Key Role of Mfd in the Development of Fluoroquinolone Resistance in *Campylobacter jejuni*

Jing Han, Orhan Sahin, Yi-Wen Barton, Qijing Zhang*

Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, United States of America

**Abstract**

*Campylobacter jejuni* is a major food-borne pathogen and a common causative agent of human enterocolitis. Fluoroquinolones are a key class of antibiotics prescribed for clinical treatment of enteric infections including campylobacteriosis, but fluoroquinolone-resistant *Campylobacter* readily emerges under the antibiotic selection pressure. To understand the mechanisms involved in the development of fluoroquinolone-resistant *Campylobacter*, we compared the gene expression profiles of *C. jejuni* in the presence and absence of ciprofloxacin using DNA microarray. Our analysis revealed that multiple genes showed significant changes in expression in the presence of a suprainhibitory concentration of ciprofloxacin. Most importantly, ciprofloxacin induced the expression of *mfd*, which encodes a transcription-repair coupling factor involved in strand-specific DNA repair. Mutation of the *mfd* gene resulted in an approximately 100-fold reduction in the rate of spontaneous mutation to ciprofloxacin resistance, while overexpression of *mfd* elevated the mutation frequency. In addition, loss of *mfd* in *C. jejuni* significantly reduced the development of fluoroquinolone-resistant *Campylobacter* in culture media or chickens treated with fluoroquinolones. These findings indicate that Mfd is important for the development of fluoroquinolone resistance in *Campylobacter*, reveal a previously unrecognized function of Mfd in promoting mutation frequencies, and identify a potential molecular target for reducing the emergence of fluoroquinolone-resistant *Campylobacter*.

**Introduction**

*Campylobacter jejuni*, a Gram-negative microaerobic bacterium, is one of the most prevalent bacterial foodborne pathogens in humans, causing more than 2 million cases of diarrhea each year in the U.S. alone [1,2,3]. As an enteric pathogen, this organism causes watery diarrhea and/or hemorrhagic colitis. *Campylobacter* infection is also the most common antecedent to Guillain-Barre syndrome, an acute flaccid paralysis that may lead to respiratory muscle compromise and death [4,5]. In developed countries, person-to-person transmission of *Campylobacter* is rare, and the main source of human *Campylobacter* infections is via food, water, or milk contaminated by *Campylobacter* [6].

Fluoroquinolone (FQ) antimicrobials are often prescribed for clinical treatment of diarrhea caused by enteric bacterial pathogens including *Campylobacter* [7,8]. However, *Campylobacter* is increasingly resistant to FQ antimicrobials, which has become a major concern for public health [9,10,11]. FQ-resistant (FQR) *Campylobacter* developed in food producing animals can be transmitted to humans via the food chain. Poultry are considered the major reservoir for *C. jejuni* and a significant source for FQR *Campylobacter* infections in humans, because the majority of domestically acquired cases of human campylobacteriosis result from consumption of undercooked chicken or food contaminated by raw chicken [2,12,13]. Although FQ antimicrobials have been banned since 2005 in poultry production in the U.S., FQR *Campylobacter* continue to persist on poultry farms [14,15,16].

The main targets of FQs in bacteria are DNA gyrase and/or topoisomerase IV [17,18]. In *Campylobacter*, the resistance to FQ antimicrobials is mediated by point mutation in the quinolone resistance-determining region (QRDR) of *gyrA* in conjunction with the function of the multidrug efflux pump CmeABC [10,19,20,21]. 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 lead to clinically relevant levels of resistance to FQ antimicrobials [19,20,22]. Specific mutations at positions Thr-86, Asp-90 and Ala-70 in GyrA have been linked to FQ resistance in *C. jejuni* [10,19,21]. When enumerated by ciprofloxacin (CIPRO)-containing plates, spontaneous FQR *Campylobacter* mutants occur at a frequency as high as $10^{-6}$ [23], suggesting that *C. jejuni* possess a high mutation rate to FQ resistance. CmeABC, an energy-dependent efflux system, contributes significantly to the intrinsic and acquired resistance to FQs in *C. jejuni* by reducing the accumulation of the antibiotics within *Campylobacter* cells [19,20,24,25]. The expression level of *cmeABC* also influences the frequencies of emergence of spontaneous FQR mutants [23].

One unique feature of FQ resistance development in *Campylobacter* is the rapid emergence of FQR mutants from a FQR susceptible population when treated with FQ antimicrobials. This has been observed in *Campylobacter*-infected animals or patients.
As a food-borne bacterial pathogen, Campylobacter jejuni is a common causative agent of gastrointestinal illnesses in humans. Development of antibiotic resistance in Campylobacter, especially to fluoroquinolone (a broad-spectrum antimicrobial), compromises clinical treatments and presents a major public health threat. It is not well understood why Campylobacter is highly adaptable to fluoroquinolone treatment or how it acquires mutations associated with fluoroquinolone resistance. Understanding the molecular mechanisms involved in the resistance development will help us to reduce the emergence of fluoroquinolone-resistant Campylobacter. Using DNA microarray and other molecular methods, as well as animal studies, we uncovered the key role of Mfd in promoting spontaneous mutations and development of fluoroquinolone resistance in Campylobacter. Mfd is a transcription-repair coupling factor involved in DNA repair and was not previously known for its role in promoting mutations conferring antibiotic resistance. Our findings not only reveal a novel function of Mfd, but also provide a potential molecular target for reducing the emergence of fluoroquinolone-resistant Campylobacter.

Fluoroquinolone Resistance in Campylobacter

Author Summary

As a food-borne bacterial pathogen, Campylobacter jejuni is a common causative agent of gastrointestinal illnesses in humans. Development of antibiotic resistance in Campylobacter, especially to fluoroquinolone (a broad-spectrum antimicrobial), compromises clinical treatments and presents a major public health threat. It is not well understood why Campylobacter is highly adaptable to fluoroquinolone treatment or how it acquires mutations associated with fluoroquinolone resistance. Understanding the molecular mechanisms involved in the resistance development will help us to reduce the emergence of fluoroquinolone-resistant Campylobacter. Using DNA microarray and other molecular methods, as well as animal studies, we uncovered the key role of Mfd in promoting spontaneous mutations and development of fluoroquinolone resistance in Campylobacter. Mfd is a transcription-repair coupling factor involved in DNA repair and was not previously known for its role in promoting mutations conferring antibiotic resistance. Our findings not only reveal a novel function of Mfd, but also provide a potential molecular target for reducing the emergence of fluoroquinolone-resistant Campylobacter.

transcript.

Results

Transcriptional analysis of C. jejuni response to FQ treatment

To understand the adaptive response of Campylobacter to FQ treatment, DNA microarray was used to analyze the transcriptional changes in C. jejuni NCTC 11168 after exposure to CIPRO. When the Campylobacter cells were treated with a subinhibitory concentration (0.06 µg/ml; 0.5× the MIC) of CIPRO for 1.5 hours, no genes showed ≥1.5-fold changes in expression, suggesting that the transcriptional response to the low dose of CIPRO was very limited. When the Campylobacter cells were treated with a suprainhibitory concentration (1.25 µg/ml; 10× the MIC) of CIPRO for 30 min, 45 genes showed ≥1.5-fold ($p<0.05$) changes in expression (Table 1), among which 13 were up-regulated and 32 were down-regulated. The up-regulated genes are involved in cell membrane biosynthesis, cellular processes, and transcription-coupled DNA repair or have unknown functions, while the majority of the down-regulated genes are involved in energy metabolisms (Table 1). Consistent with the lack of LexA, the core genes involved in SOS responses in other bacteria, such as recA, uvrA, ruvB, and ruvC, did not show significant changes in expression. The expression of other genes involved in DNA repair and recombination also did not change significantly. These findings indicate that C. jejuni does not mount a typical SOS response or upregulate the general DNA repair system in the early response to CIPRO treatment. Notably, cj1085c, a homolog of mfd, was upregulated in the presence of CIPRO. Two up-regulated genes (UV-induced mutagenesis), and SOS-controlled error-prone DNA polymerases [40,49,50]. These observations suggest that Campylobacter may not have the typical SOS response system. In light of this possibility, it is intriguing to determine how Campylobacter copes with FQ treatment and what facilitates the emergence of FQR mutants in Campylobacter.
Table 1. Genes differentially expressed in the presence of ciprofloxacin.

| Gene ID and Functional Category | P-Value | Q-Value | n-Fold change | Microarray | qRT-PCR |
|-------------------------------|---------|---------|--------------|------------|---------|
| **Cell membrane**             |         |         |              |            |         |
| CJ0205 uppP, undecaprenyl-diphosphatase | 0.0135  | 0.130143 | 1.59         | 6.1        |         |
| CJ0735 putative periplasmic protein | 0.0186  | 0.14811  | 1.70         | NT*        |         |
| CJ0824 uppS, undecaprenyl diphosphate synthase | 0.0099  | 0.120356 | 1.52         | 2.1        |         |
| CJ1351 pDA, phospholipase A | 0.0046  | 0.094812  | 2.02         | 2          |         |
| CJ033 putative integral membrane protein | 0.0033  | 0.086471  | 1.52         | NT         |         |
| CJ0179 exbB1, biopolymer transport protein | 0.0412  | 0.217646  | 1.88         | NT         |         |
| CJ0486 putative sugar transporter | 0.0043  | 0.091967  | 1.52         | NT         |         |
| CJ0553 putative integral membrane protein | 0.0106  | 0.121714  | 1.59         | NT         |         |
| CJ0834c ankyrin repeat-containing possible periplasmic protein | 0.0089  | 0.110719  | 1.51         | NT         |         |
| CJ1013c putative cytochrome C biogenesis protein | 0.0046  | 0.094812  | 2.02         | NT         |         |
| CJ0033 putative integral membrane protein | 0.0033  | 0.086471  | 1.52         | NT         |         |
| CJ0179 exbB1, biopolymer transport protein | 0.0412  | 0.217646  | 1.88         | NT         |         |
| CJ0486 putative sugar transporter | 0.0043  | 0.091967  | 1.52         | NT         |         |
| CJ0179 exbB1, biopolymer transport protein | 0.0412  | 0.217646  | 1.88         | NT         |         |
| **DNA replication, recombination and repair** |         |         |              |            |         |
| CJ1085c mfd, transcription-repair coupling factor | 0.0029  | 0.082832  | 1.57         | 2.2        |         |
| CJ0718 dnaE, DNA polymerase III, alpha chain | 4.07E-05 | 0.052459  | 1.62         | 2          | 1.98    |
| **Cellular process and energy metabolism** |         |         |              |            |         |
| CJ0041 putative flagellar hook-length control protein | 0.0357  | 0.204764  | 1.93         | NT         |         |
| CJ065c fok, putative 2-amino-4-hydroxy-6-hydroxymethyldehydroyldiphosphate pyrophosphokinase | 0.0117  | 0.129371  | 1.54         | NT         |         |
| CJ1030c lepA, GTP-binding protein homolog | 0.0057  | 0.210454  | 1.54         | NT         |         |
| CJ1280c putative ribosomal pseudouridine synthase | 0.0252  | 0.076881  | 1.50         | NT         |         |
| CJ1672c gltA, biosynthetic arginine decarboxylase | 0.0167  | 0.142007  | 1.54         | 2          | 1.96    |
| CJ0227 argD, acetylornithine aminotransferase | 0.0208  | 0.151677  | 1.57         | NT         |         |
| CJ1264c sdaA, L-serine dehydratase | 0.0046  | 0.094812  | 1.58         | NT         |         |
| CJ0814 hypothetical protein | 0.0002  | 0.067622  | 1.75         | NT         |         |
| **Unknown function**          |         |         |              |            |         |
| CJ0163c hypothetical protein | 0.0204  | 0.151393  | 1.60         | NT         |         |
| CJ0814 hypothetical protein | 0.0002  | 0.067622  | 1.99         | NT         |         |
| CJ0995c hypothetical protein | 0.0233  | 0.160562  | 1.50         | NT         |         |
genes, uppP and uppS, encode products involved in cell wall production [56,57], while pldA encodes an outer membrane phospholipase that has been implicated in hemolysis, capsular production, and virulence [58,59]. According to the Q values, the identified genes would have an estimated false discovery rate (FDR) of 20%. However, quantitative real-time RT-PCR confirmed all of the 11 genes selected from the microarray list (Table 1), suggesting that the actual FDR is lower than the estimation.

Characteristics of Mfd

Cj1085c (978aa) was annotated as Mfd [48] and shows 31.5% amino acid identity to the E. coli Mfd protein (1148 aa). In addition, it contains the characteristic domains conserved in Mfd proteins, such as the ATP/GTP-binding site motif and the superfamily II helicase motif. Mfd in other bacteria has been shown to be involved in strand-specific DNA repair by displacing lesion-stalled RNA polymerase and recruiting enzymes involved in recombination events [54,60]. The mfd locus is highly conserved in Campylobacter and is present in all Campylobacter species and C. jejuni strains that have been sequenced to date. The Mfd proteins in different Campylobacter species share 57–79% identity to the Mfd in C. jejuni NCTC 11168. Within C. jejuni, the Mfd proteins are 98–100% homologous among different strains. The mfd gene is located in the middle of a gene cluster, whose transcription is in the same direction (partially shown in Fig. 1A). The downstream gene Cj1084c encodes a putative ATP/GTP binding protein, while the upstream gene Cj1086c encode a hypothetical protein [48]. It is unknown if mfd and its flanking genes form an operon, but it appeared that Cj1086c and mfd were co-transcribed because a RT-PCR product spanning both ORFs was amplified (data not shown).

Expression levels of mfd influence the frequency of emergence of spontaneous FQ-resistant mutants

Since mfd was the only DNA repair related gene that showed a significant change in expression in the early response of C. jejuni to CIPRO treatment (Table 1), we examined its role in the emergence of spontaneous FQR mutants in Campylobacter. Firstly, the mfd gene was inactivated by insertional mutagenesis (Fig. 1B). As shown in Fig. 2, the mfd mutant (JH01) showed a approximately
100-fold reduction in the frequencies of emergence of spontaneous FQR mutants detected using plates containing three different concentrations (1, 2, and 4 μg/ml, respectively) of CIPRO. Complementation of the mfd mutant in trans by a plasmid-carried mfd restored the frequencies of mutant emergence to the wild-type level (JH02 in Fig. 2). As determined by qRT-PCR, the expression level of mfd in the complemented construct [JH02] was fully restored (1.7× the wild-type level), pRY112 alone (without the cloned mfd gene) did not complement the mfd mutant in the mutation frequency (data not shown). These results indicate that Mfd contributes significantly to the rate of spontaneous mutations to FQR resistance.

Secondly, we determined if the enhanced expression of mfd increases the mutation frequency. For this purpose, we constructed strain JH03, which was a wild-type 11168 strain containing an extra copy of mfd carried on a shuttle plasmid. In JH03, the mRNA of mfd increased 3.8 times compared with that in 11168 as determined by qRT-PCR. When compared with the wild-type 11168, the frequency of emergence of FQR mutants from JH03 increased about 10-fold (Fig. 2). The increase was reproducible in multiple experiments and was statistically significant (P<0.05). These results indicated that overexpression of mfd increases the frequency of emergence of spontaneous FQR mutants.

Given that there is only one nucleotide between the mfd gene and its downstream gene cj1084c, it was prudent to determine if the mfd mutation resulted in a polar effect on the expression of cj1084c. RT–PCR showed that cj1084c was transcribed at a comparable level in both the mfd mutant and the wild-type NCTC 11168 (Fig. 1C). RT-PCR was also performed using 10-fold serial dilutions of the RNA template, which yielded comparable results between the two strains (data not shown). PCR without the reverse transcriptase did not yield a product (Fig. 1C), indicating that the mRNA templates had no DNA contamination. These results suggested that the insertional mutation in the mfd gene did not cause an apparent polar effect on expression of the downstream gene. This finding plus the complementation data (Fig. 2) strongly indicate that loss of Mfd is responsible for the observed reduction in the mutation frequency in JH01.

Loss of mfd does not affect the susceptibility of C. jejuni to antibiotics

To examine if the reduction in the emergence of spontaneous FQR mutants is caused by the increased susceptibility of the mfd mutant to CIPRO, we compared the MICs of several antibiotics in the mfd mutant with those in the wild type. Our results did not reveal any differences between the mutant and the wild type in their susceptibility to the tested antibiotics including erythromycin, ampicillin, streptomycin, and CIPRO (data not shown). In addition, there was no apparent difference in growth kinetics between the wild-type and the mfd mutant either in MH broth (without antibiotics) or in MH broth supplemented with a subinhibitory concentration (0.06 μg/ml) of CIPRO (Fig. 3). The growth rates of the mfd over-expressing strain [JH03] and the complemented mutant [JH02] were also similar to that of the wild type (Fig. 3). Thus, the reduced spontaneous mutation rate to FQ resistance in the mfd mutant was not attributable to decreased growth rate or increased susceptibility to antibiotics. In addition, the CIPRO-resistant colonies examined for gyrA mutations all carried the C257T mutation in gyrA and had a CIPRO MIC of >32 μg/ml regardless of the backgrounds (11168 or JH01) from which the mutants were selected.

Mfd contributes to the emergence of FQR Campylobacter under in vitro treatment

FQR Campylobacter mutants emerge rapidly from a FQ-susceptible population once treated with FQ antimicrobials [19,26,27,28,29,30]. To determine if Mfd influences the development of FQR Campylobacter under selection pressure, we conducted in vitro growth experiments, in which C. jejuni was treated with a suprainhibitory concentrations of CIPRO (4 μg/ml). In the first treatment experiment, 10⁷ CFU of bacterial cells were inoculated into each flask containing 100 ml MH broth with 4 μg/ml of CIPRO, yielding an initial cell density of 10⁷ CFU/ml. At the beginning of the treatment, 1–3 CFU/ml of FQR mutants were detected in the flasks inoculated with 11168, while no FQR mutants were detected in the cultures inoculated with JH01 (Fig. 4A). One day after the initiation of the treatment, the numbers of FQR mutants in the 11168 cultures grew to a level ranging from a few hundreds to a few thousands
Mfd affects the emergence of FQR mutants in vivo

To determine if Mfd influences the emergence of FQR Campylobacter during in vivo therapeutic treatment, broiler chickens were infected with 11168 or JH01 and then treated with enrofloxacin administered in drinking water (50 ppm). The birds in both groups were quickly colonized by C. jejuni after inoculation (Fig. 5). Before the treatment with enrofloxacin, all birds were colonized by Campylobacter and the colonization levels (CFU/g feces) were similar in both groups (p>0.05). One day after initiation of the treatment, the number of colonized chickens and the levels of colonization decreased drastically in both groups, with Campylobacter detectable only in three chickens that were inoculated with the wild-type strain (Fig. 5A). After that, the numbers of Campylobacter in both groups rebounded. On day 3 after the initiation of the treatment, all of the birds in the 11168 group were re-colonized by Campylobacter and remained colonized until the end of the experiment. For the group inoculated with JH01, 6 of the 11 birds became positive with Campylobacter on day 3 after initiation of the treatment (Fig. 5A) and 3 birds remained negative until the end of the experiment. On days 3, 5 and 7 after initiation of the treatment, the average colonization level of the JH01-inoculated group was approximately 3 log units lower than that of the 11168-inoculated group (Fig. 5A) and the differences were statistically significant (p<0.05). The number of FQR Campylobacter in each chicken was also monitored. Prior to the treatment, no FQR C. jejuni was detected in any of the chickens (Fig. 5B). On day 1 after initiation of the treatment, the three Campylobacter-positive birds of the 11168-inoculated group still carried FQ-susceptible Campylobacter. However, FQR C. jejuni appeared on day 3 in all birds of the 11168-inoculated group and in some birds of the JH01-inoculated group (Fig. 5B). Comparison of the total Campylobacter counts (Fig. 5A) with the numbers of FQR Campylobacter (Fig. 5B) revealed that the birds were re-colonized by FQR mutants after initiation of the treatment. The average numbers of FQR Campylobacter in the JH01-inoculated group were approximately 3 log units lower than that of the 11168-inoculated group (Fig. 5A) and the differences were statistically significant (p<0.05). These results indicate that loss of Mfd significantly reduced the rates of emergence of FQR Campylobacter in enrofloxacin-treated chickens.

Representative Campylobacter isolates obtained at different sampling times from both groups were tested for CIPRO MICs using E-test strips. The result showed that before treatment all the tested isolates from both groups were susceptible to CIPRO (MICS = 0.094–0.125 μg/ml). The majority of the tested isolates from day 1 after initiation of the treatment were still susceptible to CIPRO (MICS = 0.094–0.5 μg/ml). On day 3 after the initiation of treatment, 21 of the 22 tested isolates (from both groups) had a CIPRO MIC of >32 μg/ml and the other one had an MIC of 8 μg/ml. Similarly, the majority (44 out of 49) of the tested isolates from days 5 and 7 had a CIPRO MIC of >32 μg/ml and the rest had MICs from 1–24 μg/ml. The MIC results further confirmed the differential plating results that the chickens were re-colonized by FQR Campylobacter.

Discussion

When Campylobacter cells were treated with a subinhibitory concentration (0.06 μg/ml, 0.5× the MIC) of CIPRO for
1.5 hours, no significant changes in gene expression were detected using the cut-off criteria defined in this study. This result was somewhat similar to the study with Haemophilus influenzae [61] in that the treatment with a low concentration of CIPRO induced few changes in gene expression, but was different from that study because several genes involved in SOS response were upregulated in Haemophilus influenzae. Prolonged treatment of Campylobacter with the subinhibitory concentration of CIPRO may reveal noticeable changes in gene expression, but culturing Campylobacter with 0.06 mg/ml of CIPRO reduces its growth rate (Fig. 3), which will make the comparison with the non-treated control unfeasible and complicate the interpretation of the microarray results. To mimic clinical treatment, C. jejuni cells were exposed to a suprainhibitory dose (1.25 μg/ml, 10× the MIC) of CIPRO. This dose is within the concentration range of CIPRO in gut contents during FQ treatment in chickens [62]. The reason that we treated the samples for 0.5 hour instead of a longer time was to detect the primary response triggered by CIPRO, instead of the secondary response caused by cell death. When Campylobacter cells were treated with this suprainhibitory dose for 0.5 hour, the expression of multiple genes was significantly altered (Table 1). Notably, the majority of the affected genes were downregulated and many of them are involved in cellular processes and energy metabolism (Table 1). This result is similar to the findings obtained with other bacteria [43,44,61] and supports the notion that reducing cellular metabolism is a common strategy utilized by bacteria to cope with antibiotic treatment.

Within bacterial cells CIPRO interacts with gyrase and DNA, blocking DNA replication and transcription [18]. When exposed to CIPRO, the expression of gyrA and gyrB in various bacteria was either altered or unchanged [43,61,63]. In this study, we found that the expression of gyrA, gyrB, and topA was not significantly affected in...
Contributors to the size of the mutant pools. This possibility DNA sequence due to transcriptional mutation [67] that resulting in an elevated level of retromutagenesis (fixed changes in mutation rates in a bacterial organism. How Mfd contributes to the key role of Mfd in promoting spontaneous emergence of spontaneous FQR mutants in increasing the adaptive mutagenesis rates [66].

In addition to transcription-coupled DNA repair, Mfd has been associated with other functions in bacteria [64]. For example, Mfd of Bacillus subtilis is involved in homologous DNA recombination and stationary-phase mutagenesis [65,66]. Inactivation of the mfd gene of B. subtilis resulted in a great reduction in the number of prototrophic revertants to Met+, His+, and Leu+ during starvation [66], indicating that Mfd promotes adaptive mutagenesis. This finding is in contrast to the known function of Mfd in mediating mutation frequency decline and could be explained by the role of Mfd in promoting transcriptional bypass and consequently increasing the adaptive mutagenesis rates [66].

In this study we found that Mfd increases the frequency of emergence of spontaneous FQR mutants in Campylobacter (Fig. 2). Furthermore, the mfd mutation also decreased the frequency of emergence of spontaneous streptomycin-resistant mutants in Campylobacter (data not shown). Together, the results convincingly showed that Mfd is an important player in modulating the mutation rates in Campylobacter. To our knowledge, this is the first report documenting the key role of Mfd in promoting spontaneous mutation rates in a bacterial organism. How Mfd contributes to the increased mutation rates in Campylobacter is unknown, but it can be speculated that transcriptional bypass mediated by Mfd may actively occur in replicating non-stressed Campylobacter populations, resulting in an elevated level of retromutagenesis (fixed changes in DNA sequence due to transcriptional mutation [67]) that contributes to the size of the mutant pools. This possibility remains to be examined in future studies. Although mfd contributes significantly to the mutation rate (Fig. 2), its expression level was not precisely proportional to the mutation frequencies. For example, expression of mfd was upregulated 3.8-fold in JH03, but its mutation frequency increased 10-fold. This difference is probably due to the fact that emergence of spontaneous mutants is a multi-step process and Mfd only contributes to one of the steps in the process. It is also possible that Mfd interacts with other proteins in modulating the mutation frequency. Thus, the changes in mfd expression level and the mutation frequency are not exactly at the same scale.

Another interesting observation of this study is the upregulation of mfd by CIPRO. The enhanced expression may be needed for transcription repair because CIPRO treatment causes DNA damage, which stalls RNA polymerase. Alternatively, the increased production of Mfd may enhance transcriptional bypass of the non-repaired DNA lesions in order to maintain cell viability and/or promote mutations for resistance. This possibility is high given the facts that massive DNA damage incurred by a suprainhibitory dose of CIPRO may overwhelm the DNA repair system and Campylobacter must maintain certain levels of transcription to survive the treatment, that Mfd contributes significantly to the mutation rates to FQ resistance (Fig. 2), and that Campylobacter does not have the error-prone DNA polymerases, such as Pol II, Pol IV, and Pol V [48]. E. coli and other bacteria have these error-prone DNA polymerases [68,69], which are repressed by LexA, but upregulated by the SOS response triggered by DNA damage. Once produced, the enzymes perform translesion DNA synthesis, allowing replication to continue without DNA repair. This special functional feature results in reduced genetic fidelity, but allows for bacterial survival under stress. The outcome of the enhanced expression of the error-prone enzymes is the increased mutation rates, which contribute to the emergence of drug resistance [70]. In the absence of a SOS response and the error-prone DNA polymerases, Campylobacter may use Mfd as an alternative pathway to increase mutation rates. Thus, enhanced expression of mfd may represent an adaptive response of Campylobacter to the stresses imposed by CIPRO treatment. How CIPRO upregulates Campylobacter Mfd is unknown and further work in this direction is warranted.

FQR Campylobacter readily emerges from a FQ-susceptible population when treated with FQ antimicrobials (Figs. 4 and 5). As shown in the in vitro experiment, the development of FQR population under CIPRO treatment is influenced by the initial cell density (Fig. 4 and the corresponding text) as well as the functional state of Mfd. Considering the differences in spontaneous mutation rate between 11168 and JH01 (Fig. 2), it was likely that the 11168 and JH01 inocula had different numbers of pre-existing FQR mutants, which were selected by CIPRO and contributed to the differences in the FQR population detected in the cultures of the two strains. The inoculum-dependent emergence of FQR mutants in both 11168 and JH01 suggests that development of FQR Campylobacter under FQ treatment involves selection of pre-existing mutants. However, the magnitude and dynamics of FQR development can not be totally explained by selection. For example, in some cultures FQR mutants were not detectable until the 2nd day of the incubation (Fig. 4). A single mutant at time zero in a culture flask would grow to a population of more than 2,000 cells in one day (the generation time of C. jejuni in MH broth is about 2 hours), which would be readily detected by the plating method on day 1. Thus, if selection was the only factor in the development of FQR Campylobacter, the latest time for detecting the pre-existing mutants in the mutant-positive flasks would be day 1 after initiation of the treatment. Obviously, this was not the case for all of the cultures because some of them did not show FQR mutants until day 2 (Fig. 4).

In addition, some cultures were negative with FQR mutants at time zero, but showed a large number of mutants at day 1, which could not be easily explained by sole selection of a few preexisting mutants from the inocula. Considering these unexplainable observations and the fact that a small fraction of the FQ-susceptible inoculum survived the killing effect as long as one day after the initiation of the treatment (data not shown), it was possible that new FQR mutants were developed during the treatment. If this occurs, Mfd may enhance the emergence of new mutants by promoting transcriptional bypass or other mechanisms, which may partly explain the differences between 11168 and JH01 in the dynamics of emergence of FQR mutants. Thus, there is a possibility that both selection of pre-existing mutants and de nova formation of mutants are involved in the development of FQR Campylobacter during treatment with FQ antimicrobials.

The role of Mfd in the development of FQR mutants was further shown by the in vivo experiment, in which Campylobacter-infected chickens were treated with enrofloxacin (Fig. 5). Previous studies have shown that therapeutic use of FQ antimicrobials in chickens promotes the emergence of FQR Campylobacter [19,27,28,29,30], which can be potentially transmitted to humans via the food chain. In this study, we showed that inactivation of mfd significantly reduced the development of FQR Campylobacter in chickens (Fig. 5). In fact, several birds in the JH01-inoculated group became negative with Campylobacter once the treatment was initiated. Since the mfd mutant did not show a growth defect in vitro (Fig. 3) and colonized chickens as efficiently as the wild-type strain (see the colonization level before treatment in Fig. 5), the observed
differences in the development of FQR mutants were not due to changes in growth characteristics. These in vitro results (Fig. 5) plus the in vivo findings (Fig. 4) clearly showed that Mfd plays an important role in the development of FQR Campylobacter mutans under the selection pressure. To our knowledge, this is the first report that documents the role of Mfd in the development of FQ resistance in a bacterial pathogen. Since Mfd is highly conserved in bacterial organisms [64], it would be interesting to know if this finding applies to other bacterial pathogens. In addition, inhibition of Mfd functions may represent a feasible approach to reducing the emergence of FQR Campylobacter.

Materials and Methods

Bacterial strains and growth conditions

C. jejuni strain NCTC 11168 (ATCC 700819) was used in this study. The strain was routinely grown in Mueller-Hinton (MH) broth (Difco) or on MH agar at 42°C under microaerobic conditions (5% O2, 10% CO2, and 85% N2). The media were supplemented with kanamycin (50 µg/ml) or chloramphenicol (4 µg/ml) as needed. Escherichia coli cells were grown at 37°C with shaking at 200 r.p.m. in Luria Bertani (LB) medium which was supplemented with ampicillin (100 µg/ml) or kanamycin (30 µg/ml) when needed.

DNA microarray and qRT-PCR

DNA microarray was used to identify genes that were differentially expressed in C. jejuni 11168 treated with CIPRO. For RNA isolation, Campylobacter cells were grown for 24 hours to the mid exponential phase (OD600=0.1-0.15) and split into two equal portions, one of which was treated with CIPRO and the other served as a non-treated control. A subinhibitory concentration (0.06 µg/ml, 0.5× the MIC) and a suprabinhibitory dose (1.25 µg/ml, 10× the MIC) of CIPRO were used in the treatments. For the treatment with 0.06 µg/ml of CIPRO, the treated and non-treated samples were incubated at 42°C for 1.5 hours under microaerobic conditions, while for the treatment with 1.25 µg/ml of CIPRO, the samples were incubated at 42°C for 30 min under microaerobic conditions. Immediately after the incubation, RNAProtect Bacteria Reagent (Qiagen, Valencia, CA) was added to the cultures to stabilize mRNA. The total RNA from each sample was extracted using the RNeasy Mini Kit (Qiagen). The purified RNA samples were treated with On-Column DNase Digestion Kit (Qiagen) followed by further treatments with DNase to remove residual DNA contamination. RNA samples were extracted from 6 independent treatments with each concentration of CIPRO. Absence of contaminating DNA in the RNA samples was confirmed by RT-PCR. The concentration of total RNA was estimated with the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and the integrity and size distribution of the purified RNA was determined by denaturing agarose gel electrophoresis and ethidium bromide staining. The quality of total RNA was further analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), which showed the good quality and integrity of the RNA samples (Data not shown).

cDNA synthesis and labeling, microarray slide (Ocimum Biosolutions) hybridization, Data collection and normalization, and statistical analysis were performed as described in a previous publication [71]. For each type of treatment (0.06 µg/ml for 1.5 hours or 1.25 µg/ml for 30 min), six microarray slides were hybridized with RNA samples prepared from 6 independent experiments. For this study, we chose p-value<0.05 and the change ≥1.5-fold as the cutoff for significant differential expression between the treated and non-treated samples. Representative genes identified by the DNA microarray were further confirmed by qRT-PCR as described in a previous work [72]. The primers used for qRT-PCR are listed in Table 2.

Insertional mutation of mfd

An isogenic mfd (cj1085c) mutant of strain NCTC 11168 was constructed by insertional mutagenesis. Primers mfd-F (5'-TGGTTGAGGAGTATGAGTTAT-3') and mfd-R (5'-AATAGCATTCTAGGAGCTTGTGTT-3') were designed from the published genomic sequence of this strain [48] and used to amplify a 1.8-kb fragment spanning the 5' region of mfd. Amplification was performed with Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA, USA). The blunt-ended PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), ligated to Smal-digested suicide vector pUC19, resulting in the construction of PUC-mfd, which was then transformed into E. coli DH5α. Since a unique EcoRV site (which generates blunt ends) occurs in the cloned mfd fragment, PUC-mfd was digested with EcoRV to interrupt the mfd gene. Primers KanNeo-F (5'-CTTATTACATATTCGCCAGTGGAT-3') and KanNeo-R (5'-GGGAACCTGAGCTTGCTGGATAT-3') were used to amplify the aphA3 gene (encoding kanamycin resistance) from the pMW10 vector [73] by using Pfu Turbo DNA polymerase (Stratagene). The aphA3 PCR product was directly

| Table 2. Oligonucleotide primers used in qRT-PCR. |
|-----------------------------------------------|
| **Primer** | **Sequence** | **Gene amplified** |
| 16s RNA F | 5'-TAC CGT GCC TGG ATG ATC TCC TA-3' | 16s RNA |
| 16s RNA R | 5'-GGA CTT AAC CCA ACA TCT CA-3' | cj0123c |
| Cj0123cF | 5'-GCC CTT GAT GTT TAG ATG TT-3' | |
| Cj0123cR | 5'-TGA AAT CAA AAG CGG TAA AAG TG-3' | |
| Cj0824F1 | 5'-CAA AGT GGC TAA CCA TGC TT-3' | cj0824 (upps) |
| Cj0824R1 | 5'-GAT TTA TGG CCG TTG GAA GA-3' | |
| Cj1351F1 | 5'-ATC CCC TTG GCA TTA GCT CT-3' | cj1351 (#PDA) |
| Cj1351R1 | 5'-TGG AAT TCC GCC TCA TCT TA-3' | |
| Cj1264cF1 | 5'-GCT TAG GGG TTC ATG GC-3' | cj1264c |
| Cj1264cR1 | 5'-CAA AGC CAA AGT ACC ACC AT-3' | |
| Cj1085cF1 | 5'-TGT TTT GTA AAC TCC ACC AG-3 | cj1085c (mfd) |
| Cj1085cR1 | 5'-ATT TGG CCC ACC AGC TCT TA-3' | |
| Cj2005F1 | 5'-GAA AAG TTG CGG CTG ATG TT-3' | cj2005 (upps) |
| Cj2005R1 | 5'-AAT TGG CAT TGC CAA GAA GC-3' | |
| Cj0537F1 | 5'-GCG TGG TGG TGG AAA TAC TAC TA-3' | cj0537 |
| Cj0537R1 | 5'-TGG AGT ATG TGG AGA ATG TTG AGA-3' | cj0718 (dnaE) |
| Cj0718F | 5'-GGACTTTGGGCTATAAAAAGTGT-3' | |
| Cj0718R | 5'-GGACTTTGGGCTATAAAAAGTGT-3' | |
| Cj1688cF1 | 5'-GCC TGA ATT GAT TGG TCC TAC AG-3' | c1688c (secY) |
| Cj1688cR1 | 5'-CCA AAT CAC ACA AAG AGG TA-3' | |
| Cj0764cF1 | 5'-TTC AGC TCG TAC AAT AAA CCC ACC CT-3' | cj0764c (spaA) |
| Cj0764cR1 | 5'-ATA ATG ACG GAG CCG CAC CTA TT-3' | |
| Cj1566cF1 | 5'-CAT AAA TTT ACC CCA AAA CAC TCC-3' | c1566c |
| Cj1566cR1 | 5'-GAG ATT TTA AAG GGG CTT TTG GT-3' | |

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ligated to EcoRV-digested pUC-mfd to obtain construct pUC-mfd-aphA3, in which the aphA3 gene was inserted within mfd (the same direction as the transcription of mfd) and the insertion was confirmed by PCR using primers mfd-F2 and Kana-intra (5’-GAA GAA CAG TAG GTG GAC GTA TTT TTT GAC TTA 3’). The pUC-mfd-aphA3 construct, which served as a suicide vector, was electroporated into C. jejuni NCTC 11168. Transformants were selected on MH agar containing 10 µg/ml of kanamycin. Inactivation of the mfd gene in the transformants by insertion of the aphA3 gene was confirmed by PCR using primers mfd-F2 and mfd-R2 (Fig. 1B). The mfd mutant of NCTC 11168 was named JH01.

Complementation of the mfd mutant in trans

The entire mfd gene including its putative ribosome binding site was amplified from strain 11168 by PCR using primers mfd-F3 (5’-CGCTTCCGGGAATATGAATAAGAGA-TACTATC-3’) and mfd-R3 (5’-GCGTTTTAATATCTTT TCQAGCTCTATAATT-3’). The underlined sequences in the primers indicate the restriction sites for SacI and SacII, respectively. The PCR product was digested with SacI and SacII, and was then cloned into the plasmid construct pRY112-pABC to generate pRY112-mfd, in which the mfd gene was fused to the promoter of cmeABC. pRY112-pABC was made by cloning the promoter sequence of cmeABC [74] to shuttle plasmid pRY112 [75]. The promoter DNA of cmeABC was amplified by primers BSF (5’-AAAGAATTTGGGATCATATG 3’) and AR2 (5’-TGAATCTAGATCAACCAGA 3’), digested with BamHI and XhoI, and cloned into pRY112. There were two reasons that we used the promoter of cmeABC in the expression of mfd. First, the 5’ end of mfd overlaps with its upstream gene and the native promoter for mfd was unknown. Second, the promoter of cmeABC is moderately active in Campylobacter [74], preventing over- or under-expression of mfd. The constructed plasmid pRY112-mfd was sequenced and confirmed that no mutations in the cloned sequence occurred. For complementation, the shuttle plasmid pRY112-mfd was transferred into JH01 by conjugation. The complemented strain was named JH02. Limited passage of JH02 in MH broth without antibiotics indicated that the complementing plasmid was stable in the construct (data not shown). The shuttle plasmid carrying the mfd gene was also transferred to wild-type 11168 to generate strain JH03 for overexpression of the mfd gene.

Growth rates in MH broth with or without CIPRO

To compare the growth kinetics of the mfd mutant with that of the wild-type, a fresh culture of each strain was inoculated into MH broth (initial cell density of OD600 = 0.05) and the cultures were incubated at 42°C under microaerobic conditions. To determine if the mutation affects C. jejuni growth with a subinhibitory concentration of CIPRO, the various strains were grown in MH broth with 0.06 µg/ml of CIPRO (0.5× the MIC). Culture samples were collected and measured for OD600 at 0, 3, 6, 12, 24 and 48 hours post-inoculation.

Antibiotic susceptibility test

The minimum inhibitory concentration (MIC) of CIPRO was determined by using E-test strips (AB Biodisk, Solna, Sweden) as described in the manufacturer’s instructions. The detection limit of the E-test for CIPRO was 32 µg/ml. The MICs of erythromycin, ampicillin and streptomycin for C. jejuni NCTC 11168, JH01, JH02, and JH03 were determined using a standard microtiter broth dilution method described previously [24]. Each MIC test was repeated at least three times to confirm the reproducibility of the MIC patterns. The antibiotics used in this study were purchased from Sigma Chemical Co. (erythromycin, ampicillin, streptomycin) or ICN Biomedicals Inc. (CIPRO).

Frequencies of emergence of spontaneous FQR mutants in vitro

Wild-type 11168, JH01, JH02 and JH03 were compared for the spontaneous mutation rates to CIPRO resistance. In each experiment, each of the 4 strains was inoculated into three flasks, each of which contained 30 ml of antibiotic-free MH broth. The cultures were incubated to the mid logarithmic phase (OD600<0.15) under microaerobic conditions. The culture in each flask was collected by centrifugation and resuspended in 1 ml of MH broth. The total CFU in each culture was measured by serial dilutions and plating on MH agar plates, while the number of FQR mutants was detected using CIPRO plates containing 1, 2 or 4 µg/ml CIPRO. The frequency of emergence of FQR mutants was calculated as the ratio of the CFU on CIPRO-containing MH agar plates to the CFU on antibiotic-free MH agar plates after 2 days of incubation at 42°C under microaerobic conditions. This experiment was repeated five times. The mutation frequency data were log-transformed for statistical analysis. One-Way ANOVA followed by Tukey test was used to determine the significance of differences in the levels of spontaneous mutation rates among the strains. The data were also analyzed by the Wilcoxon rank-sum test to allow for non-normality. For the comparisons discussed in Results, the conclusion of the two tests was the same at significance level 0.05.

Sequence analysis of the QRDR of gyrA

Representative FQR colonies were selected for determination of the point mutations in gyrA. The QRDR of gyrA was amplified by PCR using primer pair GyrAF1 (5’-CAACTGGTTTG-TAGCCCTTTG-3’) and GyrAR1 (5’-AATTTCTACA-TAGCCCTACG-3’) [76]. The amplified PCR products were purified with the QiAquick PCR purification kit (Qiagen) prior to sequence determination. DNA sequence analysis was carried out using an automated ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA) and analyzed by the Omega 2.0 (Oxford Molecular Group) sequencing analysis software.

In vitro treatment with CIPRO

To determine if Mfd affects the development of FQR mutants under treatment with CIPRO, wild-type 11168 and JH01 were treated in MH broth with 4 µg/ml (32× the MIC) of CIPRO. Wild-type 11168 and JH01 were grown on antibiotic-free MH agar plates under microaerobic conditions. After 20 hours of incubation, the cells were collected and resuspended in MH broth for inoculation. Three treatment experiments were conducted using three different initial cell densities. In experiment 1, each strain was inoculated into 3 100-ml flasks with MH broth containing 4 µg/ml of CIPRO and the initial cell density was 10^8 CFU/ml. The cultures were incubated microaerobically at 42°C. Aliquots of the cultures were collected at different time points (0, 1, 2, 3 days post-inoculation) and plated onto regular MH plates for enumeration of the total bacterial number and onto MH plates containing 4 µg/ml of CIPRO for counting FQR colonies. In experiments 2 and 3, the cultures were treated in the same way, but the initial cell densities were 10^6 and 10^7 CFU/ml, respectively. Experiment 1 was repeated three times, while experiments 2 and 3 were each repeated twice.
The transcription level of Cj1084c

To determine if the insertional mutation in mfd affected the expression of the downstream gene Cj1084c (encoding a possible ATP/GTP-binding protein), RT-PCR was performed to assess the expression of Cj1084c. Total RNA was isolated from C. jejuni 11168 and JH01 using the RNeasy Kit (Qiagen). The purified RNA samples were treated with On-Column DNase Digestion Kit (Qiagen) followed by further treatments with DNase to remove DNA contamination. The Cj1084c-specific primers Cj1084cF (5’TCTGCTCTAGATACTT3’) and Cj1084cR (5’ACCTGCTTCTTA3’) were used to amplify a 450 bp region of the gene in a conventional one-step RT-PCR by using the SuperScript III One-Step RT-PCR kit (Invitrogen). An RT-PCR mixture lacking the RT was included as a negative control.

Emergence of FQR mutants in enrofloxacin-treated chickens

To examine if mfd plays a role in the emergence of FQR Campylobacter during in vivo FQ treatment, a chicken experiment was performed using 11168 and JH01. Day-old broiler chickens (Ross×Cobb) were obtained from a commercial hatchery and randomly assigned to 2 groups (11 birds per group). Each group of chickens was maintained in a sanitized wire-floored cage. Feed and water were provided ad libitum. Prior to inoculation with Campylobacter, the birds were tested negative for Campylobacter by culturing cloacal swabs. At day 3 of age, the two groups of chickens were inoculated with 11168 and JH01, respectively, at a dose of 10⁶ CFU/chick via oral gavage. Six days after the inoculation, the birds were treated with 50 ppm enrofloxacin. The treatment was administered in drinking water and lasted for five consecutive days. During the treatment, only medicated water was given to the birds to ensure enough consumption. Cloacal swabs were collected periodically before and after enrofloxacin treatment until the end of the experiment. Each swab was serially diluted in MH broth and plated onto two different types of MH plates: one containing Campylobacter-specific growth supplements (SR 004E and SR117 E; Oxoid Ltd., Basingstoke, England) for the enumeration of total Campylobacter cells and the other containing 4 µg/ml of CIPRO in addition to the same selective agents and supplements to recover FQR Campylobacter in each chicken. At each sampling time, at least one Campylobacter colony from each chicken was selected from the regular MH agar plates (no CIPRO) for the determination of CIPRO MICs using the E-test (AB Biodisk). The colonization data (CFU/g feces) were log-transformed and used for statistical analysis. The significance of differences in the level of colonization between the two groups was determined using Student’s t-test, Welch’s t-test to allow for non-constant variation across treatment groups, and the Wilcoxon rank-sum test to allow for non-normality. The conclusion of all three tests was the same at significance level 0.05.

Microarray data accession number

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database and the accession number is GSE10471.

Author Contributions

Conceived and designed the experiments: JH QZ. Performed the experiments: JH OS YB. Analyzed the data: JH OS YB QZ. Contributed reagents/materials/analysis tools: QZ. Wrote the paper: JH QZ.

References

1. Allos B (2001) Campylobacter jejuni Infections: Update on Emerging Issues and Trends. Clin Infect Dis 32: 1201–1206.
2. Tauxe RV (2002) Emerging foodborne pathogens. Int J Food Microbiol 78: 31–41.
3. Samuel M, Vugia D, Shallow S, Marcus R, Segler S, et al. (2004) Epidemiology of sporadic Campylobacter Infection in the United States and Declining Trend in Incidence. FoodNet 1996–1999. Clin Infect Dis 30: S165–S174.
4. Nachamkin I, Allos BM, Ho T (1998) Campylobacter Species and Guillain-Barré Syndrome. Clin Microbiol Rev 11: 557–567.
5. Koga M, Gilbert M, Takahashi M, Li J, Koike S, et al. (2006) Comprehensive Analysis of Bacterial Risk Factors for the Development of Guillain-Barré Syndrome after Campylobacter jejuni Enteritis. J Infect Dis 195: 547–553.
6. Friedman CR, Neimann J, Wegener HC, Tauxe RV (2000) Epidemiology of Campylobacter jejuni infections in the United States and other industrialized nations. In: Nachamkin I, Blair MJ, eds. Campylobacter 2nd ed. Washington, DC: ASM Press. pp 121–150.
7. Takkenen J AA, Robstad O, Breuer T (2001) European Survey on Campylobacter surveillance and diagnosis 2001. Euro Surveill 6: 207–213.
8. Oldfield EJ, Wallace MR (2001) The role of antibiotics in the treatment of infectious diarrhea. Gastroenterology Clinics of North America 30: 817–835.
9. Gupta A, Nelson JM, Barrett TJ, Tauxe RV, Rossiter SP, et al. (2004) Fluoroquinolone-resistant Campylobacter isolates from conventional and antibiotic-free chicken products. Environ Health Perspect 113: 557–560.
10. Hooper DC (2001) Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis 7: 337–341.
11. Deica K, Zhao X (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones. Med Mol Biol Rev 67: 377–392.
12. Lao N, Sahin O, Lin J, Michel LO, Zhang Q (2003) In vivo selection of Campylobacter jejuni isolates with high levels of fluoroquinolone resistance associated with gyrC mutations and the function of the CmeABC efflux pump. Antimicrob Agents Chemother 47: 390–394.
13. Leung Tongkum T, Morishita TY, Ison AJ, Huang S, McDermott PF, et al. (2006) Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of Campylobacter spp. in poultry. Appl Environ Microbiol 72: 3600–3607.
14. Price LB, Johnson E, Vailes R, Silbergeld E (2005) Fluoroquinolone-resistant Campylobacter isolates from conventional and antibiotic-free chicken products. Environ Health Perspect 113: 557–560.
15. Luangtrongkum T, Morishita TY, Ison AJ, Huang S, McDermott PF, et al. (2006) Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of Campylobacter spp. in poultry. Appl Environ Microbiol 72: 3600–3607.
16. Price LB, Johnson E, Vailes R, Silbergeld E (2005) Fluoroquinolone-resistant Campylobacter isolates from conventional and antibiotic-free chicken products. Environ Health Perspect 113: 557–560.
17. Hooper DC (2001) Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis 7: 337–341.
18. Deica K, Zhao X (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones. Med Mol Biol Rev 67: 377–392.
19. Lao N, Sahin O, Lin J, Michel LO, Zhang Q (2003) In vivo selection of Campylobacter jejuni isolates with high levels of fluoroquinolone resistance associated with gyrC mutations and the function of the CmeABC efflux pump. Antimicrob Agents Chemother 47: 390–394.
20. Leung Tongkum T, Morishita TY, Ison AJ, Huang S, McDermott PF, et al. (2006) Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of Campylobacter spp. in poultry. Appl Environ Microbiol 72: 3600–3607.
27. Zhang Q, Lin J, Pereira S (2003) Fluoroquinolone-resistant Campylobacter in animal reservoirs: dynamics of development, resistance mechanisms and ecological fitness. Anim Health Res Rev 4: 63–71.
28. Grigg DJ, Johnson MM, Frost JA, Humphrey T, Jørgensen F, et al. (2005) Incidence and mechanism of ciprofloxacin resistance in Campylobacter spp. isolated from commercial poultry flocks in the United Kingdom before, during, and after fluoroquinolone treatment. Antimicrob Agents Chemother 49: 699–707.
29. van Boven M, Veldman KT, de Jong MCM, Mevin DJ (2003) Rapid selection of quinolone resistance in Campylobacter jejuni but not in Escherichia coli in individually housed broilers. J Antimicrob Chemother 52: 719–723.
30. McDermott P, Bodeis S, English L, White D, Walker R, et al. (2002) Ciprofloxacin resistance in Campylobacter jejuni evolves rapidly in chicken treated with fluoroquinolones. J Infect Dis 185: 837–840.
31. Aarestrup FM, Wegener HC (1999) The effects of antibiotic usage in food animals on the development of antimicrobial resistance of importance for humans in Campylobacter and Escherichia coli. Microb Infect 1: 639–644.
32. Piddock LJ (1995) Quinolone resistance and Campylobacter spp. J Antimicrob Chemother 36: 891–898.
33. Kauterin H, Renkonen OV, Kosonen TU (1991) Emergence of fluoroquinolone resistance in Campylobacter jejuni and Campylobacter coli in subjects from Finland. Antimicrob Agents Chemother 35: 2065–2069.
34. Ruiz J, Goniz P, Marco F, Gallardo F, Mirelis B, Jimenez d. A, Vila J (1998) Increased resistance to quinolones in Campylobacter jejuni: a genetic analysis of gyrA gene mutations in quinolone-resistant clinical isolates. Microb Immunol 42: 223–226.
35. Saenz Y, Zarazaga M, Lantero M, Gastanastes MJ, Baquero F, et al. (2000) Antibiotic resistance in Campylobacter strains isolated from animals, foods, and humans in Spain in 1997–1998. Antimicrob Agents Chemother 44: 267–271.
36. Sanchez R, Fernandez-Baca V, Diaz MD, Munoz P, Rodriguez-Creixems M, et al. (1994) Evolution of susceptibilities of Campylobacter spp. to quinolones and macrolides. Antimicrob Agents Chemother 38: 1879–1882.
37. Smith KE, Bender JB, Ostholm MT (2000) Antimicrobial resistance in animals and relevance to human infections. In: Nachamkin I, Blaser MJ, eds. Campylobacter 2nd ed. Washington, DC: ASM Press. pp 483–495.
38. Van Loosdrecht M, Doube G, De Zutter I, Dumont JM, Lammerse C, et al. (2001) Antimicrobial susceptibilities of Campylobacter strains isolated from food animals in Belgium. J Antimicrob Chemother 47: 235–240.
39. Nachamkin I, Ung H, Li M (2002) Increasing fluoroquinolone resistance in Campylobacter jejuni, Pennsylvania, USA, 1982–2001. Emerg Infect Dis 8: 1501–1503.
40. Isehanger DW, Hoge CW, Srijan A, Pitarangsi C, Vithayasai N, et al. (2002) Comparative antibiotic resistance of diarrheal pathogens from Vietnam and Thailand, 1996–1999. Emerg Infect Dis 8: 175–180.
41. Boommar S, Morita Y, Fujita M, Sugank L, Suthavarakom K, et al. (2007) Serotypes, antimicrobial susceptibility, and gyrA gene mutation of Campylobacter jejuni isolates from humans and chickens in Thailand. Microb Immunol 51: 531–537.
42. Power EG, Phillips I (1992) Induction of the SOS gene (umuC) by 4-quinolone antibacterial drugs. J Med Microbiol 36: 76–82.
43. Cirz RT, O’Neill BM, Hammond JA, Head SR, Romeberg FE (2006) Defining the Pseudomonas aeruginosa sigma E response and its role in the global response to the antibiotic ciprofloxacin. J Bacteriol 188: 7101–7110.
44. Cirz RT, Jones MB, Gingles NA, Minogue TD, Jarrahi B, et al. (2007) Complete genomes of multiple Campylobacter species. PLoS Biol 5: e176.
45. Lin J, Akiba M, Sahin O, Zhang Q (2005) CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in Campylobacter jejuni. J Bacteriol 187: 7147–7154.
46. Yao S, Rojo F, Ogasawara N, Nakai S, Alonso JC (1996) The Mtd Protein of Bacillus subtilis encodes an undecaprenyl pyrophosphate synthetase and its parent to ciprofloxacin. Antimicrob Agents Chemother 50: 269–278.
47. Savery NJ (2007) The molecular mechanism of transcription-coupled DNA repair. Trends Microbiol 15: 326–333.
48. Apfel CM, Takacs B, Fountoulakis M, Stieger M, Keck W (1999) Use of mutagens to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential umuC gene. J Bacteriol 181: 483–492.
49. Marrer E, Satoshi AT, Johnson MM, Piddock LJ (2006) Global transcription analysis of the responses of a fluoroquinolone-resistant Staphylococcus pneumoniae mutant and its parent to ciprofloxacin. Antimicrob Agents Chemother 50: 269–278.
50. Ross C, Tsuyuki C, Pedraza-Reyes M, Sung H-M, Yashin RE, et al. (2006) Novel role of mfd effects on stationary-phase mutagenesis in Bacillus subtilis. J Bacteriol 188: 7532–7520.
51. Blackwell SK, Theorell T, Bockman LR, Woodgate R, Goodman MF (2001) Roles of DNA repair genes in maintaining competence for transformation in Bacillus subtilis. Genetics 158: 1101–1111.
52. George DL, Winkin EM (1974) Slow excision repair in an mfd mutant of Escherichia coli B/r. Mol Gen Genet 133: 283–291.
53. Selby CP, Sancar A (1994) Mechanisms of transcription-repair coupling and mutation frequency decline. Microbiol Mol Biol Rev 58: 317–329.
54. Selby CP, Sancar A (1995) Molecular mechanism of transcription-coupled DNA repair. J Biol Chem 270: 30106–30113.
55. Istivan TS, Coloe PJ (2006) Phospholipase A in gram-negative bacteria and its role in pathogenesis. Microbiol 152: 1263–1274.
56. Grant KA, Belandia IU, Dekker N, Richardson PT, Park SF (1997) Molecular characterization of mfd, the structural gene for a phospholipase A from Campylobacter coli, and its contribution to cell-associated hemolysis. Infect Immun 65: 1172–1180.
57. Gmuender H, Kuratli K, Di Padova K, Gray CP, Keck W, et al. (2002) Gene expression changes triggered by exposure of E. coli B/r having a slow rate of dimer excision. Mutat Res 28: 127–130.
58. Marrer E, Satoh AT, Johnson MM, Piddock LJ (2006) Global transcription analysis of the responses of a fluoroquinolone-resistant Staphylococcus pneumoniae mutant and its parent to ciprofloxacin. Antimicrob Agents Chemother 50: 269–278.
59. Pham P, Ramazanjee S, Woodgate R, Goodman MF (2001) Roles of DNA polymerases V and II in SOS-induced error-prone and error-free repair in Escherichia coli. Proc Natl Acad Sci USA 98: 8350–8354.
60. Sancar A, Gao Z, Frokhous N, Li Y, Blackwell SK, et al. (2003) Mutations in the recQ homolog of human DNA helicase II lead to increased mutagenesis. Proc Natl Acad Sci USA 100: 7322–7327.
61. Bhatnagar M, Kelkar A, Bhargava VN, Thakkar N, Asokan R, et al. (2004) Identification of Campylobacter jejuni gyrA gene and characterization of quinolone resistance mutations. Antimicrob Agents Chemother 37: 453–467.