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In Vivo Transmission of an IncA/C Plasmid in *Escherichia coli* Depends on Tetracycline Concentration, and Acquisition of the Plasmid Results in a Variable Cost of Fitness

Timothy J. Johnson,[a] Randall S. Singer,[a] Richard E. Isaacson,[a] Jessica L. Danzeisen,[a] Kevin Lang,[a] Kristi Kobluk,[a] Bernadette Rivet,[a] Klaudyna Borewicz,[a] Jonathan G. Frye,[b] Mark Englen,[b] Janet Anderson,[a] Peter R. Davies[c]

Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, Minnesota, USA[a]; Bacterial Epidemiology and Antimicrobial Resistance Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Athens, Georgia, USA[b]; Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, Saint Paul, Minnesota, USA[c]

IncA/C plasmids are broad-host-range plasmids enabling multidrug resistance that have emerged worldwide among bacterial pathogens of humans and animals. Although antibiotic usage is suspected to be a driving force in the emergence of such strains, few studies have examined the impact of different types of antibiotic administration on the selection of plasmid-containing multidrug resistant isolates. In this study, chlortetracycline treatment at different concentrations in pig feed was examined for its impact on selection and dissemination of an IncA/C plasmid introduced orally via a commensal *Escherichia coli* host. Continuous low-dose administration of chlortetracycline at 50 g per ton had no observable impact on the proportions of IncA/C plasmid-containing *E. coli* from pig feces over the course of 35 days. In contrast, high-dose administration of chlortetracycline at 350 g per ton significantly increased IncA/C plasmid-containing *E. coli* in pig feces ($P < 0.001$) and increased movement of the IncA/C plasmid to other indigenous *E. coli* hosts. There was no evidence of conjugal transfer of the IncA/C plasmid to bacterial species other than *E. coli*. *In vitro* competition assays demonstrated that bacterial host background substantially impacted the cost of IncA/C plasmid carriage in *E. coli* and *Salmonella*. *In vitro* transfer and selection experiments demonstrated that tetracycline at 32 μg/ml was necessary to enhance IncA/C plasmid conjugative transfer, while subinhibitory concentrations of tetracycline *in vitro* strongly selected for IncA/C plasmid-containing *E. coli*. Together, these experiments improve our knowledge on the impact of differing concentrations of tetracycline on the selection of IncA/C-type plasmids.

The plasmid incompatibility group IncA/C is a broad-host-range plasmid group that has emerged worldwide among a wide variety of animal-source and environmental bacteria, including those of fish (1, 2), food animals (3–6), companion animals, humans (6–9), and water (2, 10). IncA/C plasmids were identified in aquatic bacterial species as early as 1959 (11). However, they have only recently emerged among *Escherichia coli* and *Salmonella enterica* of humans and production animals, and their discovery among these bacteria coincides with their possession of large numbers of genes encoding antimicrobial resistance (12–14). These genes encode resistance to multiple classes of antimicrobials, including aminoglycosides, carbapenems, newer-generation cephalosporins, penicillins, sulfonamides, tetracyclines, phenicols, and heavy metals. In addition to their propensity to acquire multidrug resistance-encoding genetic modules, IncA/C plasmids have an apparently broad host range (15). IncA/C plasmids have been increasingly identified among human clinical *Salmonella* and *E. coli* in the United States, and evidence suggests that similar plasmids and strains are found in beef, poultry, and pork products and live animals (16–18).

The emergence of IncA/C plasmids among *Enterobacteriaceae* of human importance is somewhat surprising because (i) bacterial species within this family are not predicted to be efficient hosts of the IncA/C plasmid based upon the Mahalanobis distance (15), (ii) certain subtypes of these plasmids, such as some bla_{CMY-2}-encoding IncA/C plasmids, are deficient in conjugative transfer (19), and (iii) these plasmids have been shown to impose a significant fitness cost to enterobacterial hosts and require selective pressure for their maintenance in bacterial populations (20). Therefore, the rapid emergence of IncA/C plasmids among *Enterobacteriaceae* implies that unexplained reasons exist for their success. This underscores the need to better understand the environmental sources of IncA/C plasmids, their bacterial reservoirs, the factors driving their selection, and their basic biology.

IncA/C plasmids are common among *E. coli* in U.S. commercial swine herds. We previously reported that IncA/C plasmids were found among *E. coli* populations associated with both postweaning (59.2%) and neonatal diarrhea (39%) in pigs (21). Analysis of a bla_{CMY-2}-encoding IncA/C plasmid isolated from a postweaning diarrhea-associated swine *E. coli*, pAPEC1990_61, highlighted the capacity for IncA/C plasmids from porcine-source *E. coli* to acquire resistance-associated genes (12). On this plasmid were three different resistance-associated clusters, encoding resistance to phenicols (*floR* and *cmlA*), tetracyclines (*tetAR*), amino-

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Address correspondence to Timothy J. Johnson, joh04207@umn.edu.
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glycosides (*strAB*, *aacC*, *aadA*, and *aadA2*), sulfonamides (*sul2*), beta-lactams (bla_{CMY-2}), quaternary ammonium compounds (*qacE*Δ*1*), and heavy metals (*mer*). Although IncA/C plasmids in *Salmonella* and *E. coli* vary considerably in their accessory (resistance) gene content, their core components are highly conserved, sharing >99% nucleotide sequence similarity within these regions. This suggests that IncA/C plasmids among *Enterobacteriaceae* share a recent ancestry but are under constant modification in their recombination "hot spots," likely due to selective pressures and/or the diversity of the mobile genetic element pool available within their environments.

One could speculate that the high prevalence of IncA/C plasmids among porcine-source *E. coli* is due to selection pressure from decades of antibiotic use for disease treatment, disease prevention, and growth promotion. Nevertheless, empirical evidence to support this hypothesis is equivocal and incomplete. Important knowledge gaps exist in how production practices, including antimicrobial use might impact the emergence and persistence of these plasmids, and how the plasmids themselves influence their own dissemination. Chlortetracycline is the most widely used antibiotic in U.S. swine production for both growth promotion and therapeutic purposes in the nursery and growing/finishing phases (22), and resistance to this antibiotic is encoded as a core component of bla_{CMY-2}-positive IncA/C plasmids (12). In the present study, controlled pig experiments and *in vitro* experiments were performed with the goals of better understanding the possible selection of IncA/C plasmids under the pressure of chlortetracycline, the impact of chlortetracycline on plasmid conjugation, and the fitness costs conferred by these plasmids to enteric bacterial hosts.

MATERIALS AND METHODS

Bacterial strain construction. A single pig commensal *E. coli* strain (pig fecal *E. coli* [PFEC]) was used for pig inoculation experiments. This strain was isolated in the present study from a convenience sample of commercial pig feces at the University of Minnesota by suspension of 1 g of fecal material in 10 ml of phosphate-buffered saline (PBS), followed by plating of 10-fold serial dilutions, selection of a lactose-utilizing isolated colony on MacConkey agar, and standard biochemical testing to confirm it as *E. coli*. The PFEC isolate was verified to be susceptible to a panel of antibiotics by using a broth microdilution MIC assay (Trek Diagnostics). To induce resistance toward nalidixic acid for selection purposes, this strain was grown overnight in 5 ml of Luria-Bertani (LB) broth. Cells were pelleted, resuspended in 100 μl of LB broth, and then plated on LB agar containing 30 μg of nalidixic acid/ml to identify a nalidixic acid-resistant PFEC strain. This derivative was then compared to the wild-type strain using standard growth curve assays in LB broth to confirm that its *in vitro* growth was not attenuated. To enable the tracking of a prototype IncA/C plasmid, pAR060302, a mini-Tn$5_{A1\text{-}04/03}$::*rfp* cassette was inserted into pAR060302 using a previously described procedure (23, 24). The system allowed tracking of pAR060302 in the pig gastrointestinal tract, and differentiation between the presence of this plasmid in its original host or after conjugative transfer to non-*E. coli* recipient bacteria. First, the mini-Tn$5_{A1\text{-}04/03}$::*rfp* cassette was mobilized into *E. coli* strain DH10B(pAR060302) using a helper plasmid and integrated into either the chromosome of DH10B or pAR060302 (25). This mixture was mated with the PFEC strain, and transconjugants were selected on media containing selective antibiotics for the recipient strain PFEC carrying pAR060302:*rfp*. The location of the mini-Tn$5_{A1\text{-}04/03}$::*rfp* cassette insertion in pAR060302 was identified via Sanger sequencing as downstream of *traG* in a noncoding region. Because *E. coli* strain PFEC contains the *lac* operon, it encodes for LacI which represses the expression of RFP due to the mini-Tn$5_{A1\text{-}04/03}$::*rfp* being under the control of LacI (25). The benefit of using this repressor is that *lac* negative cells that have acquired pAR060302:*rfp* fluoresce under blue light, which was verified prior to the experiments by transferring and analyzing pAR060302:*rfp* in *S. enterica* serovar Heidelberg.

For fitness cost experiments, a series of wild-type *E. coli* and *S. enterica* isolates were selected that lacked an IncA/C plasmid and were pan-susceptible to a panel of antibiotics based upon broth microdilution MIC assay (Trek Diagnostics; Table 1). *S. enterica* isolates were provided by the USDA-ARS Bacterial Epidemiology and Antimicrobial Resistance Research Unit, Athens, GA. *E. coli* isolates were used from previously characterized collections (26, 27). These strains were spontaneously mutated for nalidixic acid resistance as described above and then compared to the wild-type strain using standard growth curve assays in LB broth to confirm that growth was not attenuated for *in vitro* conditions; each strain was then mated individually with *E. coli* K-12 possessing pAR060302 using a standard conjugation protocol (28).

For measurements of conjugative frequency, matings were performed in the presence of increasing concentrations of tetracycline. To create the recipient, *E. coli* K-12 was modified at *galK* with a tetracycline resistance cassette (FRT-*tetRA*-FRT). This was done to assess the effects of tetracycline on conjugal transfer in the absence of any selective pressure on growth. To do so, PCR was used to amplify the FRT-*tetRA*-FRT cassette

TABLE 1 Bacterial strains used for fitness cost experiments

Species and strain	Serotype or phylogenetic group	Source
S. enterica		
TJ2001	Kentucky	Unknown
TJ2002	Kentucky	Unknown
TJ2003	Heidelberg	Egg
TJ2004	Heidelberg	Cattle
TJ2005	Heidelberg	Chicken
SL486	Heidelberg	Human
TJ2006	Enteritidis	Chicken
TJ2007	Enteritidis	Ready-to-eat meat
TJ2008	Enteritidis	Cattle
TJ2009	Newport	Unknown
SL317	Newport	Human
TJ2010	Typhimurium var. O5–	Unknown
TJ2011	Typhimurium var. O5–	Unknown
TJ2012	Typhimurium	Unknown
TJ2013	Typhimurium	Unknown
TJ2014	Typhimurium	Unknown
TJ2015	Derby	Swine
TJ2016	Derby	Ready-to-eat meat
TJ2017	4,[5],12:i:–	Swine
TJ2018	4,[5],12:i:–	Chicken
E. coli		
TJ1256	A	Human
TJ327	A	Chicken
TJ88	A	Chicken
VA10	A	Human
TJ1192	B1	Human
TJ570	B1	Chicken
TJ82	B1	Chicken
VA32	B1	Human
TJ1208	B2	Human
TJ192	B2	Human
VA22	B2	Human
TJ1125	D	Human
TJ1287	D	Human
VA62	D	Human

TABLE 2 Experimental design for controlled pig studies

Group	No. of pigs	Challenge	Treatment[a]	Room
1	5	None	None	1
2	5	*E. coli* PFEC(pAR060302:rfp)	None	2
3	5	*E. coli* PFEC(pAR060302:rfp)	CTC, 50 g/ton (low dose)	3
4	5	*E. coli* PFEC(pAR060302:rfp)	CTC, 350 g/ton (high dose)	4

[a] High-dose treatment involved 350 g/ton of chlortetracycline (CTC) for 7 days, followed by 50 g/ton of CTC for 14 days, which was then repeated.

that was inserted into the chromosomal *tufA* gene of *Salmonella enterica* serovar Typhimurium strain LT2 (provided by Diarmaid Hughes, Uppsala University) using primers with flanking *E. coli galK* sequence (F, 5′-AGTCAGCGATATCCATTTTCGCGAATCCGGAGTGTAAGAAGAATTCGAGCTCGGTACCCG-3′; R, 5′-CGGTACGGCTGACCATCGGGTGCCAGTGCGGGAGTTTCGTGCTATGACCATGATTACGCC-3′). *E. coli* K-12 carrying pSIM5 (temperature-sensitive origin of replication and chloramphenicol resistant) (29) was grown overnight at 32°C with shaking. The next day, a new culture was incubated in a 32°C water bath with shaking and then heat induced for 15 min at 42°C. Cells were made competent and electroporated with 3 μl of PCR product according to the manufacturer's instructions (MicroPulser electroporator; Bio-Rad, Hercules, CA). Colonies recovered on LB agar containing tetracycline (12.5 μg/ml) were confirmed by PCR, and the pSIM5 plasmid was removed by growing the culture at 37°C overnight and selecting colonies that were sensitive to Cm. This served as the recipient for conjugative frequency assays. The donor for these assays was *E. coli* DH10B harboring pAR060302, which is also resistant to tetracycline at similar levels.

Animal experiment design. Controlled animal experiments were conducted in BSL-2 isolation rooms at the University of Minnesota. Animal experiments were approved under the University of Minnesota IACUC, protocol 1203B12022. Experimental design involved four groups (Table 2). Each group contained five commercial pigs, 28 days of age, purchased from a commercial vendor and verified prior to the inoculations to lack enterobacteria with resistance phenotypes typical of IncA/C plasmid carriage (ampicillin, chloramphenicol, and kanamycin) using fecal flora screening on LB agar and tryptic soy agar. Pigs were collectively housed ($n = 20$) for 7 days with control feed to acclimate to their new environment and share a source of microbes and then separated by group into four isolation rooms. At 35 days of age (day −2 of the experiment), pigs were orally inoculated with 10^8 CFU of *E. coli* strain PFEC(pAR060302:*rfp*) or with PBS. Pigs were again inoculated the same way 2 days later, at day 0 of the experiment. After inoculation, groups 1 and 2 continued to receive control feed. Group 3 received control feed with 50 g/ton chlortetracycline (CTC; ~55 μg/g of feed) for the duration of the experiment (through day 35 postinoculation), representing typical application for growth promotion purposes (referred to throughout as "low dose"). Group 4 received control feed with 350 g/ton CTC (~386 μg/g of feed) for 7 days, followed by in-feed treatment with 50 g/ton CTC for 14 days and then a second dose of 350 g/ton CTC in feed for 7 days, followed by 50 g/ton CTC in feed for the duration of the experiment (referred to throughout as "high dose"). The protocol for group 4 represented consecutive and repeated treatments for disease, with application for growth promotion in between disease treatment doses of CTC via feed.

Bacterial isolation and analysis. Rectal fecal collections were performed at days −2, 0, 3, 7, 14, 21, 28, and 35 postinoculation. One gram of feces from each animal were suspended in PBS and 10-fold serial dilutions were performed. These dilutions were then plated on MacConkey agar to enumerate suspect *E. coli* bacteria. The primary goal was to analyze the proportions of total *E. coli* in each group that harbored pAR060302. To do so, colonies were compared on the following media: (i) media lacking antibiotics for total *E. coli* counts, (ii) media containing ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), and kanamycin (50 μg/ml) to select for *E. coli* carrying pAR060302:*rfp*, (iii) and media containing ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), and nalidixic acid (30 μg/ml) to specifically select for the donor strain PFEC carrying pAR060302:*rfp*. This yielded the amount of recoverable PFEC(pAR060302:*rfp*), the approximate proportion of transconjugant *E. coli* from the fecal flora carrying pAR060302:*rfp*, and the approximate proportions of these two populations relative to total *E. coli*. At selected time points, samples were also plated on MacConkey agar containing tetracycline (20 μg/ml) to select for tetracycline-resistant colonies. To further characterize transconjugants from the fecal flora (nondonor *E. coli*) that acquired pAR060302:*rfp in vivo*, *E. coli* phylogenetic typing was used to assess the clonal diversity following the interpretive approach described by Clermont et al. (30).

A secondary goal was to assess pAR060302 conjugation into non-*E. coli* recipient commensal bacteria. Fecal samples collected above were also cultured on LB agar and tryptic soy agar. These media were supplemented with ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), and kanamycin (50 μg/ml). Plates with isolated colonies were analyzed under blue light to identify non-*E. coli* that acquired pAR060302:*rfp* since these bacteria will fluoresce.

PCR targeting plasmid pAR060302 was used to verify the presence of the plasmid's replication initiation gene in all recovered colonies. PCR conditions are previously described (26), and IncA/C primers were used (F, 5′-GAGAACCAAAGACAAAGACCTGGA-3′; R, 5′-ACGACAAACCTGAATTGCCTCCTT-3′).

Competition experiments. To assess the cost of carriage of pAR060302 in different *E. coli* and *Salmonella* hosts, growth competitions between wild-type strains and corresponding pAR060302-carrying derivatives were performed in LB broth using a previously published protocol (31). Briefly, colonies from each strain were initially grown individually in LB broth at 37°C overnight with shaking. On day 0, bacteria were pelleted and suspended in equal volumes of PBS, and then inoculated at a volume of 25 μl each for competing strains into 5 ml of sterile LB broth. Competitions were performed for 48 h at 37°C, with passage at 24 h of 5 μl of the competition into 5 ml of sterile LB broth. At 48 h, 10-fold serial dilutions were performed and plated on LB agar containing nalidixic acid (30 μg/ml) for total bacterial counts and on LB agar containing nalidixic acid (30 μg/ml) and chloramphenicol (25 μg/ml) to select for strains containing pAR060302. To rule out conjugative transfer as a factor in the competitions, an additional competition was performed between *E. coli* K-12 containing pAR060302 and a rifampin-resistant derivative of *E. coli* K-12 lacking pAR060302; then, serial dilutions and plating was performed on LB agar containing rifampin (100 μg/ml) and chloramphenicol (25 μg/ml). These strains were also mated to demonstrate the transmissibility of pAR060302 between donor and recipient. All competition experiments were performed a minimum of three times. Fitness cost was calculated as follows: $\log_2(a/b) - \log_2(c/d)/\log_2(a/b)$, where a = the number of plasmid-free cells at 48 h, b = the number of plasmid-free cells at 0 h, c = the number of plasmid-containing cells at 48 h, and d = the number of plasmid-containing cells at 0 h.

To assess the impact of subinhibitory levels of tetracycline on pAR060302 selection, *in vitro* competitions were performed between *E. coli* K-12 and *E. coli* K-12 harboring pAR060302. Competitions were performed as described above, except that they were performed for 96 h, with passage every 24 h, and they were performed in the presence of 0, 0.0625, 0.125, 0.25, and 0.5 μg of tetracycline/ml in LB broth. All of these concentrations were below the MIC of tetracycline for the donor and recipient strains. Competition experiments were performed six times.

Conjugative frequency experiments. To assess the impact of increasing concentrations of tetracycline on the transfer frequency of pAR060302, matings were performed between *E. coli* DH10B harboring pAR060302 and *E. coli* K-12 lacking pAR060302 but possessing resistance to tetracycline. Donor and recipient strains were grown overnight in LB broth at 37°C with shaking. After overnight growth, 50 μl of the donor

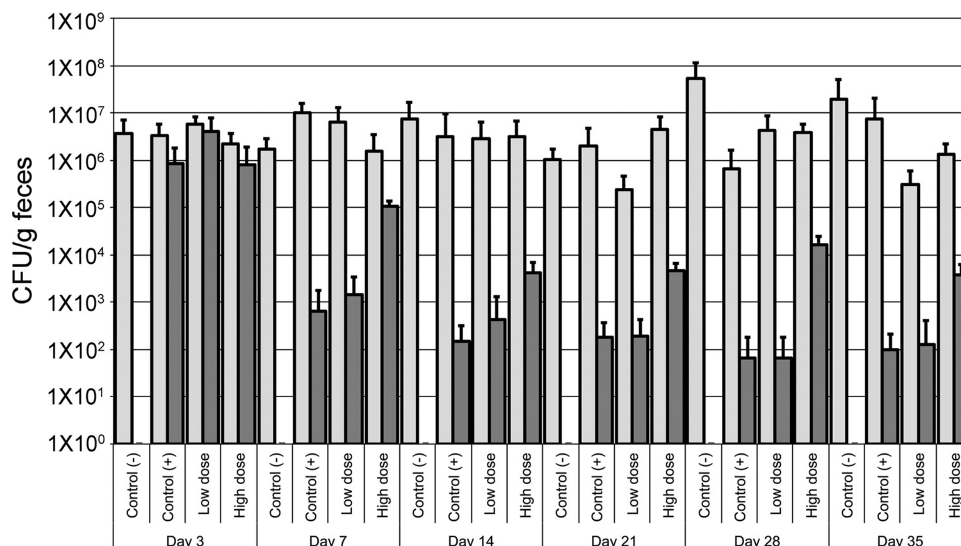

FIG 1 Enumeration of total and IncA/C plasmid-containing *E. coli* in pig feces over 35 days postinoculation. The *y* axis represents the CFU per gram of feces. Days represent sampling days postinoculation with IncA/C plasmid-containing *E. coli*. Light gray bars indicate total *E. coli* counts, and dark gray bars indicate IncA/C plasmid-containing *E. coli* counts. "Control (−)" indicates an noninoculated control, and "Control (+)" indicates an IncA/C+ *E. coli*-inoculated control without antibiotic treatment.

strain was inoculated into 5 ml of sterile media containing 0, 2, 4, 8, 16, and 32 μg of tetracycline/ml and allowed to grow at 37°C with shaking for 2 h. Then, donor and recipient strains were spotted together on LB agar containing the same respective concentrations of tetracycline in equal volumes of 50 μl. Plates were air dried, and matings were performed for 2 h at 37°C. After matings, each plate was washed with 1 ml of PBS and 10-fold serial dilutions were performed and plated on LB agar containing rifampin (100 μg/ml) plus chloramphenicol (30 μg/ml) for donor cells and nalidixic acid (30 μg/ml) plus chloramphenicol (30 μg/ml) for transconjugants. These experiments were performed in triplicate.

Statistics. To evaluate the effect of the antibiotic treatment on total *E. coli*, plasmid-bearing *E. coli*, and the proportion of total *E. coli* with the plasmid in the *in vivo* experiments, a mixed-model procedure was utilized that included a between-subjects factor (treatment group) and a within-subjects factor (day of sampling). Differences between consecutive days were assessed with repeated contrasts. Total *E. coli* counts and total *E. coli* counts containing plasmid were log transformed. Proportions were arcsine square root transformed. Significance levels were set at $\alpha = 0.05$ for all analyses. Analysis of variance was performed for fitness cost comparisons. Standard statistical software was used (SPSS, v22.0; SPSS, New York, NY).

RESULTS

High-dose chlortetracycline administration, but not low-dose administration, selected *in vivo* for IncA/C plasmid-containing *E. coli*. Fecal contents from 20 pigs were assessed at six time points for proportions of IncA/C plasmid-containing *E. coli* after inoculation with a pig fecal *E. coli* strain harboring the multidrug resistance-encoding IncA/C plasmid, pAR060302 (Fig. 1). At day 3 postinoculation, no significant differences ($P > 0.1$) were found in the total IncA/C plasmid-containing *E. coli* count or in the proportion of total *E. coli* containing the IncA/C plasmid among the three groups that were inoculated with plasmid-containing strain. However, at all subsequent time points, the high-dose treatment group had significantly higher IncA/C plasmid-containing *E. coli* counts and proportions of IncA/C plasmid-containing *E. coli* compared to the low-dose and nontreated inoculation groups ($P < 0.001$). The low-dose treatment group never had significantly different IncA/C plasmid-containing *E. coli* counts or proportions compared to the inoculated control group ($P > 0.1$).

Tetracycline-resistant *E. coli* were indigenous to conventional pigs. We also assessed the proportion of tetracycline-resistant *E. coli* at days 3, 7, and 21 of the experiment (Fig. 2). At all time points examined, the proportion of tetracycline-resistant *E. coli* approached the total *E. coli* counts, and these counts did not differ significantly, indicating that pigs were colonized with tetracycline-resistant *E. coli* prior to inoculations, and these proportions were maintained postinoculation. However, final pig masses were significantly greater for the low-dose and high-dose treatment groups compared to either control group ($P = 0.03$ and $P < 0.01$, respectively; Table 3).

IncA/C plasmid transfer between *E. coli* occurred *in vivo* under chlortetracycline application at high-dose levels. At days 3, 7, 21, and 35 postinoculation, 96 *E. coli* colonies were selected from each experimental group and time point (1,536 colonies total) that displayed resistance to kanamycin, ampicillin, and chloramphenicol, indicating possession of an IncA/C plasmid. These colonies were replica plated to LB agar containing nalidixic acid to differentiate between the donor inoculum strain and possible transconjugants that occurred through conjugation in the pig gastrointestinal tract. As a negative control, fecal samples from the noninoculated control group were plated on the same selective media to confirm the absence of resistant colonies in fecal isolates from noninoculated pigs. At day 3 postinoculation, no colonies were identified that were not donor inoculum (nalidixic acid resistant). However, at days 7, 21, and 35, colonies were identified in all pigs from the high-dose treatment group that were apparent transconjugants (Table 4). No apparent transconjugants were identified from the low-dose treatment group at any time point. Putative transconjugant *E. coli* from the high-dose treatment group were further characterized for *E. coli* phylogenetic type (Table 4). From this analysis, transconjugants were identified from all

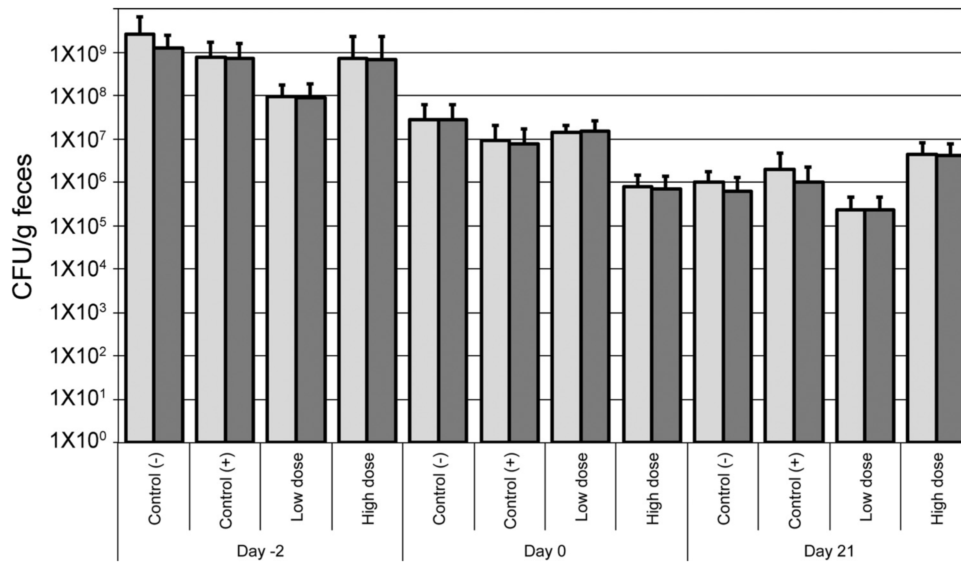

FIG 2 Enumeration of total and tetracycline-resistant *E. coli* in pig feces at selected time points. See the Fig. 1 legend for a description.

four phylogenetic groups. These isolates were further examined to confirm possession of an IncA/C plasmid using a plasmid-specific PCR for the IncA/C replication initiation gene (21). All isolates examined using this approach were IncA/C positive.

In vivo **IncA/C plasmid transfer to non-*E. coli* was not detectable.** At each time point, colonies were also examined on LB agar and TSA agar containing kanamycin, ampicillin, and chloramphenicol for potential non-*E. coli* transconjugants that had acquired the inoculated IncA/C plasmid. Colonies were present on both types of media at all time points. However, none of these colonies fluoresced, indicating that the IncA/C plasmid had not transferred outside of *E. coli*. Some of the nonfluorescing colonies were selected, restreaked on MacConkey agar, and confirmed to be *E. coli*.

IncA/C plasmid fitness cost varies within, and between, *E. coli* and *Salmonella*. Fourteen *E. coli* strains and 20 *Salmonella* strains were examined for the cost of IncA/C plasmid carriage using relative-fitness-cost calculations obtained via competition experiments (Fig. 3). For each competition, the wild-type bacterial strain was competed with its IncA/C plasmid-containing derivative. Significantly different costs of IncA/C plasmid carriage were observed between serovars of *S. enterica* ($P = 0.016$) and between phylogenetic groups of *E. coli* ($P = 0.003$). *S. enterica* serovar Enteritidis isolates displayed the greatest cost of carriage for pAR060302, while *S. enterica* serovar Typhimurium isolates displayed the least cost of carriage for pAR060302. In *E. coli*, the cost of carriage of pAR060302 was greatest in phylogenetic group B1 isolates, while there was an apparent benefit for carriage of pAR060302 in phylogenetic group A isolates. For comparison purposes, the cost of carriage of IncI1 plasmid pCVM29188_101 (32, 33) and the cost of cocarriage for IncA/C and IncI1 plasmids together was also determined in the *E. coli* recipients (Fig. 4). Notably, in all phylogenetic backgrounds, fitness cost was significantly different for carriage of both IncA/C and IncI1 plasmids together compared to the cost for carriage of either plasmid alone ($P < 0.05$). In general, the cost of IncA/C plasmid carriage was greater than the cost of IncI1 plasmid carriage. Growth curves of each individual *E. coli* strain with or without IncA/C and IncI1 plasmids were also examined (data not shown). In nearly all isolates, the patterns of growth mimicked the competition experiment results. That is, the wild-type strain in each case grew the fastest in LB broth, and the IncA/C plasmid-containing strain grew the slowest. However, when both IncA/C and IncI1 plasmids were cocarried in the same strain, they grew better than strains singly carrying an IncA/C plasmid.

TABLE 3 Starting and ending weights of the pigs used in this study

Group	Starting wt (lb) and SE		Ending wt (lb) and SE		
	Avg wt	SE	Avg wt	SE	*P*
1	15.6	0.7	38.6	3.9	NA[a]
2	15.9	1.3	40.8	5.0	0.46
3	15.6	0.7	48.2	6.9	0.03
4	15.2	0.9	53.4	8.2	<0.01

[a] NA, not applicable.

Tetracycline at higher levels enhances conjugal transfer of pAR060302. The effect of increasing concentration of tetracycline on pAR060302 conjugal transfer was determined using matings between *E. coli* DH10B containing pAR060302 and *E. coli* K-12: *tetG* (Fig. 5). The purpose of this approach was to examine the effects of tetracycline on conjugal transfer independent of selection for tetracycline-resistant bacteria. No significant differences were observed between control and treatments of <32 μg of tetracycline/ml applied to the donor (preconjugation) and on the mating substrate. However, at 32 μg of tetracycline/ml, an approximately 3- to 4-fold increase was observed in conjugative frequency.

Low-dose concentrations of tetracycline select for pAR 060302-containing *E. coli in vitro*. To determine whether subinhibitory concentrations of tetracycline select for pAR060302-containing *E. coli*, *in vitro* competition experiments were performed between *E. coli* K-12 containing pAR060302 and a rifampin-resistant derivative of *E. coli* K-12 (Fig. 6). Prior to these experiments,

TABLE 4 Summary of transconjugants analyzed from pig inoculation experiments[a]

Days postinoculation	Expt group	No. of colonies examined	% transconjugant colonies	% transconjugants belonging to:			
				Phylotype A	Phylotype B1	Phylotype B2	Phylotype D
3	Control (+)	96	0	NA	NA	NA	NA
	Low dose	96	0	NA	NA	NA	NA
	High dose	96	0	NA	NA	NA	NA
7	Control (+)	96	0	NA	NA	NA	NA
	Low dose	96	0	NA	NA	NA	NA
	High dose	96	24	52	35	4	9
21	Control (+)	96	0	NA	NA	NA	NA
	Low dose	96	0	NA	NA	NA	NA
	High dose	96	29	46	36	11	7
35	Control (+)	96	0	NA	NA	NA	NA
	Low dose	96	0	NA	NA	NA	NA
	High dose	96	32	42	26	13	19

[a] NA, no putative transconjugant colonies identified. "Control (+)" indicates an *E. coli*-inoculated control without antibiotic treatment.

both strains were shown to be tolerant to tetracycline up to concentrations of 1 μg/ml. At time zero, nearly equal proportions of the plasmid-containing and plasmid-lacking strains were recovered from the mixture used for all competitions. In the absence of tetracycline, the plasmid-lacking strain outcompeted the pAR060302-containing strain at approximately 10:1. However, in the presence of ≥0.0625 μg of tetracycline/ml, the plasmid-containing strain outcompeted the plasmid-lacking strain at nearly 10:1.

DISCUSSION

The purpose of this study was to evaluate the dynamics of IncA/C plasmid fitness, selection, and transmissibility in *E. coli* and *Salmonella*. The first goal was to better understand the impact of low versus high doses of chlortetracycline on IncA/C plasmid selection and transfer in commercial pigs. Notably, continuous low-dose administration of chlortetracycline in feed at a concentration of 50 g/ton had no observable impact on the proportions of IncA/C plasmid-containing *E. coli* over the course of 35 days after inoculation with an IncA/C plasmid-containing commensal strain. The inclusion of high-dose pulses of chlortetracycline at levels of 350 g/ton in feed significantly increased the proportion of IncA/C plasmid-containing *E. coli* at all postinoculation time points. Nearly all of the indigenous *E. coli* in the pigs were already resistant to tetracycline. Had this experiment been performed in pigs lacking tetracycline-resistant *E. coli*, the results may have been different, and this is as suggested by our *in vitro* data demonstrating that very low subinhibitory concentrations of tetracycline still strongly selected for IncA/C plasmid-containing strains (Fig. 6). However,

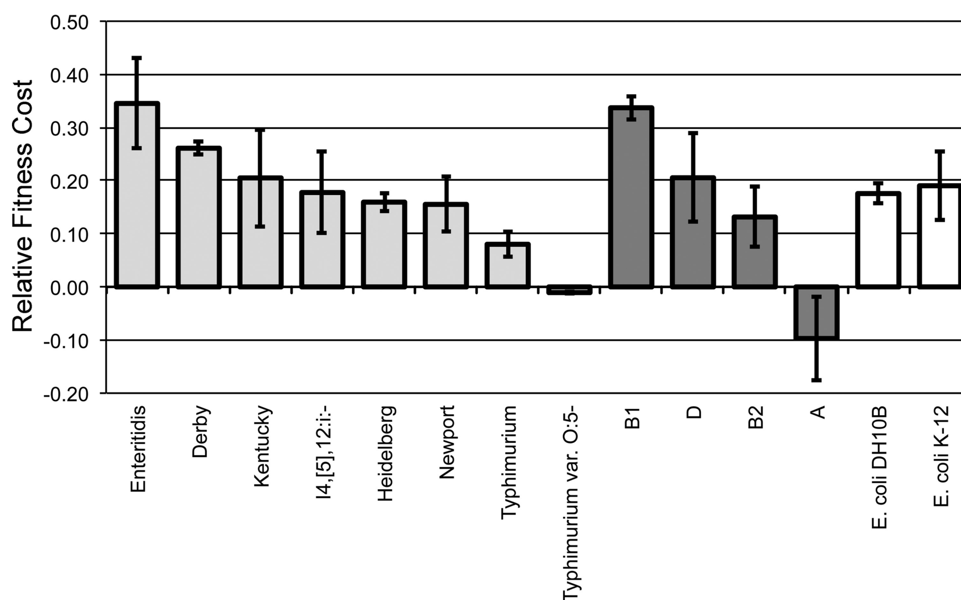

FIG 3 Relative fitness cost of IncA/C plasmid carriage on various *Salmonella* and *E. coli* recipients. A positive value indicates a cost for plasmid carriage, whereas a negative value indicates a benefit for plasmid carriage. Serotype or phylogenetic group is displayed below each bar. Error is expressed as the standard error of the mean. Each bar represents multiple isolates examined.

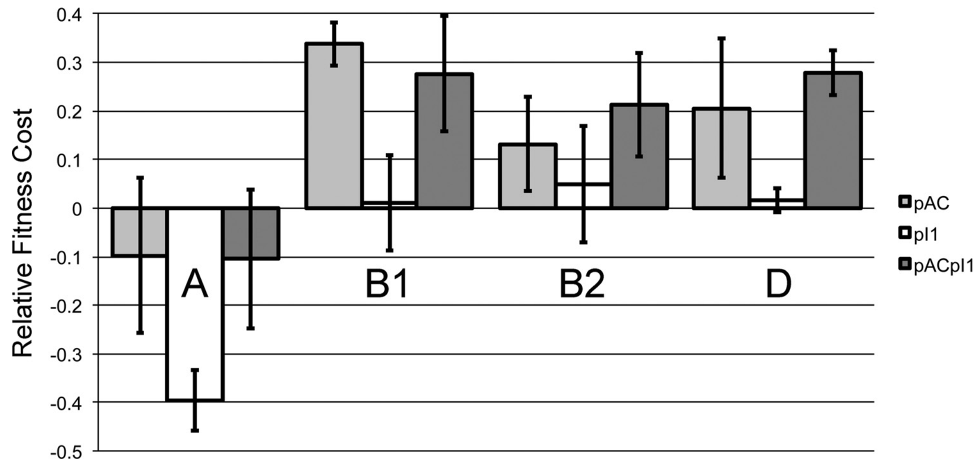

FIG 4 Relative fitness cost of plasmid combinations in various *E. coli* hosts. A positive value indicates a cost for plasmid carriage, whereas a negative value indicates a benefit for plasmid carriage. Multiple isolates from each phylogenetic group were examined for cost of carriage of an IncA/C plasmid alone (pAC), an IncI1 plasmid alone (pI1), or cocarriage of both plasmids (pACpI1).

data from the Minnesota Veterinary Diagnostic Laboratory indicates that *E. coli* isolates in pigs are nearly always resistant to tetracycline (personal communication with the University of Minnesota Veterinary Diagnostic Laboratory), as does other work (34). Therefore, this experiment likely reflects a more realistic scenario where tetracycline-resistant *E. coli* are already dominant in the pig gastrointestinal tract. It can be concluded, then, that low-dose administration of chlortetracycline alone in commercial pigs is not likely to exert biologically significant selection or coselection for IncA/C plasmids.

Other studies in cattle have examined the coselective properties of chlortetracycline on *E. coli* displaying resistance to tetracycline and ceftiofur. In one study, the administration of chlortetracycline at 22 mg/kg to cattle decreased the recovery of ceftiofur-resistant *E. coli* (35). In a more recent study by the same research group, similar regimens of therapeutic chlortetracycline treatment in cattle greatly increased both the counts of ceftiofur-resistant *E. coli* and the quantities of bla_{CMY-2}, a gene found on IncA/C plasmids conferring ceftiofur resistance (36). The latter study used a more complex design also implementing therapeutic ceftiofur treatment. Notably, both studies used therapeutic doses of chlortetracycline. Based on these results, it was concluded that preferential selection for chromosome-encoded *tetB*-containing *E. coli* may have occurred in the absence of ceftiofur pressure, whereas in the second study the application of both chlortetracycline and ceftiofur resulted in coselection for *tetA* and bla_{CMY-2}, both encoded on IncA/C plasmids. Our results also suggest a preference for *tetB*-containing commensal *E. coli*, although the presence of *tetB* was not confirmed in the present study. While we cannot here conclusively rule out the possibility of coselective advantages, there was no direct selective advantage for IncA/C plasmid-containing *E. coli* strains in pigs receiving low doses of chlortetracycline in feed.

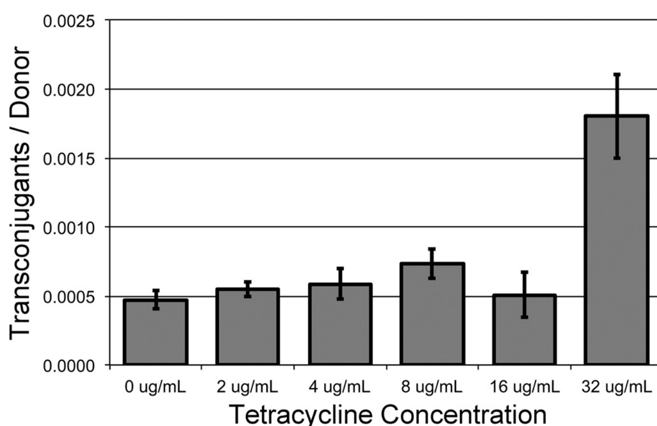

FIG 5 Frequency of conjugal transfer in the presence of increasing concentrations of tetracycline, presented as transconjugants/donor (*y* axis).

Our data indicate that high-dose administration of chlortetracycline resulted in increased movement of the IncA/C plasmid to other indigenous *E. coli* hosts via conjugation. We were unable to observe evidence of such plasmid movement in our inoculated control or low-dose groups. It is possible that plasmid transfer did occur within these groups but was below limits of detection. Nevertheless, there was certainly evidence of plasmid transfer in the high-dose treatment group. There is previous evidence of enhancement of conjugal transfer after exposure to tetracyclines (37), and the *in vitro* experiments demonstrated 3- to 4-fold enhancement of conjugal transfer at 32 μg of tetracycline/ml. Previous work has demonstrated that at 400 g/ton administered in feed, the bioavailable concentration of chlortetracycline in the pig colon is 56 μg/ml (38). Extrapolating from those data, we would estimate bioavailable concentrations of chlortetracycline of 49 μg/ml at our high dose of 350 g/ton and of 7 μg/ml at the low-dose treatment of 50 g/ton. Therefore, the *in vivo* results are consistent with *in vitro* conjugative frequency data indicating that enhanced IncA/C plasmid conjugal transfer occurs at tetracycline concentrations exceeding 32 μg/ml.

We hypothesized that other bacterial species reservoirs exist for IncA/C plasmids in the gastrointestinal tract. However, we were unable to detect any evidence of conjugal transfer of the IncA/C plasmid between our donor strain and the indigenous flora in these studies. Appropriate controls were included in each experiment, and the ability of this plasmid to transfer to non-*E. coli* hosts was validated in pilot experiments using non-*E. coli* re-

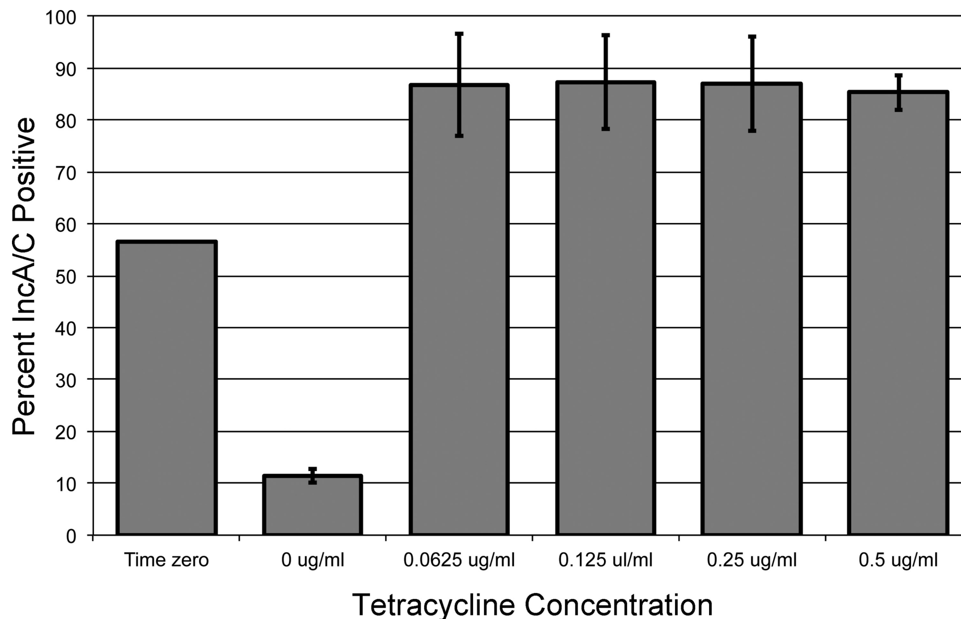

FIG 6 Competition experiments between pAR060302-containing and pAR060302-lacking *E. coli* K-12 in the presence of increasing subinhibitory concentrations of tetracycline. The *y* axis depicts the percentage of pAR060302-containing colonies recovered relative to plasmid lacking colonies. Time zero represents the starting material for all concentrations mixed at equal doses, which was then quantified via direct plating.

cipient bacteria. Thus, it is unlikely that the absence of detectable conjugative transfer was due to an inability of the plasmid to transfer. It appears that conjugal transfer may play less of a role in IncA/C plasmid dissemination between bacterial species compared to clonal expansion and dissemination within bacterial populations. Although the existing literature supports the notion that IncA/C plasmids have a broad host range (14, 39–41), it is likely that there are bottlenecks in the dissemination of this plasmid type dependent on conjugal compatibility between bacterial species. This differs from other plasmid types, such as IncP plasmids (23, 31), and it has important implications regarding the basic biology of these plasmids and their dissemination.

Another salient finding from the present study was that bacterial host background substantially impacts the cost of plasmid carriage. *E. coli* strains belonging to phylogenetic group A were better suited to carry plasmids than other *E. coli* types. It has previously been shown that *E. coli* belonging to phylogenetic group A are more amenable to acquiring drug resistance genes and phenotypes than other phylogenetic groups (42), and the PFEC strain used here was also a phylogenetic group A strain. However, the mechanisms enabling *E. coli* of phylogenetic group A to be more amenable to plasmid and resistance gene acquisition are currently unknown. Also, few studies have examined the effects of plasmid acquisition on multiple strains among diverse *E. coli* phylogenetic groups (43). It appears that IncA/C plasmids are also better suited for some *Salmonella* hosts than others, and this is reflected in the clinical literature where these plasmids are more prevalent among some serovars of *Salmonella* than others (16, 18, 44–47). This underscores the importance of bacterial host background on the cost of plasmid carriage, even between strains of the same species.

Cocarriage of IncI1 and IncA/C plasmids did not confer an additive fitness cost effect on the host. In fact, many strains actually benefited from carrying two plasmids versus a single plasmid. This form of epistasis has been previously described for plasmid carriage (48, 49). The fact that most porcine-source *E. coli* isolates appear to harbor multiple plasmids (21) suggests that multiple-plasmid carriage compared to single-plasmid carriage may be a beneficial trait. Again, the mechanisms driving positive epistasis related to coinfecting plasmids are unknown but certainly deserve greater attention.

In summary, we found no evidence of IncA/C plasmid transmission or strain selection in pigs in the presence of low-dose chlortetracycline, which is among the most widely used growth promoter antibiotics in commercial pigs. We cannot conclude from our findings that prolonged administration of antimicrobials at low-dose concentrations in feed does not result in detectable selection or coselection of resistance-encoding plasmids. However, we can conclude that while short-term exposure to low-dose levels of chlortetracycline in pig feed does not result in the selection of IncA/C plasmid-containing *E. coli*, selection can occur with short-term administration of high-dose levels. Although a great deal of literature has focused on the selection of drug-resistant bacteria in animal agriculture, surprisingly few experiments have been designed to parse the impact of different drugs, different concentrations, and different durations of administration on the selection of specific types of genetic elements. More work of this nature is greatly needed to better understand the mechanistic aspects of selection of plasmid-encoded multidrug resistance in commensal bacteria of production animals.

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