



# Development of Fluoroquinolone Resistance through Antibiotic Tolerance in *Campylobacter jejuni*

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**ABSTRACT** Antibiotic tolerance not only enables bacteria to survive acute antibiotic exposures but also provides bacteria with a window of time in which to develop antibiotic resistance. The increasing prevalence of *Campylobacter jejuni* isolates resistant to clinically important antibiotics, particularly fluoroquinolones (FQs), is a global public health concern. Currently, little is known about antibiotic tolerance and its effects on resistance development in *C. jejuni*. Here, we show that exposure to ciprofloxacin or tetracycline at concentrations 10 and 100 times higher than the MIC induces antibiotic tolerance in *C. jejuni*, whereas gentamicin or erythromycin treatment causes cell death. Interestingly, FQ resistance rapidly develops in *C. jejuni* after tolerance induction by ciprofloxacin and tetracycline. Furthermore, after tolerance is induced, alkyl hydroperoxide reductase (AhpC) plays a critical role in reducing FQ resistance development by alleviating oxidative stress. Together, these results demonstrate that exposure of *C. jejuni* to antibiotics can induce antibiotic tolerance and that FQ-resistant (FQ<sup>R</sup>) *C. jejuni* clones rapidly emerge after tolerance induction. This study elucidates the mechanisms underlying the high prevalence of FQ<sup>R</sup> *C. jejuni* and provides insights into the effects of antibiotic tolerance on resistance development.

**IMPORTANCE** Antibiotic tolerance compromises the efficacy of antibiotic treatment by extending bacterial survival and facilitating the development of mutations associated with antibiotic resistance. Despite growing public health concerns about antibiotic resistance in *C. jejuni*, antibiotic tolerance has not yet been investigated in this important zoonotic pathogen. Here, our results show that exposure of *C. jejuni* to ciprofloxacin or tetracycline leads to antibiotic tolerance development, which subsequently facilitates the emergence of FQ<sup>R</sup> *C. jejuni*. Importantly, these antibiotics are commonly used in animal agriculture. Moreover, our study suggests that the use of non-FQ drugs in animal agriculture promotes FQ resistance development, which is crucial because antibiotic-resistant *C. jejuni* is primarily transmitted from animals to humans. Overall, these findings increase our understanding of the mechanisms of resistance development through the induction of antibiotic tolerance.

**KEYWORDS** *Campylobacter jejuni*, antibiotic tolerance, fluoroquinolone resistance, oxidative stress

Antibiotic tolerance refers to the ability of an entire population of susceptible bacteria to withstand antibiotic treatment for prolonged periods of time (1). This is explicitly different from persistence, which occurs in a subpopulation of bacterial clones (2). Whether achieved through tolerance or persistence, extended survival of pathogenic bacteria under antibiotic treatment can cause prolonged and/or recurrent infections, resulting in adverse clinical outcomes and treatment failure (1, 2). Furthermore, the extended survival of tolerant

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and persistent bacteria under antibiotic treatment can provide a window of time for antibiotic-resistant bacteria to emerge (3, 4).

*Campylobacter* spp., particularly *Campylobacter jejuni*, is a leading bacterial cause of gastroenteritis, accounting for 400 to 500 million cases of diarrhea worldwide per year (5). *C. jejuni* colonizes the gastrointestinal tracts of a wide range of animals and is transmitted to humans mainly through foodborne routes (6). In addition, the increasing prevalence of *Campylobacter* isolates resistant to clinically important antibiotics is a serious public health concern. Importantly, the prevalence of fluoroquinolone-resistant (FQ<sup>R</sup>) *Campylobacter* is increasing at an alarming rate and has significantly compromised the efficacy of this critically important antibiotic class (7). Approximately 28.5% of campylobacteriosis cases in the United States are associated with FQ resistance (8), leading to adverse patient outcomes (9, 10). Other countries have a much higher prevalence of FQ<sup>R</sup> *Campylobacter*, for example, 76% in Italy (11), 87% in China (12), and 89% in Thailand (13). The World Health Organization (WHO) classifies FQ<sup>R</sup> *Campylobacter* as one of the high-priority pathogens for which new antimicrobials should be developed (14).

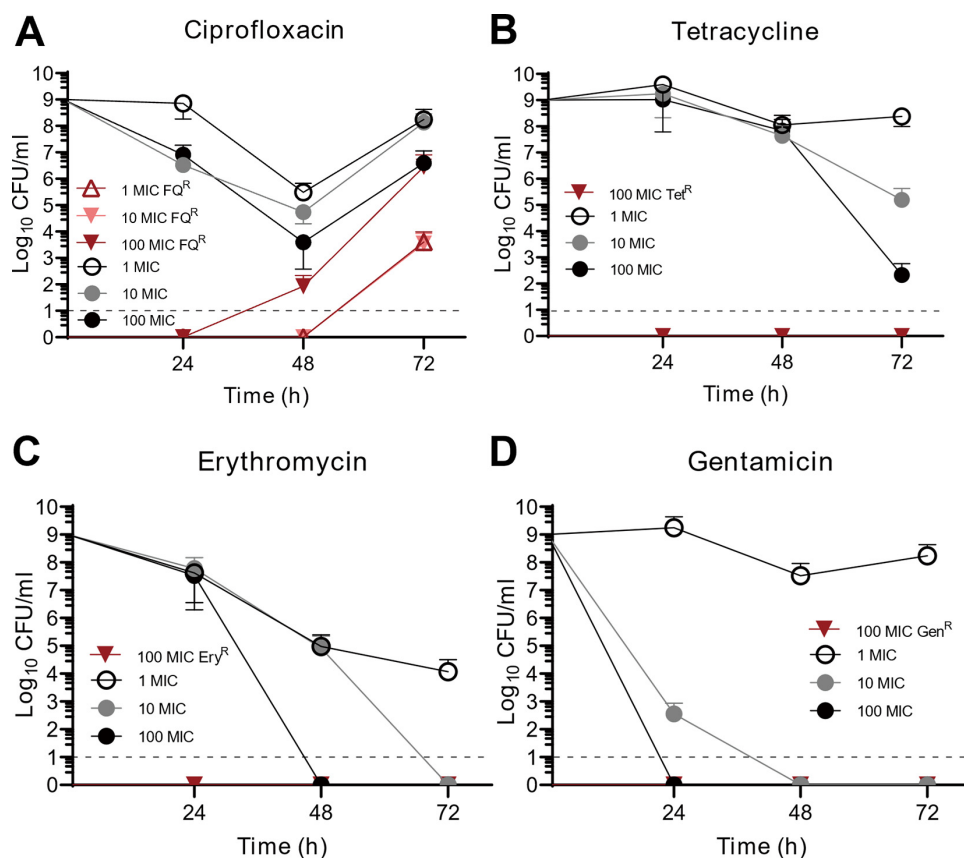
Despite public health concerns about antibiotic resistance in *Campylobacter*, little is known about whether *Campylobacter* can develop antibiotic tolerance and how tolerance may affect the development of antibiotic resistance. In this study, we demonstrate that *C. jejuni* develops antibiotic tolerance by exposure to high concentrations of ciprofloxacin (an FQ drug) or tetracycline. Importantly, FQ resistance is rapidly developed through antibiotic tolerance induced by FQs and non-FQ antibiotics. Our results suggest that antibiotic tolerance plays an important role in the development of FQ resistance in *C. jejuni*.

## RESULTS

### Tolerance induction in *C. jejuni* by exposure to high concentrations of antibiotics.

We hypothesized that *C. jejuni* develops tolerance during antibiotic exposure. To test this hypothesis, we utilized several different antibiotics: ciprofloxacin, erythromycin, tetracycline, and gentamicin. These were chosen because *C. jejuni* is likely to be exposed to these antibiotics during treatment of human campylobacteriosis or in livestock production (15–18). FQs (e.g., ciprofloxacin) are the most commonly used oral antibiotic for empirical treatment of gastroenteritis (15), erythromycin is the drug of choice for treating campylobacteriosis, and gentamicin and tetracyclines are alternative drugs for treating systemic infections with *Campylobacter* (16). In addition, tetracyclines are widely used for food-producing animals, such as cattle, one of the major natural hosts of *C. jejuni* (17–19). To evaluate bacterial survival during antibiotic treatment, we conducted time-kill assays by treating *C. jejuni* cultures with these antibiotics at concentrations 10 and 100 times higher than the MIC and measuring the viability of *C. jejuni* after washing out the drug.

We found that *C. jejuni* survived treatment with ciprofloxacin or tetracycline (Fig. 1). Conversely, treatment with either gentamicin or erythromycin led to bacterial killing (Fig. 1). Translation inhibitors, such as tetracycline and erythromycin, are generally considered bacteriostatic but exhibited bactericidal activity when used at high concentrations (Fig. 1B and C). Resistance, tolerance, and persistence are differentiated primarily based on the kinetics of time-kill assays (1, 2, 20). Resistant bacteria survive and grow in the presence of an antibiotic. In contrast, tolerant cells can survive but not replicate in the presence of an antibiotic (1, 2, 20). Tolerance applies to an entire bacterial population, whereas persistence, or heterotolerance, is used to describe a tolerant subpopulation of cells (1, 2, 20, 21). Thus, in a time-kill assay, tolerant bacteria survive for long periods of time, compared to susceptible bacteria. However, persistence shows an initial killing pattern similar to that of susceptible bacteria, followed by a long survival duration, which generates a biphasic killing curve (1, 2, 20, 22). Our results show a pattern of delayed bacterial killing by these antibiotics, which is indicative of tolerance and not persistence (Fig. 1). Interestingly, treatment with high concentrations of ciprofloxacin did not lead to immediate bacterial killing. *C. jejuni* growth was initially inhibited but resumed after 48 h due to the emergence of FQ<sup>R</sup> *C. jejuni* (Fig. 1A). These results show that tolerance can be induced in *C. jejuni* by exposure to high concentrations of ciprofloxacin

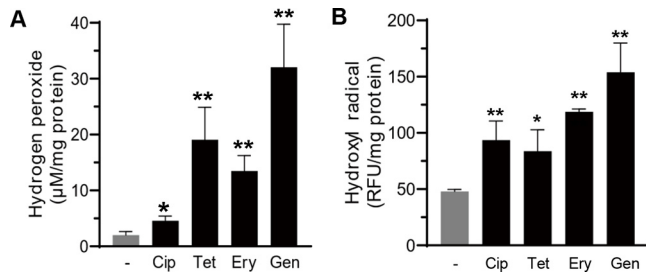


**FIG 1** Induction of antibiotic tolerance in *C. jejuni* by exposure to high concentrations of ciprofloxacin (A), tetracycline (B), erythromycin (C), and gentamicin (D). The results show the means and standard deviations of the results of three independent experiments. Black lines indicate the total *C. jejuni* levels, and red lines show the levels of *C. jejuni* resistant to the antibiotic used for the treatment. Antibiotic concentrations were calculated based on the MICs of ciprofloxacin, tetracycline, erythromycin, and gentamicin in *C. jejuni* NCTC 11168, which were 0.063  $\mu\text{g}/\text{mL}$ , 0.031  $\mu\text{g}/\text{mL}$ , 1  $\mu\text{g}/\text{mL}$ , and 0.5  $\mu\text{g}/\text{mL}$ , respectively. The dotted lines indicate the limits of detection. Tet<sup>R</sup>, tetracycline resistant; Ery<sup>R</sup>, erythromycin resistant; Gen<sup>R</sup>, gentamicin resistant.

or tetracycline, and FQ resistance rapidly develops during the window of time provided by antibiotic tolerance.

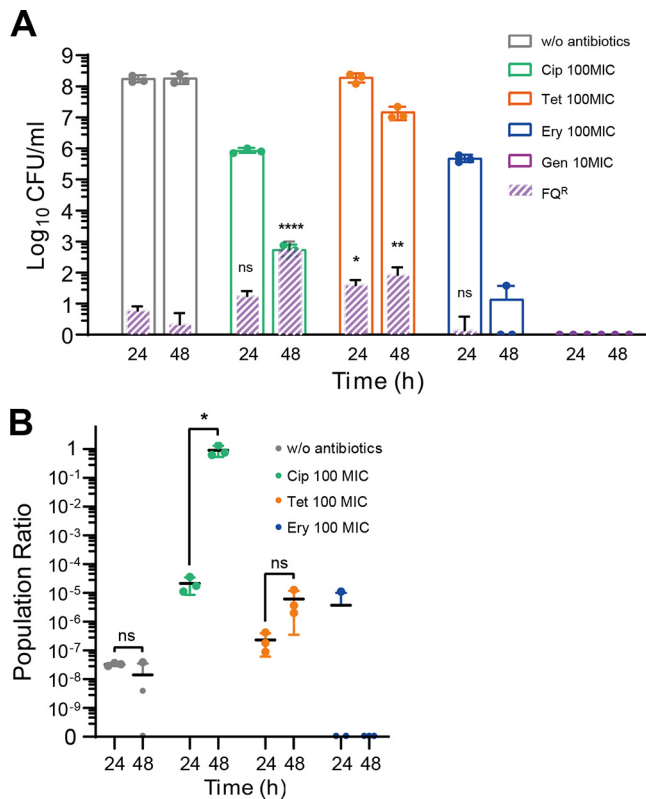
**Increases in oxidative stress by exposure to high concentrations of antibiotics in *C. jejuni*.** Since oxidative stress is a general mechanism for bacterial lethality by bactericidal antibiotics (23), we hypothesized that *C. jejuni* must overcome increased oxidative stress after acute antibiotic exposure to maintain tolerance. To test the hypothesis, we measured hydrogen peroxide and hydroxyl radicals in *C. jejuni* during exposure to high levels of antibiotics. As predicted, the levels of hydrogen peroxide and hydroxyl radicals were significantly elevated after antibiotic treatment (Fig. 2). Notably, hydroxyl radical levels were significantly increased by treatment with 10 $\times$  MIC of erythromycin or gentamicin (Fig. 2B), which likely accounts for the rapid killing of *C. jejuni* by these antibiotics (Fig. 1C and D). Compared to these antibiotics, treatment with ciprofloxacin or tetracycline induced the formation of hydroxyl radicals at lower levels (Fig. 2). These results show that the level of hydroxyl radical formation correlates with whether antibiotic treatment leads to bacterial killing or induces antibiotic tolerance in *C. jejuni*.

**FQ resistance development by exposure to tolerance-inducing antibiotics in *C. jejuni*.** Oxidative stress during antibiotic treatment leads to increased DNA damage and subsequent mutations (23). Since antibiotic treatment increased hydroxyl radical formation in *C. jejuni*, we hypothesized that the frequency of FQ resistance mutations would increase. To test this hypothesis, we exposed *C. jejuni* to 100 $\times$  MIC ciprofloxacin, 100 $\times$  MIC tetracycline, 100 $\times$  MIC erythromycin, and 10 $\times$  MIC gentamicin. Interestingly, FQ<sup>R</sup> *C. jejuni* clones rapidly emerged after exposure to tolerance-inducing antibiotics (Fig. 3A). Remarkably, the number of FQ<sup>R</sup> cells



**FIG 2** Formation of hydrogen peroxide (A) and hydroxyl radicals (B) by antibiotic exposure in *C. jejuni*. The levels of hydrogen peroxide and hydroxyl radicals were measured after exposure to 10× MIC of ciprofloxacin (Cip; 0.63 µg/mL), tetracycline (Tet; 0.31 µg/mL), erythromycin (Ery; 10 µg/mL), and gentamicin (Gen; 5 µg/mL) for 24 h. The results show the means and standard deviations of an experiment with three samples. The experiments were repeated three times and produced similar results. Statistical analysis was conducted using Student’s *t* test in comparison with an untreated control. RFU, relative fluorescence units; \*, *P* < 0.05; \*\*, *P* < 0.01.

arising during tetracycline treatment was significantly higher than that in a control without antibiotic treatment (Fig. 3A). The ratio of FQ<sup>R</sup> to total *C. jejuni* was significantly increased in the presence of ciprofloxacin over 48 h (Fig. 3B). This suggests that FQ<sup>R</sup> *C. jejuni* populations are enriched in the presence of ciprofloxacin during antibiotic tolerance. The emergence of FQ<sup>R</sup> *C. jejuni* was not consistently observed in the presence of antibiotics with strong bactericidal activity. Gentamycin treatment rapidly killed *C. jejuni*, and erythromycin treatment



**FIG 3** Development of fluoroquinolone (FQ) resistance during antibiotic tolerance in *C. jejuni*. (A) The emergence of FQ-resistant (FQ<sup>R</sup>) *C. jejuni* cells under treatment with 100× MIC of ciprofloxacin (Cip; 6.3 µg/mL), tetracycline (Tet; 3.1 µg/mL), and erythromycin (Ery; 100 µg/mL) and 10× MIC of gentamicin (Gen; 5 µg/mL). Gen was treated at a lower concentration due to its strong bactericidal activity. The results show the means and standard deviations of the levels of the total *C. jejuni* population (empty bars) and the FQ<sup>R</sup> *C. jejuni* population (patterned filled bars) of the results from three independent experiments. Statistical analysis was performed using Student’s *t* test in comparison with an untreated control at the same sampling time. ns, nonsignificant; \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*\*, *P* < 0.0001. (B) Enrichment of FQ<sup>R</sup> *C. jejuni* during antibiotic tolerance. The results show the ratio of FQ<sup>R</sup> *C. jejuni* to the total *C. jejuni*. Statistical analysis was conducted using Student’s *t* test. ns, nonsignificant; \*, *P* < 0.05.

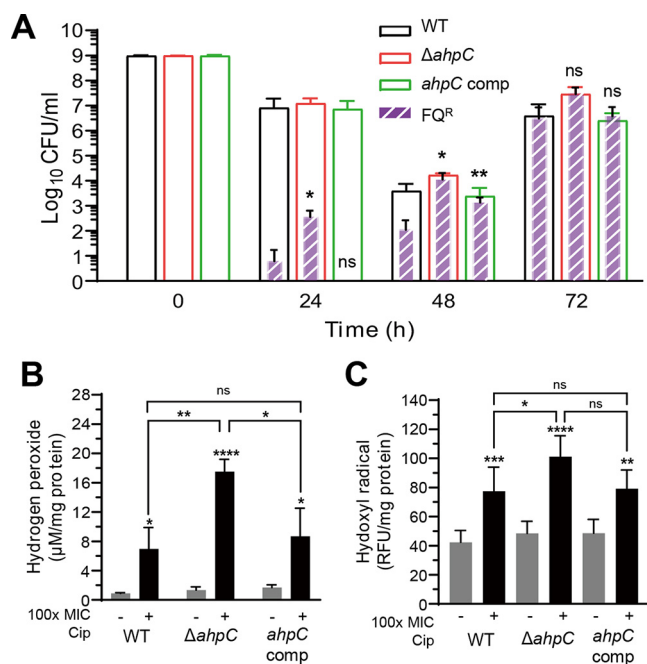
induced the emergence of FQ<sup>R</sup> *C. jejuni* isolates sporadically (one in three experiments) after 24 h but not after 48 h, likely due to bacterial killing by erythromycin (Fig. 3B).

FQ<sup>R</sup> *C. jejuni* clones can emerge by spontaneous DNA mutations in the absence of antibiotics. Time-kill assays begin with large numbers of bacterial cells, and it is possible that FQ<sup>R</sup> mutants exist in that initial population. We observed FQ<sup>R</sup> mutants present in the control without antibiotic treatment (Fig. 3). However, antibiotic treatment further increased the levels of FQ<sup>R</sup> mutants compared to the control, both in the presence of antibiotic selective pressure (i.e., ciprofloxacin treatment) and its absence (i.e., tetracycline) (Fig. 3). Ciprofloxacin treatment can enrich FQ<sup>R</sup> *C. jejuni* clones that emerge by spontaneous DNA mutations prior to and during antibiotic exposure. In contrast, tetracycline treatment would not enrich FQ<sup>R</sup> clones due to the lack of selection pressure. Despite this, we observed that FQ<sup>R</sup> *C. jejuni* was more prevalent in the tetracycline-treated culture than in a nontreated control (Fig. 3). These results suggest that tetracycline treatment induces a higher rate of spontaneous mutations than that under untreated control conditions. Moreover, the emergence of FQ<sup>R</sup> *C. jejuni* was more frequent during exposure to 100× MIC ciprofloxacin than to 10× MIC ciprofloxacin (Fig. 1A), indicating that the increased oxidative stress induced by a higher concentration of ciprofloxacin may promote the emergence of FQ<sup>R</sup> *C. jejuni*. In addition, we also observed that tolerance-inducing antibiotics increased oxidative stress and FQ resistance development in other *C. jejuni* strains, including ATCC 33291 and ATCC 33560, which is used as a quality control strain for antimicrobial susceptibility testing (see Fig. S1 in the supplemental material). Altogether, these results suggest that FQ resistance rapidly develops in *C. jejuni* during antibiotic tolerance induced by FQs and non-FQ antibiotics.

#### **AhpC reduces FQ resistance development in *C. jejuni* during antibiotic tolerance.**

Our results suggest that oxidative stress during antibiotic exposure leads to the emergence of FQ<sup>R</sup> *C. jejuni* in antibiotic-tolerant cells. *C. jejuni* harbors a single copy of genes encoding alkyl hydroperoxide reductase (AhpC), catalase (KatA), and superoxide dismutase (SodB) (24), which are all involved in the detoxification of different reactive oxygen species. Using *ahpC*, *kataA*, and *sodB* knockout mutants, we examined the contribution of each antioxidant enzyme to the prevention of FQ resistance development in *C. jejuni* during antibiotic tolerance. Remarkably, an  $\Delta$ *ahpC* mutation significantly increased the frequency of FQ resistance development compared to that in the wild type (WT) (Fig. 4A). We observed no effect of  $\Delta$ *kataA* and  $\Delta$ *sodB* mutations compared to WT (Fig. S2). In the  $\Delta$ *ahpC* mutant, ciprofloxacin treatment markedly increased the accumulation of hydrogen peroxide (Fig. 4B), a substrate of AhpC, and hydroxyl radicals (Fig. 4C). These results suggest that increased oxidative stress facilitates the development of FQ resistance during antibiotic tolerance in *C. jejuni* and that AhpC reduces the development of FQ resistance by alleviating oxidative stress.

**Oxidative stress response regulators modulating *ahpC* transcription affect FQ resistance development during antibiotic tolerance in *C. jejuni*.** To further confirm the role of AhpC in FQ resistance development during antibiotic tolerance, we used mutants defective in the regulation of oxidative stress responses. *C. jejuni* uses PerR (25) and CosR (26) to respond to oxidative stress. PerR is a repressor of *ahpC* transcription (25, 27). CosR is a response regulator that positively regulates *ahpC* transcription (26). Thus, a  $\Delta$ *perR* mutation increases *ahpC* transcription by derepression, and CosR overexpression increases *ahpC* transcription by positive regulation. We measured the development of FQ resistance after inducing antibiotic tolerance in a CosR overexpression strain (Fig. 5A) and a  $\Delta$ *perR* mutant (Fig. 5B). The frequency of FQ resistance development was substantially reduced in the  $\Delta$ *perR* and CosR overexpression mutants. However, when *ahpC* was deleted in these mutants, the frequency of FQ resistance was increased to levels similar to those of the WT (Fig. 5), confirming that AhpC prevents FQ resistance development during antibiotic tolerance. Moreover, we observed that the level of *ahpC* transcription was significantly increased in *C. jejuni* during treatment with high concentrations of antibiotics (Fig. S3). This indicates that the antioxidation function of AhpC is required during antibiotic tolerance in *C. jejuni*. These results demonstrate that AhpC plays an important role in preventing the development of FQ resistance through tolerance induction in *C. jejuni*.

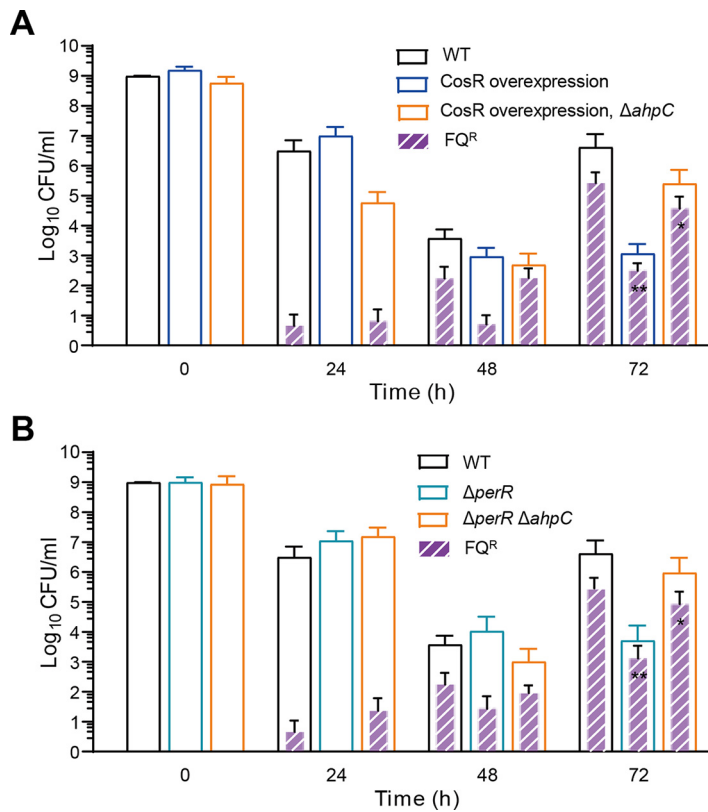


**FIG 4** Enhanced development of fluoroquinolone (FQ) resistance in an  $\Delta ahpC$  mutant during antibiotic tolerance induced by ciprofloxacin. (A) Significant increase in FQ resistance development in an  $\Delta ahpC$  mutant during antibiotic tolerance induced by 100 $\times$  MIC of ciprofloxacin (6.3  $\mu$ g/mL). The results show the means and standard deviations of the levels of total *C. jejuni* (empty bars) and FQ<sup>R</sup> *C. jejuni* (patterned filled bars) of the results from three independent experiments. Statistical analysis was conducted using Student's *t* test in comparison with WT. ns, nonsignificant; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; *ahpC* comp, *ahpC*-complemented strain. Increased production of hydrogen peroxide (B) and hydroxyl radicals (C) in an  $\Delta ahpC$  mutant. The + and - signs indicate the presence and absence of 100 $\times$  MIC of ciprofloxacin (6.3  $\mu$ g/mL), respectively. Statistical analysis was conducted using Student's *t* test. ns, nonsignificant; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

## DISCUSSION

The data in this study show that antibiotic tolerance induced by exposure to FQs and non-FQ drugs promotes the development of FQ resistance in *C. jejuni*. Studies demonstrate that antibiotic tolerance or persistence induced by intermittent antibiotic exposures or drug combinations precedes the emergence of antibiotic resistance mutations in *Escherichia coli* and *Staphylococcus aureus* (3, 4). In *E. coli*, FQs induce the SOS response in persister cells and increase DNA mutations through the induction of error-prone DNA polymerases (28). However, *C. jejuni* lacks SOS response systems and error-prone DNA polymerases. Instead, our data demonstrate that AhpC plays a critical role in preventing FQ resistance development. AhpC is involved in the detoxification of organic peroxides and low physiological levels of hydrogen peroxide (29). Deletion of *ahpC* results in the accumulation of hydrogen peroxide and hydroxyl radicals (Fig. 4B and C), which increases the frequency of FQ resistance development during antibiotic tolerance (Fig. 4A). Hydrogen peroxide is generated by the accidental autoxidation of nonrespiratory flavoproteins (30, 31) and dismutation of superoxide (32) and is converted to hydroxyl radicals through the iron-catalyzed Fenton reaction (32). Thus, enzymatic degradation of hydrogen peroxide by AhpC can reduce oxidative DNA damage by toxic hydroxyl radicals and consequently decrease FQ resistance development during antibiotic tolerance in *C. jejuni* (Fig. 6). In *E. coli*, antibiotic treatment can lead to cell death without involving the formation of hydrogen peroxide under anaerobic conditions (33). It is not fully understood how reactive oxygen species (ROS) affect antimicrobial killing in *C. jejuni*. *C. jejuni* is an obligate microaerophile that grows optimally at low oxygen concentrations (34, 35). We observed that the level of hydrogen peroxide increased during exposure to high concentrations of antibiotics (Fig. 2A). Given these findings, antimicrobial lethality is likely to be associated with ROS generation during antibiotic treatment in *C. jejuni*, which may determine whether antibiotic treatment leads to bacterial killing or tolerance induction.

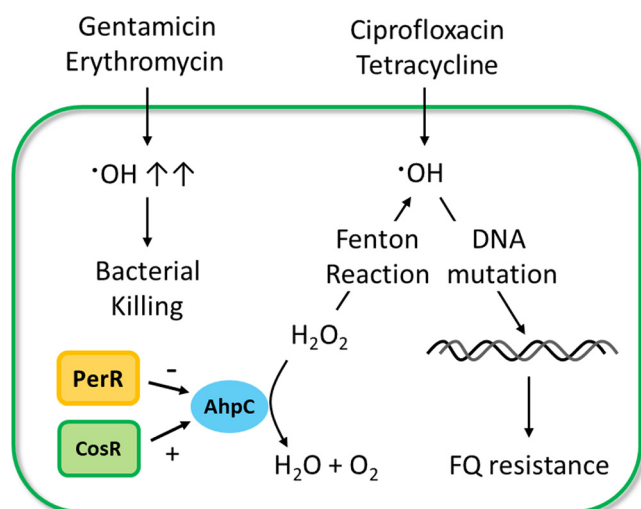




**FIG 5** Effects of *ahpC* regulation on FQ resistance development in *C. jejuni* during antibiotic tolerance induced by ciprofloxacin. (A) FQ resistance development in a CosR overexpression strain and a CosR overexpression strain with a  $\Delta$ *ahpC* mutation. (B) FQ resistance development in a  $\Delta$ *perR* mutant and a  $\Delta$ *perR*  $\Delta$ *ahpC* mutant. The concentration of ciprofloxacin is 100 $\times$  MIC (6.3  $\mu$ g/mL). The results show the means and standard deviations of the levels of total *C. jejuni* (empty bars) and FQ<sup>R</sup> *C. jejuni* (patterned filled bars) of the results from three independent experiments. Statistical analysis was conducted using two-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

Exposure to high concentrations of antibiotics significantly increased the level of *ahpC* transcription (see Fig. S3 in the supplemental material). Interestingly, the level of *ahpC* transcription was higher in cells treated with tolerance-inducing antibiotics (i.e., ciprofloxacin and tetracycline) than in those treated with bacteria-killing antibiotics (i.e., erythromycin and gentamicin), although the bacteria-killing antibiotics produced more hydroxyl radicals than the tolerance-inducing antibiotics (Fig. 2C). There are two possibilities for this observation. First, the regulation of *ahpC* transcription may be critical for bacterial killing processes by antibiotics. Without significant upregulation of *ahpC*, *C. jejuni* may be more susceptible to antibiotics, which may result in bacterial killing. The other possibility is that due to the bactericidal activity of the bacteria-killing antibiotics, *C. jejuni* is already likely to undergo the process of bacterial death, which may slow down cellular responses to stress conditions.

FQ<sup>R</sup> *C. jejuni* rapidly emerged after tolerance induction, but the change in the size of the FQ<sup>R</sup> *C. jejuni* population was dependent on the antibiotic used for tolerance induction (Fig. 3). FQ<sup>R</sup> *C. jejuni* appeared within 24 h under tetracycline treatment and remained at similar levels at 48 h (Fig. 3B). During ciprofloxacin treatment, the population of FQ<sup>R</sup> *C. jejuni* significantly increased over time (Fig. 3B). This can be ascribed to selective pressure exerted by ciprofloxacin. Ciprofloxacin treatment killed FQ-susceptible *C. jejuni* cells over time, increasing the ratio of FQ<sup>R</sup> to total *C. jejuni* (Fig. 3B). Growth was arrested in tolerant cells during antibiotic treatment; therefore, the increase in FQ<sup>R</sup> *C. jejuni* cells may be due to an increased frequency in resistant cells during extended antibiotic exposure. It is also possible that selective enrichment increased the number



**FIG 6** Schematic diagram of FQ resistance development through tolerance induction in *C. jejuni*. Exposure to high concentrations of gentamicin or erythromycin induces the formation of hydroxyl radicals at high levels, which can lead to cell death. Ciprofloxacin and tetracycline exposures do not generate lethal levels of hydroxyl radicals and cause DNA mutations, conferring FQ resistance. AhpC decreases the level of hydroxyl radicals by degrading hydrogen peroxide, which can be converted to hydroxyl radicals by the Fenton reaction. The *ahpC* transcription is negatively and positively regulated by PerR and CosR, respectively.

of FQ<sup>R</sup> *C. jejuni* cells over time, because the FQ<sup>R</sup> *C. jejuni* populations did not increase significantly during tetracycline treatment (Fig. 3B). These data suggest that *C. jejuni* may not be in complete dormancy during antibiotic tolerance. Further studies are needed to understand the physiology of antibiotic-tolerant *C. jejuni*.

FQs are the oral antibiotic most commonly used to treat various bacterial infections (15, 36). FQs bind to DNA gyrase and topoisomerase IV, generate double-stranded breaks in DNA, and lead to bacterial cell death (37, 38); thus, DNA mutations leading to structural changes in topoisomerase IV and DNA gyrase are the major cause of FQ resistance (39, 40). However, genes encoding topoisomerase IV are absent in *C. jejuni* (16, 24), and mutations in DNA gyrase subunit B do not mediate FQ resistance in *C. jejuni* (41). Instead, *C. jejuni* develops FQ resistance by single-step point mutations in GyrA (DNA gyrase subunit A) (41, 42). FQ treatment of chickens colonized by *C. jejuni* does not completely eliminate *C. jejuni*; instead, FQ<sup>R</sup> *C. jejuni* cells emerge within a few days of FQ treatment, increasing the number of *C. jejuni* cells (36). This is the same pattern of bacterial killing and FQ resistance development through antibiotic tolerance in our study (Fig. 1A), suggesting that antibiotic tolerance may contribute to FQ resistance development *in vivo*.

The high prevalence of FQ<sup>R</sup> *Campylobacter* has been ascribed mainly to the use of FQs in agriculture, because *C. jejuni* is typically transmitted from food-producing animals to humans (16, 42, 43). Considerable efforts have been made to curb FQ resistance in *Campylobacter* by banning FQs in the poultry production and agriculture sectors in the United States (44). However, FQ resistance in *Campylobacter* continues to increase (45). This has been attributed to the fitness benefits of FQ resistance in *C. jejuni*. Once *C. jejuni* develops FQ resistance, FQ<sup>R</sup> *C. jejuni* outcompetes FQ-sensitive *C. jejuni* in the intestines of chickens, the primary reservoir for *C. jejuni*, even in the absence of FQs (46). This results in a high prevalence of FQ<sup>R</sup> *C. jejuni* in chickens. Our results show that the induction of antibiotic tolerance is an important mechanism for the rapid development of FQ resistance in *C. jejuni*, even when treated with non-FQ antibiotics.

It is noteworthy to mention that the interplay between resistance development and enrichment can collectively contribute to antibiotic resistance. Tetracyclines can stimulate the development of FQ resistance but do not enrich the population of FQ<sup>R</sup> *C. jejuni* (Fig. 3). FQs play a major role in enriching the population of FQ<sup>R</sup> *C. jejuni* by selectively inhibiting FQ-sensitive populations (Fig. 3). Tetracyclines were used in agriculture for years prior to the



approval of FQs for control of early mortality in broiler chickens (47). However, significant increases in FQ<sup>R</sup> *Campylobacter* isolates are mainly attributed to the agricultural use of FQs (48, 49). Based on the findings in this study, we can speculate that non-FQ drugs used in agriculture also facilitate the emergence of FQ<sup>R</sup> *C. jejuni* more frequently than baseline spontaneous *gyrA* mutations and that the use of FQs in agriculture has resulted in substantial increases in the prevalence of FQ<sup>R</sup> *C. jejuni* through selective enrichment. A recent study from Australia showed that a subpopulation of *Campylobacter* isolates from chickens exclusively resistant to FQs was detected, although Australia has never allowed for the use of FQs in chicken production (50). Compared to FQ resistance in other countries that ever used FQs in poultry production, e.g., 87% in China (12), 89% in Thailand (13), and 85.6% in South Korea (51), ciprofloxacin resistance was less frequently (14.8%) detected in Australia (50). Based on our findings, we speculate that non-FQ drugs used in agriculture may facilitate the emergence of subpopulations of FQ<sup>R</sup> *Campylobacter* and that the absence of FQs in poultry production has not enriched the population of FQ<sup>R</sup> *Campylobacter* in Australia. Future epidemiological studies investigating the association of the use of tetracyclines with FQ resistance are needed to validate this hypothesis.

Antibiotic tolerance induced by non-FQ drugs, particularly tetracycline, can facilitate the emergence of FQ<sup>R</sup> *C. jejuni*. Tetracyclines are most widely used in agriculture, accounting for 66% and 31% of marketed agricultural antimicrobials in the United States (17) and the European Union (18), respectively. In the United States, the use of tetracyclines as growth promoters is no longer allowed, and they can be used only for therapeutic purposes. However, tetracyclines represent the largest volume of domestic sales of medically important antibiotics approved for use in food-producing animals, and about 3,948 tons of tetracyclines were sold and distributed for veterinary purposes in the United States in 2020 (17). When applied as medication in feed, more than 70% of tetracyclines are unmetabolized and excreted from animals into the environment (52). This increases the chances of bacterial exposure to high levels of tetracyclines, both inside and outside animals. Many countries widely use tetracyclines as in-feed antibiotics for growth promotion, disease prevention, and treatment (53). Although FQs have been banned in livestock production in many countries, the widespread use of tetracycline or other tolerance-inducing antibiotics in animals can contribute to the development of FQ resistance in *C. jejuni*.

In summary, our results demonstrate that antibiotic tolerance is crucial for developing FQ resistance by enabling *C. jejuni* to survive extensive exposure to antibiotics. Antibiotic treatment elevates oxidative stress, leading to DNA damage and a subsequent increase in mutation frequency. Among the antioxidant enzymes available in *C. jejuni*, AhpC plays a major role in preventing the development of FQ resistance. Overall, the findings in this study provide novel insights into the molecular mechanisms of antibiotic tolerance and resistance development and may explain the high prevalence of FQ resistance in *C. jejuni*.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *C. jejuni* NCTC 11168 was used as the WT in this study. The isogenic knockout mutants of  $\Delta$ *ahpC* (54),  $\Delta$ *katA* (55),  $\Delta$ *sodB* (55), and  $\Delta$ *perR* (56) and a CosR overexpression strain (26) were reported in our previous studies. A  $\Delta$ *perR*  $\Delta$ *ahpC* double mutant and a CosR overexpression strain with  $\Delta$ *ahpC* were constructed by transforming the  $\Delta$ *perR* mutant and the CosR overexpression strain with the genomic DNA of the  $\Delta$ *ahpC* mutant using natural transformation (57). The *C. jejuni* strains were routinely grown at 42°C in Mueller-Hinton (MH) medium (Difco) under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>).

**Antibiotic tolerance assay.** Overnight cultures of *C. jejuni* on MH agar plates were resuspended in 5 mL of MH broth in a 14-mL round-bottom tube (BD Falcon, USA) to an optical density at 600 nm (OD<sub>600</sub>) of 0.08. The bacterial suspension was incubated with shaking at 200 rpm under microaerobic conditions. After 7 h, antibiotic exposure was initiated by adding 1× MIC, 10× MIC, or 100× MIC of antibiotics (ciprofloxacin, tetracycline, erythromycin, and gentamicin). The concentrations were determined based on the MIC of the WT (i.e., *C. jejuni* NCTC 11168). The cultures were also treated with 1× MIC of antibiotics to compare the effects of a relatively lower concentration of antibiotics on tolerance. The MICs of ciprofloxacin, tetracycline, erythromycin, and gentamicin in *C. jejuni* NCTC 11168 were 0.063 μg/mL, 0.031 μg/mL, 1 μg/mL, and 0.5 μg/mL, respectively, as determined with a microdilution susceptibility test. Since these MICs show a wide concentration range, we could not use a fixed concentration for the four antibiotics. For instance, 1 μg/mL is equivalent to 15.9× MIC of ciprofloxacin, 32.3× MIC of tetracycline, 1× MIC of erythromycin, and 2× MIC of gentamicin in *C. jejuni* NCTC 11168. The growth of *C. jejuni* can be seriously affected by 1 μg/mL (i.e., 15.9× MIC) of ciprofloxacin but not by 1 μg/mL (i.e., 1× MIC) of erythromycin. Using the same fixed concentration for different antibiotics can generate different levels of antimicrobial activity. Thus, we decided the concentration of antibiotic treatment

based on the MIC. For sampling, 1.2 mL of *C. jejuni* cultures were harvested and washed with fresh MH medium. After washing, *C. jejuni* cells were resuspended in 100  $\mu$ L of MH broth and spread for enumeration onto MH agar plates and MH agar plates supplemented with 1  $\mu$ g/mL ciprofloxacin. Colonies growing on MH agar plates supplemented with ciprofloxacin were randomly picked up and subjected to a broth microdilution susceptibility test to confirm resistance (58).

**Hydroxyl radical measurement.** *C. jejuni* cells were treated with high concentrations of antibiotics for 24 h as described above. *C. jejuni* cells were washed twice with phosphate-buffered saline (PBS) and concentrated 10-fold. The assay was conducted according to the manufacturer's instructions (Molecular Probes HPF, Thermo Scientific, USA). Briefly, 100  $\mu$ L of a sample was placed onto a 96-well plate (black opaque; Corning, USA). Hydroxyphenyl fluorescein (HPF) solution was diluted to a final concentration of 5  $\mu$ M. The fluorescence at excitation/emission (ex/em) values of 530/590 nm was measured using a plate reader (Varioskan Flash; Thermo Fisher Scientific) with gentle shaking at 25°C. Measured fluorescence signals were normalized to protein concentrations determined using a Bradford assay.

**Hydrogen peroxide measurement.** The level of hydrogen peroxide formation under antibiotic treatment was measured with the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's protocol. *C. jejuni* cells were exposed to antibiotics for 24 h as described above. The samples were washed twice, concentrated 10-fold, and placed onto a 96-well plate (black opaque; Corning). The working solution (10 mM Amplex Red reagent, 10 U/mL Horseradish peroxidase stock solution, and reaction buffer) was added to each sample solution. The mixture was incubated at room temperature for 30 min, and fluorescence was detected at ex/em 530/590 nm using a plate reader (Varioskan Flash; Thermo Fisher Scientific). The hydrogen peroxide concentration was determined by comparing it with a standard curve prepared with known hydrogen peroxide concentrations and normalized to protein concentrations determined using a Bradford assay.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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B.J. conceptualized the study. M.P., J.K., and J.F. performed the experiments. B.J. and S.R. supervised the experiments. B.J., M.P., and K.S.L. analyzed the results. B.J. wrote the manuscript. M.P. prepared the figures.

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We declare no competing interests.

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