Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure

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***Campylobacter jejuni*, a major foodborne human pathogen, has become increasingly resistant to fluoroquinolone (FQ) antimicrobials. By using clonally related isolates and genetically defined mutants, we determined the fitness of FQ-resistant *Campylobacter* in chicken (a natural host and a major reservoir for *C. jejuni*) in the absence of antibiotic selection pressure. When monoinoculated into the host, FQ-resistant and FQ-susceptible *Campylobacter* displayed similar levels of colonization and persistence in the absence of FQ antimicrobials. The prolonged colonization in chickens did not result in loss of the FQ resistance and the resistance-conferring point mutation (C257 → T) in the *gyrA* gene. Strikingly, when coinoculated into chickens, the FQ-resistant *Campylobacter* isolates outcompeted the majority of the FQ-susceptible strains, indicating that the resistant *Campylobacter* was biologically fit in the chicken host. The fitness advantage was not due to compensatory mutations in the genes targeted by FQ and was linked directly to the single point mutation in *gyrA*, which confers on *Campylobacter* a high-level resistance to FQ antimicrobials. In certain genetic backgrounds, the same point mutation entailed a biological cost on *Campylobacter*, as evidenced by its inability to compete with the FQ-susceptible *Campylobacter*. These findings provide a previously undescribed demonstration of the profound effect of a resistance-conferring point mutation in *gyrA* on the fitness of a major foodborne pathogen in its natural host and suggest that the rapid emergence of FQ-resistant *Campylobacter* on a worldwide scale may be attributable partly to the enhanced fitness of the FQ-resistant isolates.**

colonization | *gyrA* mutation | poultry

Antimicrobial resistance in bacterial pathogens has become a serious threat to public health. There is a general notion that the acquisition of drug resistance, particularly the resistance mediated by chromosomal mutations, entails a biological cost for pathogens, resulting in reduced fitness in the absence of antibiotic selection pressure (1, 2). However, evidence has accumulated that *in vivo*-selected or clinically derived isolates may develop compensatory mutations that reduce the fitness cost associated with antimicrobial resistance (3–7). Different environments (*in vitro* vs. *in vivo*) may select for different compensatory mutations and varied levels of restoration of fitness (8). Even without compensatory mutations, drug-resistant mutants may show little or no fitness cost (9). One important finding revealed by previous studies is that clinically derived drug-resistant isolates display diverse fitness changes, with some showing a biological cost and others showing no cost or even enhanced fitness (4, 10). The retention of ecological fitness in resistant pathogens creates a significant barrier for the elimination of resistant organisms by natural selection.

Campylobacter jejuni, a Gram-negative microaerobic bacterium, is a common causative agent of human enterocolitis (11). For antibiotic treatment of campylobacteriosis, erythromycin and fluoroquinolone (FQ) are the drugs of choice (12). However, *Campylobacter* has become increasingly resistant to FQ antimicrobials. Although FQ-resistant (FQ^R) *Campylobacter* occurs in clinical settings (13, 14), poultry are considered a significant source of FQ^R *Campylobacter*, because FQ antimicrobials have been used for the production of meat chickens, and the majority of domestically acquired cases of human campylobacteriosis result from consumption of undercooked chicken or food contaminated by raw chicken (11, 15). Therefore, reducing the occurrence and spread of FQ^R *Campylobacter* in poultry is considered an important step in the control of foodborne campylobacteriosis. Although it is known that FQ^R mutants rapidly occur during treatment with FQ antimicrobials (13, 16, 17), it is unclear whether the selected resistant *Campylobacter* is able to persist and compete with FQ-susceptible (FQ^S) *Campylobacter* in the absence of antibiotic selection pressure.

Campylobacter resistance to FQ antimicrobials is conferred by point mutations in the *gyrA* gene and the function of the multidrug efflux pump CmeABC (12, 16, 18). *gyrA* encodes the A subunit of DNA gyrase, which is a key enzyme involved in DNA replication and transcription (19). Resistance-conferring mutations in *gyrA* render the enzyme unsusceptible to the action of FQ antimicrobials. Acquisition of high-level FQ resistance in *Campylobacter* does not require stepwise accumulation of point mutations in *gyrA*. Instead, a single point mutation in *gyrA* can create clinically relevant levels of resistance to FQ antimicrobials (16, 20–22). Specific mutations at positions T86, D90, and A70 in GyrA were linked to FQ resistance in *C. jejuni* (12, 16, 22). Specifically, the T86I change [mediated by the C257 → T (C257T) mutation in the gene] is the most commonly observed mutation in FQ^R *Campylobacter* isolates and has been associated with high-level [ciprofloxacin minimal inhibitory concentration (MIC) ≥ 16 μg/ml] resistance to FQ, whereas the D90N and T86K mutations are less common and are associated with intermediate-level FQ resistance (16, 20–22). No mutations in *gyrB* have been associated with FQ resistance in *Campylobacter* (18, 23, 24), and the lack of *parC* (16, 18, 23–26) in *Campylobacter* has excluded the role of *parC* mutations in *Campylobacter* resistance to FQ antimicrobials. Although the function of multidrug efflux pump CmeABC is essential for FQ resistance, overexpression of *cmeABC* is not observed in FQ^R isolates (16).

The simplicity of the FQ resistance mechanism (single-step point mutations in *gyrA*) in *Campylobacter* and the easy access to its natural host (chicken) have provided an ideal system to address the impact of resistance-conferring mutations on the

Abbreviations: cfu, colony-forming units; CI, competition index; DAI, days after inoculation; FQ, fluoroquinolone; FQ^R, FQ-resistant; FQ^S, FQ-susceptible; MH, Mueller–Hinton; MIC, minimal inhibitory concentration.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY196924 and AY621065).

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Table 1. Characteristics of *Campylobacter* strains used

Strains	Isolation date		Cipro MIC, μg/ml	Mutation in *gyrA*
	DAI	DIQT		
S3B			0.125	None
FQ^{S}*				
62101S1	5	N/T	0.125	None
62101S2	5	N/T	0.125	None
62301S1	7	N/T	0.125	None
62301S2	7	N/T	0.125	None
62501S4	9	N/T	0.125	None
62501S5	9	N/T	0.125	None
70201S1	16	N/T	0.125	None
70201S16	16	N/T	0.125	None
FQ^{R}†				
62101R26	5	1	>32	C257T
62101R31	5	3	>32	C257T
62301R33	7	5	>32	C257T
62301R38	7	5	>32	C257T
62501R31	9	7	>32	C257T
62501R32	9	7	>32	C257T
70201R31	16	14	>32	C257T
70201R35	16	14	>32	C257T
Defined mutants				
62101S1R1‡			>32	C257T
62301S2R1§			>32	C257T
62301S2R2§			>32	C257T
62301S2R3¶			>32	C257T

DAI, DAI with strain S3B; DIQT, days after initiation of quinolone (enrofloxacin) treatment; Cipro, ciprofloxacin; N/T, not treated with enrofloxacin.
*S3B derivatives isolated at different DAI from the control group of chickens inoculated with S3B, but not treated with enrofloxacin.
†S3B derivatives isolated at different DAI from the group of chickens inoculated with S3B and then treated with 50 ppm enrofloxacin, which was initiated at DAI 4 and continued until DAI 8.
‡62101S1 transformed with the PCR product of *gyrA* containing the C257T mutation.
§62301S2 transformed with the PCR product of *gyrA* containing the C257T mutation.
¶62301S2 transformed with the genomic DNA of 62301R33.

ecological fitness of antimicrobial-resistant pathogens. Understanding the fitness of FQ^{R} *Campylobacter* in its natural host will help us to determine whether the resistant pathogen is able to transmit and persist in the absence of antibiotic selection pressure, which will be useful for predicting and preventing the spread of FQ^{R} *Campylobacter* in animal reservoirs. Toward this end, we determined the fitness of FQ^{R} *Campylobacter* in chickens by using *in vivo*-derived clonally related isolates and isogenic mutants. It was revealed that the *in vivo*-derived FQ^{R} *Campylobacter* isolates outcompeted the majority of their clonally related FQ^{S} *Campylobacter* in the absence of antibiotic selection pressure, and the enhanced fitness in the resistant mutants was directly linked to the C257T mutation in the *gyrA* gene. These findings indicate that the single point mutation not only confers a high-level resistance to FQ antimicrobials but also modulates the *in vivo* fitness of *Campylobacter*.

Materials and Methods

Bacterial Strains and Culture Conditions. The *C. jejuni* strains used in this study are listed in Table 1. The parent strain S3B originally was isolated from chicken feces by this laboratory and is sensitive to FQ antimicrobials (ciprofloxacin MIC = 0.125 μg/ml). In a previous study (16) conducted to determine the emergence of FQ^{R} *Campylobacter* in chickens in response to the treatment with enrofloxacin, S3B was inoculated into several groups of chickens, some of which were treated subsequently with enrofloxacin, whereas others remained untreated as controls. Series of *Campylobacter* isolates were collected from the treated or nontreated chickens. Some of the isolates were used for the pairwise competitions conducted in this study (Table 1). The selected isolates on the same day after inoculation (DAI) were from different chickens. The *Campylobacter* strains were grown routinely in Mueller–Hinton (MH) broth or on MH agar plates at 42°C under microaerophilic conditions. When needed, the culture medium was supplemented with ciprofloxacin (4 μg/ml).

PCR and Construction of Genetically Defined Mutants. PCR was used to amplify different regions of *gyrA* and *gyrB* (see Table 2, which is published as supporting information on the PNAS web site, for primer sequences) from various isolates. Natural transformation was used to generate genetically defined mutants containing the C257T mutation in *gyrA*. Briefly, the *gyrA* sequence containing the specific C257T mutation was amplified by PCR from isolates 62101R26 or 62301R33 by using primers GyrAF1 and GyrAR1 (Table 2). The PCR products containing the C257T T mutation were used to transform 62101S1 and 62301S2. In addition, the genomic DNA purified from 62301R33 (containing the C257T mutation in *gyrA*) also was used to transform 62301S2. Natural transformation was performed as described by Wang and Taylor (27) by using 3 μg of donor DNA (PCR products or genomic DNA). Transformants were selected by plating on MH plates supplemented with ciprofloxacin (4 μg/ml). Multiple transformants were obtained from each transformation, and representative clones including 62101S1R1, 62301S2R1, 62301S2R2, and 62301S2R3 (Table 1) were used in pairwise competition experiments. The *gyrA* and *gyrB* genes in the transformants used for the *in vivo* competitions were fully sequenced, confirming the presence of the C257T mutation in *gyrA* and the lack of any mutations in *gyrB*.

***In Vivo* Colonization and Persistence of *Campylobacter*.** Specific pathogen-free White Leghorns were used to determine the colonization and persistence of different *Campylobacter* strains in the absence of antibiotic selection pressure. The birds were hatched in the specific pathogen-free chicken facility of the Food Animal Health Research Program of Ohio State University. Before use, all chickens were screened for *Campylobacter* by culturing cloacal swabs using MH agar plates containing *Campylobacter*-specific growth supplements and selective agents (Oxoid, Basingstoke, U.K.). No *Campylobacter* was detected in any of the birds before inoculation. Groups I (n = 18) and II (n = 18) were inoculated with isolate 62301R33 or S3B, respectively, at a dose of 10^7 colony-forming units (cfu) per chicken via oral gavage. Nonmedicated feed was given to the chickens ad libitum. FQ antimicrobials are not approved for use in feed additives in the U.S., and the feed extracts did not affect the growth rate of FQ^{S} or FQ^{R} *Campylobacter* in culture medium (data not shown). After inoculation, fecal samples were collected from each bird by means of cloacal swabs at different time points. Total *Campylobacter* colonies and FQ^{R} colonies in each sample were determined by using the *Campylobacter* selective plates and ciprofloxacin-containing (4 μg/ml) plates, respectively, as described in ref. 16.

***In Vivo* Competition.** Newly hatched broiler chickens were obtained from commercial hatcheries. The chickens were tested negative for *Campylobacter* by culturing cloacal swabs before use. Seven to 10 chickens were used for each group. For competition experiments, each pair of *Campylobacter* isolates were grown separately in MH broth for 48 h under microaerophilic conditions and mixed in various ratios before given to chickens. The actual cfu in each inoculation mixture was determined by plating on MH agar plates (for total cfu) and MH agar plates with 4 μg/ml of ciprofloxacin (for FQ^{R} cfu). Each chicken received 100

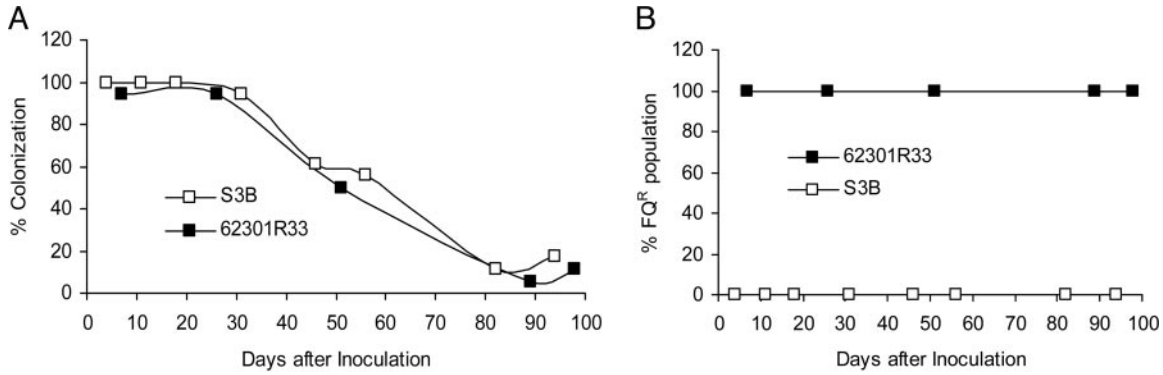

Fig. 1. Colonization and persistence of FQ^R *Campylobacter* in the chicken host. (*A*) The number of chickens colonized with *Campylobacter* in each group (n = 18). Each data point represents the percentage of chickens colonized at each sampling time. The detection limit of the plating method is 100 cfu per g of fecal contents. (*B*) The resistance rates of *Campylobacter* isolated from each group. Ten to 15 isolates from each group at each sampling time were randomly selected and tested with ciprofloxacin Etest strips. MICs of ≥4.0 μg/ml are regarded as resistant.

μl of the mixture with a cell density of $\approx 10^7$ cfu via oral gavage. Control groups were monoinoculated with either a FQ^R or FQ^S isolate. After inoculation, cloacal swabs were collected from the chickens at different intervals for culturing *Campylobacter*. As described above, each fecal suspension was serially diluted in MH broth and plated simultaneously onto two different types of culture media: conventional *Campylobacter* selective plates for recovering the total *Campylobacter* colonies and the selective MH plates supplemented with 4.0 μg/ml ciprofloxacin for recovering FQ^R *Campylobacter* colonies. Competition index (CI) was calculated as described by Khachatryan *et al.* (28) using the following formula: CI = (X − Y)/(X + Y), where X is the number of FQ^R colonies and Y is the number of FQ^S colonies. A positive CI value indicates that the FQ^R strain is more fit than the FQ^S population, whereas a negative CI value indicates that the FQ^S strain is more fit than the FQ^R population. When the CI is close to 1 or −1, it shows the dominance by FQ^R *Campylobacter* or FQ^S *Campylobacter*, respectively. Within a group, the CI values of individual birds across time points were used to test the null hypothesis (CI = 0) that there was no difference between the competing populations by using Student's t test. Repeated-measures ANOVA was used to compare the differences in CI values across groups. To confirm the results from the differential plating, 10–15 *Campylobacter* colonies were selected randomly for each group from the conventional selective plates (no ciprofloxacin) at each sampling time and tested for antibiotic susceptibility with Etest strips (AB Biodisk, Solna, Sweden). The selected colonies also were used for pulsed-field gel electrophoresis, which was performed according to procedures described in ref. 29.

Antimicrobial Susceptibility Test. The MIC of ciprofloxacin was determined by using Etest strips (AB Biodisk) as described in the manufacturer's instructions and in ref. 16. The FQ^S S3B isolate with a ciprofloxacin MIC of 0.125 μg/ml was used as an internal control for testing the isolates obtained from the chickens. The detection limit of the Etest for ciprofloxacin was 32 μg/ml. The MIC of cholic acid was measured by using a broth macrodilution method as described in ref. 30.

GenBank Accession Nos. The assigned GenBank accession nos. for *gyrA* and *gyrB* in strain S3B are AY196924 and AY621065, respectively.

Results

Characteristics of FQ^R *Campylobacter*. As determined by pulsed-field gel electrophoresis using *kpn*I- or *Sma*I-digested genomic DNA, the isolates used for pairwise competitions in this study all showed a genetic fingerprint identical to that of S3B (see Fig. 3, which is published as supporting information on the PNAS web site). Except for the C257T mutation (resulting in the T86I change) in the *gyrA* gene, no other mutations were detected in *gyrA* and *gyrB* of the FQ^R isolates. These findings plus the fact that *Campylobacter* lacks *parC*/*parE* suggested that no compensatory mutations occurred in the genes targeted by FQ antimicrobials in the FQ^R isolates. Compared with the clonally related or isogenic FQ^S *Campylobacter*, the FQ^R isolates did not show any growth defect or enhancement when separately cultured in MH broth with no added antibiotics (data not shown), indicating that the acquisition of FQ resistance in *Campylobacter* did not change its growth rate in culture medium. This finding was consistent with a previous observation made with *in vitro*-selected spontaneous FQ^R mutants of *C. jejuni* UA535 (20). Because motility is a key factor for *Campylobacter* colonization of chickens (31), we compared the motility of various *Campylobacter* isolates by using 0.4% Bacto agar (Difco). The FQ^R and FQ^S isolates were equally motile under the conditions used in this study (data not shown). Because the multidrug efflux pump CmeABC is essential for *in vivo* adaptation by mediating bile resistance in the intestinal tract (32), we also determined the expression level of CmeABC in the *Campylobacter* isolates. Immunoblotting indicated that the FQ^S and FQ^R isolates produced similar amounts of CmeABC proteins when grown in MH broth with no added antibiotics (data not shown). The MIC of cholic acid was also the same for both FQ^R and FQ^S *Campylobacter*. These observations indicated that the acquisition of FQ resistance did not cause obvious changes in the genotypic and phenotypic characteristics of *Campylobacter* when assayed *in vitro*.

***In Vivo* Persistence of FQ^R *Campylobacter* in the Absence of Antibiotic Selection Pressure.** To determine whether FQ^R *Campylobacter* was able to colonize and persist in chicken (a natural host for *Campylobacter*) in the absence of antibiotic selection pressure, specific pathogen-free chickens were infected with either 62301R33 or S3B. After the inoculation, the chickens were quickly colonized by the strains. The colonization maintained at a high level from DAI 4–31 in both groups and then decreased gradually, with only two or three chickens positive for *Campylobacter* at the end of the experiment (Fig. 1*A*). The number of *Campylobacter* shed in feces also showed similar trends between the two groups (see Fig. 4, which is published as supporting information on the PNAS web site), ranging from as high as 10^8 cfu/g of feces between DAI 4–31 to 0–10^5 cfu/g of feces at the

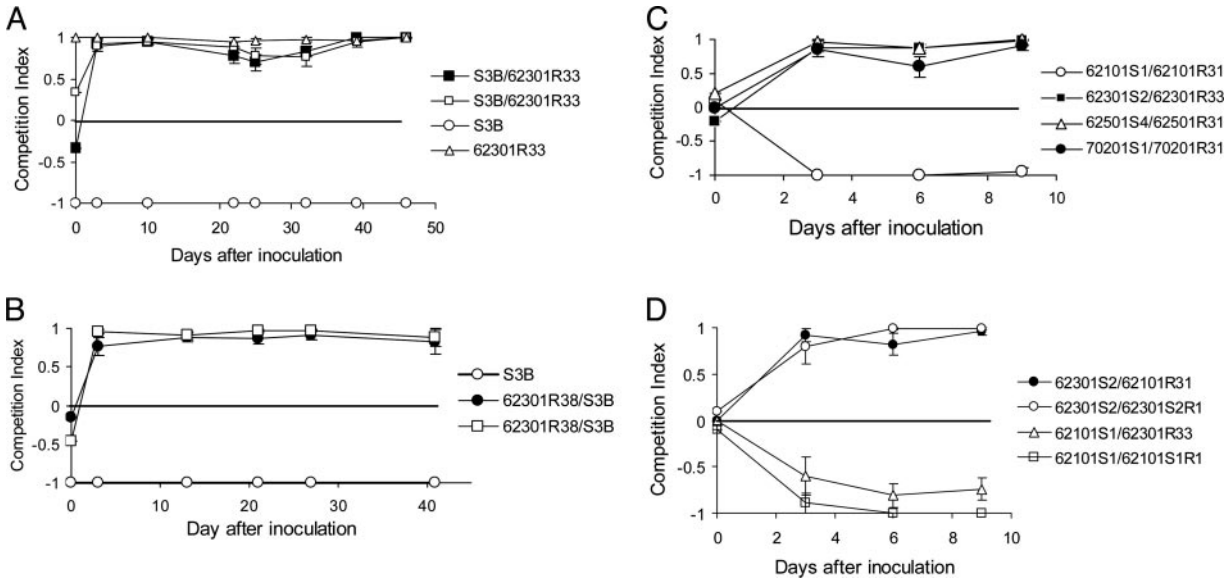

Fig. 2. Pairwise competition between FQ^R and FQ^S *Campylobacter* in the chicken host. Each data point represents the mean CI ± SE. Some SEs are smaller than the symbols. Seven to 10 birds were used in each group.

end of the experiment. Both differential plating and testing of individual colonies with Etest showed that all of the isolates from the group inoculated with 62301R33 were FQ^R (ciprofloxacin MIC > 32 μg/ml), and all of the isolates from the group inoculated with S3B were FQ^S (ciprofloxacin MIC = 0.125 μg/ml; Fig. 1*B*). Sequencing of the *gyrA* gene in the representative isolates collected from the group inoculated with 62301R33 revealed the same C257T mutation in all of the isolates. Pulsed-field gel electrophoresis analysis of the genomic DNA of the isolates showed a macrorestriction profile identical to that of 62301R33. These findings indicated that FQ^R *Campylobacter* colonized the chicken host efficiently and did not lose the *gyrA* mutation and its associated resistance to FQ for a prolonged period in the absence of antimicrobial selection pressure.

***In Vivo* Competition.** To determine whether FQ^R *Campylobacter* was ecologically fit *in vivo*, we first compared the *in vivo* derived FQ^R isolates 62301R33 and 62301R38 with the parent strain S3B by using pairwise competitions. After inoculation with either a single isolate or mixed populations, all of the groups were colonized by *Campylobacter* within 3 days (Fig. 2 *A* and *B*). No apparent differences in the colonization rate (the number of chickens colonized and the number of *Campylobacter* shed in feces) were observed among the groups, regardless of the FQ-resistance profile of the inoculum. Differential plating by using ciprofloxacin-containing plates showed that the group inoculated with S3B alone shed homogeneous FQ^S *Campylobacter*, whereas the groups inoculated with 62301R33 or 62301R38 shed homogeneous FQ^R *Campylobacter* during the entire course of the experiment. However, in the groups inoculated with mixtures of S3B/62301R33 or S3B/62301R38, the FQ^R population outcompeted the FQ^S *Campylobacter* ($P < 0.001$). The two groups for each pair of isolates yielded the same results regardless of the initial ratios (27–67% of FQ^R *Campylobacter*) of the two populations in the inoculum (Fig. 2 *A* and *B*). Once becoming dominant, the resistant population persisted in the chickens until the end of the experiments, which lasted for 42–46 days. Testing of randomly selected *Campylobacter* colonies (10 colonies per group per time point) by using Etest confirmed the results of differential plating. Together, these findings demonstrated that when coinoculated into chickens, the FQ^R *Campylobacter* outcompeted the FQ^S parent strain and quickly became the dominant population in the chickens.

To further examine the fitness of FQ^R *Campylobacter*, a series of clonally related isolates derived from S3B-inoculated chickens were used for pairwise competition experiments (Table 1 and Fig. 2*C*). Each of the pairs contained a FQ^R and a FQ^S isolate, which were collected from the same experimental study at the same sampling time point (Table 1). All of these isolates were cultured fewer than three passages in MH broth before being used for the *in vivo* competition experiments. Among the eight pairs of isolates, FQ^R *Campylobacter* outcompeted the FQ^S population ($P < 0.01$) in six (62301S2/62301R33 and 62301S1/62301R38, collected at DAI 7; 62501S4/62501R31 and 62501S5/62501R32, collected at DAI 9; and 70201S1/70201R31 and 70201S16/70201R35, collected at DAI 16) of the pairs, whereas the FQ^S *Campylobacter* outcompeted FQ^R ($P < 0.001$) in the two pairs (62101S1/62101R31 and 62101S2/62101R26) of isolates collected at DAI 5. The CI values of the groups inoculated with 62101S1/62101R31 or 62101S2/62101R26 were significantly different from the CI values of the groups inoculated with other pairs ($P < 0.001$). Representative results with four of the pairs were shown in Fig. 2*C*. Independent replication experiments by using pair 62301S2/62301R33 or 62101S1/62101R31 further confirmed the competition outcomes. Within each pair, the dominance by the "winner" population happened within 3 days after the inoculation (Fig. 2*C*), suggesting that the competition occurred quickly in the infected birds. Together, these results indicated the six FQ^R isolates collected at DAI 7, 9, and 16 were biologically more fit than their clonally related FQ^S populations when coinoculated into chickens, whereas the two FQ^R isolates derived at DAI 5 could not compete with the FQ^S isolates derived from the same DAI.

The different competition outcomes between the *Campylobacter* isolates collected at DAI 5 and those collected at DAI 7, 9, and 16 might be due to the appearance of compensatory mutations in the later FQ^R isolates or subtle genetic differences between the FQ^S isolates collected at different time points. To test which possibility was most likely, we conducted the exchange of clinical isolates in pairwise competitions in chickens. If fitness-enhancing compensatory mutations had occurred in the later FQ^R isolates, we expected that 62301R33 would be able to compete with 62101S1. As shown in Fig. 2*D*, when 62301R33

(collected at DAI 7) was paired with 62101S1 (collected at DAI 5), the resistant population could not compete with the sensitive isolate ($P < 0.01$), although the same FQ^R isolate outcompeted the FQ^S isolate 62301S2 (Fig. 2*C*). Conversely, when 62101R31 was paired with 62301S2, the resistant isolate showed a clear fitness advantage ($P < 0.001$; Fig. 2*D*), even though 62101R31 was unable to compete with 62101S1 (Fig. 2*C*). This finding demonstrated that the FQ^R isolates collected at DAI 5 and 7 showed the same phenotype in chickens, which did not support the hypothesis that compensatory mutations had occurred in the later FQ^R isolates. This observation also suggested that the FQ^S isolates collected at DAI 5 and 7 might have subtle genetic differences, which could influence the outcomes of the competition between FQ^R and FQ^S *Campylobacter*.

To determine whether *in vitro* passage could affect the *in vivo* competition, isolates 62301S2 and 62301R33 were subjected to subculture in MH broth with no added antibiotics for 10 consecutive passages (≈160 generations). Then, the two isolates were mixed and inoculated into chickens (10 chickens per group). Again, 62301R33 outcompeted 62301S2 in the inoculated birds ($P < 0.001$). No significant differences ($P > 0.05$) were observed between the CI values of the group inoculated with the low-passage mixture and those of the group inoculated with the high-passage mixture, indicating that the limited *in vitro* passage did not change the outcome of the *in vivo* competition.

The *gyrA* Mutation Is Directly Linked to the *in Vivo* Fitness Change. To determine whether the fitness changes observed with the FQ^R isolates was due to the resistance-conferring *gyrA* mutation, the specific C257T mutation was introduced into 62101S1 and 62301S2 by using natural transformation to create genetically defined mutants (Table 1). These isogenic mutants were paired with the recipient strains for pairwise competition in chickens. When 62301S2R1 was paired with 62301S2, it rapidly outcompeted 62301S2 ($P < 0.001$; Fig. 2*D*) in two independent experiments. Furthermore, two additional isogenic mutants, 62301S2R2 and 62301S2R3, which were obtained from independent transformation experiments, also outcompeted 62301S2 ($P < 0.001$), indicating that the C257T mutation in *gyrA* was responsible for the fitness enhancement in the FQ^R isolates. There was no significant difference ($P > 0.05$) between the CI values of the groups inoculated with the isogenic pairs and the CI values of the group inoculated with 62301S2/62301R33, further suggesting that compensatory mutation was not involved in the competition of 62301R33 with 62301S2. When 62101S1R1 (an isogenic transformant of 62101S1) was paired with 62101S1, the FQ^R population was outcompeted by the FQ^S population ($P < 0.001$; Fig. 2*D*), indicating that introduction of the C257T mutation into the 62101S1 background reduced the *in vivo* fitness of the isolate. These results directly linked the fitness changes in the FQ^R isolates to the *gyrA* mutation and highlight the diverse effect of the mutation on *Campylobacter* fitness in different genetic backgrounds.

Discussion

This work represents the first report, to our knowledge, that documents the profound effect of a resistance-conferring mutation in *gyrA* on the fitness of FQ^R foodborne pathogens in animal reservoirs. Traditionally, studies assessing the fitness of drug-resistant pathogens mainly were conducted *in vitro* by using culture media, and only a few were performed *in vivo* by using animal models (4, 8, 28, 33, 34). Because the results from *in vitro* competition experiments do not necessarily reflect the outcomes of *in vivo* competition (4, 34), assessing the impact of antibiotic resistance on fitness should be ideally performed in the natural host of a pathogen. In this study, we examined the persistence and ecological fitness of FQ^R *Campylobacter* in chickens in the absence of selection pressure by using clonally related isolates and genetically defined mutants. The results clearly indicate that acquisition of FQ resistance in *Campylobacter* does not impair its colonization and persistence in chickens in the absence of antibiotic selection pressure (Fig. 1). Prolonged colonization *in vivo* did not result in the reversion or loss of the specific *gyrA* mutation (C257T), indicating that the resistance-conferring mutation can be stably maintained in the chicken host. This finding is consistent with the report that FQ^R *Campylobacter* was present in chicken flocks with no history of usage of FQ antimicrobials (35). Because the production cycle for broiler chickens is typically 6–7 weeks, findings from this study suggest that, once present in a flock, FQ^R *Campylobacter* will be able to stay in the flock until the slaughter age, potentially contaminating the chicken carcasses in processing plants.

As determined by pairwise competition experiments, the *in vivo*-derived FQ^R isolates outcompeted the parent strain and the majority of their clonally related FQ^S populations (Fig. 2 *A–C*). The enhanced fitness in FQ^R *Campylobacter* cannot be explained by compensatory mutations but is directly linked to the resistance-conferring mutation in *gyrA*. This conclusion is based on the following facts: (*i*) the FQ^R isolates had no compensatory mutations in GyrA and GyrB, which are the targets of FQ antimicrobials; (*ii*) exchange of FQ^R isolates collected at different time points did not change the outcome of the competitions (Fig. 2*D*); and (*iii*) introduction of the specific *gyrA* mutation (C257T) into the FQ^S isolates by natural transformation reproduced the competition outcomes of the clonally related FQ^R isolates (Fig. 2*D*). Any compensatory mutations, if they had existed in the *in vivo*-derived FQ^R isolates, should be eliminated in the genetically defined mutants because they were constructed by using donor DNA that only contained the specific C257T mutation in *gyrA*. The fitness advantage in *Campylobacter* conferred by the *gyrA* mutation is in contrast to the fitness costs incurred from antimicrobial resistance in other bacteria, in which compensatory mutations within or outside of the target genes are often needed to partially restore their fitness (1, 5, 8, 34).

One interesting finding of this study is the diverse effect of the C257T mutation in *gyrA* on *Campylobacter* fitness. When the C257T mutation was introduced into the 62101S1 background, it incurred a biological cost because the isogenic transformant (62101S1R1) was outcompeted by 62101S1 (Fig. 2*D*). In contrast, the same mutation enhanced *Campylobacter* fitness in the 62301S2 background. Isolates 62101S1 and 62301S2 were clonally related and obtained from the same group of chickens at DAI 5 and 7, respectively, after inoculation with S3B (16). Although they had identical *gyrA* and *gyrB* sequences and did not show any apparent differences as determined by pulsed-field gel electrophoresis, there might be some subtle genetic dissimilarities in the two FQ^S isolates, affecting the outcome of the competition. At this stage, it is unclear what the differences are and how the differences occurred in the chickens. Nevertheless, findings from this study suggest that, depending on the genetic background of the recipient strain, the C257T mutation can either enhance or reduce the fitness of *Campylobacter* in the chicken host.

The exact mechanisms involved in the competition in chickens are unknown. It should be pointed out that when separately inoculated into chickens at a dose of 10^7 cfu per bird, FQ^R and FQ^S *Campylobacter* showed similar levels of colonization and persistence in the host (Figs. 1 and 2). This observation indicates that, without competition, FQ^S and FQ^R *Campylobacter* are equally capable of colonizing chickens. When coinoculated into chickens at the same time, the two populations tended to exclude each other, resulting in the dominance of either population (Fig. 2). The outcompetition of one by another may not be attributed to differences in growth rates, cell motility, or CmeABC expression. In addition, we measured the competence for natural transformation in 62101S2, 62101R26, 62301S2, and 62301R33.

By using an independent selection marker (chloramphenicol resistance) inserted in *C. jejuni* genome as the donor DNA, we found that the four isolates had similar transformation frequencies (data not shown), indicating that the FQ^R and FQ^S *Campylobacter* were comparable in their ability to take up exogenous DNA and suggesting that the competition outcome is unlikely due to differences in genetic competence of the tested strains.

Regardless of the mechanism involved in the *in vivo* competition, it can be concluded that the observed fitness changes in *Campylobacter* likely are due to the effect of the C257T mutation in *gyrA* (Fig. 2*D*). DNA gyrase consists of two subunits (GyrA and GyrB) assembled into a functional A2B2 holoenzyme, which catalyzes ATP-dependent negative supercoiling of DNA and plays an important role in DNA replication, transcription, and recombination (19). FQ antimicrobials form a stable complex with the enzyme and trap the enzyme on DNA, inhibiting DNA replication and transcription. Many bacterial pathogens have developed resistance to FQ antimicrobials by acquiring point mutations in DNA gyrase (36). It has been known in *Escherichia coli* that resistance-conferring mutations in GyrA can affect the supercoiling activity of the enzyme (37). Because DNA supercoiling is important for gene expression, it is possible that certain resistance-associated mutations in DNA gyrase may affect the transcription and expression of multiple or a subset of genes, which may consequently affect the fitness of the resistant pathogen in the intestinal tract. It will be interesting to determine whether the single C257T point mutation affects DNA supercoiling and gene expression in *Campylobacter* and whether the altered gene expression influences *Campylobacter* fitness *in vivo*.

Spontaneous point mutations conferring FQ resistance occur in *gyrA* of *Campylobacter* at a frequency of $\approx 5 \times 10^{-8}$ when cultured in conventional media (20). If this mutation rate also applies to the *in vivo* infection, the number of spontaneous FQ^R mutants in FQ^S-infected chickens would be too low to be detected by the direct-plating method, which has a detection limit of 100 cfu/g of feces. Even though the spontaneous mutant may have a fitness advantage, it is unlikely that a single mutant can compete with 10^8 FQ^S *Campylobacter* without the help of antibiotic selection pressure. It is possible that a "critical mass" is required for FQ^R *Campylobacter* to successfully compete with FQ^S *Campylobacter* in the absence of selection pressure. Findings from this study were obtained by using a simplified system (clonally related isolates or isogenic mutants directly inoculated into chickens) and should be interpreted in the context of the complex nature of *Campylobacter* colonization and transmission in animal reservoirs, which are affected by multiple factors including bacterial virulence determinants, environmental conditions, management practices, and host immune status. The interaction of the resistance-conferring mutation (C257T) in *gyrA* with other bacterial factors in influencing *Campylobacter* fitness and the ability of FQ^R *Campylobacter* to transmit under different conditions as well as in the presence of genetically diverse competing strains remain to be determined in future studies. Nevertheless, results from this study suggest that the fitness enhancement conferred by the single point mutation in GyrA of FQ^R *Campylobacter* may have helped the emergence and spread of FQ^R isolates on poultry farms and in clinical settings. This possibility is supported by the fact that the majority of FQ^R *Campylobacter* isolates derived from animal reservoirs or human patients bear the C257T mutation in *gyrA* (21, 38, 39). Our findings also suggest that FQ^R *Campylobacter* may be able to persist in the animal reservoirs even without the usage of FQ antimicrobials, highlighting the need for effective measures to control the emergence and spread of FQ^R *Campylobacter*.

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