bla_{CMY-2}-Positive IncA/C Plasmids from *Escherichia coli* and *Salmonella enterica* Are a Distinct Component of a Larger Lineage of Plasmids[▽][†]

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Large multidrug resistance plasmids of the A/C incompatibility complex (IncA/C) have been found in a diverse group of Gram-negative commensal and pathogenic bacteria. We present three completed sequences from IncA/C plasmids that originated from *Escherichia coli* (cattle) and *Salmonella enterica* serovar Newport (human) and that carry the cephamycinase gene bla_{CMY-2}. These large plasmids (148 to 166 kbp) share extensive sequence identity and synteny. The most divergent plasmid, peH4H, has lost several conjugation-related genes and has gained a kanamycin resistance region. Two of the plasmids (pAM04528 and peH4H) harbor two copies of bla_{CMY-2}, while the third plasmid (pAR060302) harbors a single copy of the gene. The majority of single-nucleotide polymorphisms comprise nonsynonymous mutations in *floR*. A comparative analysis of these plasmids with five other published IncA/C plasmids showed that the bla_{CMY-2} plasmids from *E. coli* and *S. enterica* are genetically distinct from those originating from *Yersinia pestis* and *Photobacterium damselae* and distal to one originating from *Yersinia ruckeri*. While the overall similarity of these plasmids supports the likelihood of recent movements among *E. coli* and *S. enterica* hosts, their greater divergence from *Y. pestis* or *Y. ruckeri* suggests less recent plasmid transfer among these pathogen groups.

Plasmids are extrachromosomal genetic elements that play key roles in the maintenance and dissemination of novel genetic traits in bacterial populations. Antibiotic resistance is one of the most important medicine-related traits that can be encoded by plasmids. Over the past decade, increasing attention has been focused on plasmids that harbor the antibiotic resistance gene bla_{CMY-2} (hereafter called bla_{CMY-2} plasmids). bla_{CMY-2} encodes an AmpC-type beta-lactamase that hydrolyzes third-generation cephalosporins (4, 36). This gene, which probably originated from *Citrobacter freundii* (2, 27, 35), has been disseminated horizontally within and between several bacterial species from the family *Enterobacteriaceae*, including important enteric pathogens (9, 14, 18, 24, 34, 35).

Plasmids that harbor bla_{CMY-2} are typically large (*ca.* 140-plus kbp), and they encode multiple drug resistance traits (7, 8, 10, 11, 34). Kang et al. (15) examined a collection of bla_{CMY-2}-positive strains and verified that the bla_{CMY-2} gene was carried on a large plasmid and that the plasmids frequently harbored two copies of the gene as part of a repeated region. Welch et al. (33) described a complete sequence from a bla_{CMY-2}-positive plasmid that originated from a strain of *Salmonella enterica* serovar Newport (strain SL254). This plasmid also carried two copies of bla_{CMY-2} and was from the A/C incompatibility plasmid group (IncA/C) (9, 21). Welch et al. (33) also reported the full sequences of IncA/C plasmids from *Yersinia pestis* (strain IP275) and from the fish pathogen *Yersinia ruckeri* (strain YR71). These three plasmids shared a plasmid backbone with a high degree of sequence similarity and conserved gene synteny, although only pSN254 harbored bla_{CMY-2}. The authors concluded that the plasmids shared a recent common ancestor. They also reported the previously unappreciated fact that IncA/C plasmids can be recovered from a range of meat products throughout the United States. The implication of this finding is that agricultural reservoirs have the potential to share these multidrug resistance plasmids with zoonotic agents of significant public health concern, including *Y. pestis*.

The objective of the current study was to closely examine several additional bla_{CMY-2} plasmids to better understand how these plasmids are changing over short periods of time. We report complete plasmid sequences for the bla_{CMY-2} plasmid from a strain of *S. enterica* serovar Newport and from two geographically separated *Escherichia coli* strains recovered from cattle. Additional IncA/C plasmid sequences are now available (17), and this permitted us to assess the genetic relatedness between IncA/C plasmids from a diverse collection of Gram-negative bacteria. We found that most changes in bla_{CMY-2} plasmids involved insertions and deletions of se-

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TABLE 1. Basic characteristics of three sequenced bla_{CMY-2} plasmids (this study) and the reference sequence, pSN254

Characteristic	pAR060302 (FJ621588)	peH4H (FJ621586)	pAM04528 (FJ621587)	pSN254[a] (NC_009140)
Mammalian host	Cow	Cow	Human	NA
Bacterial host	E. coli	E. coli	S. Newport	S. Newport
Strain	AR060302	H4H	AM04528	SN254
Year of isolation	2002	2002	1998	NA
Antibiotic resistance phenotype[b]	AM-AMC-C-CAZ-GM-S-SUL-TE	AM-AMC-C-CAZ-GM-K-S-SUL-TE	AM-AMC-C-CAZ- S-SUL-TE	AM-AMC-C-CAZ-GM-S-SUL-TE[c]
Size (bp)	166,531	148,106	158,195	176,473
%GC	53.1	48.3	51.9	52.8
Conjugation efficiency[d]	1.3×10^{-3}	0.0	0.0	NA
Giles type[e]	A	C	C	C[f]
No. of copies of bla_{CMY-2}	1	2	2	2
floR region[g]	Yes	Yes	Yes	Yes
aadA region[g]	Yes	Yes	No	Yes
kan region[g]	No	Yes	No	No
merA region[g]	Yes	Yes	Yes	Yes

[a] The pSN254 sequence was published by Welch et al. (33). NA, not applicable.

[b] AM, ampicillin; AMC, amoxicillin-clavulanic acid; C, chloramphenicol; CAZ, ceftazidime; GM, gentamicin; K, kanamycin; S, streptomycin; SUL, sulfasoxazole; TE, tetracycline.

[c] The antibiotic resistance phenotype is inferred from reference 33 and the plasmid sequence.

[d] Conguation efficiencies for plasmids that were first transferred to E. coli DH10B by electroporation.

[e] Giles type based on plasmid RFLP after PstI digestion (13).

[f] The Giles type for pSN254 is presumed to be group C based on sequence similarity with pAM04528.

[g] The floR, aadA, kan, and merA regions are shown in Fig. 1.

quences related to antibiotic resistance. Analysis of eight IncA/C plasmids showed that bla_{CMY-2} plasmids harbored by E. coli and S. enterica are relatively distinct from IncA/C plasmids described from other taxa.

MATERIALS AND METHODS

Strains used in this study. E. coli strain H4H (the source of plasmid peH4H) was originally isolated from a dairy cow in Washington State by the Washington State University College of Veterinary Medicine Field Disease Investigation Unit (Pullman, WA) (Table 1). E. coli strain AR060302 (the source of plasmid pAR060302) was originally isolated from a dairy calf in Illinois as part of a project investigating antibiotic resistance in production environments (R. S. Singer, unpublished data). S. enterica serovar Newport strain AM04528 (the source of pAM04528) was provided by the Centers for Disease Control and Prevention (Atlanta, GA) and originated from a case of human salmonellosis in Kansas. E. coli DH10B and DH5α were used as host strains for plasmid isolation and preparation for sequencing. Plasmids were categorized by the restriction fragment length polymorphism (RFLP) classification system described by Giles et al. (13) and with additional restriction digests. Antibiotic susceptibility was assessed using agar diffusion according to Clinical and Laboratory Standards Institute guidelines (3, 23), and MICs for a range of antibiotics were determined using an automated Sensititer assay (Trek Diagnostic Systems, Cleveland, OH) at the Washington Animal Disease Diagnostic Laboratory (Pullman, WA). To estimate conjugation efficiency, plasmids were first transferred to E. coli strain DH10B by electroporation (29). Single colonies were grown overnight (3 ml LB broth at 37°C; 200-rpm shaker), and 10 μl was then mixed with 10 μl overnight culture of a recipient strain (E. coli strain DH5α; nalidixic acid resistant [Nal^r]). This mixture was spread onto a nitrocellulose membrane overlaid onto LB agar without antibiotics. After overnight incubation (37°C), the culture mat was diluted with 0.5 ml phosphate-buffered saline (PBS) and spread onto LB agar plates with florfenicol (50 μg/ml) and nalidixic acid (20 μg/ml) and incubated overnight (37°C). The conjugation efficiency was expressed as the number of transconjugants divided by the number of donor bacteria. A subset of putative transconjugants was verified for antibiotic resistance phenotype as described above and for plasmid transfer by plasmid profiling (10).

Plasmid sequencing and annotation. Plasmids were shotgun sequenced from purified plasmid preparations (30). Plasmid pAR060302 was transconjugated to E. coli DH5α in broth culture, and one transconjugant was submitted to Agencourt Bioscience Corp. (Beverly, MA) for plasmid purification, library construction, and sequencing. Plasmids pAM04528 and peH4H were transformed into E.

coli DH10B and purified by cesium chloride gradient (30). After the cloning, the resulting plasmid libraries were sequenced either in house or through services provided by Amplicon Express (Pullman, WA). Plasmid sequences were assembled using phrap and Sequencher (Gene Codes Corp., Ann Arbor, MI); direct plasmid sequencing and PCR were used to close gaps. A combination of PCR and RFLP experiments were used to test ambiguous assemblies. Preliminary annotation was done using an annotation pipeline that included Glimmer 2 (12) for gene detection and BLAST and PFam database comparisons for initial gene identification. In addition, the pipeline employed RBS Finder (31), which was used in comparisons with database information to verify coding region start sites. Curation of the annotation was completed manually using Artemis ver. 10 (28), and pSN254 (GenBank NC_009140) was used as the reference sequence for nomenclature and repeat identification. Descriptive information about each plasmid was summarized with the aid of Vector NTI (Invitrogen Corp., Carlsbad, CA).

Phylogenetic analysis. Our phylogenetic analysis considered the three new sequences presented in our study compared with pSN254 (originating from S. enterica serovar Newport strain SN254; GenBank NC_009140 [33]) with an additional comparison of plasmids from Yersinia pestis (pIP1202; GenBank CP000603 [33]), Yersinia ruckeri (pYR1; GenBank CP000602 [33]), and Photobacterium damselae subsp. piscicida (pP91278 and pP99-018; GenBank AB277724 and AB277723 [17]). Because plasmids are typically a mosaic of sequences from multiple, potentially divergent sources, a sequence-based phylogenetic analysis must necessarily focus on gene sequences that are "conserved" between plasmids. The challenges are to identify these sequences in an efficient manner and to devise a reasonable definition for "conserved," because 100% conservation would produce no differentiation and genes that are "too divergent" can generate exaggerated differences. As detailed below, our approach to this challenge was to identify all the genes common to every plasmid, choose a threshold value to select conserved genes, concatenate these genes in the same order for each plasmid, and perform an alignment of the resulting concatenated sequences.

For a set S of n plasmids $S = (p^1, p^2, \ldots, p^n)$, a conserved gene among these plasmids can be represented by a tuple $G_i = (g_{i1}, g_{i2}, \ldots, g_{in})$, where g_{ij} denotes a specific gene i from the jth plasmid. Assuming there are m_j genes for the jth plasmid, then there are $m_1 m_2 m_3 \ldots m_n$ possible tuples. We used BLAST (1) to obtain similarity scores for each pair of genes in a tuple where the score was calculated as follows: (length of matching sequence) × (BLAST identity score)/ (length of reference gene + length of matching sequence gene), which gives a maximum score of 0.5. If all scores in a tuple were larger than a chosen threshold value, we considered the gene represented by the tuple G_i to be conserved. We
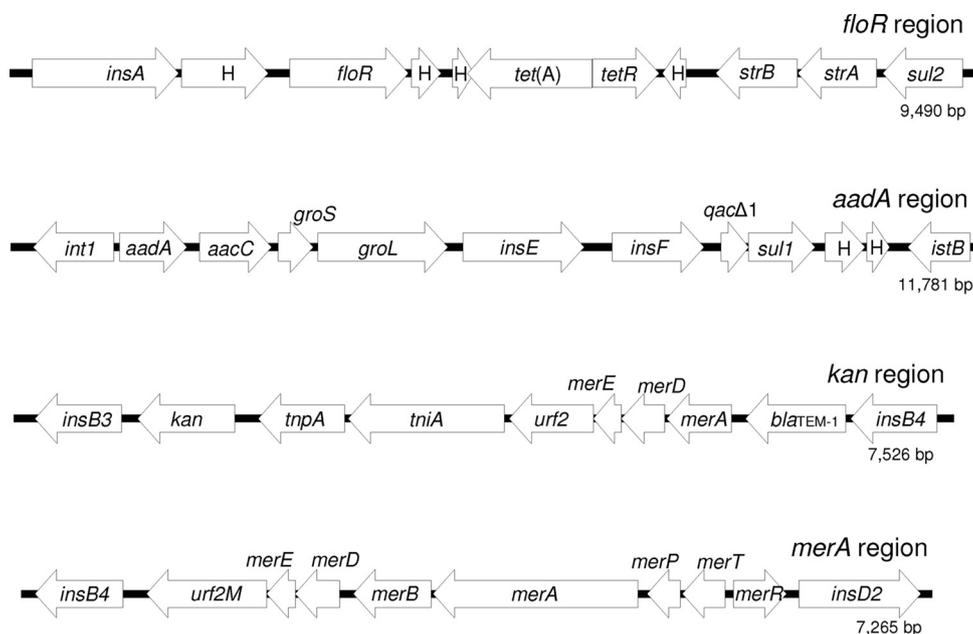
FIG. 1. Major accessory gene elements found in IncA/C plasmids peH4H, pAM04528, pAR060302, and pSN254. Shown are the *floR* resistance region (found in all four plasmids), the *aadA* resistance region (found in peH4H, pAR060302, and pSN254), the *kan* resistance region (found in peH4H only), and the *merA* resistance region (found in all four plasmids). H, hypothetical open reading frames.

then concatenated the respective genes g_{ij} for each plasmid. For K conserved genes $G_1 = (g_{11}, g_{12}, \ldots, g_{1n})$, $G_2 = (g_{21}, g_{22}, \ldots, g_{2n})$, ..., $G_K = (g_{K1}, g_{K2}, \ldots, g_{Kn})$, we have $P_1 = [g_{11}g_{21} \ldots g_{K1}]$, $P_2 = [g_{12}g_{22} \ldots g_{K2}]$, and ..., $P_n = [g_{1n}g_{2n} \ldots g_{Kn}]$, where P_j is the concatenation of all conserved genes for the jth plasmid. P_1 to P_n were aligned and analyzed using MEGA4 (32) with a maximum composite likelihood method underlying a neighbor-joining algorithm. This method assumes equality of nucleotide substitution patterns and mutation rates among lineages; however, some substitution sites are likely to be under selective pressure while others probably represent neutral mutations. Relationships within distinct clusters of the tree potentially can be made more accurate by repeating the procedure described above for the subgroups of plasmids in a cluster, and this process can be repeated until no subgroups remain.

Obtaining BLAST scores for all combinations of gene pairs is computationally impractical for more than a few plasmids. Instead, one plasmid can be selected and BLAST scores can be obtained for the genes of all the other plasmids relative to the selected reference. We have shown empirically that for a given set of plasmids the choice of reference plasmid does not alter the outcome of the analysis. Finally, when one gene from the reference plasmid had two or more similar genes in another plasmid or when two or more genes from the reference plasmid had a similar gene in another plasmid, these genes were considered duplicates and were removed from the list of conserved genes.

Nucleotide sequence accession numbers. The plasmid sequences were archived at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) under accession numbers FJ621586, FJ621587, and FJ621588.

RESULTS AND DISCUSSION

Basic characteristics of *bla*$_{CMY-2}$ plasmids. Plasmids pAM04528, peH4H, pAR060302, and pSN254 (33) ranged in size between *ca.* 148 kbp and 176 kbp and encoded up to 11 antibiotic resistance genes, in addition to a mercury resistance operon (Table 1 and Fig. 1). Disc diffusion tests verified that all three plasmids conveyed multidrug resistance phenotypes, with pAR060302 conveying additional resistance to gentamicin compared to pAM04528 and peH4H conveying additional resistance to both gentamicin and kanamycin compared to pAM04528. MICs demonstrated consistent resistance phenotypes (data not shown). The H4H strain was also resistant to

trimethoprim due to the presence of a resistance gene that was harbored by a coresident plasmid, and strain AR060302 was also resistant to kanamycin, but this trait appeared to be encoded on the chromosome (data not shown).

Restriction digests and Southern blots demonstrated that pAR060302 most closely resembles Giles type A (13) while the remaining plasmids most closely resemble Giles type C (data not shown). Sequence analysis demonstrated that antimicrobial resistance genes were grouped into four main regions (Fig. 1) in addition to the region that encompasses *bla*$_{CMY-2}$. Each region includes multiple genes and potentially more than one insertion sequence, integrase, or tranposase. The "*floR* region" includes *floR*, *tet*(A), *strA*, *strB*, and *sul2*. The "*aadA* region" includes *aadA*, *aacC*, and two heat-shock chaperones (*groS* and *groL*), followed by a 3-prime conserved region of a class I integron composed of *qacΔ*, *sul1*, and two hypothetical open reading frames. The "*kan* region" includes *kan* and a truncated mercury resistance operon plus *bla*$_{TEM-1}$. All four *bla*$_{CMY-2}$ plasmids carry a Tn21 mercury resistance operon (19) and the *floR* region, whereas the *aadA* and *kan* regions were not present in all cases (Fig. 2).

Aside from the absence of the *aadA* region from pAM04528, the only other differences between this plasmid and the previously published pSN254 were a single-nucleotide polymorphism (SNP) found in a noncoding region of the plasmid (Table 2) and an inverted *tnpA* (see below). Plasmid peH4H differed the most from pSN254, with two major deletions associated with *tra* genes (Fig. 2), addition of the *kan* region, 9 SNPs, an 842-bp alteration of the *aadA* coding region, and insertion of a tandem repeat (Table 2). Three of six nonsynonymous changes occurred in the *floR* sequence, which is clearly a nonrandom distribution relative to the entire peH4H sequence. *floR* encodes an efflux pump that exports both flo-
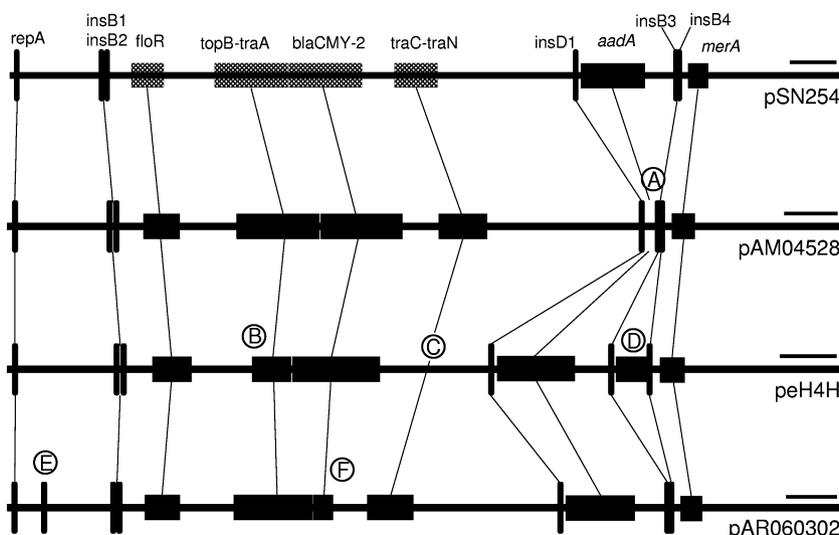
FIG. 2. Schematic diagram of full-length, IncA/C, and bla_{CMY-2} plasmids. (A) The *aadA* region is absent from pAM04528. (B) The *traD*, *traL*, *traE*, and *traK* genes are absent from peH4H. (C) A 22-kbp region downstream from the bla_{CMY-2} region is absent from peH4H. (D) The *kan* region is present in peH4H between *insB3* and *insB4*. (E) IS*1294* is found only in pAR060302. (F) Only a single copy of bla_{CMY-2} is present in pAR060302. The portions of the sequence that are not highlighted are identical in these plasmids, except for minor differences noted in Table 2. Bars, 10,000 bp.

rfenicol and chloramphicol (6). The concentration of nonsynonymous SNPs at this locus is consistent with selection for functional changes for this trait. Plasmid pAR060302 had an additional transposase insertion (Fig. 2) plus 4 SNPs, including 3 nonsynonymous SNPs in the *floR* sequence, 1 of which was different from the peH4H SNPs at this locus (Table 2).

Comparison of bla_{CMY-2} regions. Plasmid pAR060302 is also unique because it harbors a single copy of bla_{CMY-2}, whereas the other three plasmids harbor two copies (Fig. 3), as verified by a double restriction enzyme digest (XbaI and EcoRI) followed by Southern blotting for the bla_{CMY-2} sequence (data not shown). There was no apparent difference in the resistance phenotype for one (pAR060302) or two (pAM04528 and peH4H) copies of bla_{CMY-2}, as judged by zone size differences

from disc diffusion tests (ceftazidime) or by results from MIC tests where the ceftiofur MIC for pAM04528 was 8 μg/ml and the MICs for the other two plasmids were >8 μg/ml.

As observed previously (15, 33), the bla_{CMY-2} region is composed of that gene, in addition to *tnpA*, *blc*, *sugE*, *dsbC*, and two hypothetical open reading frames (Fig. 3). This region is identical in pSN254 and pAM04528, except that our assembly showed *tnpA* as inverted relative to pSN254. The *tnpA* found in the bla_{CMY-2} region is an IS*Ecp1* insertion sequence that has been associated with several other plasmid-encoded, expanded-spectrum β-lactamases (16, 20, 26). The size (>13,000 bp) of the bla_{CMY-2} region, combined with a high degree of sequence symmetry, makes it very difficult to confirm the directionality of *tnpA* for pSN254 and pAM04528. While the re-

TABLE 2. Summary of single-nucleotide and small-scale polymorphisms relative to the pSN254 sequence

Position (bp)	Name	DNA	Amino acid	Position in:			Comments
				pAM04528	pAR060302	peH4H	
14859	Hyp. protein[a]	G→A	P→L	NA[b]	16547	NA	
25054	Hyp. protein	C→T	A→V	NA	NA	25054	
25968	*floR*	G→A	V→M		27656	25968	
26148	*floR*	C→T	P→S	NA	NA	26148	
26212	*floR*	T→A	F→Y	NA	27900	26212	
26235	*floR*	C→A	P→T	NA	27923	NA	
126062–126419	*aadA*	Multiple	~50% divergence	NA	NA	91634–92476	5′ region, *aadA* sequence, spanning 842 bp
127040	Repeat region	A→C	Noncoding	NA	NA	92621	*aadA* flanking region
132430	*insF*	G→A	Synonymous	NA	NA	98011	
160, 25	Putative exonuclease	T→C	S→P	NA	NA	131846	
161189	Repeat sequence	Insertion	Noncoding	NA	NA	132811–132820	TTTTTCTCTCT
171923	Noncoding	A→T	Noncoding	153,663	NA	NA	
174236	Hyp. protein	"A" insertion	Frameshift	NA	164292	145867	
176058	*stbA*	A→G	Synonymous	NA	NA	147690	

[a] Hyp. protein, hypothetical protein or conserved hypothetical protein.
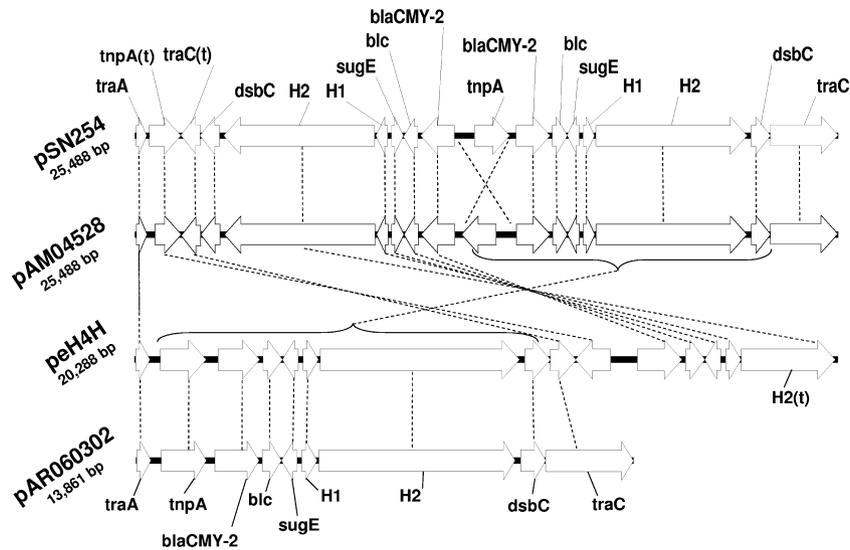[b] NA, not applicable.

FIG. 3. Schematic diagram showing different bla_{CMY-2} regions. The primary element consists of eight genes downstream of *traA*, as shown for pAR060302. A second copy of this segment (inverted) is inserted immediately upstream of the original segment in pSN254 and pAM04528. Note that the proposed reversal in directionality of *tnpA* is suspect due to the sequence symmetry in this region (see Discussion). A duplicated bla_{CMY-2} region is also present in peH4H as a tandem repeat downstream of the original element. Gene names with a (t) suffix are truncated relative to the full length shown for pAR060302. H1 and H2 refer to hypothetical open reading frames.

peated bla_{CMY-2} regions in pSN254 and pAM04528 are tandem and encode in opposite directions, the tandem repeat in peH4H encodes in the same direction, but the 5-prime repeated region is truncated (Fig. 3).

The addition of tandem repeats of the bla_{CMY-2} region is likely due to transposition rather than homologous exchange

because for all duplicated bla_{CMY-2} regions, the first copy of the bla_{CMY-2} region is flanked by a short sequence at the 3-prime terminus (ATTTCCCTA), and this short sequence is nearly identical at the 5-prime terminus of the single (pAR060402) or repeated bla_{CMY-2} region (ATTTCCTTA). The latter short sequence is also found at the termini of du-
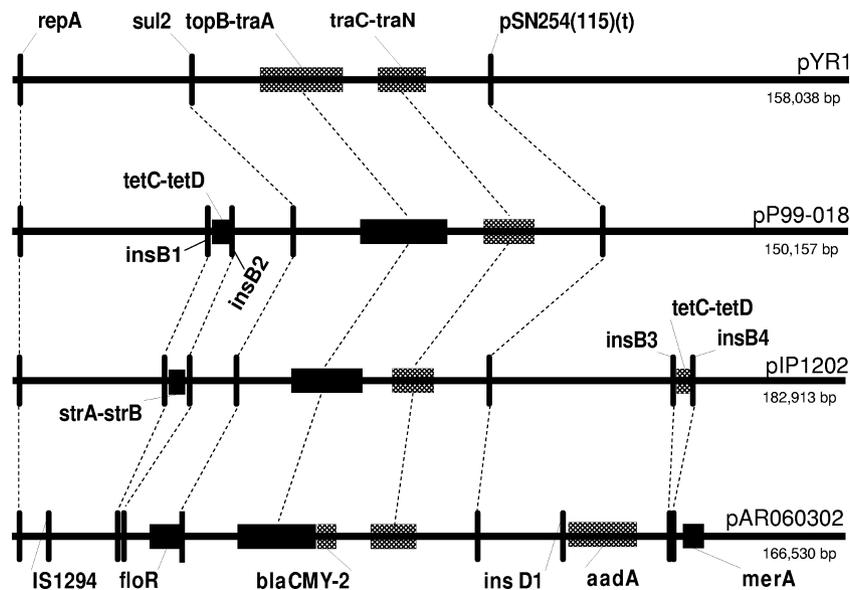
FIG. 4. Schematic diagram highlighting several conserved segments and variable regions relative to antimicrobial resistance for pAR060302 and other IncA/C plasmids from *Yersinia ruckeri* (pYR1) (33), *Photobacterium damselae* (pP99-018) (17), and *Y. pestis* (pIP1202) (33). Relative to pYR1, plasmid pP99-018 (also see pP91278 [17]) has the addition of *insB1* and *insB2* sequences associated with *tet*(C) and *tet*(D). The *tet*(C) and *tet*(D) genes are relocated in pIP1202 (between *insB3* and *insB4*), while *strA* and *strB* are found between *insB1* and *insB2*. In contrast, the bla_{CMY-2} plasmids (from pSN254, peH4H, pAM04528, and pAR060302) harbor no inserts between *insB1* and *insB2* (Fig. 2); if present, the region between *insB3* and *insB4* is occupied by the *kan* region. In addition, these plasmids include a *floR* region, a *merA* region, and, with the exception of pAM04528, an *aadA* region (associated with insD1), as shown for pAR060302. Locus pSN254(115) is shown here as a reference point, and this sequence is identical for all plasmids considered in this study except for a 3-prime truncation in pYR-1 and a 5-prime truncation in peH4H.

plicated bla_{CMY-2} regions in pSN254, pAM04528, and peH4H. All four bla_{CMY-2}-negative plasmids included in our study have the ATTTCC**C**TTA sequence located adjacent to *traA*, and the proximal sequence located "downstream" from this region is also conserved across all of the plasmids, indicating that this short flanking sequence is a probably a transposon insertion site that is conserved in the IncA/C plasmid backbone. There are no deletions of existing plasmid sequence associated with tandem duplication of the bla_{CMY-2} region, and therefore it is unlikely that homologous exchange produced the tandem repeat.

Theoretical models indicate that plasmids will only be retained over the short term if they convey fitness advantages on the host bacterium or if the rate of horizontal transmission exceeds the rate of segregation or competitive loss (5). Clearly, antibiotic selection pressure could select for expansion and dissemination of these plasmids in human and animal populations, although further work is needed to identify the primary variables driving this process. In our hands, plasmid pAR060302 self-conjugated with relatively high efficiency (1.3×10^{-3} conjugations per donor cell) (Table 1). The failure of self-conjugation for peH4H is probably explained by the loss of multiple *tra* genes (Fig. 2), but a comparison of *tra* genes between pAR060302 and pAM04528 showed no loss of *tra* genes or base mutations in *tra* genes that might explain this difference in function. The only difference involving *tra* genes was partial duplication of *traC* associated with the duplication of the blaCMY-2 region (Fig. 3). Given the sequence conservation between pAM04528 and pSN254, we speculate that the latter also lacks the ability to self-conjugate.

Genetic relationships between plasmids. The high degree of sequence similarity and synteny makes it almost certain that the four bla_{CMY-2} plasmids examined in this study were derived from a common ancestor, although it is not possible to know with certainty which of the bla_{CMY-2} plasmids most closely resembles the ancestral state. Plasmid pAR060302 has a single copy of the bla_{CMY-2} region and retains the *aadA* region, suggesting that pAR060302 is representative of the most ancestral sequence, but there are multiple scenarios that could be derived depending on how the large antibiotic-resistance-encoding sequences were lost or gained.

There are a number of differences between the bla_{CMY-2} plasmids and other IncA/C plasmids, and we have highlighted some of the more interesting differences in Fig. 4. Plasmid pYR1 from *Y. ruckeri* appears to be the most dissimilar, with only a single antibiotic resistance gene (*sul2*), and one common reference point is present in truncated form in the pYR1 sequence [pSN254(115)]. Plasmid pP99-018 from *Photobacterium damselae* subsp. *piscicida* acquired *tet*(C) and *tet*(D), flanked by transposase genes *insB1* and *insB2*. These transposase genes appear to have been duplicated in pIP1202 from *Y. pestis* with *tet*(C) and *tet*(D). *strA* and *strB* appeared between transposase genes *insB1* and *insB2*. The pAR060302 sequence shows that insertions have vacated the region between *insB1* and *insB2*, and in addition to insertion of the bla_{CMY-2}, region there has been the addition of the *aadA* and *merA* regions (Fig. 4).

We used a sequence conservation-based method to build a tree for the eight IncA/C plasmids, and we conducted multiple
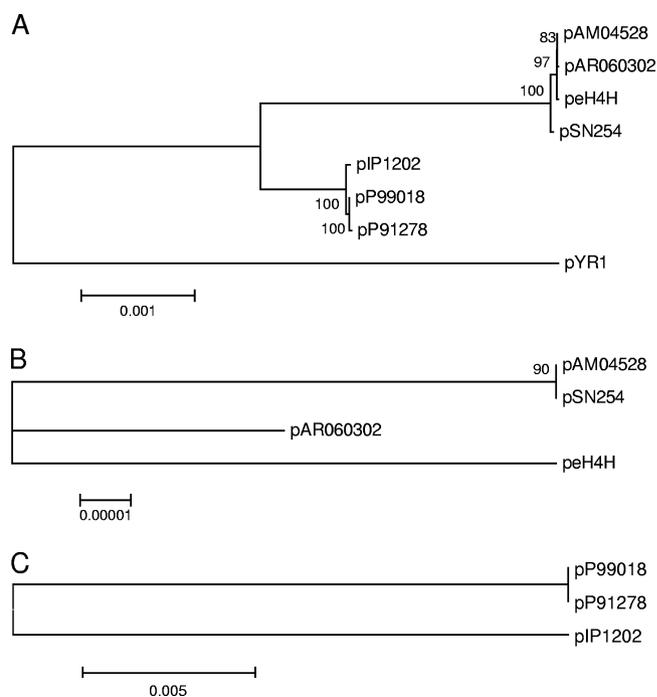

FIG. 5. (A) Bootstrap consensus tree for a neighbor-joining algorithm showing relative genetic relatedness between eight IncA/C plasmids. The tree was based on sequences from 91 conserved genes (BLAST score threshold = 0.3) (see Table S1 in the supplemental material; see Materials and Methods for details), with pIP1202 arbitrarily chosen as the reference plasmid. (B) Subtree for bla_{CMY-2} plasmids based on 25 conserved genes (BLAST score threshold = 0.3) (see Table S2 in the supplemental material; see Materials and Methods for details), with pSN254 used as the reference sequence. (C) Subtree for three additional plasmids based on 49 conserved genes (BLAST score threshold = 0.3; see Table S3 in the supplemental material; see Materials and Methods for details), with pIP1202 used as the reference sequence. Neighbor-joining consensus trees were calculated from 500 bootstrap iterations, with numbers at nodes representing percent correspondence. The scale bars represent the number of base substitutions per site (32).

numerical experiments to ensure that tree construction was robust. There are three distinct subgroups within the main tree, with one subgroup represented by plasmids from *E. coli* and *S. enterica* and a second subgroup represented by plasmids from *Y. pestis* and *P. damselae* (Fig. 5A). The *Y. ruckeri* plasmid was distal to these two subgroups. Conserved genes were identified within the two plasmid subgroups (see Tables S2 and S3 in the supplemental material), and sequence analysis confirmed that pAM04528 and pSN254 were virtually identical while peH4H was the most divergent member of one subgroup (Fig. 5B). Plasmid pIP1202 from *Y. pestis* was the most divergent member of the second subgroup (Fig. 5C). The *Y. pestis* plasmid (pIP1202) is most closely related to the IncA/C plasmids from *Photobacterium damselae*. This analysis supports the likelihood of recent dissemination of bla_{CMY-2} plasmids among *E. coli* and *S. enterica* hosts, but their greater divergence from *Y. pestis* or *Y. ruckeri* suggests less recent plasmid transfer among these pathogen groups. The last is a marine fish pathogen with no obvious ecological overlap with *Y. pestis*. At present, it is not possible to determine whether this plasmid clade originated from marine or terrestrial systems. The pIP-1202-like plasmids are disseminating in marine systems,

judging by a recent report of *Vibrio cholerae* strains harboring these plasmids (25). bla_{CMY-2} plasmids have been detected in *Aeromonas salmonicida* isolated from Atlantic salmon (*Salmo salar*) (22). While the full plasmid sequence was not reported, PCR and some sequence data suggest that the bla_{CMY-2} plasmids detected in *A. salmonicida* closely match the pSN254 sequence (33). If this is the case, then it appears that there has been successful dissemination of bla_{CMY-2} plasmids first documented in mammalian enteric flora to an aquatic ecosystem.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Barlow, M., and B. G. Hall.** 2002. Origin and evolution of the AmpC beta-lactamases of *Citrobacter freundii*. Antimicrob. Agents Chemother. **46:**1190–1198.
3. **Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. **45:**493–496.
4. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, and H. Giamarellou.** 1996. Characterization of the plasmidic beta-lactamase CMY-2, which is responsible for cephamycin resistance. Antimicrob. Agents Chemother. **40:**221–224.
5. **Bergstrom, C. T., M. Lipsitch, and B. R. Levin.** 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. Genetics **155:**1505–1519.
6. **Braibant, M., J. Chevalier, E. Chaslus-Dancla, J. M. Pages, and A. Cloeckaert.** 2005. Structural and functional study of the phenicol-specific efflux pump FloR belonging to the major facilitator superfamily. Antimicrob. Agents Chemother. **49:**2965–2971.
7. **Carattoli, A.** 2003. Plasmid-mediated antimicrobial resistance in *Salmonella enterica*. Curr. Issues Mol. Biol. **5:**113–122.
8. **Carattoli, A., V. Miriagou, A. Bertini, A. Loli, C. Colinon, L. Villa, J. M. Whichard, and G. M. Rossolini.** 2006. Replicon typing of plasmids encoding resistance to newer beta-lactams. Emerg. Infect. Dis. **12:**1145–1148.
9. **Carattoli, A., F. Tosini, W. P. Giles, M. E. Rupp, S. H. Hinrichs, F. J. Angulo, T. J. Barrett, and P. D. Fey.** 2002. Characterization of plasmids carrying CMY-2 from expanded-spectrum cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. Antimicrob. Agents Chemother. **46:**1269–1272.
10. **Daniels, J. B., D. R. Call, and T. E. Besser.** 2007. Molecular epidemiology of bla_{CMY-2} plasmids carried by *Salmonella enterica* and *Escherichia coli* isolates from cattle in the Pacific Northwest. Appl. Environ. Microbiol. **73:**8005–8011.
11. **Davis, M. A., D. D. Hancock, T. E. Besser, J. B. Daniels, K. N. Baker, and D. R. Call.** 2007. Antimicrobial resistance in *Salmonella enterica* serovar Dublin isolates from beef and dairy sources. Vet. Microbiol. **119:**221–230.
12. **Delcher, A. L., D. Harmon, S. Kasif, O. White, and S. L. Salzberg.** 1999. Improved microbial gene identification with GLIMMER. Nucleic Acids Res. **27:**4636–4641.
13. **Giles, W. P., A. K. Benson, M. E. Olson, R. W. Hutkins, J. M. Whichard, P. L. Winokur, and P. D. Fey.** 2004. DNA sequence analysis of regions surrounding bla_{CMY-2} from multiple *Salmonella* plasmid backbones. Antimicrob. Agents Chemother. **48:**2845–2852.
14. **Huang, I. F., C. H. Chiu, M. H. Wang, C. Y. Wu, K. S. Hsieh, and C. C. Chiou.** 2005. Outbreak of dysentery associated with ceftriaxone-resistant *Shigella sonnei*: first report of plasmid-mediated CMY-2-type AmpC beta-lactamase resistance in *S. sonnei*. J. Clin. Microbiol. **43:**2608–2612.
15. **Kang, M. S., T. E. Besser, and D. R. Call.** 2006. Variability in the region downstream of the blaCMY-2 beta-lactamase gene in *Escherichia coli* and *Salmonella enterica* plasmids. Antimicrob. Agents Chemother. **50:**1590–1593.
16. **Karim, A., L. Poirel, S. Nagarajan, and P. Nordmann.** 2001. Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1. FEMS Microbiol. Lett. **201:**237–241.
17. **Kim, M. J., I. Hirono, K. Kurokawa, T. Maki, J. Hawke, H. Kondo, M. D. Santos, and T. Aoki.** 2008. Complete DNA sequence and analysis of the transferable multiple-drug resistance plasmids (R plasmids) from *Photobacterium damselae* subsp. *piscicida* isolates collected in Japan and the United States. Antimicrob. Agents Chemother. **52:**606–611.
18. **Koeck, J. L., G. Arlet, A. Philippon, S. Basmaciogullari, H. V. Thien, Y. Buisson, and J. D. Cavallo.** 1997. A plasmid-mediated CMY-2 beta-lactamase from an Algerian clinical isolate of *Salmonella senftenberg*. FEMS Microbiol. Lett. **152:**255–260.
19. **Liebert, C. A., R. M. Hall, and A. O. Summers.** 1999. Transposon Tn21, flagship of the floating genome. Microbiol. Mol. Biol. Rev. **63:**507–522.
20. **Literacka, E., B. Bedenic, A. Baraniak, J. Fiett, M. Tonkic, I. Jajic-Bencic, and M. Gniadkowski.** 2009. *bla*CTX-M genes in *Escherichia coli* strains from Croatian hospitals are located in new (*bla*CTX-M-3a) and widely spread (*bla*CTX-M-3a and *bla*CTX-M-15) genetic structures. Antimicrob. Agents Chemother. **53:**1630–1635.
21. **Llanes, C., P. Gabant, M. Couturier, and Y. Michel-Briand.** 1994. Cloning and characterization of the Inc A/C plasmid RA1 replicon. J. Bacteriol. **176:**3403–3407.
22. **McIntosh, D., M. Cunningham, B. Ji, F. A. Fekete, E. M. Parry, S. E. Clark, Z. B. Zalinger, I. C. Gilg, G. R. Danner, K. A. Johnson, M. Beattie, and R. Ritchie.** 2008. Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. J. Antimicrob. Chemother. **61:**1221–1228.
23. **NCCLS.** 2003. Performance standards for antimicrobial susceptibility testing, 13th informational supplement, 13th ed. Approved standard M100-S13. NCCLS, Wayne, PA.
24. **Odeh, R., S. Kelkar, A. M. Hujer, R. A. Bonomo, P. C. Schreckenberger, and J. P. Quinn.** 2002. Broad resistance due to plasmid-mediated AmpC beta-lactamases in clinical isolates of *Escherichia coli*. Clin. Infect. Dis. **35:**140–145.
25. **Pan, J. C., R. Ye, H. Q. Wang, H. Q. Xiang, W. Zhang, X. F. Yu, D. M. Meng, and Z. S. He.** 2008. *Vibrio cholerae* O139 multiple-drug resistance mediated by *Yersinia pestis* pIP1202-like conjugative plasmids. Antimicrob. Agents Chemother. **52:**3829–3836.
26. **Park, Y. J., S. Y. Kim, J. K. Yu, S. I. Kim, Y. Uh, S. G. Hong, L. Jongwook, and H. S. Kwak.** 2009. Spread of *Serratia marcescens* coharboring $aac(6')$-Ib-cr, *bla*(CTX-M), *armA*, and *bla*(OXA-1) carried by conjugative IncL/M type plasmid in Korean hospitals. Microb. Drug Resist. **15:**97–102.
27. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type beta-lactamases. Antimicrob. Agents Chemother. **46:**1–11.
28. **Rutherford, K., J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajandream, and B. Barrell.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944–945.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning, a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. **Suzek, B. E., M. D. Ermolaeva, M. Schreiber, and S. L. Salzberg.** 2001. A probabilistic method for identifying start codons in bacterial genomes. Bioinformatics **17:**1123–1130.
32. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
33. **Welch, T. J., W. F. Fricke, P. F. McDermott, D. G. White, M. L. Rosso, D. A. Rasko, M. K. Mammel, M. Eppinger, M. J. Rosovitz, D. Wagner, L. Rahalison, J. E. Leclerc, J. M. Hinshaw, L. E. Lindler, T. A. Cebula, E. Carniel, and J. Ravel.** 2007. Multiple antimicrobial resistance in plague: an emerging public health risk. PLoS One **2:**e309.
34. **Winokur, P. L., A. Brueggemann, D. L. DeSalvo, L. Hoffmann, M. D. Apley, E. K. Uhlenhopp, M. A. Pfaller, and G. V. Doern.** 2000. Animal and human multidrug-resistant, cephalosporin-resistant salmonella isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase. Antimicrob. Agents Chemother. **44:**2777–2783.
35. **Wu, S. W., K. Dornbusch, G. Kronvall, and M. Norgren.** 1999. Characterization and nucleotide sequence of a *Klebsiella oxytoca* cryptic plasmid encoding a CMY-type beta-lactamase: confirmation that the plasmid-mediated cephamycinase originated from the *Citrobacter freundii* AmpC beta-lactamase. Antimicrob. Agents Chemother. **43:**1350–1357.
36. **Zhao, S., D. G. White, P. F. McDermott, S. Friedman, L. English, S. Ayers, J. Meng, J. J. Maurer, R. Holland, and R. D. Walker.** 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.