

Characterization of Plasmids Carrying CMY-2 from Expanded-Spectrum Cephalosporin-Resistant *Salmonella* Strains Isolated in the United States between 1996 and 1998

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Sequencing of DNA from 15 expanded-spectrum cephalosporin (e.g., ceftriaxone)-resistant *Salmonella* isolates obtained in the United States revealed that resistance to ceftriaxone in all isolates was mediated by *cmv-2*. Hybridization patterns revealed three plasmid structures containing *cmv-2* in these 15 isolates. These data suggest that the spread of *cmv-2* among *Salmonella* strains is occurring through mobilization of the *cmv-2* gene into different plasmid backbones and consequent horizontal transfer by conjugation.

Salmonellosis is primarily a food-borne disease that affects an estimated 1.4 million people each year in the United States (14). Expanded-spectrum cephalosporins (e.g., ceftriaxone and cefotaxime) are the antimicrobial agents of choice in the treatment of pediatric patients with invasive *Salmonella* infections (9). Until recently, resistance to expanded-spectrum cephalosporins was rarely reported among *Salmonella* strains (8). Review of 1996 data from the National Antimicrobial Resistance Monitoring System (NARMS) in the United States identified only 1 (0.1%) ceftriaxone-resistant *Salmonella* isolate among 1,272 human *Salmonella* isolates. However, by 1999 almost 2% of *Salmonella* isolates were ceftriaxone resistant, as determined by review of 1999 NARMS data (6). Comparisons of these ceftriaxone-resistant isolates found divergent strains, indicating multiple probable sources. The isolates either were different serotypes or, among patients infected with *Salmonella enterica* serotype Typhimurium, were distinguishable by their pulsed-field gel electrophoresis patterns, thus demonstrating that these ceftriaxone-resistant human isolates did not represent the epidemic spread of a clonal strain (6). This study was undertaken to confirm the identity of the β -lactamase conferring resistance to expanded-spectrum cephalosporins and characterize the associated plasmids from the apparently sporadic human *Salmonella* isolates collected through NARMS from 1996 to 1998.

MATERIALS AND METHODS

The 15 bacterial strains used in the study are listed in Table 1. Thirteen of the isolates were obtained by the Centers for Disease Control and Prevention through NARMS. These 13 isolates represented 87% of the total expanded-spectrum cephalosporin-resistant *Salmonella* isolates ($n = 15$) obtained by the

Centers for Disease Control and Prevention from 1996 to 1998 (6). Isolate SS034 was isolated in Nebraska, whereas isolate 922 was isolated in Ohio. Susceptibility testing of the *Salmonella* isolates and the *Escherichia coli* transconjugants and transformants was performed by the disk diffusion methodology according to NCCLS standards (16). The MIC for the pACYC184 construct containing *cmv-2* was tested by the E-test (AB Biodisk, Solna, Sweden) methodology. The MICs of ceftiofur (kindly provided by Pharmacia/Upjohn) were determined by broth microdilution (15, 17). Plasmid DNA was extracted either by the method of Kado and Liu (10) or with the Concert Purification Midi kit (Life Technologies, Milan, Italy) and digested with *Pst*I (Roche, Indianapolis, Ind.). Conjugation and transformation experiments were performed as described previously with *E. coli* C600N (ampicillin susceptible, nalidixic acid resistant) and *E. coli* DH5 α as hosts (Gibco BRL, Bethesda, Md.) (2, 18, 19). Transformants were selected on Luria-Bertani agar (Difco, Detroit, Mich.) containing 50 μ g of ampicillin (Sigma) per ml. All ceftriaxone-resistant C600N and DH5 α transconjugants and transformants were subsequently named C6 or DH followed by the appropriate wild-type *Salmonella* strain designation (e.g., C6/SS034 and DH/4656).

Southern blot hybridizations were performed by standard methods (19) with a *cmv-2*-specific DNA probe labeled with [α -³²P]dCTP with an RTS RadPrimer DNA Labeling kit (Life Technologies). DNA sequencing was performed with primers derived from known sequences and an ABI Prism model 377 sequencer (Perkin-Elmer Biosystems, Foster City, Calif.). The primers and DNA probes used to detect potential class 1 integrons have been described previously (4). Primers 92 (CCGTTTGTCAACACAGTAC [forward]) and 52 (TTGCAGCTT TCAAGAATGCGCC [reverse]) were used to amplify full-length *bla*_{cmv}. Primer 92 was designed by using the sequence from the intercistronic region between *ampC* and *ampR* in *Citrobacter freundii* (GenBank accession no. X76636). Primer 52 was designed from the known *cmv-2* sequence. Plasmid vectors pCRII (Invitrogen, Carlsbad, Calif.) and pACYC184 (5) were used in cloning experiments. Isoelectric focusing was performed at room temperature on a mini isoelectric focusing gel system (model 111; Bio-Rad, Richmond, Calif.) (13). The isoelectric points of unknown β -lactamases were estimated by comparison with those of TEM-1, SHV-3, SHV-5, and CMY-2.

RESULTS AND DISCUSSION

The antibiotic resistance phenotypes of the 15 strains under study are shown in Table 1. All isolates were resistant to ampicillin, ceftriaxone, ceftiofur, and cefoxitin. After mating experiments with C600N, 7 of 15 isolates were able to transfer decreased susceptibilities to ceftriaxone to C600N (Table 1). For those strains for which a transconjugant with decreased

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TABLE 1. Ceftriaxone-resistant *Salmonella* strains used in the study^a

| Strain | Serotype | State in which strain was isolated | Antibiotic resistance phenotype | Tra | Transfer phenotype | Integron | Plasmid type |
|--------|----------|------------------------------------|-----------------------------------|-----|-----------------------------------|----------------|--------------|
| 922 | Typhim. | Ohio | ACSSuTGmToKTpCroXnlF _x | Pos | ACSSuTGmToKTpCroXnlF _x | ND | A |
| 2039 | Typhim. | California | ACSSuTGmToKTpCroXnlF _x | Pos | ACSSuTGmToKTpCroXnlF _x | In-t4 In-t6 | A |
| 2152 | Typhim. | California | ACSSuTGmToKCroXnlF _x | | NT | In-t4 In-t6 | A |
| SS034 | Typhim. | Nebraska | ACSSuTGmToKCroXnlF _x | Pos | ACSSuTGmToCroXnlF _x | In-t6 | A |
| 4204 | Typhim. | New York | ACSSuTGmToKCroXnlF _x | | ACSSuTCroXnlF _x | In-t6 | A |
| 2855 | Typhim. | Oregon | ACSSuTKTpCroXnlF _x | | NT | In-t5 | A |
| 3977 | Typhim. | Kansas | ACSSuTKCroXnlF _x | | ACSSuTCroXnlF _x | In-t6 | A |
| 4501 | Typhim. | Colorado | ACSSuTKCroXnlF _x | | NT | In-t6 | A |
| 4656 | Typhim. | New York | ACSSuTCroXnlF _x | | ACSSuTCroXnlF _x | Neg | C |
| 4528 | Newport | Kansas | ACSSuTCroXnlF _x | | NT | Neg | C |
| 2668 | Typhim. | Colorado | ACSSuTKCroXnlF _x | Pos | ACroXnlF _x | In-t6 | B |
| 4255 | Typhim. | Massachusetts | ASCroXnlF _x | Pos | ACroXnlF _x | Neg | B |
| 3430 | Typhim. | Massachusetts | ACroXnlF _x | Pos | ACroXnlF _x | Neg | B |
| 4287 | Typhim. | Massachusetts | ACroXnlF _x | | ACroXnlF _x | Neg | B |
| 1358 | Thompson | Connecticut | ACroXnlF _x | Pos | ACroXnlF _x | Neg | B |

^a Abbreviations: A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfisoxazole; T, tetracycline; Gm, gentamicin; To, tobramycin; K, kanamycin; Tp, trimethoprim; Cro, ceftriaxone; Xnl, ceftiofur; Fx, Cefoxitin; Typhim, Typhimurium; Tra, self-transferred plasmids by conjugation; NT, no ceftriaxone-resistant transformants isolated; ND, not done; Pos, positive; Neg, negative

susceptibility to ceftriaxone was not isolated, plasmid DNA was isolated and used to transform *E. coli* DH5 α . From the transformation experiments, an additional four transformants with reduced susceptibilities to ceftriaxone were isolated. The MIC of ceftriaxone was 8 to 32 μ g/ml for all *E. coli* C600N transconjugants and DH5 α transformants (hereafter these *E. coli* transconjugants and transformants will be referred to as ceftriaxone resistant). By isoelectric focusing, all ceftriaxone-resistant *E. coli* transconjugants and transformants expressed a β -lactamase (pI >9.0) that comigrated alongside CMY-2 (data not shown). In addition, primers specific for *bla*_{cm_y} amplified an appropriate 631-bp DNA product from all ceftriaxone-resistant *E. coli* transconjugants and transformants and from the four *Salmonella* strains for which a transformant or transconjugant was not isolated (strains 2152, 2855, 4501, and 4528) (data not shown). Other resistance factors cotransferred with ceftriaxone resistance in 6 of 11 transconjugants or transformants (Table 1). Two strains (strains 922 and 2039) transferred all resistance factors to their corresponding *E. coli* transconjugant. The remaining five transconjugants or transformants were resistant only to β -lactam antibiotics.

The sequence of the *bla*_{cm_y} gene obtained by PCR amplification was determined. DNA sequencing revealed that all strains encoded *cm_y*-2, and no sequence divergence was detected in any strain. The DNA sequence found in the U.S. isolates was identical to the original *cm_y*-2 sequence described

in *Klebsiella pneumoniae* (3), yet it was different from the *cm_y*-2-like sequence described in a ceftriaxone-resistant *Salmonella* serotype Senftenberg strain isolated in Algeria (11). Compared with the U.S. isolates, the Algerian isolate had three base pair changes within the first 50 bp, and two of these changes resulted in amino acid changes, suggesting that the *cm_y*-2 gene disseminating throughout the United States is distinct from that in Algeria.

To further demonstrate that CMY-2 alone is responsible for mediating expanded-spectrum cephalosporin resistance in these isolates, CMY-2 was cloned by first amplifying *cm_y*-2 from C6/SS034 with primers 92 and 52 and cloning it into pACYC184. As shown in Table 2, all strains were resistant or intermediate to ceftriaxone, ceftazidime, cefotaxime, ceftiofur, and cefoxitin. Both SS034 and DH/pNF10 were resistant to aztreonam; however, C6/SS034 was susceptible to aztreonam, perhaps due to the lower plasmid copy number or genomic background differences between C600N and DH5 α . All strains were susceptible to cefepime and imipenem. Both C6/SS034 and DH/pNF10 were susceptible to piperacillin-tazobactam, as tazobactam is a known inhibitor of *cm_y*-2 (3). The fact that strain SS034 also produces TEM-1 may have contributed to its resistance to piperacillin-tazobactam (7).

Plasmid DNA was isolated from the 11 *E. coli* transconjugants and transformants and the 4 wild-type *Salmonella* strains that did not yield a ceftriaxone-resistant transconjugant or

TABLE 2. β -Lactam MICs for CMY-2-containing strains

| Isolate | MIC (μ g/ml) ^a | | | | | | | | |
|-------------|--------------------------------|-------|-------|-----|------|------|-------|-----|------|
| | CRO | CAZ | CTX | XNL | FEP | TZP | IPM | ATM | FOX |
| SS34 | >256 | >256 | >256 | 32 | 2 | >256 | 0.25 | 32 | >256 |
| C6/SS34 | 16 | 32 | 8 | 16 | 0.50 | 4 | 0.25 | 4 | 64 |
| DH/pACYC184 | 0.062 | 0.125 | 0.062 | 0.5 | 0.25 | 0.50 | 0.125 | 0.5 | 2 |
| DH/pNF10 | 64 | >256 | 64 | 8 | 0.5 | 4 | 0.5 | 32 | 64 |

^a Abbreviations: CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; XNL, ceftiofur; FEP, cefepime; TZP, piperacillin-tazobactam; IPM, imipenem; ATM, aztreonam; FOX, cefoxitin.

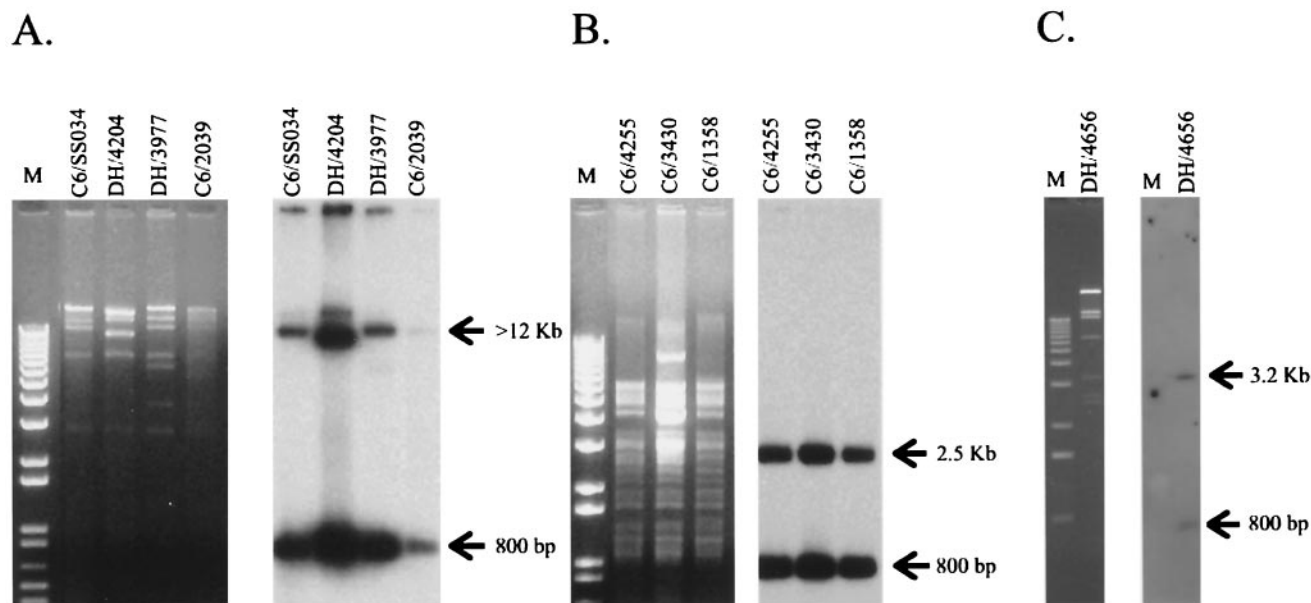


FIG. 1. Restriction analysis (left panels in the pairs of panels) and *cmv-2* Southern hybridization (right panels in the pairs of panels). *PstI*-digested plasmids were extracted from *E. coli* C6/SS034, DH/4204, DH/3977, and C6/2039 (type A hybridization pattern) (A); *E. coli* C6/4255, C6/3430, and C6/1358 (type B hybridization pattern) (B); and *E. coli* DH/4656 (type C hybridization pattern) (C). A 1-kb marker (KiloBase DNA marker; Pharmacia Biotech, Milan, Italy) (A and B) and a 12-kb ladder (Gibco BRL) (C) were used as standards.

transformant, and the plasmid DNA was probed with a *cmv-2*-specific probe (amplified with primers 92 and 52). This analysis demonstrated that *cmv-2* was encoded on large plasmids (ca. 60 to 75 kb) in each strain (data not shown). The *cmv-2*-containing plasmids isolated from the *E. coli* transconjugants and transformants were additionally subjected to restriction endonuclease digestion with *PstI* (Roche) since a single *PstI* restriction site is present within *cmv-2* (3), and the digests were analyzed by Southern hybridization with *cmv-2* as a probe. Three *PstI* restriction fragment length polymorphism hybridization groups, referred to as types A, B, and C (Fig. 1), were observed. The *cmv-2* probe hybridized to bands of approximately 12 kb and 800 bp (type A) and 2.5 kb and 800 bp (type B) for 8 and 5 of 15 *cmv-2*-containing plasmids, respectively (Fig. 1A, type A, and Fig. 1B, type B). For strains 4528 (wild type) and DH/4656, the *cmv-2*-specific probe hybridized to a 3.2-kb fragment and an 800-bp fragment (Fig. 1C, type C). *PstI* digestion of type B plasmids, which encode resistance only to β -lactam antibiotics, suggested that these plasmids were highly related. Plasmids with the type A or C hybridization pattern transferred resistance to at least four antibiotics (streptomycin, chloramphenicol, tetracycline, and sulfonamides), in addition to ceftriaxone (Table 1), but had different *PstI* restriction fragment length polymorphism patterns (Fig. 1). The significance of the conserved *cmv-2* hybridization pattern in these plasmids is not known.

These data suggest that *cmv-2* is being transferred among *Salmonella* strains by plasmid transfer to different genomic backbones as well as by independent acquisition of *cmv-2* by different plasmid backbones, most of which carry multiple antibiotic resistance determinants. The mechanism of transfer and acquisition of *cmv-2* is unknown; however, it appears that *cmv-2* is not encoded within a cassette that inserts into a class

1 integron. Experiments for the detection of class 1 integrons were performed by both PCR amplification (12) and Southern hybridization by using the integrase gene as a probe (data not shown) (4). Class 1 integrons were detected in eight strains (Table 1); however, the *cmv-2* gene was not included as an integron-borne gene cassette. Isolates 2039, SS034, 2152, 4204, 3977, 4501, and 2668 all contained an integron (In-t6) that carries the *aadA2* gene cassette, which confers resistance to streptomycin and spectinomycin. Isolates 2039 and 2152 also carried an additional integron (In-t4) that encodes the *cmlA* and *aadB* gene cassettes, which confer resistance to chloramphenicol and kanamycin, respectively. One isolate, isolate 2855, contained a larger integron (In-t5) that carries the *dfpA1* and *aadA2* gene cassettes, which encode trimethoprim and streptomycin-spectinomycin resistance, respectively. Integrons were located on *cmv-2*-carrying plasmids only in isolates SS034 and 2039.

The results of this study demonstrate the emergence and spread of a CMY-2 β -lactamase in *Salmonella* strains isolated from humans in the United States. The ceftriaxone resistance reported in porcine, bovine, and human *Salmonella* isolates in Iowa and Nebraska was also mediated by *cmv-2* (20, 7). The emergence of ceftriaxone resistance among *Salmonella* strains isolated from food animals supports the transfer of ceftriaxone-resistant *Salmonella* strains from food animals to humans (21, 1). In this study, we demonstrated that CMY-2 alone can mediate resistance to expanded-spectrum cephalosporins, including ceftiofur, by cloning the *cmv-2* gene into pACYC184. Although the reasons for the emergence of resistance to expanded-spectrum cephalosporins in humans remain uncertain, the emergence of resistance in food animals may play a role. The increased prevalence of ceftriaxone-resistant *Salmonella* strains in food animals may in turn be related to the veterinary

use of ceftiofur, an expanded-spectrum cephalosporin used only in veterinary medicine. Further studies are warranted to determine the risk factors for dissemination of *cmy-2*-mediated resistance and to determine whether limiting the use of ceftiofur in food animals, along with improvements in food processing methods, might reduce the potential for dissemination of ceftriaxone resistance.

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