Plasmid-borne florfenicol and ceftiofur resistance encoded by the floR and bla\textsubscript{CMY-2} genes in *Escherichia coli* isolates from diseased cattle in France

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This study was designed to determine the genetic basis of florfenicol and ceftiofur resistance in *Escherichia coli* isolates recovered from French cattle. In these isolates, ceftiofur resistance was conferred by bla\textsubscript{CMY-2} located on three distinct conjugative plasmids on a specific DNA fragment, ISEcp1-bla\textsubscript{CMY-2}-sugE. Two of the plasmids also carried the floR gene conferring resistance to florfenicol. The floR gene was shown to be associated with the insertion sequence ISCR2. Mobile elements appear to contribute to the mobilization of floR and bla\textsubscript{CMY-2} genes in *E. coli*. The presence of bla\textsubscript{CMY-2} and floR on the same plasmid highlights the potential risk for a co-selection of the bla\textsubscript{CMY-2} gene through the use of florfenicol in food animal production.

**INTRODUCTION**

In numerous reported cases, resistance to extended-spectrum cephalosporins (ESCs), such as ceftriaxone and ceftiofur, in *Salmonella enterica* and *Escherichia coli* is conferred by chromosomal or plasmid-borne AmpC \textbeta-lactamases. The \textbeta-lactamase CMY-2 has been described worldwide as the most prevalent plasmid-borne AmpC \textbeta-lactamase in *S. enterica* and *E. coli* (Alvarez \textit{et al.}, 2004; Li \textit{et al.}, 2007).

The association of bla\textsubscript{CMY-2} gene and genes conferring resistance to other antimicrobials, such as aminoglycosides, sulphonamides, tetracyclines and phenicol, on the same plasmid could also contribute to selecting multidrug resistance phenotypes (Doublet \textit{et al.}, 2004).

Florfenicol is a fluorinated structural analogue of chloramphenicol and thiamphenicol approved exclusively for veterinary use. The florfenicol-resistance gene floR has been characterized on different mobile genetic elements, on the *Salmonella* genomic island 1 (SGI1) in various *S. enterica* serovars and *Proteus mirabilis*, on conjugative and non-conjugative plasmids in *E. coli* and *S. enterica* serovars Typhimurium and Newport, as well as on the chromosome in *E. coli* (Boyd \textit{et al.}, 2008; Mulvey \textit{et al.}, 2006). The floR gene has also been reported as part of a transposon on a plasmid of a bovine *E. coli* strain, suggesting that this insertion sequence contributes to the diffusion of the floR gene between different plasmids and also the chromosome (Doublet \textit{et al.}, 2005). This transposition process has been shown to occur via a circular intermediate form. Characteristics of this Tn\textsubscript{floR} are very similar to those associated with site-specific integrating transposons such as Tn554, Tn5406 and Tn558, i.e. production of circular form, no inverted repeat at their termini and presence of 6–7 bp sequences at their left and right junctions. However, it has recently been demonstrated that this circular intermediate form may be the result of an homologous recombination between insertion sequence ISCR2 and its truncated form located on both sides of the floR gene (Toleman \textit{et al.}, 2006). The presence of these insertion sequences would play a role in the transposition of floR but would not constitute a transposable element.

Data have been reported on *S. enterica* isolates in the United States and France which harboured both bla\textsubscript{CMY-2} and floR on the same plasmid (Alcaine \textit{et al.}, 2005; Doublet \textit{et al.}, 2004; Egorova \textit{et al.}, 2008). Here, we characterized plasmids carrying floR and bla\textsubscript{CMY-2} in bovine *E. coli* isolates recovered in 2003 and 2004. We also investigated the mobile genetic elements potentially involved in the capture and dissemination of these genes by analysing the genetic environments of floR and bla\textsubscript{CMY-2} on the plasmids.

**Abbreviation:** ESC, extended-spectrum cephalosporin.
METHODS

Bacterial isolates. The bovine E. coli isolate 13688 was collected in 2003 from a lung of a calf with clinical signs reported as respiratory disease. Two other bovine E. coli isolates, 13954 and 13956, were obtained from faeces of cattle suffering from digestive disease (diarrhoea) in 2003 and 2004, respectively (Table 1). These isolates were collected through the RESAPATH network and initial screening of ESC-resistant isolates was done using cefotiofur as an indicator.

E. coli J53 (pro met azi) was used as a control for mating experiments.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was determined by the disc diffusion method on Mueller–Hinton agar according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (http://www.sfm.asso.fr) with 32 antimicrobial drugs (Sirot et al., 1996). E. coli ATCC 25922 was used as a control in antimicrobial susceptibility testing. The MICs for chloramphenicol and florfenicol were determined by the standard agar doubling dilution method using primers previously described (Arcangioli et al., 1999; Eckert et al., 2004). The PCR products of blaTEM, blaSHV, and floR were sequenced on both strands by Cogenics (Meylan, France). The nucleotide sequences were analysed with the BLAST program (Altschul et al., 1997).

Phylogenetic grouping. Phylogenetic grouping of the E. coli isolates was performed by amplification by PCR of two genes, chuA and yjaA, and an anonymous DNA fragment, TspE4.C2 (Clermont et al., 2000).

Conjugation and plasmid analysis. Mating experiments were carried out in liquid media with E. coli K-12 J53 (pro met azi) used as recipient strain and E. coli isolates 13688, 13954 and 13956 used as donor strains. Transconjugants were selected on brain heart infusion agar supplemented with cefotiofur (2 mg l⁻¹) and sodium azide (500 mg l⁻¹). Plasmid DNA of the E. coli transconjugants was obtained as described by Takahashi & Nagano (1984) and was then subjected to restriction mapping with two restriction endonucleases, BglII and EcoRI, to evaluate the relatedness of plasmids.

A PCR-based replicon typing method was performed on each blaCMY-2-carrying plasmid to determine plasmid incompatibility groups (Carattoli et al., 2005).

Analysis of the genetic environments of blaCMY-2 and floR resistance genes. The presence of floR and blaCMY-2 on the same plasmid and their genetic environment were studied by Southern blotting of BglII-digested plasmidic DNA using as probes floR and blaCMY-2 genes and the AmpA-floRNA gene cluster (hereafter described as ΔISR2-flor-ISR2), obtained from the floR plasmid pEF03 (Doubelt et al., 2002). The genetic environment of blaCMY-2 and floR was assessed by PCR mapping with primers Tn-F/AmpC-R, AmpC-F/SugE-R, AmpC-F/AmpC-R and Tn-F/SugE-R to amplify the different regions of the element carrying blaCMY-2 (Su et al., 2006) and with primers floRCirc1 and floRCirc2 to detect the circular intermediate of the floR-carrying element (Doublet et al., 2005).

RESULTS AND DISCUSSION

E. coli isolates 13688, 13954 and 13956 were resistant to amoxicillin, amoxicillin–clavulanic acid, cefotiofur, cefoxitin, cefazidime, kanamycin, gentamicin, streptomycin, apramycin, tetracycline, chloramphenicol, nalidixic acid, enrofloxacin, trimethoprim and sulphonamides and displayed intermediate resistance to cefotaxime and aztreonam by the disc diffusion method. Resistance to florfenicol was also observed in E. coli strains 13688 and 13956. All three strains showed susceptibility to cefepime, cefquinome and amikacin (Table 1). Phylogenetic grouping assigned the E. coli isolates 13688 and 13956 to Groups A and B1, respectively, which most commensal strains belong to. E. coli 13954 belonged to Group D, which is a source of extraintestinal pathogenic E. coli.

Identification of resistance genes by PCR and DNA sequencing in the three isolates showed that resistance to ESCs was conferred by the blaCMY-2 gene. The narrow-spectrum β-lactamase gene blaTEM-1 was also detected in these three E. coli strains.

Table 1. Characteristics of the blaCMY-2-carrying Escherichia coli strains analysed in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year</th>
<th>Origin</th>
<th>AmpC β-lactamase identified</th>
<th>MIC (mg l⁻¹)‡</th>
<th>Additional resistances†</th>
<th>Replicon typing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CHL</td>
<td>FFC</td>
<td>CHL</td>
<td>GEN</td>
</tr>
<tr>
<td>13954</td>
<td>2003</td>
<td>Cattle, faeces</td>
<td>CMY-2</td>
<td>–</td>
<td>–</td>
<td>KAN, GEN, S, APRA, TET, CHL, NAL, ENR, TMP, SUL</td>
</tr>
<tr>
<td>13954-†</td>
<td>2003</td>
<td>Cattle, faeces</td>
<td>CMY-2</td>
<td>–</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td>13956</td>
<td>2004</td>
<td>Cattle, faeces</td>
<td>CMY-2</td>
<td>256</td>
<td>256</td>
<td>KAN, GEN, S, APRA, TET, CHL, FFC, NAL, ENR, TMP, SUL</td>
</tr>
<tr>
<td>13956-†</td>
<td>2004</td>
<td>Cattle, faeces</td>
<td>CMY-2</td>
<td>256</td>
<td>256</td>
<td>FFC, CHL, TET, TMP, SUL</td>
</tr>
<tr>
<td>13688</td>
<td>2003</td>
<td>Calf, lung</td>
<td>CMY-2</td>
<td>256</td>
<td>256</td>
<td>KAN, GEN, S, APRA, TET, CHL, FFC, NAL, ENR, TMP, SUL</td>
</tr>
<tr>
<td>13688-†</td>
<td>2003</td>
<td>Calf, lung</td>
<td>CMY-2</td>
<td>256</td>
<td>256</td>
<td>FFC, CHL, APRA, GEN, TET, TMP, SUL</td>
</tr>
</tbody>
</table>

*CHL, Chloramphenicol; FFC, florfenicol.
†KAN, Kanamycin; GEN, gentamicin; S, streptomycin; APRA, apramycin; TET, tetracycline; CHL, chloramphenicol; NAL, nalidixic acid; ENR, enrofloxacin; TMP, trimethoprim; SUL, sulphonamides; FFC, florfenicol.
‡Transconjugants.


"bla\textsubscript{CMY-2} has been shown to be the most prevalent type of plasmid AmpC \(\beta\)-lactamase in members of the \textit{Enterobacteriaceae} of animal origin (Li \textit{et al.}, 2007). The dissemination of this resistance gene throughout bacterial populations can occur by the spread of an epidemic clone or by plasmid transfer. In this study, macrorestriction profiles of the three \textit{E. coli} strains DNA were distinct. The three isolates were probably not epidemiologically linked according to epidemiological data (data not shown).

Then, to assess the transfer of plasmid-borne \(\text{bla}\textsubscript{CMY-2}\)-determining experiments were carried out with the three strains. Transconjugants were obtained on plates supplemented with cefotiofur (2 mg l\(^{-1}\)) and were subjected to antimicrobial susceptibility testing. All transconjugants (13688-1, 13954-1 and 13956-1) showed a phenotypic \(\beta\)-lactam-resistance profile (resistance to amoxicillin, amoxicillin plus clavulanic acid, cefalotin, ceftazidime, cefoxitin, cefotaxime and cefotiofur) related to the presence of \(\text{bla}\textsubscript{CMY-2}\) on conjugative plasmids. In addition, two of the three transconjugants tested (13688-1 and 13956-1) were also resistant to sulphonamides, trimethoprim and tetracycline. Moreover, the \textit{E. coli} transconjugants 13688-1 and 13956-1 were also resistant to chloramphenicol and florfenicol, as were their parental strains, both with MICs of 256 mg l\(^{-1}\) (Table 1). Analysis of EcoRI (data not shown) and \(Bgl\text{II}\) restriction profiles of the \(\text{bla}\textsubscript{CMY-2}\)-carrying plasmids in strains 13688-1, 13954-1 and 13956-1 showed that the three plasmids were different from each other and also different from the previously described \(\text{bla}\textsubscript{CMY-2}\) and \(\text{floR}\)-carrying plasmid isolated from the human \textit{S. enterica} serovar Typhimurium strain 2039 and from the \(\text{floR}\)-carrying plasmid recovered from the bovine \textit{E. coli} strain BN10660 (Fig. 1a) (Doublet \textit{et al.}, 2002, 2004), suggesting an independent acquisition of the \(\text{floR}\) and \(\text{bla}\textsubscript{CMY-2}\) genes on these plasmids. This result differs from others recently reported in France in food animals where identical \(\text{bla}\textsubscript{CTX-M-1}\)-carrying plasmids were encountered in \textit{E. coli} strains recovered from poultry, cattle and swine (Girlich \textit{et al.}, 2007; Meunier \textit{et al.}, 2006).

In members of the \textit{Enterobacteriaceae}, the spread of \(\text{bla}\textsubscript{CMY-2}\) seems to be ensured by the acquisition of the \(\text{bla}\textsubscript{CMY-2}\)-carrying structure ISEcp1-\(\text{bla}\textsubscript{CMY-2}\)-\(\text{blc}\textsubscript{E}\)-\(\text{sug}\textsubscript{E}\) (Su \textit{et al.}, 2006). Overlapping PCR mapping confirmed that an identical structure to that described in \textit{S. enterica} serovar Choleraesuis strain SC-B67 (Su \textit{et al.}, 2006) was detected in transconjugants 13688-1 and 13956-1, in contrast to transconjugant 13954-1, in which only a truncated form of ISEcp1 was located upstream of \(\text{bla}\textsubscript{CMY-2}\) (Fig. 2a). The presence of \(\text{bla}\textsubscript{CMY-2}\) on these plasmids was also confirmed by Southern blotting using a \(\text{bla}\textsubscript{CMY-2}\) probe, which yielded a single \(Bgl\text{II}\) fragment of around 5 kb (Fig. 1c). Thus, even if the plasmids carrying \(\text{bla}\textsubscript{CMY-2}\) are different in the three \textit{E. coli} isolates, the similarity of the regions surrounding the gene strongly suggests a transfer of \(\text{bla}\textsubscript{CMY-2}\) among different plasmids in \textit{E. coli} strains. Further to the acquisition of the \(\text{bla}\textsubscript{CMY-2}\) gene, plasmids can be transmitted within the same species or between different genera of bacteria as it has been reported between \textit{E. coli} and \textit{S. enterica} (Winokur \textit{et al.}, 2001).

The florfenicol resistance of transconjugants 13688-1 and 13956-1 was conferred by the presence of the \(\text{floR}\) gene. The regions adjacent to \(\text{floR}\) were studied by Southern blotting of plasmid DNA digested by \(Bgl\text{II}\) with the \(\Delta\text{ISCR2-flor-ISCRI}\) gene cluster used as a probe (Fig. 1b). Results indicated that \(\text{floR}\) and the flanking regions were well-conserved in the two transconjugants 13688-1 and 13956-1 and were similar to the structure initially described in \textit{E. coli} strain BN10660 (Fig. 2b) (Doublet \textit{et al.}, 2005).

A PCR-based replicon typing method showed that two of the \(\text{bla}\textsubscript{CMY-2}\) plasmids isolated from \textit{E. coli} strains 13688-1

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**Fig. 1.** \(Bgl\text{II}\) restriction patterns of plasmids (a), Southern blot hybridization with the \(\Delta\text{ISCR2-flor-ISCRI}\) probe of \(Bgl\text{II}\)-digested plasmids (b) and Southern blot hybridization with the \(\text{bla}\textsubscript{CMY-2}\) probe of \(Bgl\text{II}\)-digested plasmids (c) from transconjugant \textit{E. coli} 13688-1 (lanes 1), transconjugant \textit{E. coli} 13954-1 (lanes 2), transconjugant \textit{E. coli} 13956-1 (lanes 3), transconjugant \textit{E. coli} 2039 TC1 (lanes 4) and transconjugant \textit{E. coli} BN10660-1 (lanes 5). Molecular mass marker: Smart Ladder (Eurogentec).
and 13956-1 were of the replicon type A/C. IncA/C plasmids harbouring \(\text{bla}_{\text{CMY-2}}\) have been reported worldwide in both humans and animals, suggesting that this IncA/C plasmid could be a successful and widely distributed plasmid circulating on different continents (Hopkins et al., 2006). In \(E.\ coli\) 13954-1, the \(\text{bla}_{\text{CMY-2}}\) plasmid was of the IncI1 replicon type.

Previous reports described the presence on IncA/C plasmids harbouring \(\text{bla}_{\text{CMY-2}}\) of genes conferring resistance to antimicrobials other than \(\beta\)-lactams, suggesting that these types of plasmids are involved in dissemination of multidrug-resistant strains (Lindsey et al., 2009; Mataseje et al., 2009). Interestingly, in transconjugant 13954-1, the IncI1 plasmid seemed not to confer additional antimicrobial drug resistance. This result was also reported by Hopkins et al. (2006), suggesting an evolution of plasmids and an ability to gain or to lose resistance determinants.

The association of \(\text{floR}\) and \(\text{bla}_{\text{CMY-2}}\) on the same IncA/C plasmid significantly increases the risk of co-selection and then horizontal dissemination and persistence of ceftiofur and florfenicol co-resistant \(E.\ coli\) in animals. Recently, a plasmid with both \(\text{floR}\) and \(\text{bla}_{\text{CMY-2}}\) was recovered from a catfish pathogen, \(Edwardsiella ictaluri\), in the United States whereas no cephalosporins are currently approved for aquaculture, suggesting that the use of florfenicol in aquaculture could contribute to the dissemination of such a multidrug resistance plasmid (Welch et al., 2009). This is of particular concern as, based on phenotypic data, it has recently been demonstrated that healthy calves are rapidly colonized after birth by ceftiofur and florfenicol co-resistant \(E.\ coli\) (Donaldson et al., 2006).

Identification of insertion sequences and transposons argues in favour of the important role played by these mobile genetic elements in the acquisition of resistance genes by chromosomes or plasmids. The presence of several antimicrobial resistance genes on the same plasmid highlights the role of co-selection in horizontal transfer of multiple antimicrobial resistance genes among bacterial pathogens.

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