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Selection Pressure Required for Long-Term Persistence of *bla*$_{CMY-2}$-Positive IncA/C Plasmids$^{\nabla}$

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Multidrug resistance *bla*$_{CMY-2}$ plasmids that confer resistance to expanded-spectrum cephalosporins have been found in multiple bacterial species collected from different hosts worldwide. The widespread distribution of *bla*$_{CMY-2}$ plasmids may be driven by antibiotic use that selects for the dissemination and persistence of these plasmids. Alternatively, these plasmids may persist and spread in bacterial populations in the absence of selection pressure if a balance exists among conjugative transfer, segregation loss during cell division, and fitness cost to the host. We conducted a series of experiments (both *in vivo* and *in vitro*) to study these mechanisms for three *bla*$_{CMY-2}$ plasmids, peH4H, pAR060302, and pAM04528. Results of filter mating experiments showed that the conjugation efficiency of *bla*$_{CMY-2}$ plasmids is variable, from $<10^{-7}$ for pAM04528 and peH4H to $\sim10^{-3}$ for pAR060302. Neither peH4H nor pAM04528 was transferred from *Escherichia coli* strain DH10B, but peH4H was apparently mobilized by the coresident trimethoprim resistance-encoding plasmid pTmpR. Competition studies showed that carriage of *bla*$_{CMY-2}$ plasmids imposed a measurable fitness cost on the host bacteria both *in vitro* (0.095 to 0.25) and *in vivo* (dairy calf model). Long-term passage experiments in the absence of antibiotics demonstrated that plasmids with limited antibiotic resistance phenotypes arose, but eventually drug-sensitive, plasmid-free clones dominated the populations. Given that plasmid decay or loss is inevitable, we infer that some level of selection is required for the long-term persistence of *bla*$_{CMY-2}$ plasmids in bacterial populations.

Plasmids found in prokaryotes are typically mobile and variable in size (1.5 to >600 kb) and are generally composed of a "mosaic" of gene sequences that are derived from many sources. This mosaic includes a "plasmid backbone" plus "accessory" genes (36). The backbone includes replication genes plus additional genes for maintenance and transfer capabilities. Accessory genes encode a wide range of phenotypic characteristics that favor survival and reproduction of the host bacterium, such as those encoding catabolic activity, synthetic activity, antimicrobial resistance, and resistance to heavy metals (44, 47). Antibiotic resistance genes encode accessory traits that pose a challenge to human and animal health. One gene of particular interest, *bla*$_{CMY-2}$, encodes an AmpC-like β-lactamase that confers resistance to expanded-spectrum cephalosporins (4, 34). *bla*$_{CMY-2}$ is often found on large (ca. 140- to 160-kb) IncA/C plasmids (*bla*$_{CMY-2}$ plasmids) from a diverse range of bacterial hosts, including *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Edwardsiella*, and *Aeromonas* (31, 35, 50). These *bla*$_{CMY-2}$ plasmid-bearing bacteria have been detected in mammals and fish without a documented or consistent pattern of antibiotic selection pressure (2, 17, 26, 31, 37, 50–52). There is a high prevalence (up to 100%) of *bla*$_{CMY-2}$ plasmids reported in commensal and pathogenic bacteria such as *Escherichia coli* and *Salmonella enterica* in cattle herds across the United States (1, 15, 30, 42, 51). The *bla*$_{CMY-2}$ genes have also been detected in *E. coli* and *Salmonella* isolates from ground meat (54). The presence of *bla*$_{CMY-2}$ plasmid-bearing *Salmonella enterica* serovars Newport and Typhimurium presumptively of animal origin has been reported in human clinical cases (17, 18).

The ability of *bla*$_{CMY-2}$ plasmids to transfer within and between bacterial populations and the high prevalence of *bla*$_{CMY-2}$ plasmids in cattle herds suggest that cattle may act as a reservoir for disseminating *bla*$_{CMY-2}$ plasmids or plasmid-bearing bacteria to other animals and human populations. In this context, it is important to note that *bla*$_{CMY-2}$ plasmids typically encode multiple antibiotic resistance traits that are already found in terrestrial and aquatic systems (6, 20, 25, 28, 30, 35, 53). Consequently, to invade new populations, *bla*$_{CMY-2}$ plasmids should encode a unique trait(s) that allows the plasmid-carrying population to expand where other plasmids cannot convey a similar selective advantage. Resistance to expanded-spectrum cephalosporins is considered the most likely trait, and the use of an expanded-spectrum cephalosporin (ceftiofur) in livestock has been implicated as an important selective pressure responsible for the expansion of *bla*$_{CMY-2}$ plasmids in the enteric flora of livestock (40, 43, 48). Nevertheless, when (juvenile and adult) cattle are treated with ceftiofur, the results have not been consistent, suggesting that ceftiofur does not present a strong selective pressure *in vivo* (9, 24, 43, 48). *bla*$_{CMY-2}$ plasmids have also been detected in places where ceftiofur and ceftriaxone are not used, such as in aquaculture farms (*Edwardsiella ictaluri* isolated from catfish) and in organic cattle farms in the United States (31, 50).

We expect that carriage of plasmids will impose a fitness cost on the host bacterium in the absence of selection (antibiotic)

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pressure (7, 21, 22, 32). *In vitro* experiments and mathematical models suggest that the persistence of plasmids over evolutionary time is determined by the combination of (i) the fitness cost to the bacterium due to the plasmid (the cost will be less if the plasmid benefits the host), (ii) the conjugation rate (transfer of the plasmid to new host bacteria), and (iii) plasmid loss due to segregation during cell division (46). While segregation control is needed to retain a plasmid within a given clonal lineage, if the fitness cost due to plasmid carriage is high, then segregation control becomes irrelevant, as the plasmid-bearing bacteria cannot compete with conspecific, plasmid-free bacteria. If plasmids impose a net fitness cost, then plasmid-free segregants will eventually "sweep" through the population and replace the resistant plasmid-bearing strains. Elimination of resistance genes themselves from a plasmid can lower the fitness burden of a plasmid (3, 11), which can also lead to a selective sweep of a less versatile plasmid.

Widespread dissemination and persistence of bla_{CMY-2} plasmids could be explained by ceftiofur use and/or by other fitness traits that compensate for the burden of plasmid carriage. To begin testing these alternatives, we conducted a series of studies to explore the roles of fitness cost, conjugation efficiency, and the segregation control system in the persistence of multidrug resistance bla_{CMY-2} IncA/C plasmids peH4H, pAR060302, and pAM04528. Our studies show that in the absence of selection pressure, these bla_{CMY-2} plasmids confer a measurable fitness cost (*in vitro* and *in vivo*) and have variable conjugation efficiencies. Long-term passage without antibiotic selection leads to decay and loss of plasmids. Therefore, for long-term persistence in a bacterial population, bla_{CMY-2} plasmids clearly require some level of selection pressure.

MATERIALS AND METHODS

We used three sequenced bla_{CMY-2}-positive IncA/C plasmids (5) for our studies, including peH4H (~148 kb, isolated from dairy cow *E. coli* strain H4H by the Field Disease Investigation Unit, College of Veterinary Medicine, Washington State University, Pullman, WA), pAM04528 (~155 kb, isolated from human *Salmonella enterica* serovar Newport strain AM04528, provided by the Centers for Disease Control and Prevention, Atlanta, GA, and referred to here as pAM), and pAR060302 (~166 kb, isolated from dairy cow *E. coli* strain AR060302 by R. Singer, University of Minnesota, Minneapolis, MN, and referred to here as pAR). The H4H strain also harbors an unsequenced plasmid, pTmpR (120 kb), that confers resistance to trimethoprim (5). The following transformants were obtained by introducing these plasmids into laboratory strain *E. coli* DH10B by electroporation (41): DH10B(peH4H), DH10B(pTmpR), DH10B(pTmpR/peH4H), DH10B(pAM), and DH10B(pAR).

Unless otherwise noted, the culture conditions were 37°C with shaking (200 rpm) and the incubation time varied as indicated. Antibiotic concentrations were 40 μg/ml trimethoprim (Sigma, St. Louis, MO), 20 μg/ml ampicillin (Fisher Biotech, Fair Lawn, NJ), 30 μg/ml chloramphenicol (MP Biomedicals, Solon, OH), 50 μg/ml florfenicol (LKT laboratories, Inc., St. Paul, MN), 8 μg/ml ceftiofur (Sigma-Aldrich, St. Louis, MO), 30 μg/ml kanamycin (Fisher Scientific, Pittsburgh, PA), 20 μg/ml nalidixic acid (MP Biomedicals, Solon, OH), 50 μg/ml rifampin (Sigma-Aldrich, St. Louis, MO), and 30 μg/ml tetracycline (GTS, San Diego, CA).

Assessing the conjugation efficiency of bla_{CMY-2} plasmids. To determine the conjugation efficiency of plasmids peH4H, pAR, pAM, and pTmpR, we used filter mating experiments. Briefly, equal quantities of overnight cultures of plasmid-bearing donor strains [DH10B(peH4H), DH10B(pTmpR), DH10B(pTmpR/peH4H), DH10B(pAR), and DH10B(pAM)] and plasmid-free recipient strain DH5α (nalidixic acid resistant [Nal^r]) were mixed and added to a nitrocellulose membrane overlaid on LB (Luria-Bertani medium; Fisher Biotech, Fair Lawn, NJ) agar plates without antibiotics. After overnight incubation (37°C), the culture was diluted with sterile phosphate-buffered saline (PBS, pH 7.0, 500 μl) and spread onto LB agar plates containing nalidixic acid, ceftiofur, and/or trimethoprim. Putative transconjugants were enumerated, and the conjugation efficiency of plasmids per donor cell was calculated by dividing the number of CFU of transconjugants by the number of CFU of donors. Each experiment was replicated independently three times. The presence of plasmids in the transconjugants was confirmed for a subset of isolates using plasmid profiles and antibiotic resistance phenotypes (8, 38).

Measurement of the relative fitness cost of bla_{CMY-2} plasmids. We employed both *in vitro* and *in vivo* competition studies to assess the relative fitness cost of bla_{CMY-2} plasmids. For *in vitro* studies, equal numbers of CFU ($\sim 10^9$) of bla_{CMY-2} plasmid-free *E. coli* DH10B and each bla_{CMY-2} plasmid-bearing strain [DH10B(peH4H), DH10B(pTmpR), DH10B(pTmpR/peH4H), DH10B(pAR), or DH10B(pAM)] were added into 5 ml LB broth. The different combinations of cultures were passaged (every 24 h, we transferred 5 μl of cultures into a fresh 5 ml of LB broth) for 8 days. On day 8, the plasmid-bearing and plasmid-free cells were enumerated by serial dilution on antibiotic-containing LB agar plates (27). The fitness cost of bla_{CMY-2} plasmids was determined as the difference in the number of cell doublings between plasmid-free and plasmid-bearing cells, relative to the number of cell doublings of plasmid-free cells (23), as follows: $\text{Cost} = [\log_2 (N_t^f/N_0^f) - \log_2 (N_t^p/N_0^p)]/[\log_2 (N_t^f/N_0^f)]$, where N_0^f and N_t^f are the numbers of plasmid-free cells on days 0 and 8 and N_0^p and N_t^p are the numbers of plasmid-bearing cells on days 0 and 8, respectively. A fitness cost above 0 indicates a possible fitness disadvantage that is associated with the carriage of bla_{CMY-2} plasmids.

For the *in vivo* experiments, we needed to compare isogenic clones with and without peH4H. Efforts to cure H4H of peH4H have produced only a single plasmid-free strain that exhibited growth defects (D. R. Call, unpublished data). Therefore, in this experiment, we used peH4H-bearing and peH4H-free *E. coli* H4H strains; the latter arose spontaneously during long-term passage experiments. Both strains were taken from a passage equivalent to ~ 2,360 generations (H4H2360). *In vivo* experiments included neonatal calves (2 to 7 days old; $n = 5$). Both strains retained trimethoprim resistance (i.e., pTmpR was present), and the peH4H-free strain was selected for nalidixic acid resistance (Nal^r) (9). Calves (fecal samples) were prescreened for the presence of trimethoprim- and nalidixic acid- or trimethoprim- and ceftiofur-resistant *E. coli* strains and found to be apparently free of bacteria with these combinations of resistance traits. Equal numbers of CFU (10^{10}) of both peH4H-free and peH4H-bearing *E. coli* H4H2360 (in 20 ml sterile PBS) were orally administrated to calves using a sterile 12-ml syringe. Fresh fecal material (~20 g) was collected from the rectum aseptically, and the numbers of CFU of the two *E. coli* H4H2360 strains were calculated using MacConkey agar plates containing selective antibiotics. Briefly, 1 g of freshly collected fecal sample was diluted in peptone-buffered saline and 10-fold dilutions were plated on selective agar plates. Competitive-index (CI) values within animals across time points were calculated (27). We used pulsed-field gel electrophoresis (PFGE) with XbaI and the PulseNet protocol (10) to verify the strain identities of H4H2360 at the start and end of the experiments. During this experiment, calves were housed individually in vivarium rooms (one calf per room) and fed fresh bulk milk from the Washington State University Dairy. Alfalfa hay, calf pellet rations, and water were provided *ad libitum*. There were no antibiotic exposures. Animal inoculation studies were approved by the Washington State University Institutional Animal Care and Use Committee.

Long-term passage of bla_{CMY-2} plasmids. To evaluate the impact of alternative mechanisms on the persistence of bla_{CMY-2} plasmids, we passaged the bla_{CMY-2} plasmid-bearing (wild-type and transformant) bacterial strains, H4H, AM04528, and AR060302 [DH10B(peH4H), DH10B(pTmpR/peH4H), DH10B(pAR), and DH10B(pAM)], in LB broth without antibiotic selection pressure by using the method of De Gelder et al. (14). Briefly a single colony from a freshly streaked freezer stock was inoculated into 5 ml LB broth and incubated at 37°C. After 24 h of incubation, 5 μl of culture was transferred into a fresh 5 ml of LB broth and the passage was continued until 4,000 generations for H4H and DH10B (peH4H- and pTmpR-carrying strains) and up to 2,600 generations for the other strains. Using CFU counts, we estimated *a priori* that each passage in this system resulted in approximately 10 generations in 24 h (the number of CFU increased from $\sim 10^6$ to $\sim 10^9$ in 24 h, which is equivalent to ~10 doublings). During long-term passage, approximately every 200 generations (20 days), we selected 48 or 96 colonies and inoculated them onto LB agar plates with and without antibiotics (ampicillin, cefoxitin-ceftiofur, florfenicol-chloramphenicol, tetracycline, kanamycin, and trimethoprim) by using a 96-pin replicator. The percentage of antibiotic resistance phenotypes in each population was subsequently calculated, and it was assumed that the assay provided an analytic sensitivity of 1 to 2 orders of magnitude (10^2). Antibiotic-resistant and -sensitive colonies were tested periodically for the presence or absence of plasmids by plasmid profile determination, followed by Southern blot hybridization using a

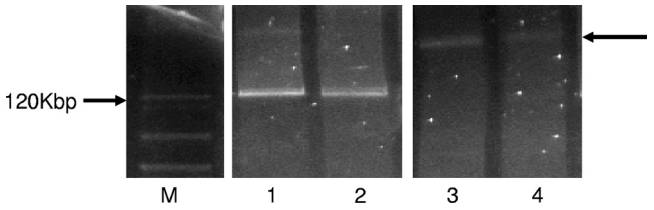

FIG. 1. Plasmid profile of H4H::DH5α transconjugants obtained from conjugation experiments. Lanes: M, BAC-Tracker supercoiled DNA ladder; 1, plasmids peH4H and pTmpR from wild-type strain H4H; 2, plasmids peH4H and pTmpR from H4H::DHα transconjugants; 3 and 4, chimera plasmids (see arrow on right) from H4H::DH5α transconjugants.

bla_{CMY-2}-specific probe and a commercial kit (Roche PCR DIG probe synthesis kit and DIG Easy Hyb; Roche Diagnostics GmbH, Penzberg, Germany) (8).

RESULTS

Conjugation efficiency varies. In the absence of antibiotic selection, the conjugation efficiency varied between plasmids. In wild-type strains, we observed low conjugation efficiencies (2.8×10^{-7} to 1.3×10^{-7} for peH4H and 1.2×10^{-8} to 1.2×10^{-7} for pAM) to moderate efficiency for pAR (9.2×10^{-4} to 2.1×10^{-3}) and pTmpR (5.6×10^{-3} to 8.0×10^{-3}). When *E. coli* DH10B was used as the donor strain, neither pH4H nor pAM transconjugants were detected while pAR and pTmpR had conjugation efficiencies similar to those observed with the wild-type strains (1.8×10^{-4} to 1.3×10^{-3} and 5.6×10^{-3} to 8.0×10^{-3}, respectively). Interestingly, while peH4H conjugation from DH10B was not observed, when we combined peH4H and pTmpR in the DH10B host, we found transconjugants (2.4×10^{-8} to 7.0×10^{-8}) that harbored either both peH4H and pTmpR or a larger plasmid that presumably represented a chimera of these two plasmids (~2/3 of the transconjugant population) (Fig. 1), consistent with pTmpR contributing to the mobilization of peH4H.

Measurable fitness cost involved with carriage of bla_{CMY-2} IncA/C plasmids. *In vitro* direct competition studies between plasmid-free and plasmid-bearing *E. coli* strains showed that there is a measurable fitness cost associated with the carriage of bla_{CMY-2} plasmids. Plasmid peH4H showed a higher cost (0.25) than plasmids pAR (0.09) and pAM (0.19). In contrast, plasmid pTmpR conferred a clear fitness advantage (−0.56) on the host and appeared to mitigate the fitness cost of peH4H (0.10) when these two plasmids were coresident (Fig. 2). The apparent advantage of pTmpR could be explained in part by "invasion" of plasmid-free competing bacteria by conjugation during the competition assay. To determine if conjugative transfer confounded the assay by creating more plasmid-bearing strains and producing an overestimated fitness advantage for pTmpR, we repeated the experiment using a recipient DH10B strain that could be distinguished from the plasmid-bearing host due to its resistance to rifampin. After 4 days of passage, we found no DH10B(pTmpR) Rif^r transconjugants in the population, indicating that conjugation did not significantly affect the original fitness results. Therefore, we conclude that in the absence of antibiotic selection, the three bla_{CMY-2} plasmids imposed a fitness cost on the *E. coli* host while pTmpR provided a fitness benefit (Fig. 2).

Plasmid peH4H does not provide an obvious fitness advantage to *E. coli* strain H4H2360 in calves. To identify the effect of plasmid peH4H on host fitness under *in vivo* conditions, we used neonatal calves to conduct competition studies with *E. coli* H4H bacteria that either had the peH4H plasmid or did not (both strains still harbored pTmpR and were isolated after 2,360 generations of *in vitro* passage); these bacteria were designated H4H2360). The results showed that in 4 out of 5 calves, the *E. coli* H4H bacteria that harbored peH4H were outnumbered by the peH4H-free bacteria and did not persist as long as their peH4H-free competitors (Fig. 3). In one calf, the peH4H-bearing bacteria remained dominant in the population. It appears that carriage of peH4H provided a fitness disadvantage from day 1 onward (4/5 calves), and thus, on average, the peH4H-bearing *E. coli* bacteria lost the competition against the peH4H-free bacteria (Fig. 3). PFGE verified that the H4H2360 bacteria at the start and end of the experiment were identical (data not shown). At 15 days postinoculation, neither type was detected in the calves, suggesting that long-term persistence in calves may be limited in the absence of antibiotic selection pressure.

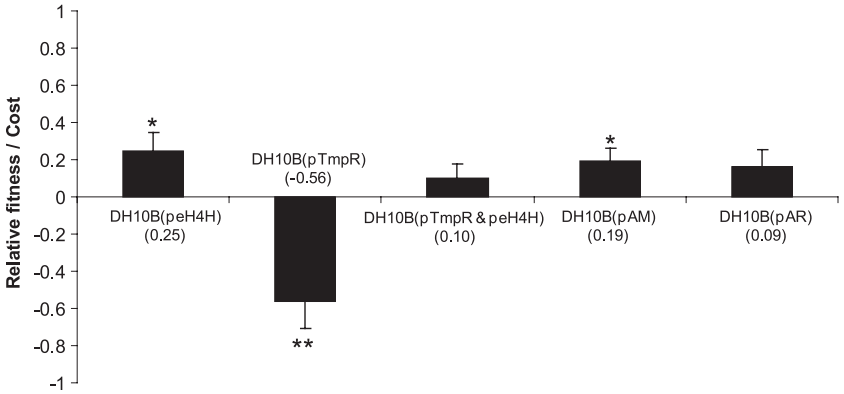

FIG. 2. Fitness cost of plasmids bla_{CMY-2} and pTmpR measured during *in vitro* competition studies with plasmid-bearing and plasmid-free *E. coli* DH10B. The *x* axis shows plasmid-bearing *E. coli* DH10B competition against plasmid-free *E. coli* DH10B, and the *y* axis shows the relative fitness or cost (see Materials and Methods). Carriage of peH4H, pAM, and pAR imposes a relative fitness disadvantage on *E. coli* DH10B, and carriage of pTmpR imposes a fitness advantage. *, $P < 0.05$; **, $P < 0.001$ (Student's *t* test).

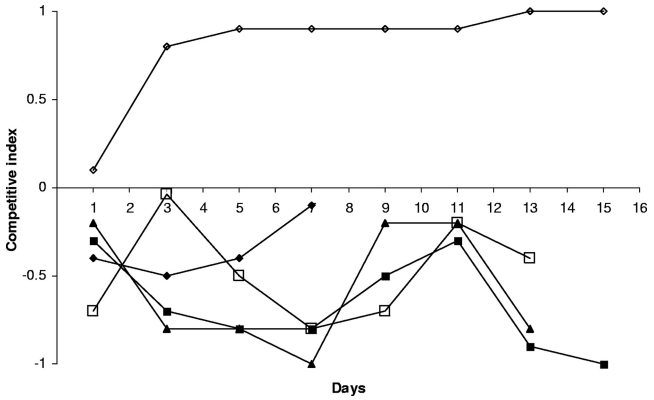

FIG. 3. CIs measured during *in vivo* competition between plasmid peH4H-bearing and peH4H-free *E. coli* H4H2360. The *x* axis shows days on which the CIs were calculated, and the *y* axis shows that the CIs ranged between −1 and +1. The different symbols represent individual animals. A CI value above 0 indicates that plasmid peH4H-bearing strain H4H dominates, while a value below 0 indicates that peH4H-free strain H4H2360 dominates. We did not find strain H4H2360 after day 7 in one animal and after day 15 in two animals.

Emergence of antibiotic-sensitive bacterial phenotypes during long-term passage. To study the effects of fitness cost, segregation control, and conjugation on plasmid persistence *in vitro*, we conducted long-term passage experiments with the three bla_{CMY-2} plasmid-bearing bacterial strains H4H, AM04528, and AR060302. The findings showed that in two trials, different antibiotic sensitivity phenotypes emerged in the three strains at different generations during the long-term passage, as might be expected for a random mutation process (Fig. 4 and 5; Tables 1 and 2). For strain H4H, kanamycin-sensitive (Kan^s) derivatives were detected (10% of the cells) at around 1,000 generations and swept through the population (~100% of the cells) by generation 1,600. Ampicillin- and ceftiofur-sensitive (Amp^s Cef^s) cells (~6% of the cells) were detected at around 1,040 generations and gradually increased their proportion to a majority level (~93% of the cells) by 4,000 generations. Tetracycline- and florfenicol-sensitive (Tet^s and Flo^s) H4H cells were detected at around 1,450 and 1,600 generations, respectively, and these phenotypes gradually increased and dominated the population (93% of the cells) by 4,000 generations. In addition, we found that at 4,000 generations, most (~93%) of the cells were sensitive to all of the antibiotics tested and we initially detected (~28% of the cells) this sensitive phenotype at ~2,140 generations. As expected from competition studies (Fig. 2), most (~100%) of the H4H cells throughout the experiment were resistant to trimethoprim and retained the pTmpR plasmid up to 4,000 generations, except for a small fluctuation (~1 to 7% of the cells picked) between 3,100 and 3,700 generations (Fig. 4). A plasmid profile followed by Southern blotting of the antibiotic-sensitive and -resistant H4H strains isolated during the passage experiment showed that the antibiotic-resistant cells had retained and the antibiotic-sensitive cells had lost plasmid peH4H (n = 6 isolates) (data not shown). In addition, identical PFGE profiles confirmed that the chromosomal background of the resistant and sensitive H4H strains was the same at the start and end of passage experiments (n = 6 isolates), indicating that sensitive strains were unlikely to be contaminants (data not shown). When *in vitro* passage experiments were done with host strain DH10B (with both pTmpR and peH4H), the antibiotic sensitivity phenotypes emerged in a pattern similar to the dynamics of the H4H phenotypes described above (Table 2).

Strains AM04528 and AR060302 showed a sharp increase in Amp^s Cef^s phenotypes at around 400 and 600 generations,

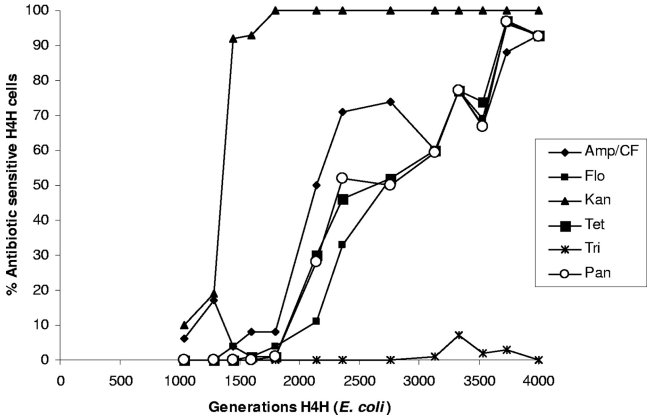

FIG. 4. Percentages of antibiotic-sensitive *E. coli* H4H phenotypes (*y* axis) in different generations (*x* axis) during long-term passage culture (without antibiotic selection). A gradual increase in antibiotic-sensitive populations was observed after 1,000 generations, and most isolates became pansensitive after 3,000 generations. In contrast, most isolates were resistant to trimethoprim and retained pTmpR even after 4,000 generations. Amp/CF, proportion of isolates sensitive to ampicillin-ceftiofur; Kan, proportion of isolates sensitive to kanamycin; Tet, proportion of isolates sensitive to tetracycline; Flo, proportion of isolates sensitive to florfenicol; Tri, proportion of isolates sensitive to trimethoprim; Pan, proportion of isolates sensitive to Amp-Cef, Kan, Tet, and Flo (Tri sensitivity was excluded from this calculation).

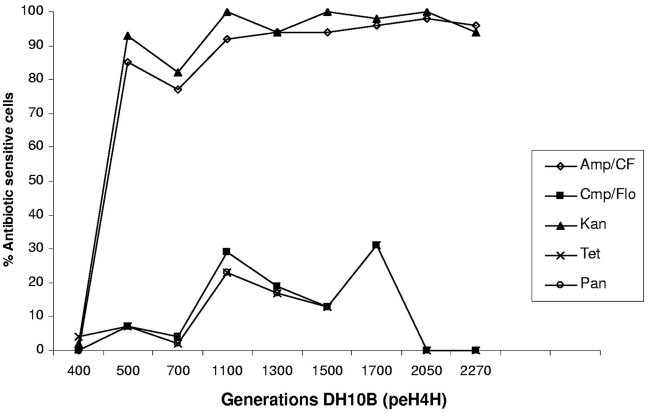

FIG. 5. Percentages of antibiotic-sensitive *E. coli* DH10B(peH4H) phenotypes (*y* axis) in different generations (*x* axis) during the long-term passage experiment. After 500 generations, there was a rapid increase (up to >80% and >90%) of ampicillin-ceftiofur- and kanamycin-sensitive DH10B(peH4H) isolates, respectively. The florfenicol- and tetracycline-sensitive subpopulations reached >80% of the culture after 1,100 generations. Amp/CF, proportion of isolates sensitive to ampicillin-ceftiofur; Kan, proportion of isolates sensitive to kanamycin; Tet, proportion of isolates sensitive to tetracycline; Flo, proportion of isolates sensitive to florfenicol; Tri, proportion of isolates sensitive to trimethoprim; Pan, proportion of isolates sensitive to Amp-Cef, Kan, Tet, and Flo (Tri sensitivity was excluded from this calculation).

TABLE 1. Percentages of bacteria of strains AM04528, AR060302, DH10B(pAM), and DH10B(pAR) with antibiotic sensitivity phenotypes in 2 independent trials during long-term passage experiments

Trial and no. of generations[a]	% of bacteria with antibiotic sensitivity phenotype															
	AM04528				AR060302				DH10B(pAM)				DH10B(pAR)			
	Amp^s Cef^s[b]	Flo^s	Tet^s	Pan^s	Amp^s Cef^s	Flo^s	Tet^s	Pan^s	Amp^s Cef^s	Flo^s	Tet^s	Pan^s	Amp^s Cef^s	Flo^s	Tet^s	Pan^s
Trial 1																
200	0	0	0	0	0	0	0	0	9	0	0	0	100	0	0	0
400	88	0	0	0	0	0	0	0	98	0	0	0	100	0	0	0
600	89	0	0	0	73	0	0	0	98	2	2	2	100	0	0	0
800	98	25	25	25	83	3	0	0	100	0	0	0	100	0	0	0
1,000	98	8	2	2	99	8	0	0	92	0	0	0	100	0	0	0
1,200	90	4	4	4	83	0	0	0	88	0	0	0	96	0	0	0
1,400	50	0	0	0	100	0	0	0	100	0	0	0	98	21	21	21
Trial 2																
400	68	0	0	0	84	0	0	0	46	0	0	0	72	0	0	0
600	86	0	0	0	86	0	0	0	88	0	0	0	86	0	0	0
800	94	0	0	0	68	0	0	0	92	0	0	0	68	0	0	0
1,000	96	0	0	0	92	0	0	0	92	0	0	0	94	0	0	0
1,200	92	48	48	46	88	12	12	0	86	18	18	18	86	22	18	18
1,400	85	96	96	96	85	0	0	0	50	100	100	50	66	0	0	0
1,600	92	90	80	80	83	90	0	68	83	85	96	83	94	80	94	80
1,800	92	92	86	86	100	88	86	86	88	88	92	88	92	94	100	92
2,000	92	92	88	88	100	85	88	85	86	88	88	86	96	88	88	88
2,200	96	96	92	92	90	85	94	85	92	92	94	92	88	92	92	88
2,400	100	75	92	75	82	100	85	82	96	88	98	82	98	92	94	92
2,600	100	98	100	98	88	88	90	88	88	96	96	88	94	96	96	94

[a] A generation is binary fission. Based on $1{:}2^{10}$ serial dilutions, we calculated that 24 h is equivalent to approximately 10 generations.

[b] Percentages of bacteria with antibiotic sensitivity phenotypes were calculated by selecting a subset of 48 to 96 isolates from the population approximately for every 200 generations and characterizing the antibiotic susceptibility profiles. Amp^s Cef^s, sensitivity to ampicillin-ceftiofur; Tet^s, sensitivity to tetracycline; Flo^s, sensitivity to florfenicol; Pan^s, sensitivity to Amp-Cef, Tet, and Flo.

respectively, in the first long-term passage trial (~88% of the cells of strain AM04528 and ~73% of the cells of strain AR060302). These populations were largely replaced by these Amp^s Cef^s AM04528 and AR060302 phenotypes by 800 and 1,000 generations, respectively (Table 1). Interestingly almost 100% of the AM04528 and AR060302 cells were resistant to tetracycline (Tet^r) and florfenicol (Flo^r), except for a minor fluctuation in the Flo^s phenotype between 800 and 1,250 generations (Table 1). Overall, the peH4H, pAM, and pAR plasmid-bearing *E. coli* DH10B populations showed similar patterns of the emergence of antibiotic sensitivity phenotypes, but these emerged more quickly than in their wild-type counterparts (Table 1).

DISCUSSION

The most persistent and successful plasmids have a strong segregation control system, a low fitness cost, and a high conjugation rate (45). We studied these traits for the apparently successful multidrug resistance bla_{CMY-2} IncA/C plasmids peH4H, pAM04528 (pAM), and pAR060302 (pAR). The conjugation efficiency of the three bla_{CMY-2} plasmids varied from $<10^{-7}$ to a moderate level (10^{-3}). The latter observation is consistent with the findings of Fricke et al. (19), who also found that the conjugation efficiency of IncA/C plasmids was moderate (10^{-3}) compared to that of more efficient plasmids such as when pB10 (64-kbp IncP-1β plasmid) was transferred to strains of *E. coli*, *Pseudomonas* spp., *Sinorhizobium meliloti*, and *Stenotrophomonas maltophilia* ($>10^{-1}$) (16).

Apparent loss of genes that are associated with conjugation and an extensively truncated *traC* gene may be the reason for the low conjugation efficiency of peH4H and pAM, respectively (5, 26, 39). No transconjugants of peH4H or pAM were detected when these plasmids resided singly in DH10B, and this host strain appeared to be a competent host considering that pAR had no reduction in conjugation efficiency from DH10B. When we added pTmpR with peH4H to the DH10B host, conjugation was successful and this indicates that the coresident plasmid pTmpR probably mobilizes peH4H into other plasmid-free bacteria. Interestingly, for at least 2/3 of these transconjugants, we found a plasmid that is larger (>165 kb) than both plasmids peH4H and pTmpR. The antibiotic resistance pattern and the plasmid profile suggest that these large plasmids are the result of a chimera of plasmids peH4H and pTmpR (Fig. 1). The efficiency of mobilization of peH4H and associated chimeras was 4 orders of magnitude less than that of pTmpR alone. Plasmid pTmpR is coresident with peH4H in wild-type strain H4H. Poole et al. (39) also reported that conjugation-deficient IncA/C plasmids could be mobilized in the presence of compatible conjugative plasmids.

In vitro direct competition studies of bla_{CMY-2} plasmid-bearing and plasmid-free *E. coli* DH10B showed a measurable fitness cost (0.09 to 0.25) associated with carriage of the three bla_{CMY-2} plasmids (peH4H, pAM, and pAR) (Fig. 2). The larger size of bla_{CMY-2} plasmids and the absence of selection pressure could be the reasons for the significant fitness cost of these bla_{CMY-2} plasmids. Similar findings were obtained by De Gelder et al., who reported that the cost of plasmid pB10 varies

TABLE 2. Percentages of strain DH10B(pTmpR/peH4H) bacteria with antibiotic sensitivity phenotypes in 2 independent trials during long-term passage experiments

Trial and no. of generations[a]	% of bacteria with phenotype					
	Amp^s Cef^s[b]	Flo^s	Kan^s	Tet^s	Tri^s	Pan^s
Trial 1						
1,300	9	0	100	0	0	0
1,420	30	0	100	0	0	0
1,650	67	0	100	0	0	0
2,000	75	0	100	0	0	0
2,370	65	65	100	73	7	65
2,700	91	79	100	95	4	79
3,000	100	92	100	93	0	92
3,270	100	91	100	91	0	91
3,400	97	85	100	92	0	85
4,000	97	85	100	92	0	85
Trial 2						
400	0	0	2	4	0	0
500	85	7	93	7	0	7
700	77	4	82	2	0	2
1,100	92	29	100	23	0	23
1,300	94	19	94	17	0	17
1,500	94	13	100	13	0	13
1,700	96	31	98	31	0	31
2,050	98	0	100	0	0	0
2,270	96	0	94	0	0	0

[a] A generation is binary fission. Based on $1:2^{10}$ serial dilutions, we calculated that 24 h is equivalent to approximately 10 generations.

[b] Percentages of bacteria with antibiotic sensitivity phenotypes were calculated by selecting a subset of 48 to 96 isolates from the population approximately for every 200 generations and characterizing the antibiotic susceptibility profiles. Amp^s Cef^s, sensitivity to ampicillin-ceftiofur; Kan^s, sensitivity to kanamycin; Tet^s, sensitivity to tetracycline; Flo^s, sensitivity to florfenicol; Tri^s, sensitivity to trimethoprim; Pan^s, sensitivity to Amp-Cef, Kan, Tet, and Flo (Tri^s was excluded from this category).

(low to high) for different bacterial hosts. This ranged from a low level (<0.03) for *E. coli* K-12 and *Sinorhizobium meliloti* to a moderate level for *Pseudomonas* spp. (0.037 to 0.15) and a high level for *Stenotrophomonas maltophilia* (0.59) (13, 23). The reduction in the fitness cost of peH4H upon coresidence with pTmpR may be due to mitigation by the benefits conferred by pTmpR (Fig. 2), although the mechanism underlying the pTmpR fitness advantage has not been determined. *In vivo* studies using neonatal calves showed a stochastic outcome where bla_{CMY-2} plasmid-bearing strain H4H2360 was dominated by its peH4H-free counterpart for 4 out of 5 calves (Fig. 3). Overall, the findings of both *in vivo* and *in vitro* competition studies indicate a measurable fitness disadvantage associated with the carriage of bla_{CMY-2} plasmids to the bacterial host. If the *in vivo* results are representative of events occurring in cattle populations, then the fitness cost associated with bla_{CMY-2} plasmids should lead to eventual loss of these plasmids from the cattle population if there are no other selection pressures for the maintenance of these traits (Fig. 2).

The long-term passage of bla_{CMY-2} plasmid-bearing bacterial strains (H4H, AR060302, and AM04528 and their DH10B counterparts) showed a slow emergence of different antibiotic sensitivity phenotypes in all of the populations tested. Similar findings were also reported in various long-term passage (500 to 1,100 generations) studies conducted with conjugative multidrug resistance plasmids such as pB10, pACYC184, and pBR322 in *E. coli* and other bacterial species (7, 13, 33, 49). The reduced prevalence of the peH4H plasmid in the population is consistent with the finding of De Gelder et al. (12), who detected the emergence of pB10-free *E. coli* K-12 at around 500 generations. In the present study, however, plasmid-free (peH4H, pAM, and pAR) strains typically did not emerge until between 800 and 1,200 generations, which is consistent with a relatively high level of plasmid stability.

The long-term passage experiments showed that in the absence of selection pressure, plasmids can shed antibiotic resistance genes, resulting in a presumably less costly plasmid. The arrangements of these antibiotic resistance genes between the mobile elements on the IncA/C backbone may contribute to the differential extrication of these genes from the plasmid backbone (5). For example, early loss of the kanamycin resistance gene may be due to its position between the two direct terminal repeats (insB3 and insB4), leading to instability of the transposable kanamycin resistance elements through homologous intermolecular recombination, as has been shown for Tn*1525* (29). Similarly, De Gelder et al. (12) reported the loss of a region containing *tet*(A) and *tetR*, which were flanked by long repeat sequences. The bla_{CMY-2}-containing sequence is flanked by inversely oriented insertion sequences (IS*Ecp1*), indicating that it may be a composite transposon that favors the extrication of bla_{CMY-2} genes from the plasmid backbone (26). The Flo^r Tet^r genes are also flanked by insertion sequences insB1 and insB2. The sequential emergence of Kan^s, Amp^s Cef^s, and Flo^s Tet^s phenotypes probably reflects a combination of the frequency of recombination (loss) and the relative fitness cost of the plasmid segments that encode these resistance traits. To examine this question further, we hybridized four isolates (representing different antibiotic resistance phenotypes) from generation 2,360 to an oligonucleotide microarray that included probes for the open reading frames from peH4H (unpublished data). As expected, insertion sequences with antibiotic resistance genes were lost in sensitive strains that still harbored a peH4H plasmid. For example, a ceftiofur-sensitive isolate lost the bla_{CMY-2} insert region. A tetracycline- and florfenicol-sensitive strain harbored a peH4H plasmid that was missing the *tet*(A)-*floR* region and the bla_{TEM-1} region, but resistance to ceftiofur and ampicillin was retained because the bla_{CMY-2} region was retained. Another isolate, which was sensitive to ampicillin, ceftiofur, tetracycline, and florfenicol, showed loss of all associated regions of the plasmid. All of these strains were sensitive to kanamycin, and three of four were missing all or part of the region encompassing *aacC*. The one exception was a kanamycin-sensitive strain that showed no apparent loss of resistance genes, but our microarray was not complete for this region and this result may reflect an inability of our microarray to detect single-nucleotide changes or small indels that could render a gene nonfunctional. In totality, these findings are consistent with earlier observations that bla_{CMY-2}-positive IncA/C plasmids differ largely by the loss or gain of resistance traits that are encoded by insertion sequences (5, 19).

Although the pattern of emergence of antibiotic-sensitive DH10B was similar in the long-term passage culture, the earlier and rapid emergence and dominance of Kan^s and Amp^s Cef^s bla_{CMY-2} plasmid-bearing *E. coli* DH10B cells compared to their wild-type counterparts is not unexpected because *E.*

coli DH10B is not the natural host of these bla_{CMY-2} plasmids (Fig. 5; Tables 1 and 2). In fact, it is surprising that the bla_{CMY-2} plasmids were retained in the populations for several hundred generations. Overall, the long-term passage culture experiments in the absence of selection suggest that the plasmids are very stable but that random mutations accumulate in the plasmid, leading to plasmid decay, and eventually the entire plasmids were lost from the populations (2, 9, 13, 24, 29). We observed some variance in the loss of different resistance phenotypes during the long-term passage trials. We attributed this variance to the timing of losses (a random process) and the different combinations of mutations coupled with the population bottleneck that occurred during every 24-h passage to fresh medium.

While long-term passage experiments indicate that bla_{CMY-2} plasmids decay or are lost completely, presumptively intact bla_{CMY-2} plasmids were recovered beyond 800 generations. Studies with strain H4H show that the fitness cost of peH4H can be mitigated in part by another plasmid. Furthermore, while peH4H-free strains dominated in the calf model, this was not the case for one calf. We surmise that even though loss of bla_{CMY-2} plasmids is a deterministic outcome, in the absence of selection, stochastic events and periodic selection (e.g., ceftiofur use) will most likely prolong persistence and this probably contributes to the successful proliferation of bla_{CMY-2} plasmids.

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