like AcrAB-ToIC, can also export other antimicrobials such as the quinolone nalidixic acid. In good accordance with the results for florfenicol and chloramphenicol, the MICs of nalidixic acid for the *B. bronchiseptica* isolates also dropped by three to seven dilution steps in the presence of PAβN (Table 1). While isolates 1, 2, 12–18 and 20 showed MICs of nalidixic acid of 64 mg/L and 128 mg/L, the isolates with the phenicol-specific efflux pumps FloR or CmlB1 and the isolates 21–23 used for control purposes had lower MICs for nalidixic acid of 16 mg/L. In the presence of PAβN, an MIC for nalidixic acid of 1–2 mg/L was determined for these *B. bronchiseptica* isolates, indicating that they may also harbour one or more efflux system(s) not further specified—putatively also of the RND family—exporting phenicols and/or nalidixic acid. Enhanced expression of RND systems in resistant isolates have been described for the AcrAB-ToIC tripartite pump from *E. coli* and *S. enterica*. In *S. enterica* these pumps conferred lower susceptibility to chloramphenicol, florfenicol and quinolones, but not to ampicillin or streptomycin. In the genome of the completely sequenced *B. bronchiseptica* isolate RB50, several putative efflux proteins have been identified. One cluster of genes shows homology to genes encoding the RND efflux system common in *E. coli*: genes encoding an AcrA homologue (CAE34795), followed by two genes encoding proteins similar to AcrB (CAE34794, CAE24793), and followed by a gene encoding a protein similar to ToIC (CAE34792). Further work is needed to clarify whether these putative efflux proteins from *B. bronchiseptica* act as a multi-drug transporter and, if so, what is the substrate spectrum of this efflux system.

In conclusion, the results of this study showed that at least two different phenicol-specific efflux pumps of the MF superfamily, encoded by the genes *floR* and *cmlB1*, but also a further unspecified efflux system, confer resistance to phenicols in *B. bronchiseptica*. These data complement recent findings on chloramphenicol resistance genes *catB2* and *catB3*, coding for chloramphenicol-inactivating enzymes, in porcine *B. bronchiseptica*.

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Transparency declarations

None to declare.

References


