August 2004

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2004, p. 2845–2852 Vol. 48, No. 8
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.8.2845–2852.2004

DNA Sequence Analysis of Regions Surrounding bla_{CMY-2} from Multiple *Salmonella* Plasmid Backbones

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Received 17 November 2003/Returned for modification 7 February 2004/Accepted 12 April 2004

The emergence in the United States of resistance to expanded-spectrum cephalosporin (e.g., ceftriaxone) within the salmonellae has been associated primarily with three large (>100-kb) plasmids (designated types A, B, and C) and one 10.1-kb plasmid (type D) that carry the bla_{CMY-2} gene. In the present study, the distribution of these four known bla_{CMY-2}-carrying plasmids among 35 ceftriaxone-resistant *Salmonella* isolates obtained from 1998 to 2001 was examined. Twenty-three of these isolates were *Salmonella enterica* serotype Newport, 10 were *Salmonella enterica* serotype Typhimurium, 1 was *Salmonella enterica* serotype Agona, and 1 was *Salmonella enterica* serotype Reading. All 23 serotype Newport isolates carried a type C plasmid, and 5, 4, and 1 serovar Typhimurium isolate carried type B, A, and C plasmids, respectively. Both the serotype Agona and serotype Reading isolates carried type A plasmids. None of the isolates carried a type D plasmid. Hybridization data suggested that plasmid types A and C were highly related replicons. DNA sequencing revealed that the region surrounding bla_{CMY-2} was highly conserved in all three plasmid types analyzed (types B, C, and D) and was related to a region surrounding bla_{CMY-5} from the *Klebsiella oxytoca* plasmid pTKH11. These findings are consistent with a model in which bla_{CMY-2} has been disseminated primarily through plasmid transfer, and not by mobilization of the gene itself, to multiple *Salmonella* chromosomal backbones.

Salmonellae are a leading cause of food-borne illness throughout the world. Although the worldwide incidence is not known, in the United States alone there are an estimated 1.4 million cases of salmonellosis each year. Nearly all of these cases (95%) are thought to be due to transmission of the bacteria in contaminated foods such as eggs, dairy products, produce, and meats (33). Typically, salmonellae cause a self-limiting gastroenteritis that does not require treatment with antibiotics. Antimicrobial agents are usually not essential for the treatment of patients with *Salmonella* infections; for such patients, antimicrobial agents may not reduce the severity of the gastrointestinal symptoms and may prolong the duration of the carrier state (2). Antimicrobial agents may be life-saving, however, for patients with severe invasive infection. Septicemia occurs in approximately 6% of the 30,000 to 32,000 culture-confirmed cases of salmonellosis in the United States annually (19, 33; http://www.cdc.gov/foodnet; http://www.cdc.gov/ncidod/dbmd/phlisdata/default.htm). Invasive infections commonly occur in children, particularly in infants. In the United States, 10% of culture-confirmed infections in which salmonellae were isolated from the blood or central nervous system occurred in infants ≤1 year old (22, 32). Expanded-spectrum cephalosporins (e.g., ceftriaxone and cefotaxime) are the antimicrobial agents of choice for invasive *Salmonella* infections of pediatric patients. Ceftriaxone is the expanded-spectrum cephalosporin used most often for pediatric patients, because its long half-life allows for a single daily administration, whereas other antibiotics require two to three daily doses. The fluoroquinolones (e.g., ciprofloxacin) are often used for treatment of salmonellosis in adults but are not approved for use in pediatric patients.

Decreased susceptibility (MIC, 16 to 32 μg/ml) or resistance (MIC, ≥64 μg/ml) to ceftriaxone within the salmonellae is a growing public health concern. Since 1991, *Salmonella* serotypes resistant to expanded-spectrum cephalosporins have been reported worldwide (1, 4, 9, 14, 35). From these isolates, a wide variety of β-lactamases belonging to Bush groups 1 and 2be have been described (10). In the United States before 1996, all reported cases of infection with ceftriaxone-resistant salmonellae were known or believed to be acquired abroad (19). However, the prevalence of ceftriaxone resistance (or decreased susceptibility) among *Salmonella* isolates from humans increased more than sevenfold from 1996 to 1998, from 1 of 1,272 (0.1%) isolates in 1996 to 5 of 2,205 (0.2%) isolates in 1997 and 9 of 1,466 (0.6%) in 1998 (14). Year-2001 data from the National Antimicrobial Resistance Monitoring System (NARMS) demonstrated that 3% of non-serotype Typhi *Salmonella* isolates from humans exhibited decreased susceptibility to ceftriaxone while 2% were resistant to ceftriaxone (12). A similar study performed in the state of Nebraska in 2000 demonstrated that decreased susceptibility to ceftriaxone reached 6.5% within the salmonellae and 18% among all *Salmonella enterica* serotype Typhimurium isolates (P. D. Fey,

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

Strain	Serotype (PFGE type)	Antibiotic resistance phenotype[a]	*E. coli* background[b]	Antibiotic resistance phenotype of *E. coli*[c]	Plasmid type	State of origin	Yr of isolation
1041	Newport (A)	ACSSuTCroFx	D10	Same as wild type	C	Nebr.	1999
5313	Newport (A)	ACSSuTCroFx			C	Minn.	1999
5501	Newport (A)	ACSSuTCroFx	DH	Same as wild type	C	N.J.	1999
5653	Newport (A)	ACSSuTCroFx			C	Fla.	1999
7073	Newport (A)	ACSSuTCroFx	D10	Same as wild type	C	Mass.	1999
7075	Newport (A)	ACSSuTCroFx			C	Mass.	1999
7079	Newport (A)	ACSSuTCroFx			C	Mass.	1999
7467	Newport (A)	ACSSuTCroFx			C	Oreg.	1999
8611	Newport (A)	ACSSuTCroFx	D10	Same as wild type	C	Tenn.	2000
8683	Newport (A)	ACSSuTCroFx	D10	Same as wild type	C	Wash.	2000
6163	Newport (B)	ACSSuSxtTCroFx			C	Ga.	1999
7040	Newport (B)	ACSSuSxtTCroFx	D10	All except Sxt	C	Mass.	1999
7041	Newport (B)	ACSSuSxtTCroFx			C	Mass.	1999
5561	Newport (C)	ACSSuTCroFx	C6	Same as wild type	C	N.Y.	1999
6100	Newport (C)	ACSSuTCroFx			C	Calif.	1999
712	Newport (D)	ACSSuSxtTCroFx	D10	All except Sxt	C	Nebr.	1999
2231	Newport (D)	ACSSuSxtTCroFx	D10	Same as wild type	C	Nebr.	2000
4962	Newport (E)	ACSSuTCroFx	D10	Same as wild type	C	Colo.	1999
5299	Newport (E)	ACSSuTCroFx			C	Colo.	1999
5924	Newport (F)	ACSSuTKCroFx	D10	Same as wild type	C	Kans.	1999
6980	Newport (G)	ACSSuTCroFx	DH	Same as wild type	C	N.J.	1999
10091	Newport (H)	ACSSuTCroFx	D10	Same as wild type	C	Md.	2001
2050	Newport (I)	ACSSuTCroFx	D10	Same as wild type	C	Nebr.	2000
5436	Typhimurium	ACroFx	D10B	Same as wild type	B	Ga.	1999
5655	Typhimurium	ACSSuTCroFx	D10	Same as wild type	C	Fla.	1999
5678	Typhimurium	ACSSuTCroFx	C6	Same as wild type	A	Kans.	1999
5927	Typhimurium	ACSSuTCroFx	C6	All except SSuT	B	Kans.	1999
5965	Typhimurium	ACroFx	C6	Same as wild type	B	Conn.	1999
6191	Typhimurium	ACroFx	C6	Same as wild type	B	Ga.	1999
6516	Typhimurium	ACroFx	C6	Same as wild type	B	N.J.	1999
1568	Typhimurium	ACSSuTCroFx	D10	Same as wild type	A	Nebr.	2000
2000	Typhimurium	ACSSuTKCroFx	D10	Same as wild type	A	Nebr.	2000
2232	Typhimurium	ACSSuTCroFx	D10	Same as wild type	A	Nebr.	2000
2042	Agona	ACSSuSxtTCroFx	D10	Same as wild type	A	Nebr.	2000
2151	Reading	ACSSuTKCroFx	D10	All except K	A	Nebr.	2000

[a] Abbreviations: A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfisoxazole; Sxt, trimethoprim-sulfamethoxazole; T, tetracycline; K, kanamycin; Cro, ceftriaxone; Fx, cefoxitin.

[b] Background to which plasmid was transferred via conjugation (C6), transformation (DH), or electroporation (D10).

[c] "All except" indicates resistance to the same antimicrobial agents as the wild-type *Salmonella* strain, except for the particular antimicrobials noted. "Same as wild type" indicates resistance to the same antimicrobial agents as the wild-type *Salmonella* strain.

S. L. Greenwood, A. R. Sambol, P. C. Iwen, M. E. Rupp, T. J. Safranek, and S. H. Hinrichs, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. 203, 2001).

In the United States, decreased susceptibility to ceftriaxone in domestically acquired *Salmonella* infections is almost exclusively mediated through the production of the *Citrobacter freundii*-derived bla_{CMY-2} cephamycinase (14, 17, 29, 36). Previous restriction fragment length polymorphism (RFLP) and Southern blot experiments have demonstrated that bla_{CMY-2} is carried on at least three separate large (>100-kb) plasmid replicons termed types A, B, and C as well as on a 10-kb plasmid described by Winokur et al. (11, 38). From 1996 to 1999, these bla_{CMY-2}-carrying plasmids were isolated most frequently from serotype Typhimurium (14). However, since 2000, bla_{CMY-2}-carrying plasmids have been isolated at a substantial rate from isolates representing an apparently highly related strain of *Salmonella enterica* serotype Newport (6, 17, 29). Decreased susceptibility to ceftriaxone was noted in 25% of all serotype Newport isolates collected from humans in 2001 through the NARMS program (12).

The purpose of this study was to determine the extent to which the four known bla_{CMY-2}-carrying plasmid replicons were found in 35 ceftriaxone-resistant *Salmonella* isolates obtained throughout the United States from 1999 to 2001. In addition, the DNA sequence that surrounded bla_{CMY-2}, which was found to be highly conserved, was analyzed for three separate plasmid replicons.

MATERIALS AND METHODS

Strains used in the study. The 35 ceftriaxone-resistant isolates used in the study are listed in Table 1. The isolates were collected from 1998 to 2001 through either NARMS or the Nebraska Public Health Laboratory. Representative *Escherichia coli* transformants carrying type A, B, and C plasmids harboring bla_{CMY-2} were as follows: C6/pNF34 (type A), DH/pNF4656 (type C), and C6/pNF1358 (type B) (11). DH/pIW759 is a type D plasmid isolated from a *Salmonella enterica* serotype Heidelberg strain (porcine origin) in Iowa (37, 38).

Plasmid transfer. Plasmids carrying bla_{CMY-2} were transferred to *E. coli* through either transformation, conjugation, or electroporation. Transformation was performed with *E. coli* DH5α (Invitrogen, Carlsbad, Calif.) according to the manufacturer's recommendations, while *E. coli* DH10B (Invitrogen) was electroporated by using the method described by Sheng et al. (31). Conjugation was performed by using *E. coli* C600N (ampicillin susceptible, nalidixic acid resistant) as previously described (3, 27). Transformants and electroporants were selected on Luria-Bertani agar (Difco, Detroit, Mich.) containing 50 μg of ampicillin

TABLE 2. Primers used to amplify junction region between each plasmid replicon and the bla_{CMY-2} region

Region	Primer sequence[a]	Amplicon size (bp)
pNF1358 upstream junction (type B)	For, GTCATCAGACCTGTGCG Rev, CGGAAATATCAAACTCGT	505
pNF1358 downstream junction (type B)	For, GTGTATTTCAGGCCAATCGC Rev, CTGGGTATTCTCTGGTGCA	659
pNF4656 upstream junction (type C)	For, GACACCTTGCCGTTAATC Rev, CGGAAATATCAAACTCGT	523
pNF4656 downstream junction (type C)	For, GCGAGACGCACTCCAGTC Rev, CTGTTGCAAATAGTCGGTG	1,378
pIW759 upstream junction (type D)	For, CGCTGAACATGAAAGGA Rev, CGGAAATATCAAACTCGT	706
pIW759 downstream junction (type D)	For, GCGAGACGCACTCCAGTC Rev, GCTGTATACGCAGTGCTTT	641

[a] Abbreviations: For; forward primer. Rev; reverse primer.

(Sigma, St. Louis, Mo.)/ml. All bla_{CMY-2} C600N, DH5α, and DH10B transconjugants, transformants, or electroporants were named either C6, DH, or D10 (standing for C600N, DH5α, or DH10B, respectively) followed by the appropriate wild-type *Salmonella* strain designation. Antimicrobial susceptibility testing of *Salmonella* and *E. coli*, as well as *E. coli* ATCC 25922 (quality control strain), was performed by using disk diffusion according to NCCLS standards (25). The following antimicrobial agents were tested: ampicillin, cefoxitin, ciprofloxacin, tetracycline, chloramphenicol, kanamycin, streptomycin, gentamicin, sulfamethoxazole, trimethoprim-sulfamethoxazole, and nalidixic acid. Susceptibility to ceftriaxone was determined by using the E-test according to the manufacturer's recommendations (AB-Biodisk, Solna, Sweden).

DNA sequencing. The plasmid-harbored bla_{CMY-2} regions from pNF4656 and pNF1358 were cloned and sequenced by first preparing plasmid DNA from *E. coli* DH/pNF4656 and *E. coli* C6/pNF1358. DNA was extracted by using a QIAGEN (Valencia, Calif.) large-construct kit. The DNA was air sheared into 1.0- to 1.5-kb fragments according to the manufacturer's recommendations (Invitrogen) and cloned into the pCR 4 blunt-TOPO vector (Invitrogen). The genomic library was transformed into *E. coli* Top10 cells (Invitrogen), and random clones (approximately 4× coverage) were sequenced on a LiCor (Lincoln, Nebr.) 4000 sequencer by using IR800-labeled T3 and T7 primers (13, 21). Contigs were generated by using Vector NTI Contig Express software (Informax, North Bethesda, Md.).

Molecular methods. All restriction enzymes used in the study were purchased from Invitrogen. Southern blot hybridizations were performed by standard methods (30) with a bla_{CMY-2}-specific DNA probe labeled with digoxigenin ddUTP (Roche, Indianapolis, Ind.). The primers used to amplify a bla_{CMY}-specific probe, or to detect the gene, have been described previously (11). The primers shown in Table 2 were used to amplify the junction regions between the plasmid sequence and IS*Ecp1* upstream of bla_{CMY-2} and between the plasmid sequence and *sugE* (type B) or *ecnR* (types C and D) downstream of bla_{CMY-2} in pNF1358 (type B), pNF4656 (type C), and pIW759 (type D).

PFGE. Genomic DNA suitable for pulsed-field gel electrophoresis (PFGE) was prepared according to standard methods outlined by PulseNet (15, 34). *Salmonella enterica* serotype Braenderup H9812 was used as a standard. The DNA embedded in agarose was digested with XbaI and electrophoresed on a CHEF DR-III instrument (Bio-Rad, Richmond, Calif.) by using the following conditions: initial switching time, 2.2 s; final switching time, 63.8 s; total time, 19 h. *Salmonella* plasmid DNA was digested with PstI and electrophoresed by using the following conditions: initial switching time, 0.1 s; final switching time, 12 s; total time, 6 h. The RFLP patterns were compared by using Bionumerics software (Applied Maths, Kortrijk, Belgium) with a 0.75% molecular weight position tolerance.

Nucleotide sequence accession numbers. The GenBank accession numbers for IS*Ecp1* and the *C. freundii ampR* and *ampC* region are AY125469 and AY125469, respectively. The accession numbers for the DNA sequences of the bla_{CMY-2} regions from pIW759, pNF1358, and pNF4656 are AY581205, AY581206, and AY581207, respectively.

RESULTS

Plasmid typing. In a previously published study, bla_{CMY-2}-carrying plasmids isolated from three *Salmonella* serotypes were placed into three categories (A, B, and C) based on RFLP and Southern blot analyses (11). Thirty-five additional ceftriaxone-resistant isolates were obtained from the NARMS program ($n = 26$) and the Nebraska Public Health Laboratory ($n = 9$) from 1999 to 2001 (Table 1). Twenty-three of these isolates belonged to serotype Newport, 10 belonged to serotype Typhimurium, and 1 each belonged to serotypes Agona and Reading. All 35 of the isolates were resistant to cefoxitin and ampicillin as well as ceftriaxone (Table 1). Thirty-one (88.5%) of 35 isolates were also resistant to chloramphenicol, streptomycin, sulfisoxazole, and tetracycline. Three (8.6%) and six (17.1%) were resistant to kanamycin and trimethoprim-sulfamethoxazole, respectively. None of the isolates were resistant to ciprofloxacin, gentamicin, or nalidixic acid. PCR experiments demonstrated that all *Salmonella* isolates encoded a bla_{CMY-2}-like β-lactamase, as predicted (data not shown) (11, 14).

Plasmid transfer experiments were performed for all 10 serotype Typhimurium isolates, isolate 2042 (serotype Agona), isolate 2151 (serotype Reading), and 14 serotype Newport isolates (representing the nine PFGE groups delineated; see below) by using either conjugation, transformation, or electroporation (Table 1). For all 26 *Salmonella* isolates, we were able to transfer plasmid DNA resulting in decreased ceftriaxone susceptibility to *E. coli* (either C600N, DH5α, or DH10B) by one of the three methods of transfer. PFGE demonstrated that all *E. coli* transconjugants, transformants, and electroporants (referred to collectively as transformants below) were of the appropriate genomic lineage and were not contaminants (data not shown). Plasmid DNA was isolated from the *E. coli* transformants, digested with PstI, electrophoresed by PFGE, and analyzed by Southern hybridization using bla_{CMY-2} as a probe. The resulting RFLP patterns from all transformants were consistent with either a type A, B, or C plasmid as previously described by Carattoli et al. (11). None of the isolates carried a type D plasmid. Figure 1 shows a PstI digest and a subsequent Southern blot after hybridization with bla_{CMY-2} of the four known plasmid types (A to D). The bla_{CMY-2} probe hybridizes to an 800-bp fragment in all four plasmid types, whereas it hybridizes to a band of >12 kb in type A plasmids, a 2.5-kb band in type B plasmids, a 3.2-kb band in type C plasmids, and a 9.3-kb band in type D plasmids. Four of the 10 serotype Typhimurium *E. coli* transformants carried type A plasmids, 5 carried type B plasmids, and 1 carried a type C plasmid. Both D10/2151 (serotype Reading) and D10/2042 (se-

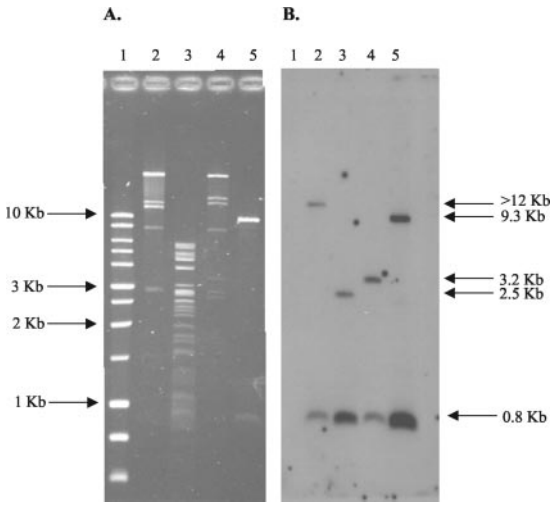

FIG. 1. (A) PstI digest of the four known bla_{CMY-2}-containing plasmid types. Lanes: 1, 1-kb ladder (Promega, Madison, Wis.); 2, C6/pNF34 (type A); 3, C6/pNF1358 (type B); 4, DH/pNF4656 (type C); 5, DH/pIW759 (type D). Sizes of the DNA ladder markers are given on the left. (B) Subsequent Southern hybridization of the gel in panel A probed with full-length bla_{CMY-2}. Sizes of bands hybridizing to the bla_{CMY-2} DNA probe, as explained in the text, are given on the right.

rotype Agona) carried type A plasmids, whereas all serotype Newport *E. coli* transformants carried type C plasmids. All *E. coli* transformants that carried type A or type C plasmids were resistant to multiple classes of antibiotics, whereas those that carried type B plasmids were resistant only to β-lactam antibiotics (ceftriaxone, cefoxitin, and ampicillin) (Table 1). In 22 of 26 isolates, the *E. coli* transformants were resistant to all of the same antibiotics as wild-type *Salmonella*.

PFGE. PFGE was performed on all ceftriaxone-resistant serotype Typhimurium and serotype Newport isolates. All serotype Typhimurium isolates had divergent PFGE patterns, as previously demonstrated with the 1996-to-1998 ceftriaxone-resistant isolates (data not shown) (14). In contrast, PFGE patterns were highly related for all serotype Newport isolates, as has been reported previously (Fig. 2) (6, 17, 29). Among the 23 serotype Newport isolates, nine distinguishable but similar RFLP patterns were identified (A to I) (Table 1). The most highly represented PFGE group was group A, which consisted of 10 isolates; group B consisted of 3 isolates, and groups C, D, and E consisted of 2 isolates each. The remaining isolates (n = 4) had unique PFGE patterns (F to I). Plasmid typing demonstrated that, regardless of PFGE type, the bla_{CMY-2}-carrying type C plasmids found in all serotype Newport isolates were very similar. Figure 3 shows plasmid DNA following digestion with PstI and a Southern blot using bla_{CMY-2} as a probe for five serotype Newport transformants (representing four of nine PFGE groups). Note that the only detectable difference between these plasmids is a ~11- to 12-kb band in C6/5561 (Fig. 3A, lane 5) and a 2.7-kb band in DH/pNF4656 (Fig. 3A, lane 2). For those isolates of serotype Newport for which no attempt was made to isolate a transformant with decreased susceptibility to ceftriaxone, wild-type plasmid DNA was isolated and digested with PstI. In all cases, Southern blot analysis with

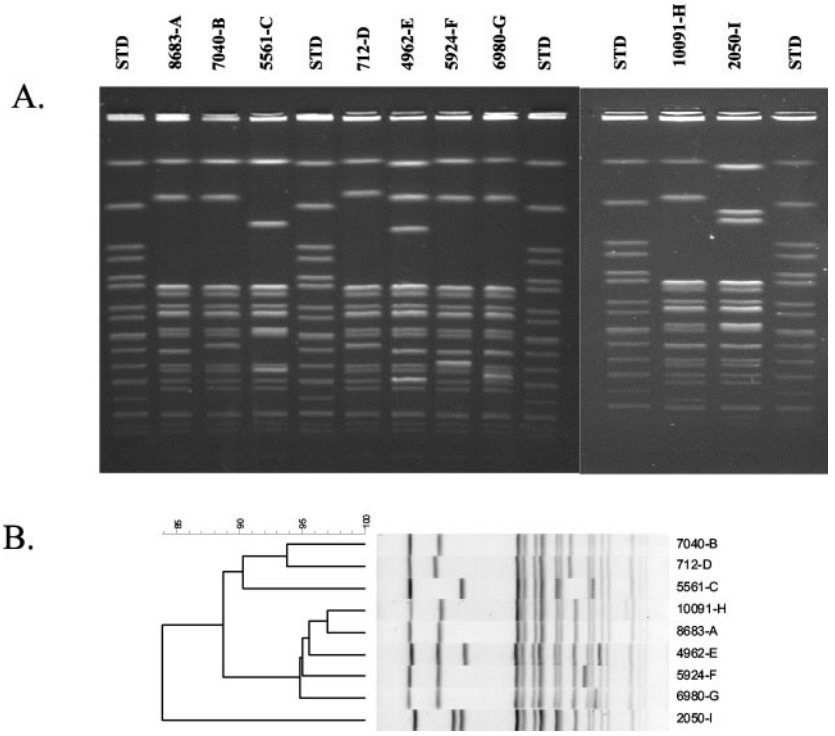

FIG. 2. (A) PFGE RFLP patterns of serotype Newport isolates resistant to expanded-spectrum cephalosporin. The strain number, followed by the PFGE group designation, is given above each lane. STD, standard. (B) Dendrogram of RFLP patterns shown in panel A following normalization and analysis with Bionumerics software. Strain numbers, followed by PFGE group designations, are given on the right.

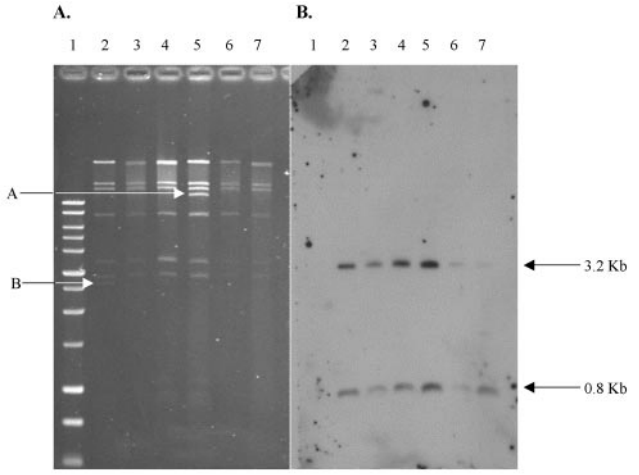

FIG. 3. (A) PstI digest of serotype Newport type C plasmids. Lanes: 1, 1-kb ladder (Promega); 2, DH/pNF4656; 3, D10/4962 (PFGE group E); 4, DH/5501 (PFGE group A); 5, C6/5561 (PFGE group C); 6, D10/7040 (PFGE group B); 7, D10/7073 (PFGE group A). Arrow A indicates a ~11- to 12-kb band in C6/5561, and arrow B indicates a 2.7-kb band in DH/pNF4656; these bands constitute the only differences in the plasmid RFLP patterns, as discussed in the text. (B) Subsequent Southern hybridization of the gel in panel A probed with full-length bla_{CMY-2}. Sizes of bands hybridizing to the bla_{CMY-2} probe, as explained in the text, are given on the right.

a bla_{CMY-2} probe demonstrated that each serotype Newport isolate carried a type C plasmid.

Relatedness of plasmid types A and C. Due to the highly similar PstI RFLP patterns of the type A and C plasmids, it was hypothesized that they were related replicons. To address this question, plasmid DNA was prepared from C6/pNF34 (type A) and DH/pNF4656 (type C) and digested with HincII. HincII-digested C6/pNF1358 was used as a negative control. As predicted, the HincII RFLP patterns were similar for type A and C plasmids, whereas that for type B was divergent (Fig. 4). Southern blot analysis, using pNF4656 as a probe, demonstrated that pNF4656 hybridized to each distinguishable HincII band in pNF34. In contrast, pNF4656 hybridized to only three HincII bands from pNF1358.

DNA sequence of region surrounding bla_{CMY-2}. Plasmid libraries from DH/pNF4656 and C6/pNF1358 were generated by using the pCR4 blunt-TOPO vector, and ~500 clones were sequenced for both the DH/pNF4656 and C6/pNF1358 libraries (~4× coverage). In contrast, the DNA sequence of the bla_{CMY-2} region from pIW759 was generated by using primers within bla_{CMY-2}, and sequencing was carried out from the native plasmid in both directions. Subsequent DNA sequencing primers were then constructed through the DNA sequence generated. After the bla_{CMY-2} gene and the regions surrounding bla_{CMY-2} were identified, it became apparent that these regions were highly conserved in all three plasmid types and had the same general organization as that already described for pTKH11, a plasmid carrying bla_{CMY-5} isolated from *Klebsiella oxytoca* (Fig. 5) (39). Including IS*Ecp1* (see below) upstream of bla_{CMY-2} to the 5′ end of the *blc* gene, there was 100% sequence identity among all three plasmid types and 99.3% sequence identity to the same region in pTKH11.

The sequences of all three plasmids immediately downstream of bla_{CMY-2} contained open reading frames (ORFs) that had 96.3 and 96.9% sequence identity, respectively, to *blc* and *sugE*, two genes found just downstream of the *C. freundii* chromosomal bla_{ampC} gene. In *C. freundii*, *blc* encodes an outer membrane lipoprotein whereas *sugE* is hypothesized to be a member of the small multidrug resistance (SMR) family of multidrug efflux systems (8, 16, 26). Therefore, neither of these genes is thought to be involved in bla_{CMY-2} regulation (39). Upstream and adjacent to *sugE* in the type C and D plasmids is partial sequence (364 bp) of an ORF that has 96.7% sequence identity to *ecnR* (599 bp), a gene which is found just upstream of *sugE* in the *C. freundii* chromosome (7). *ecnR* encodes the response regulator for *ecnAB*, which acts as an antidote-toxin gene pair involved in programmed cell death. Adjacent to and downstream of the truncated *ecnR*, there is a 405-bp region that is shared by type C and D plasmids. No significant identity to any known bacterial DNA sequence was found in this region. Adjacent to this common region, an IS*26* insertion sequence was found in the type C plasmid but not in the type D plasmid (23). BLAST searches demonstrated that DNA sequence generated from pIW759 outside of the bla_{CMY-2} region was not found in the DNA sequence from pNF4656 or pNF1358, suggesting that the type D plasmid is an independent replicon and was not once part of a composite plasmid with either a type B or a type C plasmid. Located just upstream of bla_{CMY-2} is an ORF with 99.8% sequence identity with IS*Ecp1* (20). IS*Ecp1* is inserted at bp 942 of the *C. freundii ampR-ampC* promoter region. The region into which IS*Ecp1* inserts is known to be an AmpR binding region (Fig. 6). The DNA sequence is divergent in all three plasmid replicons immediately adjacent to the left inverted repeat of IS*Ecp1*.

***bla*$_{CMY-2}$ region junction.** By using known sequence upstream and downstream of the bla_{CMY-2}-carrying region (Fig. 5), primers were generated (Table 2) to amplify the junction regions between the plasmid sequence and IS*Ecp1* on one end (left end) and *sugE* (type B) or *ecnR* (types C and D) and the plasmid sequence on the other (right end). Because sequence downstream of IS*26* was not available (sequencing of pNF4656

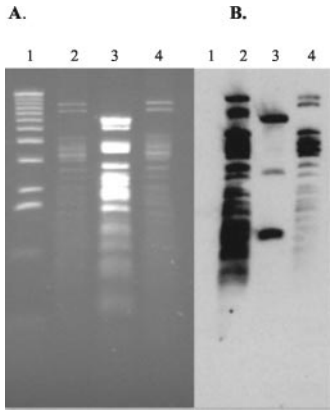

FIG. 4. (A) HincII digest of plasmid types A, B, and C. Lanes; 1, 1-kb ladder (BRL, Bethesda, Md.); 2, C6/pNF34 (type A); 3, C6/pNF1358 (type B); 4, DH/pNF4656. (B) Subsequent Southern hybridization of gel in panel A probed with whole pNF4656 DNA.

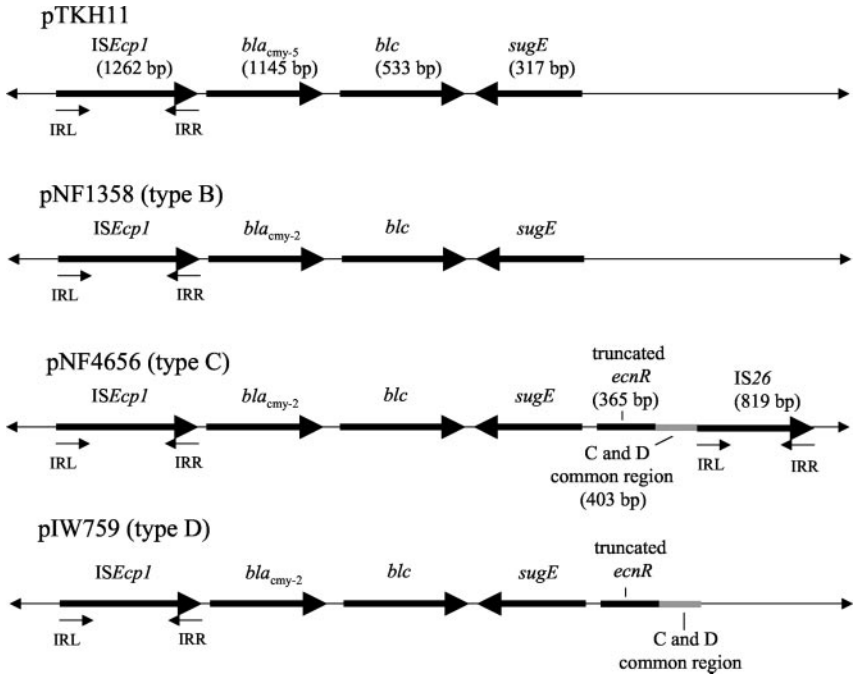

FIG. 5. Map of bla_{CMY-2} regions from plasmid types B, C, and D in comparison with that from pTKH11. IRL, left inverted repeat; IRR, right inverted repeat. *blc*, *sugE*, and *ecnR* encode bacterial lipocalin, a small multidrug resistance protein, and the response regulator for the enterocidin gene, respectively.

is not complete), the right-end junction primer for the type C plasmid was designed from sequence within IS*26*. Specific left-end primers designed from pNF4656 (type C) generated the expected products in all 23 type C plasmids and all 6 type A plasmids (data not shown). In contrast, the right-end primers designed from pNF4656 did not amplify an appropriate product in any of the 23 type C plasmids or in any of the 6 type A plasmids. Both right-end and left-end primers designed from

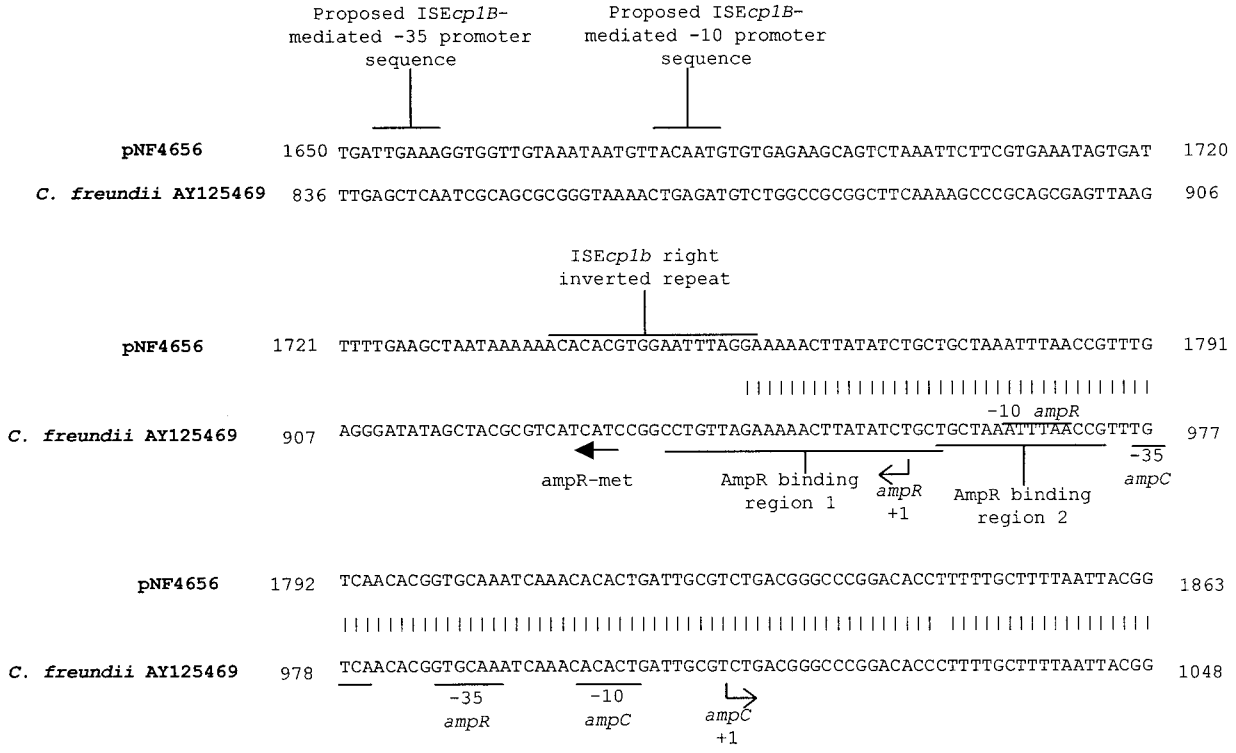

FIG. 6. Comparison of region upstream of bla_{CMY-2} in pNF4656 and the *ampR-ampC* promoter region from *C. freundii*.

pNF1358 amplified the expected products in all five type B plasmids. Left-end and right-end primers specific for pIW759 (type D) did not amplify a product in any type A, B, or C plasmid (data not shown).

DISCUSSION

The purpose of this study was to determine the extent to which the four characterized plasmid replicons that carry bla_{CMY-2} were found within 35 expanded-spectrum cephalosporin-resistant salmonellae collected from 1999 to 2001. These four plasmid types (types A to D) have been characterized previously in two separate studies (11, 38). In the present study, transfer of bla_{CMY-2} through conjugation to *E. coli* C600N was predictably successful only when a type B plasmid was being transferred. Conjugal transfer of type A and C plasmids was less successful, and transfer frequencies were extremely low (10^{-8}). It is not known whether the majority of type A and C plasmids were defective in conjugal transfer or whether these observations are an experimental artifact. It was determined, however, that electroporation using the method of Sheng et al. (31) was an extremely reliable method of transferring bla_{CMY-2}-containing plasmids from salmonellae to *E. coli* DH10B.

The observation that similar bla_{CMY-2}-carrying plasmids were observed in two temporally distinct strain sets (the 1996-to-1998 set and the present strain set of 1999 to 2001) suggests that bla_{CMY-2} has moved to separate genomic backgrounds through plasmid transfer, as opposed to transfer of the element itself to separate plasmids or the chromosome (11). Within serotype Typhimurium, the most prevalent serotype isolated from humans, three plasmid types (types A, B, and C) were found in multiple genomic backgrounds. In contrast, only one bla_{CMY-2}-containing plasmid type, type C, was found within the population of highly related ceftriaxone-resistant serotype Newport isolates. No isolates were found to contain a type D plasmid. Preliminary data suggest that the smaller pIW759-like plasmids are more common within *E. coli* populations than within salmonellae (P.L. Winokur, unpublished data). Since our Southern hybridization data suggest that plasmid types A and C are highly related, it appears that two variants of the same plasmid are mostly responsible for the dissemination of bla_{CMY-2} within ceftriaxone-resistant salmonellae in the United States (30 of 35 isolates belong to either type A or type C). Primers designed to identify the junction regions between the bla_{CMY-2} region and plasmid sequence demonstrate that the junction near IS*Ecp1* is conserved in type A and C plasmids. However, downstream, near *ecnR*, all 30 type A or C plasmids diverge from the representative type C plasmid, pNF4656. This result is not unexpected, since one of the primers used to amplify the right-end junction from pNF4656 was designed within a mobile element, IS*26*. Further DNA sequencing is needed to determine whether the right-end junction sequence within type A and C plasmids is conserved. One potential reason for the RFLP differences seen between type A and C plasmids is the fact that type A plasmids contain at least twice as many IS*26* elements as type C plasmids (data not shown).

Recently, Poirel et al. reported that IS*Ecp1B*, an insertion sequence element highly related to IS*Ecp1*, was found upstream of $bla_{CTX-M-19}$ in a *Klebsiella pneumoniae* isolate obtained in Vietnam (28). It was hypothesized, but not experimentally demonstrated, that IS*Ecp1B* was responsible for the movement of $bla_{CTX-M-19}$, because a separate inverted repeat with consistent nucleotide identity to the right inverted repeat of IS*Ecp1B* was found downstream of $bla_{CTX-M-19}$. In addition, primer extension studies demonstrated the presence of a promoter element just upstream of the right inverted repeat that directed the transcription of $bla_{CTX-M-19}$. Therefore, IS*Ecp1B* was hypothesized to be responsible not only for the movement of $bla_{CTX-M-19}$ but also for its expression.

The DNA sequence comparison between the *ampR-ampC* intergenic promoter region in *C. freundii* and the region upstream of bla_{CMY-2} in pNF4656 is shown in Fig. 6. This analysis demonstrated that IS*Ecp1* inserted into AmpR binding region 1 in pNF4656. DNA binding studies have demonstrated that AmpR binds to region 1 but binds to region 2 only in the presence of region 1 (5, 18). These data strongly suggest that AmpR would not be able to activate or induce transcription from bla_{CMY-2} in pNF4656 if present. The proposed promoter from *C. freundii* bla_{ampC} is present in pNF4656, but it is not known what effect IS*Ecp1* has on its function. The promoter sequence found within IS*Ecp1B*, which has been shown to drive the transcription of $bla_{CTX-M-19}$, is also found in IS*Ecp1* upstream of bla_{CMY-2}. The effect of this putative promoter sequence on bla_{CMY-2} transcription is unknown and warrants further study. In contrast to what was found for the relationship of IS*Ecp1B* and $bla_{CTX-M-19}$, no separate inverted repeat was found downstream of the bla_{CMY-2} region in any of the three plasmids sequenced. Multiple experiments attempting to demonstrate mobility of the bla_{CMY-2} region from pNF1358 to other plasmids were not successful (data not shown).

In conclusion, this work demonstrates that bla_{CMY-2} is spread primarily through plasmid transfer and not through the mobilization of a transposon or integron to multiple plasmid replicons and chromosomes. These findings were unexpected, because Morosini and colleagues have demonstrated that salmonellae carrying a plasmid-encoded, *Enterobacter cloacae*-derived AmpC β-lactamase had a reduction in growth rate and were less invasive by an in vitro cell invasion assay (24). The pathogenesis of *Salmonella* spp. is dependent on the organism's ability to adhere to and invade intestinal epithelial cells. Therefore, one would predict that the prevalence of AmpC-producing salmonellae would be very low, because these strains would be less physiologically fit. Alternatively, the predicted decreased fitness may be counterbalanced by the selective pressure of antimicrobial usage, compensatory mutations within certain genomic backgrounds of *Salmonella*, varying effects on invasiveness and/or fitness associated with different species-derived AmpC β-lactamases, and/or regulation of the bla_{ampC} gene itself (i.e., promoter strength).

ACKNOWLEDGMENT

W.P.G. is supported through a USDA National Needs Fellowship.

ADDENDUM IN PROOF

Hossain et al. have recently described an *S. enterica* serotype Typhimurium strain that carried a bla_{CMY-7} gene (A. Hossain, M. D. Reisbig, and N. D. Hanson, J. Antimicrob. Chemother.

53: 964–970, 2004). A promoter within an IS*Ecp1*-like element, which was located just upstream of bla_{CMY-7} at the identical nucleotide position as described in the present study, was shown to drive transcription of bla_{CMY-7}.

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