

# Molecular Epidemiology of *bla*<sub>CMY-2</sub> Plasmids Carried by *Salmonella enterica* and *Escherichia coli* Isolates from Cattle in the Pacific Northwest<sup>∇</sup>

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**Restriction analyses of *bla*<sub>CMY-2</sub>-bearing plasmids and *Salmonella* and *Escherichia coli* hosts identified (i) shared highly similar plasmids in these species in rare cases, (ii) a clonal host-plasmid relationship in *Salmonella enterica* serotype Newport, and (iii) a very high diversity of strain types and plasmids among commensal *E. coli* isolates.**

Nontyphoid *Salmonella enterica* subsp. *enterica* (NTS) causes approximately 36,000 confirmed cases of food-borne illness in the United States and an estimated 1.4 million unreported cases each year (9, 17, 25). A trend toward increasing antimicrobial resistance to broad-spectrum cephalosporins in some NTS serovars has raised concern because these drugs are regarded as first-line therapy in pediatric salmonellosis (11). The most recent surveillance data indicate that 0.6% of U.S. human NTS isolates are resistant to ceftriaxone and 3.4% are resistant to ceftiofur (a veterinary broad-spectrum cephalosporin) (7). The predominant mechanism of cephalosporin resistance in NTS in the United States is a cephamycinase encoded by plasmid-borne *bla*<sub>CMY-2</sub> genes (11, 27, 28, 30). *bla*<sub>CMY-2</sub>, which likely originated from the chromosomal AmpC locus of *Citrobacter freundii* (1, 19), has been observed in plasmids in several species of the *Enterobacteriaceae* (2–4, 15, 16, 18, 27, 29).

Commensal bacteria may serve as a reservoir of plasmid-borne antimicrobial resistance genes for pathogens, and there is evidence that plasmid transfer occurs readily between *Escherichia coli* and *S. enterica*. For example, a phylogenetic analysis of F plasmid-specific genes from reference collections of *S. enterica* and *E. coli* found several examples in which *finO* and *traD* sequence variants were shared between the two species (6). Furthermore, *bla*<sub>CMY-2</sub> Southern blot experiments with plasmids from *E. coli* and *S. enterica* have revealed similarities among isolates, suggesting that sequences (in addition to the *bla*<sub>CMY-2</sub> open reading frame) are shared among the plasmids harbored by these microbial genera (27, 28).

Cattle could represent an important niche for transfer of *bla*<sub>CMY-2</sub> plasmids between *E. coli* and NTS. Both bacterial genera inhabit the bovine gastrointestinal tract, and selection pressure favoring cephalosporin resistance is ubiquitous in some cattle production systems due to the frequent use of ceftiofur (13). For example, between 2001 and 2003, the percentage of ceftiofur-resistant NTS rose more rapidly among

isolates from U.S. cattle than in those from human, chicken, turkey, and swine hosts (8). The predominance of *bla*<sub>CMY-2</sub>-mediated cephalosporin resistance among NTS and *E. coli* isolates from cattle (20, 24, 30) led us to investigate the relationship between *bla*<sub>CMY-2</sub> plasmids and the two microbial genera. Assessing the diversity of host chromosomal and plasmid DNAs from commensal isolates of *E. coli* and clinical isolates of *S. enterica* permitted us to evaluate whether *bla*<sub>CMY-2</sub> dissemination in this ecological niche is clonal or due to epidemic plasmid spread and whether the nature of this process differed in a pathogen (*S. enterica*) and a potential reservoir of antimicrobial resistance genes (*E. coli*).

*E. coli* isolates from 46 animals originating in 14 herds and *S. enterica* isolates from 48 animals with salmonellosis originating in 47 herds were chosen to represent the bovine commensal flora and a major bovine pathogen, respectively. All isolates were obtained from cattle in Washington state or Idaho between 2001 and 2003. All isolates gave an amplicon of the appropriate length when tested by PCR using *bla*<sub>CMY-2</sub>-specific primers described by Zhao et al. (30) (Table 1).

*E. coli* and *S. enterica* serotypes were determined by the Gastroenteric Disease Center (University Park, PA) and the National Veterinary Services Laboratory (Ames, IA), respectively. Isolates were assessed for pulsed-field gel electrophoresis (PFGE) type in accordance with PulseNet protocols (23). Plasmids were isolated by electroporation into *E. coli* DH10B and prepared for PstI restriction fragment length polymorphism (pRFLP) typing using previously described methods (12, 21). pRFLP typing and *bla*<sub>CMY-2</sub> Southern blotting were performed as described by Giles et al. (12) but using continuous voltage (7.2 V/cm for 1.5 h). Agar diffusion susceptibility testing was performed in accordance with CLSI standards (10). Gel images were analyzed using Bionumerics (Applied Maths, Belgium) with optimization and tolerance settings determined by the minimum values required to classify a standard plasmid (which was included with each gel) as indistinguishable from itself in an unweighted pair group method with arithmetic mean (UPGMA) analysis.

*S. enterica* included four serotypes: Newport (*n* = 35), Typhimurium (*n* = 5), Dublin (*n* = 7), and Muenster (*n* = 1); PFGE patterns were highly similar within serotypes (Fig. 1).

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TABLE 1. *bla*<sub>CMY-2</sub> containing *S. enterica* and *E. coli* isolates

Isolate	Serotype <sup>a</sup>	PFGE type	Resistance phenotype <sup>b</sup>		Plasmid type	<i>bla</i> <sub>CMY-2</sub> fragment size (kb) <sup>d</sup>	Conjugative <sup>e</sup>	Yr
			Source strain	Transformant				
S8126	Dublin	D1	AMP CHL GEN KAN SXT TET SSS STR CAZ	Same as donor <sup>c</sup>	3	20	N	2003
S8133	Dublin	D1	AMP CHL GEN KAN SXT TET SSS STR CAZ	Same as donor	3	20	N	2002
S8244	Dublin	D2	AMP CHL TET SSS STR CAZ	Same as donor	7	20	N	2003
S8247	Dublin	D1	AMP CHL GEN KAN TET SSS STR CAZ	AMP CHL KAN TET SSS CAZ	3	20	Y <sup>f</sup>	2003
S8253	Dublin	D2	AMP CHL TET SSS STR CAZ	Same as donor	7	20	N	2003
S8274	Dublin	D1	AMP CHL GEN KAN TET SSS STR CAZ	Same as donor	3	20	N	2003
S8282	Dublin	D3	AMP CHL GEN KAN TET SSS STR CAZ	Same as donor	3	20	N	2002
S7651	Muenster	M1	AMP CHL GEN KAN SXT TET SSS STR CAZ	Same as donor	2	3.2	N	2002
S7140	Newport	N3	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7276	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7333	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7465	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7497	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7656	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7658	Newport	N8	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7661	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7677	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7748	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7885	Newport	N7	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7889	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7891	Newport	N11	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7893	Newport	N2	AMP TET SSS STR CAZ	Same as donor	33	3.2	N	2002
S7894	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7906	Newport	N10	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7909	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7918	Newport	N13	AMP TET SSS STR CAZ	Same as donor	31	3.2	N	2002
S7926	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7935	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S7936	Newport	N12	AMP CHL GEN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	18	3.2	N	2002
S8118	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2002
S8124	Newport	N3	AMP CHL TET SSS STR CAZ	Same as donor	32	3.2	N	2002
S8129	Newport	N3	AMP CHL TET SSS STR CAZ	Same as donor	13	20	N	2003
S8132	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2003
S8151	Newport	N9	AMP CHL TET SSS STR CAZ	Same as donor	13	20	N	2003

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TABLE 1—Continued

Isolate	Serotype <sup>a</sup>	PFGE type	Resistance phenotype <sup>b</sup>		Plasmid type	<i>bla</i> <sub>CMY-2</sub> fragment size (kb) <sup>d</sup>	Conjugative <sup>e</sup>	Yr
			Source strain	Transformant				
S8242	Newport	N4	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2003
S8245	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2003
S8257	Newport	N5	AMP TET SSS STR CAZ	Same as donor	31	3.2	N	2003
S8258	Newport	N1	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2003
S8277	Newport	N4	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2003
S8280	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2003
S8281	Newport	N2	AMP CHL TET SSS STR CAZ	Same as donor	18	3.2	N	2003
S8912	Newport	N6	AMP TET SSS STR CAZ	Same as donor	30	3.2	N	2003
S8922	Newport	N11	AMP TET SSS STR CAZ	Same as donor	32	3.2	N	2003
S7655	Typhimurium	T2	AMP CHL KAN SXT TET SSS STR CAZ	Same as donor	14	20	N	2002
S7907	Typhimurium	T4	AMP CHL KAN SXT TET SSS STR CAZ	Same as donor	13	20	N	2002
S8238	Typhimurium	T1	AMP CHL TET SSS STR CAZ	Same as donor	29	3.2	N	2003
S8240	Typhimurium	T5	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL SXT TET SSS CAZ	11	20	N	2003
S8914	Typhimurium	T3	AMP CHL KAN TET SSS STR CAZ	Same as donor	13	20	N	2003
E1128	O2:H42	E23	AMP CHL TET SSS STR CAZ	Same as donor	13	20	N	2002
E1216	O33:H4	E2	AMP CHL GEN SXT TET SSS STR CAZ	AMP CHL SXT TET SSS CAZ	10	20	Y	2002
E1246	n:H30	E4	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL SXT TET SSS CAZ	13	20	Y	2002
E1291	O159:H4	E24	AMP CHL TET SSS STR CAZ	Same as donor	10	20	Y	2001
E1445	O35:H16	E25	AMP CHL KAN SXT TET SSS STR CAZ	Same as donor	24	20	Y	2001
E1455	O145:+	E26	AMP CHL GEN KAN TET SSS STR CAZ	Same as donor	23	3.2	Y	2001
E1473	O35:H16	E27	AMP CHL KAN SXT TET SSS STR CAZ	Same as donor	24	20	Y	2001
E2275	O66:H25	E28	AMP CHL GEN KAN SXT TET SSS STR CAZ	Same as donor	1	3.2	N	2001
E2323	O154:H30	E13	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	16	3.2	N	2002
E2331	n:H9	E29	AMP CHL TET SSS STR CAZ	Same as donor	17	3.2	N	2002
E2346	O154:H30	E14	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	17	3.2	N	2002
E2358	n:H42	E30	AMP CHL GEN KAN TET SSS STR CAZ	Same as donor	23	3.2	N	2002
E2379	n:H48	E31	AMP CHL GEN KAN TET SSS STR CAZ	Same as donor	24	3.2	N	2002
E2449	n:H42	E32	AMP CHL TET SSS STR CAZ	Same as donor	13	20	Y	2002
E2469	O20:H30	E33	AMP CHL KAN SXT TET SSS STR CAZ	Same as donor	20	20	Y	2002
E2517	n:H38	E34	AMP CHL GEN KAN SXT TET SSS STR CAZ	Same as donor	5	3.2	Y	2002
E2530	O111:H8	E11	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL SXT TET SSS CAZ	26	1	N	2002
E2588	n:H11	E19	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL GEN KAN TET SSS CAZ	19	20	N	2002
E2639	O8w:H16	E18	AMP CHL KAN TET SSS STR CAZ	AMP CAZ	2	2.9	N	2002
E2654	n:H38	E35	AMP CHL KAN SXT TET SSS STR CAZ	Same as donor	24	20	N	2002

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TABLE 1—Continued

Isolate	Serotype <sup>a</sup>	PFGE type	Resistance phenotype <sup>b</sup>		Plasmid type	<i>bla</i> <sub>CMY-2</sub> fragment size (kb) <sup>d</sup>	Conjugative <sup>e</sup>	Yr
			Source strain	Transformant				
E2676	O11:H15	E5	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	16	3.2	N	2002
E2679	n:H5or56	E20	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL GEN KAN TET SSS CAZ	21	3.2	N	2002
E2699	n:H28	E21	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL KAN SUL CAZ	22	20	Y	2002
E2713	n:H4	E15	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	13	20	N	2002
E2759	O20:H18	E3	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL KAN SXT TET SSS CAZ	24	20	Y	2002
E2769	n:H4	E9	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL SXT TET SSS CAZ	18	3.2	Y	2002
E525	O20:H30	E22	AMP CHL KAN SXT TET SSS STR CAZ	Same as donor	20	20	Y	2002
E6875	O2:H42	E16	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	17	3.2	N	2002
E6900	a:5or56	E12	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL GEN TET SSS CAZ	15	3.2	N	2003
E7119	O2:H42	E17	AMP CHL KAN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	15	3.2	N	2003
E7132	O116w:H9	E36	AMP CHL SXT TET SSS STR CAZ	Same as donor	16	20	N	2003
E7140	O116w:H9	E37	AMP CHL TET SSS STR CAZ	Same as donor	8	11, ~20	N	2003
E7196	O101:H9	E6	AMP CHL GEN KAN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	10	20	Y	2002
E7212	n:H9	E7	AMP CHL GEN KAN TET SSS STR CAZ	AMP CHL TET SSS CAZ	13	20	Y	2003
E7292	O117:H4	E8	AMP CHL GEN SXT TET SSS STR CAZ	AMP CHL TET SSS CAZ	34	3.2	N	2003
E7403	O145:H12	E10	AMP CHL GEN KAN TET SSS STR CAZ	AMP CHL GEN TET SSS CAZ	7	3.2, 11	N	2003
E793	O20:H30	E1	AMP CHL KAN SXT TET SSS STR CAZ	AMP CAZ	6	3.2	Y	2003
E8100	O10:H42	E39	AMP CHL TET SSS STR CAZ	Same as donor	27	1, 3.2	N	2003
E8142	n:H9	E41	AMP CHL SXT TET SSS STR CAZ	Same as donor	13	20	Y	2003
E8166	n:H9	E42	AMP CHL SXT TET SSS STR CAZ	Same as donor	10	20	N	2003
E8188	O159:H4	E43	AMP CHL TET SSS STR CAZ	Same as donor	26	20	N	2003
E8430	O159:H4	E44	AMP CHL TET SSS STR CAZ	Same as donor	26	20	N	2003
E8431	O159:H4	E45	AMP CHL TET SSS STR CAZ	Same as donor	28	20	N	2003
E8603	n:n	E46	AMP CAZ	Same as donor	4	2.9, 4, 5	Y	2003
E8618	O159:H4	E38	AMP CHL TET SSS STR CAZ	Same as donor	12	20	N	2003
E8625	O159:H4	E40	AMP CHL TET SSS STR CAZ	Same as donor	25	20	N	2003

<sup>a</sup> n, negative; + positive, novel antigen; a, autoagglutination; w, weak agglutination.

<sup>b</sup> AMP, ampicillin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; STR, streptomycin; SSS, triple sulfa; CAZ, ceftazidime.

<sup>c</sup> "Same as donor" is with respect to all resistances except streptomycin, as the recipient strain (DH10B) is intrinsically resistant to streptomycin.

<sup>d</sup> A *bla*<sub>CMY-2</sub>-positive PstI fragment of 850 bp was present for all plasmids, in addition to the larger fragment reported in this column.

<sup>e</sup> N, no; Y, yes.

<sup>f</sup> Transconjugants were observed only when the helper plasmid pRK2013 was included in the mating mixture.

Sixteen serotypes were identified among the 28 *E. coli* isolates that were typeable for both O and H antigens, but PFGE patterns were markedly more diverse than in the *Salmonella* spp.; each of the 46 isolates displayed a unique pattern (data not shown). The differences in serotype and PFGE diversities

between the two genera may reflect the sources of the isolates: clinical versus commensal bacteria from healthy animals. Pathogenic *Salmonella* spp. have been described as inherently clonal (5, 14), whereas relatively little is known about the genetic diversity of nonpathogenic *E. coli* isolates from animal

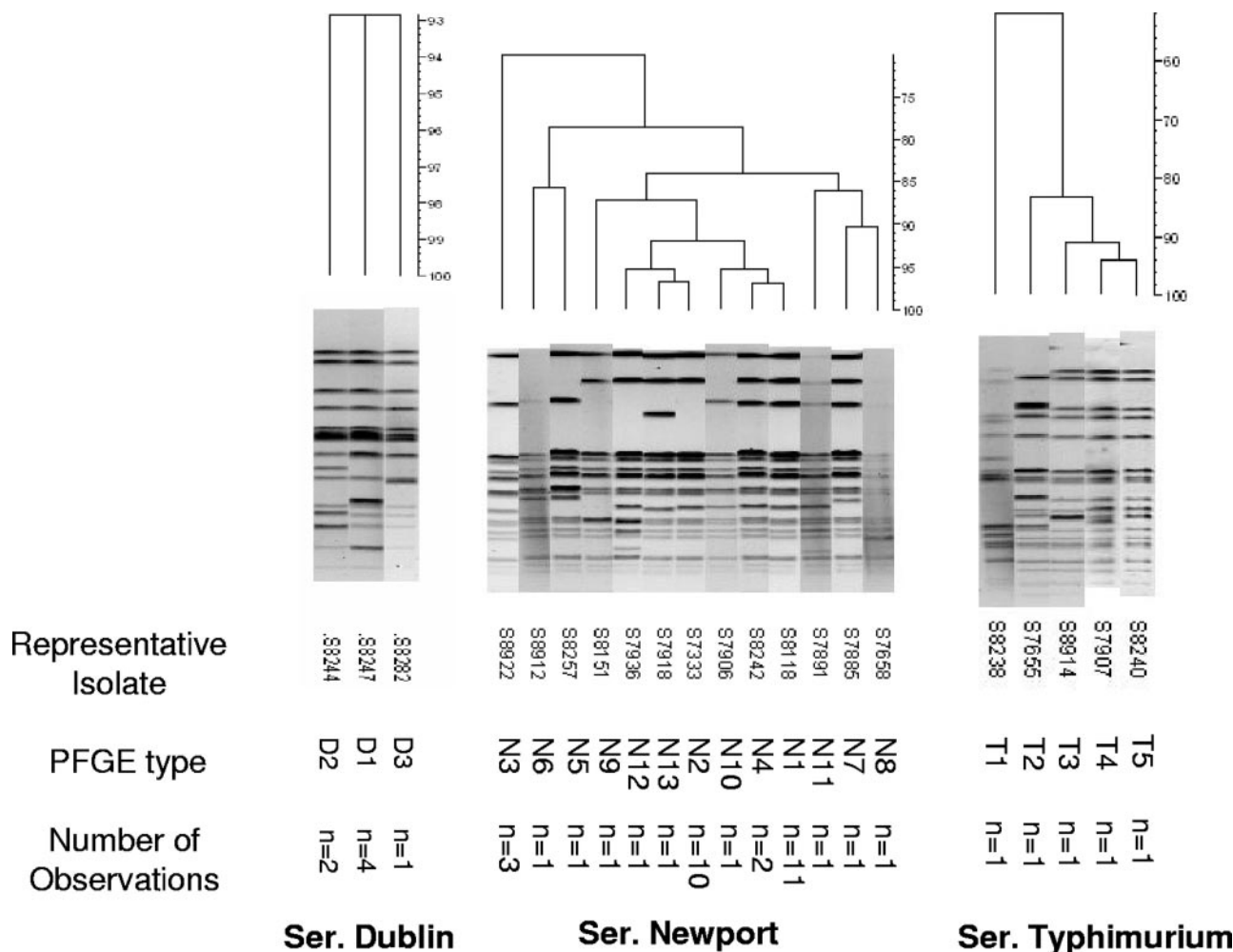


FIG. 1. UPGMA dendrograms with negative images of ethidium bromide-stained gels from XbaI PFGE of *bla*<sub>CMY-2</sub>-positive *S. enterica* from cattle following normalization and analysis with Bionumerics software. The PFGE type designations correspond to those referred to in Table 1. Ser., serotype.

sources. Winokur et al. found diverse PFGE patterns among 55 *bla*<sub>CMY-2</sub> commensal *E. coli* isolates from clinical veterinary specimens (28). Taken together, these findings suggest that *bla*<sub>CMY-2</sub>-bearing commensal *E. coli* isolates are not strongly clonal at the serotype and PFGE levels, regardless of the clinical status of the source.

Forty pRFLP patterns were observed among the 94 isolates; however, repeatability analyses using a subset of 14 plasmids demonstrated that consistent self-grouping was observed only at the 90% similarity level. Thus, we considered plasmids with ≥90% similarity indistinguishable, resulting in the designation of 34 unique patterns. Four reference plasmids (A, B, C, and D), described by Giles et al. (12), were included as positive controls. Twelve pRFLP patterns were observed in more than one bacterial isolate, and two patterns were observed in both genera, consistent with exchange of some plasmids among commensal *E. coli* and *S. enterica* isolates (Fig. 2). Eighty-five plasmids had *bla*<sub>CMY-2</sub> Southern blot fragments identical to the A or C patterns previously described and were conserved within pRFLP types, consistent with horizontal-transfer activity (Fig. 3).

Each *Salmonella* serotype tended to be associated with a specific plasmid variant. Notably, for *S. enterica* serovar Newport, 26 of 35 isolates originated from 26 different herds but shared a single pRFLP type (type 18). Using the model of plasmid-bacterial-host associations proposed by Souza and Eguiarte (22), the relationship between *S. enterica* serovar Newport and its plasmids could be described as clonal, implying that *bla*<sub>CMY-2</sub> plasmids in this serotype were largely disseminated with an epidemic host bacterium. In contrast, the diversity of plasmids from *E. coli* was high, reflecting the high level of PFGE and serotype diversity observed between the isolates. The exceptions to this observation were five pRFLP types (10, 13, 16, 17) associated with multiple serologically distinguishable host strains, consistent with the idea of epidemic plasmids (22).

Although there was evidence of interspecies sharing of plasmids, the predominance of only two plasmid variants (A and C) in *Salmonella* isolates from an animal niche containing a plethora of *E. coli*-borne plasmid variants (presumably available for transfer to *Salmonella*) was conspicuous, suggesting that the major mechanisms of *bla*<sub>CMY-2</sub> dissemination differ



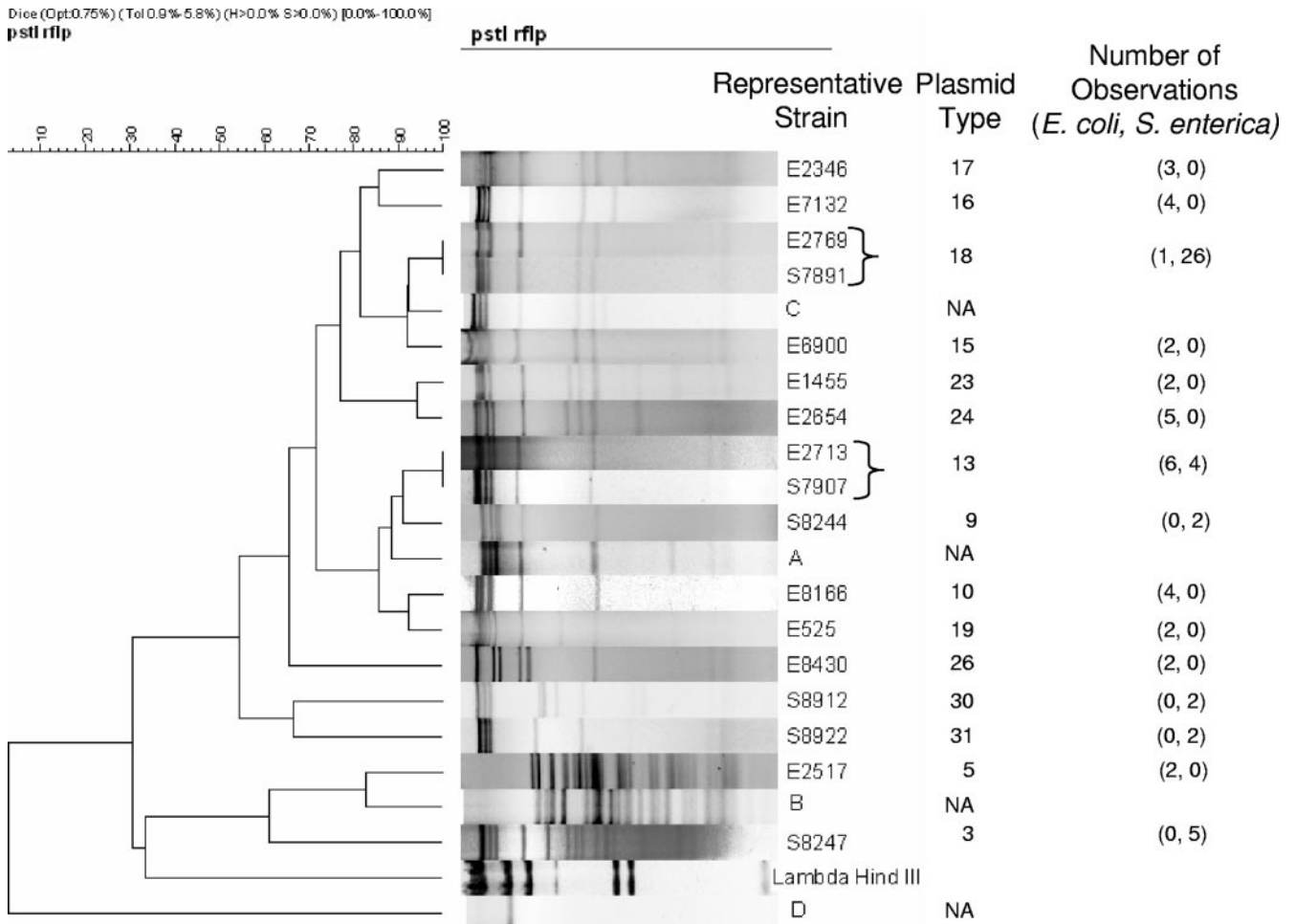


FIG. 2. UPGMA dendrogram with a negative image of an ethidium bromide-stained PstI plasmid RFLP gel after normalization in Bionumerics software. The bracketed strains indicate identical RFLP patterns from plasmids isolated from both *E. coli* and *S. enterica*. Plasmid type designations correspond to those in Table 1. Reference A, B, C, and D plasmids (12) were included as controls.

between *S. enterica* and *E. coli*. This pattern is consistent with a recent study by Welch et al., who found greater diversity of plasmids among *E. coli* isolates than among the *S. enterica* isolates, using PCR primer sets representing 13 widely spaced

loci from an entirely sequenced IncA/C *bla*<sub>CMY-2</sub> plasmid from *S. enterica* serovar Newport (26). Our observation that isolates limited to a solitary niche (cattle) are similarly diverse suggests a model of *bla*<sub>CMY-2</sub> dissemination in which insertions and

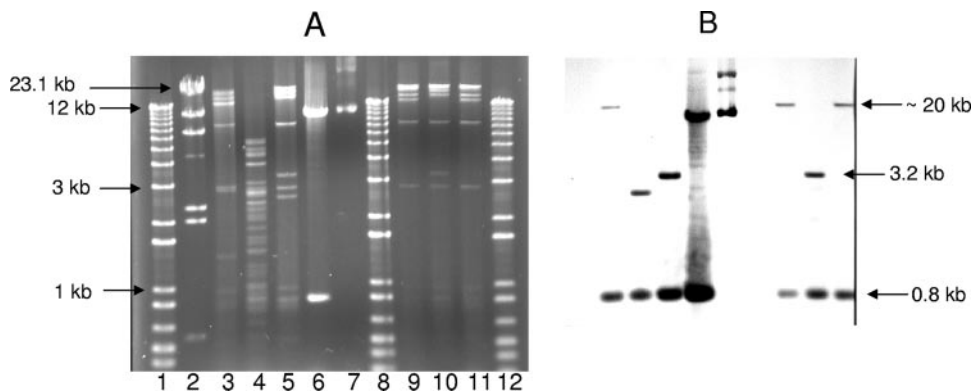


FIG. 3. (A) pRFLP of A, B, C, and D reference plasmids, followed by three examples of *bla*<sub>CMY-2</sub> plasmids from *S. enterica* isolates from Washington state cattle. Lanes: 1, 8, and 12, 12-kb ladder; 2,  $\lambda$ HindIII markers; 3, 4, 5, and 6, A, B, C, and D reference plasmids (12); 9, 10, and 11, pS7907, pS7909, and pS8129; 7, undigested reference plasmid D. (B) Southern hybridization of the gel from panel A with a full-length CMY-2 probe.

deletions that occur during promiscuous plasmid sharing among *E. coli* isolates occasionally result in plasmids that are successful in a *Salmonella* host (such as pFLP types 13 and 18). Also consistent with this model, conjugation experiments revealed that 40% of *E. coli* plasmids (versus 2% of *Salmonella* plasmids) were able to transfer or be mobilized to a Nal<sup>r</sup> DH5 $\alpha$  recipient. The subsequent success of a *Salmonella* host/plasmid clone, then, is likely modulated by diverse factors, including virulence, infectivity, and environmental persistence, as well as antimicrobial selection pressures. Factors that promote inter-species exchange of antimicrobial-resistance plasmids and enhance dissemination of *S. enterica* clones merit further study.

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