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FEMS Microbiology Letters 233 (2004) 301–305

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Plasmid-mediated florfenicol and ceftriaxone resistance encoded by the *floR* and bla_{CMY-2} genes in *Salmonella enterica* serovars Typhimurium and Newport isolated in the United States

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Received 9 February 2004; received in revised form 26 February 2004; accepted 26 February 2004

First published online 7 March 2004

Abstract

Multidrug resistance plasmids carrying the bla_{CMY-2} gene have been identified in *Salmonella enterica* serovars Typhimurium and Newport from the United States. This gene confers decreased susceptibility to ceftriaxone, and is most often found in strains with concomitant resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline. The bla_{CMY-2}-carrying plasmids studied here were shown to also carry the florfenicol resistance gene, *floR*, on a genetic structure previously identified in *Escherichia coli* plasmids in Europe. These data indicate that the use of different antimicrobial agents, including phenicols, may serve to maintain multidrug resistance plasmids on which extended-spectrum cephalosporin resistance determinants co-exist with other resistance genes in *Salmonella*.

Keywords: *Salmonella*; Multidrug resistance; Phenicols; Cephalosporins; Plasmid; Conjugation

1. Introduction

Salmonella enterica and *Escherichia coli* exhibiting resistance to extended-spectrum cephalosporins are an emergent problem worldwide. Until 1996, resistance to extended-spectrum cephalosporins was rarely reported among *Salmonella*. In 2000, the emergence of domestically acquired infections by extended-spectrum cephalosporin-resistant *Salmonella* carrying a plasmid-mediated CMY-2 AmpC β-lactamase was reported by the National Antimicrobial Resistance Monitoring System (NARMS) in the United States [1]. Molecular and phenotypic analysis of extended-spectrum cephalosporin-resistant strains revealed several distinct serovars and chromosomal DNA patterns, suggesting that this resistance phenotype is present among genetically diverse strains [1–3]. It has been recently demonstrated that the bla_{CMY} genes in *Salmonella* reside on different large plasmids, which were divided into three groups (type A, B and C) on the basis of restriction patterns and bla_{CMY-2} gene hybridization profiles [4].

The occurrence of CMY-2-mediated cephalosporin resistance in *Salmonella* has now been reported in Canada [5], Spain [6], Romania [7], and Taiwan [8]. In 2002, the US Centers for Disease Control and Prevention investigated an outbreak of multidrug-resistant *S. enterica* serovar Newport (*S.* Newport) infections associated with eating raw or undercooked ground beef

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0378-1097/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Microbiological Societies.
doi:10.1016/j.femsle.2004.02.023

[9]. This multidrug resistance phenotype included resistance to streptomycin, sulfamethoxazole, tetracycline, ampicillin, chloramphenicol and decreased susceptibility to extended-spectrum cephalosporins. Researchers in Pennsylvania, USA have also identified plasmid-mediated extended-spectrum cephalosporin resistance conferred by the bla_{CMY-2} gene in *S.* Newport strains isolated from cattle [2].

The prevalence of extended-spectrum cephalosporin resistant (including ceftiofur-resistance) *Salmonella* from food animals has increased (URL: http://www.arru.saa.ars.usda.gov). Ceftiofur is an extended-spectrum cephalosporin approved for use in veterinary medicine [10,11], and emergence of these strains may be related to use of this drug. Since the bla_{CMY} genes confer decreased susceptibility to both ceftiofur and ceftriaxone, the use of ceftiofur has the potential to select for *Salmonella* cross-resistant to ceftriaxone [4]. However, more detailed scientific analyses are needed to determine the extent of non-cephalosporin antimicrobial resistance determinants that may be present on the bla_{CMY-2}-carrying plasmids as well. In fact, many plasmids that contain bla_{CMY-2} genes have been reported to confer additional resistance to aminoglycosides, phenicols, tetracyclines and sulfonamides. Ceftriaxone-resistant *Salmonella* strains recently isolated from animals in Canada [5] and retail foods in the US [11] have also been reported to be resistant to florfenicol. Florfenicol, a veterinary fluorinated analog of thiamphenicol and chloramphenicol, was approved by the United States Food and Drug Administration (FDA) in 1996 for treatment of bovine respiratory pathogens such as *Mannheimia haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus*. Florfenicol resistance has recently been identified among veterinary *E. coli* isolates isolated from calves with diarrhea, and the *floR* resistance gene, located on conjugative and non-conjugative plasmids, has been identified among florfenicol-resistant *E. coli* from poultry and cattle isolated in the US and Europe [12–14]. The *floR* gene has also been identified in the *Salmonella* genomic island 1 (SGI1) of *S.* Typhimurium Definitive Type 104 (DT104) and of other *S. enterica* serovars, i.e. Agona, Albany, and Paratyphi B [15–17]. A plasmid encoding a highly related *floR* gene (*pp-flo*) was previously identified in Japan from *Photobacterium damselae* subsp. *piscicida* [18]. Analysis of the DNA sequences upstream and downstream of the *floR* gene in *E. coli* plasmids revealed the presence of two open reading frames (orfA′ and orfA) that show considerable homology to transposase proteins, suggesting that the *floR* gene could be located within a mobile genetic element [12].

Since there have been increasing reports of bla_{CMY-2}-carrying plasmids that possess additional resistance phenotypes, including phenicol resistance, we sought to determine possible genetic linkages of the florfenicol resistance gene, *floR*, with bla_{CMY-2} genes conferring resistance to extended-spectrum cephalosporins, and to compare the florfenicol resistance genes and their flanking regions with other previously described florfenicol resistance genotypes.

2. Materials and methods

Seven isolates were chosen among the bla_{CMY-2}-positive unrelated strains isolated from humans through the NARMS project at CDC in the 1996–1999 period, and from retail foods by the Center for Veterinary Medicine, US Food and Drug Administration in 2000, using the following criteria: they were resistant to florfenicol, showing a minimal inhibitory concentration

Table 1
Ceftriaxone-resistant *Salmonella* strains used in this study

Strain	Serovar	Antibiotic resistance phenotype	Ff MIC[b] (μg/ml)	Cm MIC (μg/ml)
2039	Typhimurium	ApCmFfSmSpSuTcTmGmKmFxCro	64	256
2039TC1[a]		ApCmFfSmSuTcTmGmKmFxCro	32	256
3977	Typhimurium	ApCmFfSmSpSuTcKmFxCro	256	>256
3977TC1		ApCmFfSmSuTcFxCro	128	128
1290	Typhimurium DT208	ApCmFfSmSuTcGmKmFxCro	128	>256
1290TC1		ApCmFfSmSuTcGmKmFxCro	32	256
1291	Typhimurium DT208	ApCmFfSmSuTcGmKmFxCro	64	>256
1291TC1		ApCmFfSmSuTcGmKmFxCro	32	256
4528	Newport	ApCmFfSmSuTcFxCro	256	256
4528TC1		ApCmFfSmSuTcFxCro	256	256
5313	Newport	ApCmFfSmSuTcFxCro	256	256
5313TC1		ApCmFfSmSuTcFxCro	256	256
7073	Newport	ApCmFfSmSuTcFxCro	256	256
7073TC1		ApCmFfSmSuTcFxCro	256	256

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Ff, florfenicol; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamides; Tc, tetracycline; Tm, trimethoprim; Gm, gentamicin; Km, kanamycin; Cro, ceftriaxone; Fx, Cefoxitin.

[a] Strains labeled as TC1 are *E. coli* transconjugant strains.

[b] MIC breakpoints of Ff and Cm: susceptible MIC $\leqslant$ 8 μg/ml; resistant MIC $\geqslant$ 32 μg/ml.

(MIC) $\geqslant$ 32 μg/ml; they were previously characterized at molecular level for the presence of plasmid located bla_{CMY-2} gene [4,5,11]; they demonstrated the transfer of florfenicol resistance by conjugation to an *E. coli* susceptible strain. The plasmids in the *S.* Typhimurium strains 2039 and 3977 and the plasmid in the *S.* Newport strain 4528 were previously classified as bla_{CMY-2}-positive type A and C, respectively [4]. The phenotypic characteristics of the selected strains are reported in Table 1. Molecular methods used to characterize the plasmids, *floR* and flanking regions were those described previously [12,15–17,19].

3. Results and discussion

All strains and their derivative *E. coli* transconjugants were confirmed to possess the *floR* gene by PCR using previously published specific primers [12]. PCR products obtained from strains 2039 and 3977 were sequenced demonstrating that the *floR* gene was almost identical (1 bp different at position 3746) to that previously described in *E. coli* BN10660 plasmid isolated from cattle in France (EMBL Accession No. AF231986), but differed from that located within the *Salmonella* Genomic Island SGI1 of *S.* Typhimurium DT104 by 20 bp (EMBL Accession No. AF261825). Furthermore, all the florfenicol-resistant *Salmonella* strains of this study were found to lack the SGI1, as determined by PCR amplification and Southern blot hybridization experiments (data not shown) [15–17].

Minimal inhibitory concentrations of both chloramphenicol and florfenicol for *Salmonella* strains showing plasmid-mediated florfenicol resistance, as well as their respective transconjugants, are reported in Table 1. Particularly high MIC levels for both florfenicol and chloramphenicol (both 256 μg/ml) were observed for the three *S.* Newport strains (4528, 5313 and 7073), and the same MIC levels were maintained by their relative transconjugants (4528TC1, 5313TC1 and 7073TC1). Lower MIC levels were observed for transconjugants obtained from the four *S.* Typhimurium strains (2039TC1, 3977TC1, 1290TC1 and 1291TC1). The origin of this discrepancy is not known but it could be due to the differential expression of the *floR* resistance gene in the *E. coli* recipient cells or additional contribution of other efflux mechanisms in the wild-type isolates. Florfenicol and chloramphenicol MICs for the host strain of *E. coli* used for conjugation were 4 μg/ml.

To gain insight into the nature of florfenicol resistance in these strains, all plasmids were purified, digested with *Bgl*I and compared with the *E. coli* plasmid BN10660 previously described to carry the *floR*-gene associated to the orfA and orfA′ genes [12]. Although the plasmids obtained from *Salmonella* strains were not identical (Fig. 1, panel (a), lanes 2–8), restriction analysis suggested that they were similar to one another but different from the *E. coli* BN10660 plasmid (Fig. 1, panel (a), lane 1). Plasmid profiles were hybridized with specific probes as described previously [4,19]. These included the bla_{CMY-2} gene and the orfA′–*floR*–orfA gene cluster, the latter obtained from the cloned *floR* plasmid pEF03 (Fig. 1). The bla_{CMY-2} probe hybridized with a *Bgl*I fragment of ca. 5 kb in the four *S.* Typhimurium strains (Fig. 1, panel (b), lanes 2–5) and with a distinct *Bgl*I band of ca. 8 kb in the three *S.* Newport strains (Fig. 1, panel (b), lanes 6–8), suggesting different localizations of the bla_{CMY-2} gene on these plasmids. As expected, the *E. coli* BN10660 plasmid was negative for the presence of the bla_{CMY-2} gene (Fig. 1, panel (b), lane 1). Hybridization with the orfA′–*floR*–orfA specific probe yielded the same pattern for all seven transconjugants and the *E. coli* BN10660 plasmid (Fig. 1, panel (c), lanes 1–8). This result indicates that the *floR*-resistance determinant and flanking transposase regions are apparently well-conserved in all the bla_{CMY-2}-carrying plasmids analysed, and are consistent with the pattern previously described on the *E. coli* BN10660 plasmid isolated from cattle in Europe [12].

In summary, we have identified the florfenicol *floR* resistance gene on bla_{CMY-2}-carrying plasmids conferring extended-spectrum cephalosporin (e.g. ceftiofur and ceftriaxone) resistance to *Salmonella* isolated from humans, animals, and foods in the US. Having these resistance genes co-exist on the same plasmid may allow the bacterial host to be positively selected by either individual antimicrobial agent. Thus, the application of a phenicol selective pressure in a food animal production setting could contribute to the dissemination of the bla_{CMY-2}-plasmids, and vice versa. The *floR* gene also confers cross-resistance to chloramphenicol, raising the possibility that chloramphenicol could have previously selected the *floR* gene long before the introduction of florfenicol for veterinary use, although chloramphenicol use in all food-producing animals has been prohibited in the US since 1984. Additional antimicrobial resistance phenotypes were also observed among tested transconjugants (e.g. aminoglycosides, sulfa drugs, tetracyclines), demonstrating the continued need to determine what other antimicrobial resistance genes co-exist on the bla_{CMY-2}-carrying plasmids in addition to those for florfenicol.

Our results also demonstrate that the *floR* gene on bla_{CMY-2}-plasmids is flanked by the orfA′ and orfA sequences consistent with the genetic organization previously described for different *E. coli* plasmids circulating in animals in Europe that lack cephalosporin resistance determinants [12]. Protein sequence homologies of the *floR*-flanking open reading frames suggest that they could be transposases. This means that the *floR* gene is likely located within a mobile element, which could facilitate its transfer from one plasmid to another or to the bacterial chromosome [19].

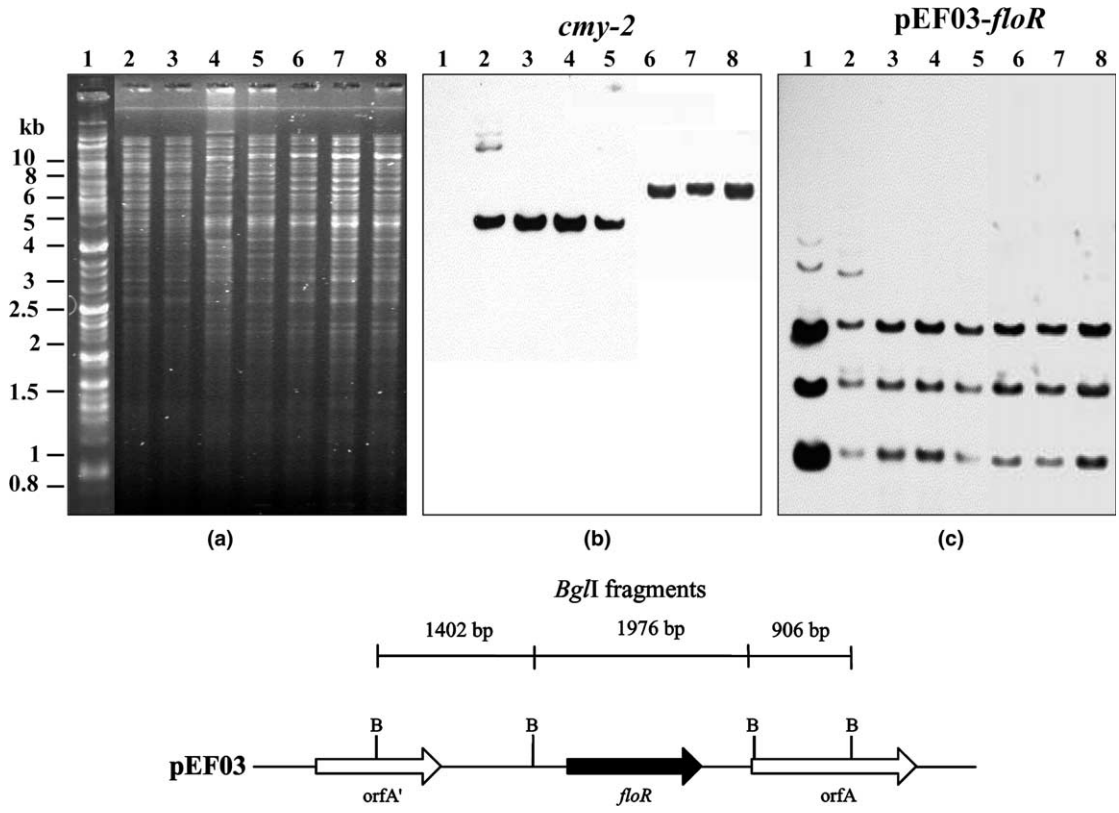

Fig. 1. (a) *Bgl*I restriction profiles of plasmids extracted from *E. coli* BN10660 (lane 1), from transconjugants 2039TC1 (lane 2), 3977TC1 (lane 3), 1290TC1 (lane 4), 1291TC1 (lane 5), 4528TC1 (lane 6), 5313TC1 (lane 7) and 7073TC1 (lane 8). (b) Southern blot hybridization with the bla_{CMY-2}probe (consisting of the bla_{CMY-2} internal PCR product) of *Bgl*I digested plasmids. (c) Southern blot hybridization with the pEF03 probe of *Bgl*I digested plasmids. A schematic view of the gene organization of the *floR* locus on the 6522 bp *Eco*RI–*Bam*HI pEF03 inset is shown below [12]. The sizes and locations of the three *Bgl*I (b) fragments are indicated. The reading frames for orfA′, *floR*, and orfA are shown as arrows.

The use of antimicrobial agents in food animals has drawn increased scientific and public scrutiny in recent years. The appearance and dissemination of multidrug-resistant foodborne bacterial pathogens is of particular concern. Once antimicrobial use puts pressure on a microbe, resistance may quickly emerge in that species [20]. The presence of more than one antimicrobial resistance gene on a plasmid allows for co-selection of multidrug resistance phenotypes. Circulation of linked resistance genes such as $bla_{CMY-2}$and *floR* among bacteria in food animal production environments is significant in light of the possibility of contamination of food products with these organisms. Our findings further highlight the potential ramifications of antimicrobial selective pressures in food animal production settings and the important interface between veterinary and human medicine.

Acknowledgements

We thank C. Mouline for expert technical assistance. This study was funded by INRA, projet Transversalité and by ISS, Progetto Ricerca Intramurale No. 2012/RI.

References

[1] Dunne, E.F., Fey, P.D., Kludt, P., Reporter, R., Mostashari, F., Shillam, P., Wicklund, J., Miller, C., Holland, B., Stamey, K., Barrett, T.J., Rasheed, J.K., Tenover, F.C., Ribot, E.M. and Angulo, F.J. (2000) Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with AmpC beta-lactamase. JAMA 284, 3151–3156.

[2] Rankin, S.C., Aceto, H., Cassidy, J., Holt, J., Young, S., Love, B., Tewari, D., Munro, D.S. and Benson, C.E. (2002) Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype Newport isolates from animals in Pennsylvania. J. Clin. Microbiol. 40, 4679–4684.

[3] Zhao, S., White, D.G., McDermott, P.F., Friedman, S., English, L., Ayers, S., Meng, J., Maurer, J.J., Holland, R. and Walker, R.D. (2001) Identification and expression of cephamycinase bla_{CMY} genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob. Agents Chemother. 45, 3647–3650.

[4] Carattoli, A., Tosini, F., Giles, W.P., Rupp, M.E., Hinrich, S.H., Angulo, F.J., Barett, T.J. and Fey, P.D. (2002) Characterization of plasmids carrying CMY-2 from extended-spectrum cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. Antimicrob. Agents Chemother. 46, 1269–1272.

[5] Allen, K.J. and Poppe, C. (2002) Occurrence and characterization of resistance to extended-spectrum cephalosporins mediated by β-lactamase CMY-2 in *Salmonella* isolated from food-producing animals in Canada. Can. J. Vet. Res. 66, 137–144.

[6] Navarro, F., Perez-Trallero, E., Marimon, J.M., Aliaga, R., Gomariz, M. and Mirelis, B. (2001) CMY-2-producing *Salmonella enterica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis* and *Escherichia coli* strains isolated in Spain. J. Antimicrob. Chemother. 48, 383–389.

[7] Miriagou, V., Filip, R., Coman, G. and Tzouvelekis, L.S. (2002) Expanded-spectrum cephalosporin-resistant *Salmonella* strains in Romania. J. Clin. Microbiol. 40, 4334–4336.

[8] Yan, J.J., Ko, W.C., Chiu, C.H., Tsai, S.H., Wu, H.M. and Wu, J.J. (2003) Emergence of ceftriaxone-resistant *Salmonella* isolates and rapid spread of plasmid-encoded CMY-2-like cephalosporinase, Taiwan. Emerg. Infect. Dis. 9, 323–328.

[9] Zansky, S., Wallace, B., Schoonmaker-Bopp, D., Smith, P., Ramsey, F., Painter, J., Gupta, A., Kalluri, P. and Noviello, S. (2002) From the centers for disease control and prevention. Outbreak of multi-drug resistant *Salmonella* Newport – United States, January–April 2002. JAMA 288, 951–953.

[10] Hornish, R.E. and Kotarski, S.F. (2002) Cephalosporins in veterinary medicine – ceftiofur use in food animals. Curr. Top. Med. Chem. 2, 717–731.

[11] White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, P.F., McDermott, S., Wagner, D.D. and Meng, J. (2001) The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N. Engl. J. Med. 345, 1147–1154.

[12] Cloeckaert, A., Baucheron, S., Flaujac, G., Schwarz, S., Kehrenberg, C., Martel, J.L. and Chaslus-Dancla, E. (2000) Plasmid-mediated florfenicol resistance encoded by the *floR* gene in *Escherichia coli* isolated from cattle. Antimicrob. Agents Chemother. 44, 2858–2860.

[13] Keyes, K., Hudson, C., Maurer, J.J., Thayer, S., White, D.G. and Lee, M.D. (2000) Detection of florfenicol resistance genes in *Escherichia coli* isolated from sick chickens. Antimicrob. Agents Chemother. 44, 421–424.

[14] White, D.G., Hudson, C., Maurer, J.J., Ayers, S., Zhao, S., Lee, M.D., Bolton, L., Foley, T. and Sherwood, J. (2000) Characterization of chloramphenicol and florfenicol resistance in *Escherichia coli* associated with bovine diarrhea. J. Clin. Microbiol. 38, 4593–4598.

[15] Boyd, D., Peters, G.A., Cloeckaert, A., Boumedine, K.S., Chaslus-Dancla, E., Imberechts, H. and Mulvey, M.R. (2001) Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. J. Bacteriol. 183, 5725–5732.

[16] Doublet, B., Lailler, R., Meunier, D., Brisabois, A., Boyd, D., Mulvey, M.R., Chaslus-Dancla, E. and Cloeckaert, A. (2003) Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster in *Salmonella enterica* serovar Albany. Emerg. Infect. Dis. 9, 585–591.

[17] Meunier, D., Boyd, D., Mulvey, M.R., Baucheron, S., Mammina, C., Nastasi, A., Chaslus-Dancla, E. and Cloeckaert, A. (2002) *Salmonella enterica* serotype Typhimurium DT 104 antibiotic resistance genomic island I in serotype Paratyphi B. Emerg. Infect. Dis. 8, 430–433.

[18] Kim, E. and Aoki, T. (1996) Sequence analysis of the florfenicol resistance gene encoded in the transferable R-plasmid of a fish pathogen, *Pasteurella piscicida*. Microbiol. Immunol. 40, 665–669.

[19] Doublet, B., Schwarz, S., Baucheron, S., Martel, J.L., Chaslus-Dancla, E. and Cloeckaert, A. (2002) Molecular analysis of chromosomally florfenicol resistant *Escherichia coli* isolates from France and Germany. J. Antimicrob. Chemother. 49, 49–54.

[20] Salyers, A.A. and Amiable-Cuevas, C.F. (1997) Why are antibiotic resistance genes so resistant to elimination? Antimicrob. Agents Chemother. 41, 2321–2325.