

# Impaired Fitness and Transmission of Macrolide-Resistant *Campylobacter jejuni* in Its Natural Host

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*Campylobacter jejuni* is a major zoonotic pathogen transmitted to humans via the food chain and is prevalent in chickens, a natural reservoir for this pathogenic organism. Due to the importance of macrolide antibiotics in clinical therapy of human campylobacteriosis, development of macrolide resistance in *Campylobacter* has become a concern for public health. To facilitate the control of macrolide-resistant *Campylobacter*, it is necessary to understand if macrolide resistance affects the fitness and transmission of *Campylobacter* in its natural host. In this study we conducted pairwise competitions and comingling experiments in chickens using clonally related and isogenic *C. jejuni* strains, which are either susceptible or resistant to erythromycin (Ery). In every competition pair, Ery-resistant (Ery<sup>r</sup>) *Campylobacter* was consistently outcompeted by the Ery-susceptible (Ery<sup>s</sup>) strain. In the comingling experiments, Ery<sup>r</sup> *Campylobacter* failed to transmit to chickens precolonized by Ery<sup>s</sup> *Campylobacter*, while isogenic Ery<sup>s</sup> *Campylobacter* was able to transmit to and establish dominance in chickens precolonized by Ery<sup>r</sup> *Campylobacter*. The fitness disadvantage was linked to the resistance-conferring mutations in the 23S rRNA. These findings clearly indicate that acquisition of macrolide resistance impairs the fitness and transmission of *Campylobacter* in chickens, suggesting that the prevalence of macrolide-resistant *C. jejuni* will likely decrease in the absence of antibiotic selection pressure.

*Campylobacter jejuni* has been recognized as one of the most common causes of human enterocolitis worldwide (2). This organism is transmitted to humans via contaminated foods of animal origin, especially undercooked poultry meat and unpasteurized milk/dairy products (2, 4). Although antibiotic treatment may not be necessary for most food-borne campylobacteriosis cases, antimicrobial therapy is warranted in patients with severe or prolonged infections (2, 12). Generally, erythromycin (Ery) and ciprofloxacin are considered the main antimicrobials for treating human campylobacteriosis (2, 12, 17). However, during the past decades *Campylobacter* has become increasingly resistant to clinically important antimicrobial agents, compromising the effectiveness of clinical therapy (17). Since antimicrobial-resistant *Campylobacter* can be transmitted from food animals to humans through the food chain, the rising resistance to antibiotics among *Campylobacter* isolates of animal origin is a concern for public health.

Ery, a 14-membered ring macrolide, as well as other 15- and 16-membered ring macrolides (e.g., azithromycin, tilmicosin, and tylosin), are of high efficacy against several important pathogens, including *Campylobacter*, *Chlamydia*, and *Mycobacterium* species (20, 21). These antimicrobials inhibit bacterial protein synthesis by binding to the 50S subunits of bacterial ribosome and have been widely used for the treatment of infections in both humans and animals for a number of years (20). The use of macrolides in food-producing animals is considered to be one of the major factors influencing the emergence of Ery-resistant (Ery<sup>r</sup>) *Campylobacter* (20). There are recent evidence indicating that the continuous use of a macrolide at subtherapeutic level in chickens results in the development of Ery resistance in *Campylobacter* (32, 34).

Although multiple mechanisms of macrolide resistance have been reported in different bacterial genus and species, modifications of the ribosomal target sites (e.g., the 23S rRNA gene and ribosomal proteins L4 and L22) and active efflux via the CmeABC efflux pump are the major mechanisms conferring macrolide res-

sistance in *Campylobacter* (13, 19, 20, 41). To date, point mutations in domain V of the 23S rRNA gene at positions 2074 and 2075, corresponding to positions 2058 and 2059 in *Escherichia coli*, respectively, have been recognized as the most common mechanism for macrolide resistance in *C. jejuni* and *Campylobacter coli* (20, 41). Among the reported resistance-associated mutations, the A2074C, A2074G, and A2075G mutations are found to confer a high-level of macrolide resistance, while other mutations in the 23S rRNA gene or the mutations in the ribosomal proteins L4 (G74D) and L22 (insertions at position 86 or 98) are shown to confer a lower level of macrolide resistance in *Campylobacter* (13, 14, 19, 20, 41).

In bacteria, the acquisition of antibiotic resistance, particularly the resistance mediated by chromosomal mutations, is frequently accompanied by a biological cost, resulting in a decrease in fitness (i.e., a reduced growth rate or a decrease in ability to compete and persist in the host and environment) of microorganisms in the absence of antibiotic selection pressure (7–9, 30, 33, 40). Even though many types of antibiotic resistance impose a biological cost on bacterial fitness, the fitness cost can be reduced at different levels through compensatory mutations (5, 10, 11, 33, 40). In addition, some resistance-conferring mutations or determinants do not incur an apparent fitness burden or even enhance the fitness of

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TABLE 1 Characteristics of *Campylobacter* strains used in this study

Strain <sup>a</sup>	Description	Ery MIC (μg/ml) <sup>b</sup>	Mutation in 23S rRNA <sup>c</sup>
<b>Ery<sup>s</sup> strains</b>			
ATCC 700819	Highly motile variant of <i>C. jejuni</i> NCTC 11168	2	None
Bd34-2*	Ery <sup>s</sup> isolate from a chicken inoculated with 700819 and treated with tylosin-containing feed	2	None
Bd41-3*	Ery <sup>s</sup> isolate from a chicken inoculated with 700819 and treated with tylosin-containing feed	2	None
<b>Clonally related Ery<sup>r</sup> strains</b>			
J.L.270*	Ery <sup>r</sup> isolate from a chicken inoculated with 700819 and treated with tylosin-containing feed	32	None
J.L.272*	Ery <sup>r</sup> isolate from a chicken inoculated with 700819 and treated with tylosin-containing feed	>512	A2074G
J.L.273*	Ery <sup>r</sup> isolate from a chicken inoculated with 700819 and treated with tylosin-containing feed	>512	A2074G
<b>Isogenic Ery<sup>r</sup> strains</b>			
T.L.101†	Laboratory-constructed Ery <sup>r</sup> transformant from 700819	>512	A2074G
T.L.102†	Laboratory-constructed Ery <sup>r</sup> transformant from 700819	>512	A2075G
T.L.103†	Laboratory-constructed Ery <sup>r</sup> transformant from 700819	>512	A2075G

<sup>a</sup> \*, Clonally related to 700819 and the dose of tylosin in the feed was 50 mg/kg of feed (34); †, transformants were from three independent transformation experiments.

<sup>b</sup> Determined by the agar dilution method.

<sup>c</sup> Corresponding to the nucleotide positions in the 23S rRNA gene of *C. jejuni* NCTC 11168.

the antibiotic resistant strains (10, 26, 31, 37, 38, 44). For example, a modeling study on antibiotic resistance revealed that some resistant bacteria, such as penicillin-resistant strains, did not show a decreased fitness in the host; instead, these resistant strains possessed an increased ability to transmit between hosts compared to the susceptible strains (6). In *C. jejuni*, it has been found that fluoroquinolone (FQ)-resistant strains, carrying the C257T mutation in the *gyrA* gene, do not show a fitness cost in its natural host (chicken). Instead, the FQ-resistant mutants possess an enhanced fitness in the absence of antibiotic selection pressure (26).

Although macrolide resistance mechanisms were well defined in *Campylobacter*, the impact of the resistance-associated mutations on *Campylobacter* fitness has not been well defined. Recently, it was shown that acquisition of Ery resistance imposes a fitness burden in *C. jejuni* in culture medium as Ery<sup>r</sup> *Campylobacter* showed a competitive disadvantage compared to erythromycin-susceptible (Ery<sup>s</sup>) *Campylobacter* in mixed cultures (25, 27). However, the fitness changes observed in laboratory media may not necessarily reflect the fitness alteration *in vivo* since the environments in animals are much more complex than in culture media (6, 11). More importantly, to facilitate the control of macrolide resistance in *Campylobacter*, it is essential to assess whether the resistance impacts *Campylobacter* fitness and transmissibility in its natural hosts. Toward this end, we used clonally related and isogenic mutants of Ery<sup>r</sup> *Campylobacter* to evaluate their fitness and transmissibility in chickens, the major animal reservoir for *C. jejuni*.

## MATERIALS AND METHODS

**Bacterial strains.** *C. jejuni* strains used in the present study are listed in Table 1. *C. jejuni* ATCC 700819 (NCTC 11168), Bd34-2, and Bd41-3 are susceptible to Ery, whereas the other strains (J.L.270, J.L.272, J.L.273, T.L.101, T.L.102, or T.L.103) exhibit low or high resistance to Ery (Table 1). The isolates Bd34-2, Bd41-3, J.L.270, J.L.272, and J.L.273 are clonally related to ATCC 700819 and were isolated from chickens that were orig-

inally challenged with the parent strain ATCC 700819 and treated with tylosin-containing feed as described in a previous study (34). Briefly, the chickens were inoculated in laboratory with *C. jejuni* ATCC 700819 at 3 days of age and provided with the medicated feed (tylosin; 50 mg/kg of feed) for the entire 41 days of the experiment. *C. jejuni* was reisolated from the inoculated chickens from cloacal swabs at different days after the inoculation. Detailed information on the experiment is described in the previous publication (34). The isogenic Ery<sup>r</sup> transformants T.L.101, T.L.102, and T.L.103 were constructed from the parent strain ATCC 700819 using natural transformation (see below). These transformants have either A2074G or A2075G mutation in the 23S rRNA gene and are highly resistant to Ery (Table 1).

**Construction of the Ery<sup>r</sup> transformants.** To construct the isogenic Ery<sup>r</sup> transformants, *C. jejuni* strains with the A2074G mutation (J.L.273) or A2075G mutation (C.T.2–2) were used to prepare donor genomic DNA for natural transformation. These Ery<sup>r</sup> *Campylobacter* strains were originally isolated from chickens and turkeys (34, 36). Genomic DNA from the Ery<sup>r</sup> strains was extracted using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) according to the manufacturer's protocol and then digested with the restriction enzyme EcoRV prior to the natural transformation experiment. This digestion was done to release the 23S rRNA gene from its flanking sequences in the donor DNA, allowing the selection of transformants that only contain mutations in the 23S rRNA gene and minimizing the cotransfer of unrelated mutations from the donor DNA to the transformants. Natural transformation was performed with a biphasic method as described by Wang and Taylor (45) using the parent strain ATCC 700819 as the recipient. Transformants were selected on Mueller-Hinton (MH) agar containing 8 μg of Ery/ml, and the A2074G or A2075G mutation in the 23S rRNA gene of the isogenic Ery<sup>r</sup> transformants was confirmed by sequence analysis. 23S rRNA gene-specific primers (5'-GTAAACGGCGGCCGTAAC TA-3' and 5'-GACCGAAGTGTCTCAGCAGC-3') were used to amplify an internal part of the domain V of the 23S rRNA gene (29). PCR amplification was performed with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 40 s, and a final extension step at 72°C for 10 min. The amplified PCR products (714 bp) were purified with the QIAquick PCR purification kit (Qiagen, Valencia,

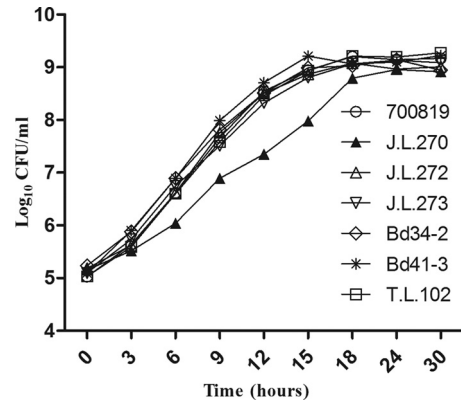
CA) prior to sequencing. DNA sequencing was conducted at the DNA facility of Iowa State University. Three transformants (T.L.101, T.L.102, and T.L.103) derived from three independent transformation experiments were used in the present study (Table 1).

**Motility assay.** Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter* strains were tested for their motility prior to inoculation into chickens. Briefly, Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter* strains grown overnight were resuspended in MH broth and adjusted to an optical density at 600 nm of 0.3. Each *Campylobacter* strain was inoculated to the center of semisolid MH motility media (0.4% MH agar) using a sterile needle. After incubation at 42°C for 48 h under microaerobic conditions, the diameter of swarming from the inoculation spot was measured in millimeters and recorded.

**In vitro growth determination.** To determine the *in vitro* growth of the parent strain ATCC 700819, clonally related Ery<sup>r</sup> strains, and isogenic Ery<sup>r</sup> transformants, a fresh culture of each *Campylobacter* strain was inoculated into MH broth and adjusted to an initial cell density of 10<sup>5</sup> CFU/ml. The cultures were incubated at 42°C with shaking (160 rpm) for 30 h under microaerobic conditions. The growth kinetics was determined by measuring the numbers of *Campylobacter* colonies (log<sub>10</sub> CFU/ml) at 0, 3, 6, 9, 12, 15, 18, 24, and 30 h postinoculation.

**Pairwise competition experiments.** Newly hatched broiler chickens from a commercial hatchery were used to determine the *in vivo* competition between Ery<sup>r</sup> and Ery<sup>s</sup> *Campylobacter* in the absence of antibiotic selection pressure. The chickens used in the present study were tested negative for *Campylobacter* by culturing cloacal swabs before use. These birds were randomly assigned to groups with 10 to 15 birds per group. Each group was inoculated with either a single or a mixture of Ery<sup>r</sup> and Ery<sup>s</sup> *Campylobacter* at 1:1 ratio via oral gavage. The *Campylobacter* strains used for chicken inoculation were grown at 42°C for 24 h under microaerobic conditions. The inoculum was given to the birds at 3 days of age with approximately 10<sup>7</sup> CFU per bird. Fecal samples were collected from each bird by means of cloacal swabs at 3, 6, and 10 days postinoculation (dpi). Each fecal sample was serially diluted in MH broth and plated onto MH agar containing *Campylobacter* selective agents and growth supplements (SR084E and SR117E; Oxoid, Basingstoke, United Kingdom) to recover the total *Campylobacter* colonies and onto MH agar containing the same selective agents and growth supplements plus 8 μg of Ery/ml to recover Ery<sup>r</sup> *Campylobacter* colonies. Colony count was performed after 48 h of incubation at 42°C under microaerobic conditions. The results of the differential plating were further confirmed by the MIC of selected isolates by the agar dilution method.

**Transmission of Ery<sup>r</sup> *Campylobacter* in chickens.** Three groups of newly hatched broiler chickens (11 to 13 birds per group) were used to assess the transmissibility of Ery<sup>r</sup> *Campylobacter* between hosts. Each group of chickens was inoculated with a single *Campylobacter* strain (10<sup>7</sup> CFU per bird) via oral gavage. The strains used in this experiment included the Ery<sup>s</sup> parent strain ATCC 700819, the isogenic Ery<sup>r</sup> transformants T.L.101 (carrying the A2074G mutation) and T.L.102 (carrying the A2075G mutation). At 5 dpi, when the colonization was established similarly in each group by the corresponding strain, eight chickens inoculated with the Ery<sup>s</sup> strain and four chickens inoculated with the Ery<sup>r</sup> strain T.L.101 were randomly selected and mingled together (2:1 ratio between chickens inoculated with Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter*, respectively). Likewise, 11 chickens inoculated with the Ery<sup>r</sup> strain T.L.102 were mingled with 5 chickens originally inoculated with the Ery<sup>s</sup> *Campylobacter* strain, giving an approximately 1:2 ratio between chickens inoculated with Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter* strains. The chickens (*n* = 8) inoculated with the Ery<sup>r</sup> *Campylobacter* strain T.L.101 that were not used for comingling studies were raised separately and served as a control for determining the *in vivo* stability of Ery<sup>r</sup> *Campylobacter* in the absence of antibiotic selection pressure. Fecal samples were collected from each bird before comingling, as well as at 7 and 14 days after comingling, using cloacal swabs. The number of Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter* colonies was determined using the differential plating method on both MH agar and MH agar containing



**FIG 1** *In vitro* growth kinetics of clonally related and isogenic *C. jejuni* strains. The cultures were incubated in MH broth at 42°C under microaerobic conditions with shaking (160 rpm). Each data point represents the mean log<sub>10</sub> CFU/ml of five technical replicates. The experiment was repeated twice and similar results were obtained. 700819, Bd34-2, and Bd41-3 are Ery susceptible, while J.L. 270, J.L.272, J.L.273, and T.L.102 are Ery resistant (see Table 1 for MIC values).

8 μg of Ery/ml as described earlier. The agar dilution method was also performed to confirm the results of the differential plating.

**Antimicrobial susceptibility test.** The MICs of Ery for *Campylobacter* colonies randomly selected at each sampling time point were determined using the agar dilution method as recommended by the CLSI (16). *C. jejuni* 33560 was used as the quality control organism, and the MIC of Ery at 8 μg/ml was used as the resistance breakpoint in the present study. Ery was obtained from Sigma Chemical Co., St. Louis, MO.

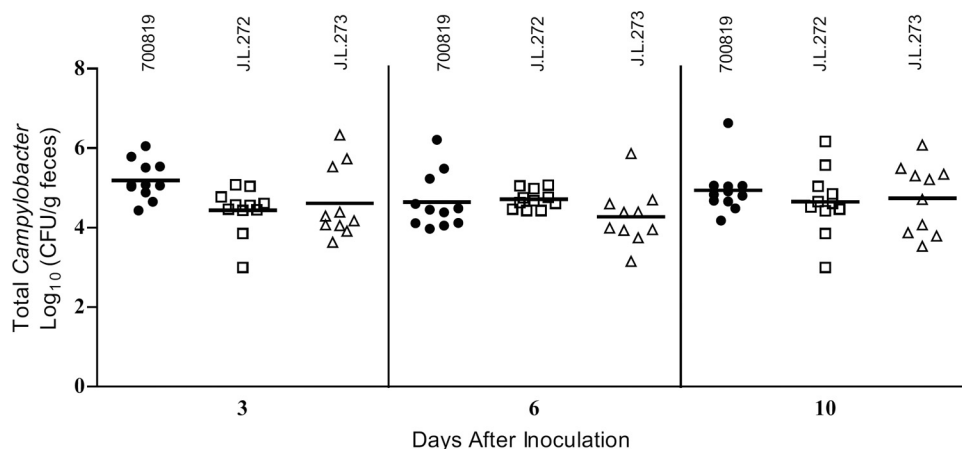
**Statistical analysis.** The significance of differences between Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter* in colonization levels at each sampling time point was determined by using Student's *t* test, Welch's *t* test to allow for nonconstant variation across treatment groups, and the Wilcoxon rank-sum test to allow for non-normality as described previously (24). Differences were considered significant at a *P* value of <0.05.

## RESULTS

**Characteristics of Ery<sup>r</sup> *Campylobacter*.** The clonally related Ery<sup>r</sup> strains (except J.L.270) and isogenic Ery<sup>r</sup> transformants carried either A2074G or A2075G mutation in all three copies of the 23S rRNA gene (Table 1). Although no specific point mutation was observed in the 23S rRNA gene of J.L.270, this Ery<sup>r</sup> strain carried a mutation in the ribosomal protein L4 (G74D). Since motility is a key factor influencing the ability of *Campylobacter* to colonize the chicken intestinal tract (18, 23, 28, 39), the motility of Ery<sup>r</sup> and Ery<sup>s</sup> *Campylobacter* strains used in the present study was investigated. The Ery<sup>r</sup> and Ery<sup>s</sup> strains were equally motile under the laboratory conditions used here (data not shown). Compared to the Ery<sup>s</sup> strains, the Ery<sup>r</sup> isolates did not show apparent differences in growth kinetics in MH broth except for J.L.270, which grew slower than the rest of strains (Fig. 1). The Ery<sup>r</sup> strains harboring either the A2074G or the A2075G mutation in the 23S rRNA gene were highly resistant to erythromycin (MICs > 512 μg/ml), while J.L.270, which carried the G74D point mutations in the L4 protein, had an Ery MIC of 32 μg/ml (Table 1). All of the Ery<sup>s</sup> isolates had an Ery MIC of 2 μg/ml (Table 1).

**In vivo competition between clonally related isolates.** To determine whether the acquisition of macrolide resistance affects the fitness of *Campylobacter* in its natural host, we conducted pairwise competition experiments in chickens using clonally related strains





**FIG 2** Colonization levels of Ery<sup>s</sup> *C. jejuni* ATCC 700819 (●) and clonally related Ery<sup>r</sup> strains J.L.272 (□) and J.L.273 (△) in chickens. Each *Campylobacter* strain was individually inoculated into chickens at the concentration of  $1.4 \times 10^6$  CFU/bird (ATCC 700819),  $5.56 \times 10^5$  CFU/bird (J.L.272), and  $2.57 \times 10^5$  CFU/bird (J.L.273). Fecal samples were collected at 3, 6, and 10 days after inoculation. Each data point represents the number of *Campylobacter* obtained from an individual chicken. The mean colonization level ( $\log_{10}$  CFU/g feces) of each group is indicated by a horizontal bar.

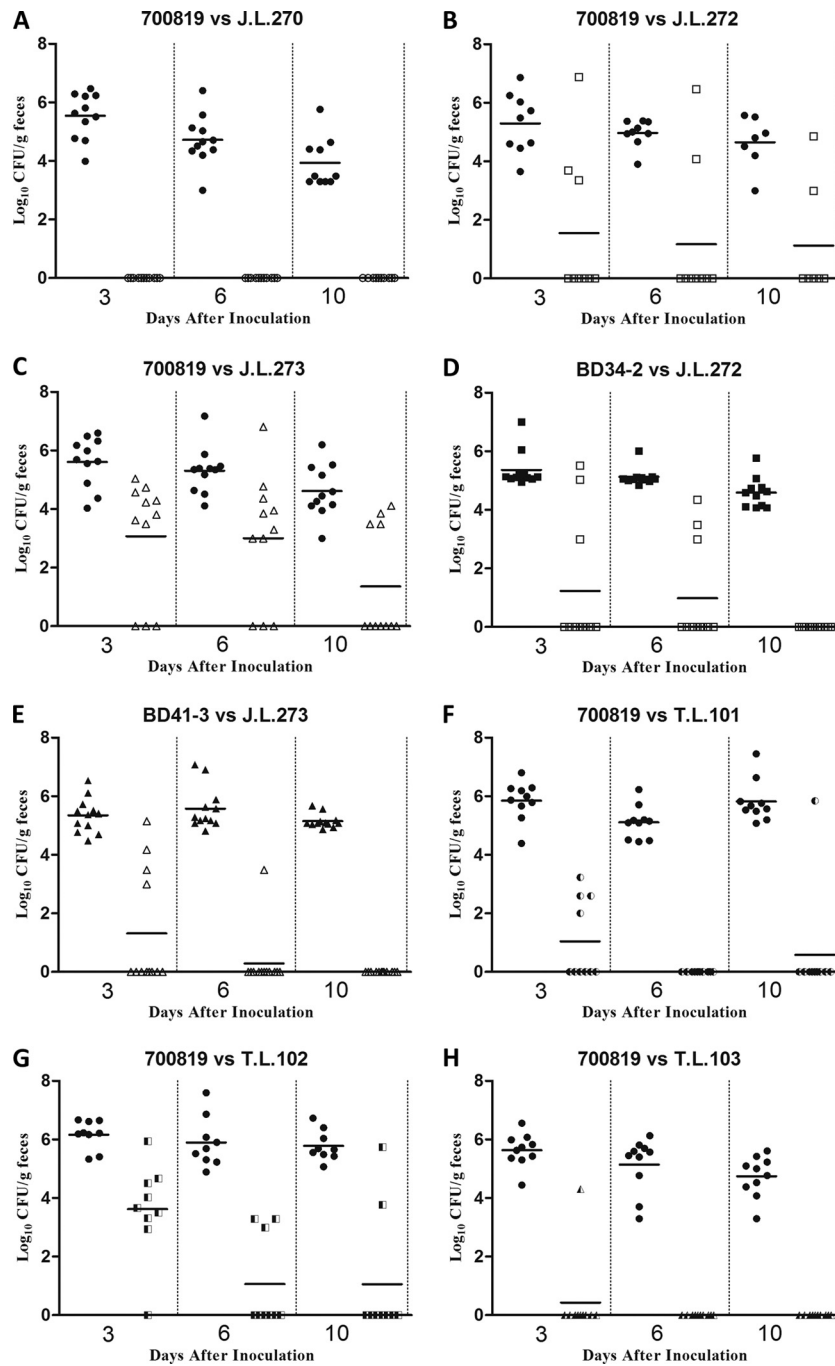
of *C. jejuni*. When the Ery<sup>s</sup> *C. jejuni* ATCC 700819 strain and its clonally related Ery<sup>r</sup> strains were individually inoculated into chickens, both Ery<sup>s</sup> and Ery<sup>r</sup> strains were able to colonize the chicken intestinal tract effectively at similar levels (Fig. 2). However, when these Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter* were concomitantly inoculated into chickens, Ery<sup>s</sup> strain outcompeted Ery<sup>r</sup> strains as early as dpi 3 (Fig. 3A, B, and C). For example, when Ery<sup>s</sup> *C. jejuni* ATCC 700819 and Ery<sup>r</sup> strain J.L.270 were coinoculated into chickens, only *C. jejuni* ATCC 700819 was detected in the chicken intestinal tract throughout the 10-day study period (Fig. 3A). Similarly, when Ery<sup>s</sup> *C. jejuni* ATCC 700819 and Ery<sup>r</sup> strain J.L.272 were coinoculated into chickens, the majority of the birds were colonized only by the Ery<sup>s</sup> strain, and the Ery<sup>r</sup> *Campylobacter* was clearly outcompeted by the Ery<sup>s</sup> strains (Fig. 3B). Although Ery<sup>r</sup> strain J.L.273 was detected in the majority of the chickens after coinoculated with Ery<sup>s</sup> *Campylobacter*, it was outnumbered by 700819 and was cleared from 7 of the 11 inoculated chickens (Fig. 3C). These results indicate that Ery<sup>r</sup> *C. jejuni* is less fit than Ery<sup>s</sup> *C. jejuni* in chickens in the absence of antibiotic selection pressure.

To confirm the fitness burden observed in Ery<sup>r</sup> *C. jejuni*, two additional pairwise competition experiments using clonally related Ery<sup>s</sup> and Ery<sup>r</sup> *C. jejuni* derived from experimentally challenged chickens (Bd34-2 versus J.L.272 and Bd41-3 versus J.L.273) were conducted. Remarkably, similar results were observed in both pairwise competition experiments, in which Ery<sup>r</sup> *C. jejuni* was outcompeted by Ery<sup>s</sup> *C. jejuni* as early as dpi 3, and no Ery<sup>r</sup> *C. jejuni* was detected in feces collected at dpi 10 from both pairwise competition groups (Fig. 3D and E). The predominance of Ery<sup>s</sup> *Campylobacter* in the coinoculated chickens was further confirmed by MIC testing of randomly selected *Campylobacter* colonies. The agar dilution test showed that 95.30% (162 of 170) of the tested *Campylobacter* colonies were susceptible to Ery (Table 2), confirming the results of the differential plating. Together, these findings demonstrated that Ery<sup>s</sup> *C. jejuni* is more fit than clonally related Ery<sup>r</sup> *C. jejuni* in chickens in the absence of antibiotic selection pressure.

**In vivo competition between isogenic isolates.** To determine whether the fitness cost observed with Ery<sup>r</sup> *C. jejuni* was associated with the specific resistance-conferring mutations in the 23S rRNA

gene, isogenic Ery<sup>r</sup> transformants were generated from the Ery<sup>s</sup> parent strain *C. jejuni* ATCC 700819 and used for pairwise competition experiments. When the Ery<sup>s</sup> parent strain and the isogenic Ery<sup>r</sup> transformant carrying the A2074G mutation in the 23S rRNA gene (T.L.101) were concomitantly inoculated into chickens, the Ery<sup>s</sup> strain quickly outcompeted T.L.101 (Fig. 3F). Although *C. jejuni* T.L.101 was isolated from four chickens at dpi 3, none of the samples collected at dpi 6 and only 1 of 10 samples from dpi 10 were positive for this Ery<sup>r</sup> *Campylobacter* strain (Fig. 3F). Likewise, when Ery<sup>r</sup> transformants carrying the A2075G mutation in the 23S rRNA gene (T.L.102 and T.L.103) and the isogenic Ery<sup>s</sup> parent strain ATCC 700819 were coinoculated into chickens, the isogenic Ery<sup>r</sup> transformants were outcompeted by the Ery<sup>s</sup> parent strain as early as dpi 3 (Fig. 3G and H). Similar to the clonally related *C. jejuni* strains, the MIC results from the agar dilution method also confirmed the results of the differential plating. All of the 55 tested *Campylobacter* colonies were susceptible to Ery (Table 2). Together, these findings strongly suggest that the fitness cost observed in Ery<sup>r</sup> *C. jejuni* is linked to the specific point mutations in the 23S rRNA gene.

**Transmission of Ery<sup>r</sup> *Campylobacter* in chickens.** To assess the ability of Ery<sup>r</sup> *Campylobacter* to transmit between chickens, we conducted a comingling experiment using three groups of chickens that were precolonized with the Ery<sup>s</sup> parent strain ATCC 700819, the isogenic Ery<sup>r</sup> transformant T.L.101, or the isogenic Ery<sup>r</sup> transformant T.L.102. Before comingling, the chickens inoculated with ATCC 700819, T.L.101, or T.L.102 were colonized at similar levels (data not shown). When chickens precolonized with ATCC 700819 (Ery<sup>s</sup>) were mingled with chickens precolonized with T.L.101 (Ery<sup>r</sup>), no Ery<sup>r</sup> *Campylobacter* was detected in the feces of Ery<sup>s</sup> inoculated chickens throughout the study period (Fig. 4A). In contrast, Ery<sup>s</sup> *Campylobacter* was detected from feces of chickens precolonized with the Ery<sup>r</sup> strain at both 7 and 14 days after comingling (Fig. 4B). Moreover, Ery<sup>s</sup> *C. jejuni* totally displaced Ery<sup>r</sup> *Campylobacter* in 2 of the 4 Ery<sup>r</sup> precolonized chickens at 14 days after comingling. Similar results were also observed when chickens precolonized with the Ery<sup>s</sup> parent strain ATCC 700819 were mingled with chickens precolonized with the isogenic Ery<sup>r</sup> transformant T.L.102. Among 5 chickens originally col-



**FIG 3** Pairwise competition between Ery<sup>s</sup> and Ery<sup>r</sup> *Campylobacter* in chickens in the absence of antibiotic selection pressure. (A to E) Competition between clonally related isolates. (A) ATCC 700819 (●) versus J.L.270 (○); (B) ATCC 700819 (●) versus J.L.272 (□); (C) ATCC 700819 (●) versus J.L.273 (△); (D) Bd34-2 (■) versus J.L.272 (□); (E) Bd41-3 (▲) versus J.L.273 (△). (F to H) Competition between isogenic strains. (F) ATCC 700819 (●) versus T.L.101 (○); (G) ATCC 700819 (●) versus T.L.102 (■); (H) ATCC 700819 (●) versus T.L.103 (▲). Each symbol represents the number of Ery<sup>s</sup> or Ery<sup>r</sup> *Campylobacter* in an individual chicken. The horizontal bars represent the mean colonization levels (log<sub>10</sub> CFU/g feces) of Ery<sup>s</sup> or Ery<sup>r</sup> strains detected at each sampling time point.

onized with Ery<sup>s</sup> *Campylobacter*, all but one were negative for Ery<sup>r</sup> *Campylobacter* at both 7 and 14 days after comingling (Fig. 4C). In contrast, 10 of 11 chickens originally colonized with Ery<sup>r</sup> *Campylobacter* were positive for Ery<sup>s</sup> strain at 7 days after comingling. At 14 days after comingling, seven of the 11 chickens precolonized by Ery<sup>r</sup> *Campylobacter* were completely replaced by the Ery<sup>s</sup> strain (Fig. 4D). Notably, the number of Ery<sup>r</sup> *Campylobacter* in the feces

of chickens precolonized with the Ery<sup>r</sup> strain T.L.102 reduced considerably, whereas the number of Ery<sup>s</sup> *Campylobacter* rapidly increased after comingling. Together, these results indicate that Ery<sup>s</sup> *Campylobacter* is highly impaired in its transmission to chickens with an established Ery<sup>s</sup> *Campylobacter* population and that it can be readily displaced by sensitive *Campylobacter* in the absence of antibiotic selection pressure.

TABLE 2 MICs of randomly selected *C. jejuni* colonies from the competition experiments

Pairwise competition	No. of isolates with an Ery MIC ( $\mu\text{g/ml}$ ) <sup>a</sup> of:					No. of Ery <sup>s</sup> and Ery <sup>r</sup> isolates <sup>b</sup> at:						Total no. of isolates (%)	
						dpi 3		dpi 6		dpi 10			
	0.25	0.5	1	2	$\geq 4$	Ery <sup>s</sup>	Ery <sup>r</sup>	Ery <sup>s</sup>	Ery <sup>r</sup>	Ery <sup>s</sup>	Ery <sup>r</sup>	Ery <sup>s</sup>	Ery <sup>r</sup>
Clonally related pairs													
700819/J.L.270	0	1	23	9	0	11	0	11	0	11	0	33 (100.0)	0 (0)
700819/J.L.272	0	0	15	7	4*	7	2	8	1	7	1	22 (84.6)	4 (15.4)
700819/J.L.273	0	1	20	8	4*	9	2	10	1	10	1	29 (87.9)	4 (12.1)
Bd34-2/J.L.272	5	6	0	26	0	11	0	12	0	14	0	37 (100.0)	0 (0)
Bd41-3/J.L.273	0	12	0	29	0	12	0	15	0	14	0	41 (100.0)	0 (0)
Total	5	20	58	79	8	50	4	56	2	56	2	162 (95.3)	8 (4.7)
Isogenic pairs													
700819/T.L.101	0	0	1	21	0	7	0	7	0	8	0	22 (100.0)	0 (0)
700819/T.L.102	0	0	3	14	0	0	0	8	0	9	0	17 (100.0)	0 (0)
700819/T.L.103	2	0	7	7	0	3	0	7	0	6	0	16 (100.0)	0 (0)
Total	2	0	11	42	0	10	0	22	0	23	0	55 (100.0)	0 (0)

<sup>a</sup> \*, The actual MICs of these isolates were  $\geq 512 \mu\text{g/ml}$ .

<sup>b</sup> The isolates were randomly selected from plating at 3, 6, and 10 days postinoculation (dpi). The breakpoint for Ery resistance is  $\geq 8 \mu\text{g/ml}$ .

To confirm the transmission of Ery<sup>s</sup> *Campylobacter* to Ery<sup>r</sup> colonized chickens, MIC testing was performed with randomly selected *Campylobacter* colonies isolated from the comingled chickens. Among *Campylobacter* colonies collected at 7 and 14 days postmingling, 62.5 and 40.0% of the colonies from chickens originally colonized with T.L.101 and T.L.102, respectively, were susceptible to Ery (Table 3). In contrast, none of the isolates from the chickens precolonized with Ery<sup>s</sup> *Campylobacter* were resistant to Ery (Table 3). These MIC data further confirmed the transmission of Ery<sup>s</sup> *Campylobacter* to chickens precolonized by Ery<sup>r</sup> *Campylobacter* and the inability of Ery<sup>r</sup> *Campylobacter* to spread to chickens with an established Ery<sup>s</sup> *Campylobacter* population.

Chickens inoculated with the Ery<sup>r</sup> strain T.L.101 that were not mingled with Ery<sup>s</sup> inoculated chickens were used as a control to assess the phenotypic stability of Ery<sup>r</sup> *Campylobacter* in chickens in the absence of antibiotic selection pressure. T.L.101 colonization in the inoculated chickens persisted for the entire experimental period (Fig. 4E). The Ery MICs for the randomly selected *Campylobacter* colonies were also  $\geq 512 \mu\text{g/ml}$  (Table 3), indicating that T.L.101 stably maintained the Ery<sup>r</sup> phenotype in the absence of antibiotic selection pressure. This result suggests that the appearance of Ery<sup>s</sup> *Campylobacter* in chickens precolonized with Ery<sup>r</sup> *Campylobacter* was not due to the reversion of the resistance phenotype.

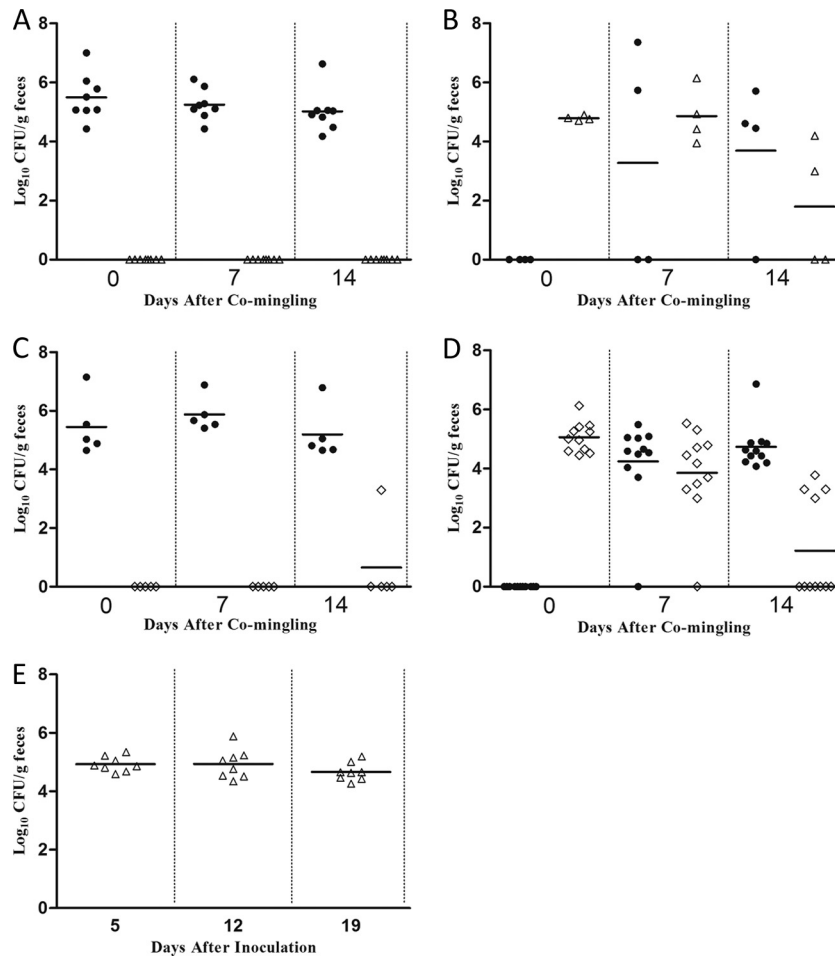
## DISCUSSION

In this study, we examined the ecological fitness of Ery<sup>r</sup> *Campylobacter* in chickens in the absence of antibiotic selection pressure by using clonally related and isogenic strains of *C. jejuni*. The results clearly indicate that acquisition of macrolide resistance entails a fitness cost for *C. jejuni* in its natural host. From the pairwise competition experiments, it was clear that Ery<sup>r</sup> *Campylobacter* was outcompeted rapidly by Ery<sup>s</sup> strains (Fig. 3). In addition, when chickens colonized with Ery<sup>r</sup> *C. jejuni* were comingled with birds colonized with Ery<sup>s</sup> *C. jejuni*, Ery<sup>s</sup> *Campylobacter* was able to transmit to and colonize in the chickens precolonized by Ery<sup>r</sup> *Campylobacter*, while Ery<sup>r</sup> *C. jejuni* failed to transmit to the chickens precolonized by Ery<sup>s</sup> *Campylobacter* (Fig. 4). Together, these

findings reveal the fitness burden of Ery<sup>r</sup> *Campylobacter* in its natural host in the absence of antibiotic selection pressure.

The use of clonally related and isogenic transformants in the chicken experiments linked the fitness burden to the point mutations in the 23S rRNA gene of Ery<sup>r</sup> mutants. However, it should be pointed out that natural transformation may not necessarily generate true isogenic mutants since other unrelated mutations might be also transferred to the transformants. To minimize this potential problem, we digested the donor DNA with EcoRV prior to transformation to release the 23S rRNA gene from the rest of the genome. In addition, we used three transformants from three independent transformations for the chicken experiments, all of which yielded the same results (Fig. 3 and Fig. 4). Furthermore, the clonally related isolates also consistently showed a significant fitness cost in the Ery<sup>r</sup> mutants. Collectively, these findings provide strong evidence that links the resistance-conferring mutation in the 23S rRNA to the reduced fitness in chickens.

It has been shown that certain mutations in the 23S rRNA gene, such as the A2074G transition, may have a negative effect on the growth rate of *Campylobacter* in culture media (19, 25). However, in the present study we found that the growth rates of the Ery<sup>r</sup> mutants carrying the A2074G or A2075G mutations were similar to that of the Ery<sup>s</sup> wild-type strain (Fig. 1). Similar to our finding, other studies (27, 34) also reported that the Ery<sup>r</sup> mutants with the A2074G transition or A2074C transversion did not show any growth defect compared to Ery<sup>s</sup> parent strains. Thus, the fitness cost for the Ery<sup>r</sup> mutants carrying mutations in the 23S rRNA genes is not attributable to a growth defect. In addition, the Ery<sup>r</sup> mutants colonized at levels similar to the Ery<sup>s</sup> strain when mono-inoculated into chickens (Fig. 2) but colonized at levels significantly lower than the Ery<sup>s</sup> strains when coinoculated into chickens (Fig. 3). These results indicate that the fitness cost was primarily due to the inability of Ery<sup>r</sup> mutants to compete with Ery<sup>s</sup> *C. jejuni*. J.L.270, which carried a mutation in the L4 protein (Table 1), grew slower than other strains (Fig. 1), and its resistance phenotype was not stable when assessed by passage in laboratory media (not shown). Thus, the fitness cost of this strain could be explained partly by the growth defect and the instability of its resistance



**FIG 4** Levels of *Campylobacter* colonization in chickens before and after comingling. (A) Colonization levels of *Campylobacter* in chickens ( $n = 8$ ) precolonized with Ery<sup>s</sup> *C. jejuni* ATCC 700819 before and after comingling with chickens ( $n = 4$ ) precolonized with Ery<sup>r</sup> transformant T.L.101. The numbers of 700819 and T.L.101 in the chickens are indicated by solid circles (●) and open triangles (△), respectively. (B) Colonization levels of *Campylobacter* in chickens ( $n = 4$ ) precolonized with Ery<sup>r</sup> transformant T.L.101 before and after comingling with chickens ( $n = 8$ ) precolonized with Ery<sup>s</sup> *C. jejuni* ATCC 700819. The numbers of 700819 and T.L.101 in the chickens are indicated by solid circles (●) and open triangles (△), respectively. (C) Colonization levels of *Campylobacter* in chickens ( $n = 5$ ) precolonized with Ery<sup>s</sup> *C. jejuni* ATCC 700819 before and after comingling with chickens ( $n = 11$ ) precolonized with Ery<sup>r</sup> transformant T.L.102. The numbers of 700819 and T.L.102 in the chickens are indicated by solid circles (●) and open diamonds (◇), respectively. (D) Colonization levels of *Campylobacter* in chickens ( $n = 11$ ) precolonized with Ery<sup>r</sup> transformant T.L.102 before and after comingling with chickens ( $n = 5$ ) precolonized with Ery<sup>s</sup> *C. jejuni* ATCC 700819. The numbers of 700819 and T.L.102 in the chickens are indicated by solid circles (●) and open diamonds (◇), respectively. (E) Colonization levels of Ery<sup>r</sup> strain T.L.101 in chickens ( $n = 8$ ) in the absence of competing Ery<sup>s</sup> *C. jejuni*. These non-mingled chickens were used as a control for the comingling study. In panels A to E, each data point represents the log<sub>10</sub> transformed CFU number/g of feces from a single bird, and the mean colonization level (log<sub>10</sub> CFU/g of feces) is indicated by a horizontal bar.

phenotype. In contrast to J.L. 270, other tested Ery<sup>r</sup> mutants stably maintained Ery resistance in both laboratory media (data not shown) and in chickens (Fig. 2 and Fig. 4E).

The fitness cost of Ery<sup>r</sup> *C. jejuni* in its natural hosts revealed in the present study is consistent with results obtained with other model systems. Two studies using *in vitro* culture systems demonstrated that Ery<sup>r</sup> *C. jejuni* was less fit than Ery<sup>s</sup> *Campylobacter* in mixed cultures (25, 27). In addition, another study showed a fitness cost of macrolide-resistant *Campylobacter* carrying an A2074C mutation in the colonization of mice (3). These studies using different systems consistently demonstrated the fitness cost of macrolide-resistant *Campylobacter*. The association between macrolide resistance and a significant burden on bacterial fitness was also observed in other bacteria. When a sequential passage of a mixed culture between macrolide-resistant and macrolide-

susceptible *Helicobacter pylori* was performed, the ratio of the resistant strain to the susceptible strain was considerably reduced per passage (31). It was also shown that clarithromycin resistance confers a fitness cost on *H. pylori* in mice and the fitness cost was reduced in clinical isolates (9). A recent study demonstrated that azithromycin resistance mutations reduced the virulence and fitness of *Chlamydia caviae* in guinea pigs (8). The reason for the reduced fitness of macrolide-resistant bacteria is unknown at present, but it is plausible to speculate that the resistance-conferring mutations in bacteria 23S rRNA gene might affect protein synthesis rates. Since macrolide antibiotics are known to inhibit protein synthesis and ribosomal assembly in bacteria (15), mutations that counteract the inhibitory effects of macrolides might alter protein synthesis, leading to a fitness disadvantage in the absence of antibiotic selection.



TABLE 3 MICs of randomly selected *C. jejuni* colonies from the comingling chickens

Comingling study ( <i>n</i> )	No. of isolates with an Ery MIC (μg/ml) <sup>a</sup> at:					No. of Ery <sup>s</sup> and Ery <sup>r</sup> isolates <sup>b</sup> at:				Total no. of isolates (%)	
	1	2	4	8	≥16	DAM 7		DAM 14		Ery <sup>s</sup>	Ery <sup>r</sup>
						Ery <sup>s</sup>	Ery <sup>r</sup>	Ery <sup>s</sup>	Ery <sup>r</sup>		
700819 and T.L.101											
700819-inoculated birds (8)	7	8	0	0	0	8	0	7	0	15 (100.0)	0 (0)
T.L.101-inoculated birds (4)	3	0	2	0	3	2	2	3	1	5 (62.5)	3 (37.5)
700819 and T.L.102											
700819-inoculated birds (5)	7	2	0	0	0	5	0	4	0	9 (100.0)	0 (0)
T.L.102-inoculated birds (11)	1	6	1	1	11	3	8	5	4	8 (40.0)	12 (60.0)
Nonmingling group											
T.L.101 (8)	0	0	0	0	17	0	9	0	8	0 (0)	17 (100.0)

<sup>a</sup> The tested isolates were randomly collected from chickens at 7 and 14 days after comingling.

<sup>b</sup> DAM, day after comingling. The breakpoint for Ery is ≥8 μg/ml.

The comingling experiments revealed an impaired transmission of Ery<sup>r</sup> *Campylobacter* to chickens precolonized by Ery<sup>s</sup> *C. jejuni* (Fig. 4). In contrast, Ery<sup>s</sup> *C. jejuni* was able to transmit to and establish colonization in chickens that were precolonized by Ery<sup>r</sup> *C. jejuni*. In some birds, the Ery<sup>r</sup> strains were totally replaced by Ery<sup>s</sup> strains after comingling. This finding implies that in the natural reservoir (chickens), where *Campylobacter* is prevalent, it is likely that Ery<sup>r</sup> *Campylobacter* encounters a difficulty in spread among birds in the absence of antibiotic selection. It should be pointed out that the Ery<sup>s</sup> *Campylobacter* isolated from the chickens previously colonized with a Ery<sup>r</sup> strain was unlikely the result of the reversion or loss of the A2074G or A2075G mutations in the 23S rRNA gene since these mutations are stable as shown in the chickens colonized with the Ery<sup>r</sup> *Campylobacter* only (Fig. 4E) and in other published work (14, 19, 27). The finding from the comingling experiments confirms and complements the results of pairwise competition experiments and indicates that Ery<sup>r</sup> *Campylobacter* is less fit than Ery<sup>s</sup> *Campylobacter* in its natural host.

Our laboratory findings reported here are consistent with the national surveillance data in the United States and Denmark. In the United States, the use of macrolide antimicrobials in animal production has been a practice for years, but the prevalence of Ery<sup>r</sup> *C. jejuni* has been at a low level (22). In Denmark, reduced use of tylosin as a growth promoter in swine led to a significant decrease in the number of Ery<sup>r</sup> *C. coli* isolated from pigs (1). Based on our laboratory observations using clonally related isolates derived from chickens and the transmission studies (Fig. 2, 3, and 4), we expect that a similar situation (i.e., outcompetition of Ery<sup>r</sup> *C. jejuni* by Ery<sup>s</sup> strains) occurs on chicken farms. However, the laboratory findings should be extrapolated to on-farm settings cautiously since many factors influence bacterial fitness. For example, the use of macrolide antimicrobials on farms would provide a selective advantage for Ery<sup>r</sup> *Campylobacter* and facilitates the maintenance of the resistant population. In addition, compensatory mutations could occur under prolonged selection, which might reduce the fitness cost associated with Ery resistance. Furthermore, the ecological fitness of *C. jejuni* can be influenced by other bacterial and environmental factors. Thus, the fitness picture of *C. jejuni* in animal reservoirs is more complex than that revealed in a laboratory setting and is likely influenced by interactions of many different factors.

The reduced fitness of Ery<sup>r</sup> *Campylobacter* is a stark contrast to

fluoroquinolone (FQ)-resistant *Campylobacter*, which can rapidly outcompete FQ-susceptible strains and can be persistently maintained in chickens in the absence of antibiotic selection pressure (37). This difference indicates that different antimicrobial resistance mechanisms have varied effects on the fitness of *Campylobacter* in animal reservoir. The fitness burden of Ery<sup>r</sup> *Campylobacter* in antibiotic-free environments, the low spontaneous mutation rate for macrolide resistance (34), and the slow process of macrolide resistance development (32, 34) may have all contributed to the relatively low prevalence of resistance to macrolide antimicrobials compared to FQ resistance in *C. jejuni*. Although withdrawal of FQ antimicrobials in the United States has thus far had a limited effect on the prevalence of FQ-resistant *Campylobacter* in poultry (35, 42, 43), management of macrolide antibiotic usage on farms is likely to be an effective way to reduce the prevalence of macrolide resistance in *Campylobacter*.

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