

Effect of Macrolide Usage on Emergence of Erythromycin-Resistant *Campylobacter* Isolates in Chickens[∇]

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In this work we conducted both in vitro and in vivo experiments to examine the development and mechanisms of erythromycin (Ery) resistance in *Campylobacter jejuni* and *Campylobacter coli*. In vitro plating revealed that both *Campylobacter* species had similar but low spontaneous mutation frequencies (3×10^{-9} to $<5.41 \times 10^{-10}$) for Ery resistance. Chickens infected with *C. jejuni* or *C. coli* were subjected to single or multiple treatments with medicated water containing tylosin (0.53 g/liter), which transiently reduced the level of *Campylobacter* colonization but did not select for Ery-resistant (Ery^r) mutants in the treated birds. However, when tylosin was given to the chickens in feed at a growth-promoting dose (0.05 g/kg feed), Ery^r mutants emerged in the birds after prolonged exposure to the antibiotic. The vast majority of the in vitro- and in vivo-selected *Campylobacter* mutants with Ery MICs of 8 to 256 $\mu\text{g/ml}$ lacked the known resistance-associated mutations in the 23S rRNA gene, while the highly resistant mutants (Ery MIC > 512 $\mu\text{g/ml}$) had the A2074G mutation in the 23S rRNA gene. Inactivation of CmeABC, a multidrug efflux pump, dramatically reduced the Ery MIC in all of the examined mutants regardless of the presence of the A2074G mutation. Together, these results reveal distinct features associated with Ery resistance development in *Campylobacter*, demonstrate the significant role of CmeABC in Ery resistance, and suggest that long-term use of a macrolide as a growth promoter selects for the emergence of Ery^r *Campylobacter* in animal reservoirs.

Thermophilic *Campylobacter* spp. (*Campylobacter jejuni* and *Campylobacter coli*) have been recognized as one of the most commonly detected bacterial pathogens causing human gastrointestinal enteritis (5). As an enteric organism, *Campylobacter* resides in the gastrointestinal tracts of domestic and wild animals (17). The majority of human *Campylobacter* infections are associated with consumption of undercooked poultry meat as well as raw milk and contaminated water (17).

Macrolides, such as erythromycin (Ery), represent the drugs of choice for treating human campylobacteriosis, but fluoroquinolones (FQs) are often used for the treatment of enteritis when a microbiological diagnosis is absent (14, 20). During the past decades, *Campylobacter* has become increasingly resistant to both classes of antibiotics, although the actual resistance rates vary in different countries (14, 20). The prevalence of FQ-resistant *Campylobacter* is high on a worldwide scale, making macrolide antibiotics especially important for treatment of *Campylobacter* infections in humans (5). However, many studies have revealed a general trend of rising Ery resistance in *Campylobacter*, especially in *C. coli* (7, 8, 14, 20, 40). The use of macrolides in food-producing animals as therapeutic or growth-promoting agents has been considered a factor in the selection of Ery-resistant (Ery^r) *Campylobacter* (20), but experimental evidence supporting this link is still limited.

Macrolide antibiotics inhibit bacterial growth by binding to prokaryotic ribosomes and interfering with protein synthesis

(16). Antibiotic modifications, target site alterations, and drug efflux are the three main mechanisms involved in macrolide resistance in bacteria (20, 27). However, no evidence has been reported for macrolide modification in *Campylobacter* (20, 39). In both *C. jejuni* and *C. coli*, point mutations in the 23S rRNA gene have been associated with macrolide resistance (14, 19, 23, 25, 33). Specifically, the resistance-associated mutations occur at base position 2074 (A2074C, A2074G, or A2074T) or 2075 (A2075G or A2075C) or both in the 23S rRNA gene of *Campylobacter*. These positions correspond to base positions 2058 and 2059, respectively, in *Escherichia coli*. There are three copies of the 23S rRNA gene in *C. jejuni* and *C. coli* (15, 37). Usually all three copies of the 23S rRNA gene are mutated, but both the wild-type and mutated alleles can coexist in a single macrolide-resistant mutant (19, 25, 33). A recent finding (10) demonstrated that modifications in the ribosomal proteins L4 (G74D) and L22 (insertions at position 86 or 98) also conferred macrolide resistance in *Campylobacter*. By insertional mutagenesis, it has been shown that the multidrug efflux pump CmeABC of *C. jejuni* contributes to intrinsic and acquired resistance to macrolides (11, 28, 33). Inhibition of CmeABC by an efflux pump inhibitor decreased the MICs of Ery (2- to 512-fold) in various *Campylobacter* isolates and also significantly decreased the frequency of emergence of Ery^r *C. jejuni* mutants (34).

Macrolides, such as tylosin and spiramycin, are used in chickens as therapeutic/prophylactic agents for the control of chronic respiratory diseases caused by mycoplasmas and as subtherapeutic agents for improving growth rates and feed efficiency in the United States (18, 20). Since chickens are frequently colonized by *Campylobacter* in the intestinal tract and since contaminated poultry meat is considered a significant

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source of food-borne campylobacteriosis in humans, it has been a concern whether exposure of chickens to therapeutic or subtherapeutic doses of macrolides promotes the emergence of macrolide-resistant *Campylobacter*, as is reported for the emergence of FQ resistance (22, 32, 35). To address this concern, we conducted *in vitro* and *in vivo* studies to examine the frequencies of emergence of Ery^r *C. jejuni* and *C. coli* under the selection pressure of macrolide usage. In addition, we investigated the resistance mechanisms associated with Ery resistance in *Campylobacter*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two *C. jejuni* strains, S3B (32) and ATCC 700819 (NCTC 11168), and four *C. coli* strains, RN14B, MR32D, AW-II-35, and AW-II-37, were used in this study. All these strains are susceptible to Ery, with Ery MICs of 0.125 µg/ml for AW-II-35 and AW-II-37 and 2 µg/ml for S3B, 700819, RN14B, and MR32D. The *Campylobacter* strains were grown routinely on Mueller-Hinton (MH) agar plates or in MH broth at 42°C under microaerobic conditions. *Campylobacter*-specific growth supplements and selective agents (Oxoid) were added to the media when needed. When required, the MH media were also supplemented with various amounts of Ery.

Frequencies of emergence of Ery^r *C. jejuni* and *C. coli* *in vitro*. *C. jejuni* and *C. coli* cultures were grown in antibiotic-free MH broth to the late logarithmic phase. The cultures were plated in duplicate on antibiotic-free MH agar plates and Ery-containing plates (2, 4, or 8 µg/ml). The plates were incubated for 2 days microaerobically at 42°C. The frequency of Ery resistance was calculated as the ratio of the numbers of CFU on Ery-containing plates to the CFU on Ery-free plates. The experiment was repeated three times.

Antibiotic susceptibility test. MICs of antibiotics were tested using the agar dilution method as recommended by the CLSI (formerly NCCLS) (13). For quality control, *C. jejuni* ATCC 33560 and the quality control range for the Ery MIC of 0.25 to 2 µg/ml were used in this study. According to the breakpoints recommended by CLSI (13), Ery MICs of ≤8 µg/ml and ≥32 µg/ml indicate susceptibility and resistance, respectively. Ery was purchased from Sigma Chemical Co., St. Louis, MO.

Sequence analysis of the 23S rRNA gene. The 23S rRNA gene sequences of *C. jejuni* and *C. coli* were amplified by PCR with gene-specific primers (5'-GTAA ACGCGGCCGTAACATA-3' and 5'-GACCGAAGTGTCTCACGACG-3') (25). The PCR conditions were as follows: 94°C for 5 min; 94°C for 30 s, 52°C for 30 s, and 72°C for 40 s for 35 cycles; and a final extension at 72°C for 10 min. The amplified PCR products were purified with the QIAquick PCR purification kit (QIAGEN) prior to sequencing. DNA sequences were determined in the DNA facilities at Iowa State University and the University of Tennessee. Sequence analysis was performed using Omega 2.0 software (Oxford Molecular Ltd.).

Immunoblotting analysis of CmeABC expression. To examine if CmeABC was overexpressed in the Ery^r isolates, the CmeA, CmeB, and CmeC proteins of Ery^r isolates S3BE2-2 and S3BE4-3 (Ery MIC = 256 and 128 µg/ml, respectively; no mutation in the 23S rRNA gene) as well as their parent strain, S3B, were analyzed by immunoblotting with antibodies specific for CmeA, CmeB, and CmeC as described previously (28).

Insertional mutagenesis of the *cmeB* gene in various isolates. Natural transformation was used to construct the isogenic *cmeB* mutants of various *Campylobacter* isolates as described previously (4). Genomic DNA of 11168B (*cmeB::kan*) was used as the donor DNA in the transformation experiments, while various *Campylobacter* isolates with different levels of resistance to Ery were used as the recipient strains. Natural transformation was performed with a biphasic method as described by Wang and Taylor (43). Transformants were selected on plates containing 30 µg/ml of kanamycin. The insertion of the kanamycin cassette into the *cmeB* gene of each recipient isolate was confirmed by PCR as described previously (28).

Treatment of *Campylobacter*-colonized chickens with medicated water containing tylosin. A chicken colonization model was used to assess the effect of high-dose tylosin treatment on the emergence of Ery^r mutants in *Campylobacter*. For this purpose, day-old broiler chickens (Ross × Cobb) were purchased from a commercial hatchery and randomly assigned to multiple groups (10 to 15 birds per group). Each group of chickens was housed in a sanitized wire floor cage and provided with nonmedicated feed *ad libitum*. Prior to inoculation with *Campylobacter*, all birds were confirmed to be free of *Campylobacter* by culturing cloacal swabs. Each *Campylobacter* strain or a mixture of two strains was used to inoculate two groups of chickens (10⁷ CFU/bird). Five days after the inoculation, one

of the two groups inoculated with the same culture (or mixture) was treated with tylosin (Tylan; soluble; Elanco Animal Health), while the other group was untreated and used as a control for the treated group. Each tylosin treatment was given to the chickens in drinking water for three consecutive days according to the dose (0.53 g/liter) approved for commercial poultry production in the United States. During the treatment, only medicated water was given to the birds to ensure enough consumption to provide tylosin at 110 mg/kg of body weight per day as indicated by the drug label. Cloacal swabs were collected from the chickens before and after tylosin treatment until the end of each experiment. Each swab was diluted serially in MH broth, and each dilution was plated onto two different types of MH plates: one containing *Campylobacter*-specific selective agents and growth supplements (SR084E and SR117E; Oxoid) to recover the total *Campylobacter* populations and the other containing 8 µg/ml Ery in addition to the same selective agents and supplements to recover the *Campylobacter* populations that were considered not susceptible to Ery. *Campylobacter* colonies were counted following 48 h of incubation at 42°C under microaerobic conditions. At each sampling time, 10 to 15 colonies (from different chickens) were collected from each group for MIC testing using the standard agar dilution method (13). Three independent experiments were conducted in total. In experiment A, *C. jejuni* S3B and 700819 were used for infecting chickens; in experiment B, the two groups of chickens were inoculated with a mixture of *C. coli* strains AW-II-35 and AW-II-37; and in experiment C, the chickens were inoculated with *C. jejuni* 700819 but the treatment group was treated three times with tylosin at a weekly interval.

Feeding of *Campylobacter*-colonized chickens with tylosin at a low dose. Two independent experiments were conducted with chickens using tylosin at the dose used for growth promotion. In each experiment, day-old broiler chickens were obtained from a commercial hatchery and the birds were randomly assigned to two groups (9 to 11 birds per group). Birds in the control group received nonmedicated feed without any antibiotic additives. Chickens in the treatment group received the same feed but supplemented with tylosin (Elanco Animal Health). The nonmedicated feed and the tylosin-containing feed were prepared by the feed mill in the Johnson Animal Research and Teaching Unit at the University of Tennessee. Instructions on the drug label were followed in the preparation of the medicated feed for growth promotion in chickens. The final concentration of tylosin in the feed was 50 mg/kg, which is an approved dose for growth promotion in poultry production in the United States. Since a withdrawal period is not required for tylosin used for growth promotion in chickens (6), the birds in the treatment group were given the medicated feed for the entire 41 days of the experiment. All of the birds were negative for *Campylobacter* prior to *Campylobacter* inoculation, as determined by culturing cloacal swabs. In the first experiment, each chicken was inoculated with a fresh culture of approximately 10⁷ CFU of *C. jejuni* ATCC 700819 via oral gavage at 3 days of age. After the inoculation, cloacal swabs were collected from each chicken at 6, 13, 20, 27, 34, and 41 days of age. Isolation of *Campylobacter* and differential plating for enumerating the proportion of the mutant colonies were conducted as described for the water medication study. In addition, individual colonies were collected from Ery-free plates for each chicken and were used for MIC tests. In the second experiment, the inoculation of chickens with *C. jejuni* ATCC 700819 was at 17 days of age and cloacal swabs were collected from the chickens at 20, 27, 34, and 41 days of age. The reason for using two different inoculation dates in the two experiments was to measure if the duration of exposure to the growth promoter affects the emergence of Ery^r *Campylobacter*. In addition, a distinct characteristic of *C. jejuni* colonization in poultry is that this organism is not detected in chickens less than 2 to 3 weeks of age under commercial broiler production conditions. Thus, inoculation of birds with *C. jejuni* at 17 days of age in the second experiment should allow us to measure the impact of the use of tylosin as a growth promoter on the development of Ery resistance in *Campylobacter* under conditions similar to commercial production. Multiple isolates with different Ery MICs were analyzed by PCR and pulsed-field gel electrophoresis (PFGE) to confirm their genetic identities. The PCR was done using primers F3 and R3, specific for the *cmp* gene encoding the major outer membrane protein, and the PFGE was performed using KpnI as described previously by Huang et al. (24).

Detection limit and statistical analysis. In all chicken experiments, the detection limit of the plating methods is approximately 100 CFU/g of feces. When no *Campylobacter* colonies were detected in a chicken, it was considered negative and arbitrarily assigned the number 0 for the purpose of calculating means and statistical analysis. Student's *t* test was used to examine the significance of differences in *Campylobacter* colonization levels (log transformed CFU/g feces) at each sampling point between the tylosin-treated and nontreated groups inoculated with the same *Campylobacter* strain. A *P* value of <0.01 was considered significant.

TABLE 1. Frequency of emergence of Ery^r *Campylobacter* in vitro under different selection pressures

Ery concn ($\mu\text{g/ml}$)	Frequency ^a for strain:			
	S3B	700819	RN14B	MR32D
4	$(3.0 \pm 3.2) \times 10^{-9}$	$<5.4 \times 10^{-10}$	$(1.1 \pm 1.2) \times 10^{-9}$	$(4.9 \pm 2.2) \times 10^{-10}$
8	$(1.2 \pm 0.9) \times 10^{-10}$	$<5.4 \times 10^{-10}$	$(5.6 \pm 2.5) \times 10^{-10}$	$(4.5 \pm 1.6) \times 10^{-10}$

^a Means of three independent experiments \pm standard deviations.

RESULTS

Frequencies of emergence of Ery^r *Campylobacter* mutants in vitro. To determine the frequencies of emergence of Ery resistance in *C. jejuni* and *C. coli* in vitro, two *C. jejuni* strains (S3B and 700819) and two *C. coli* strains (RN14B and MR32D) grown in antibiotic-free MH broth were plated on MH agar plates containing 4 or 8 $\mu\text{g/ml}$ erythromycin. As shown in Table 1, the frequencies of emergence of Ery resistance in both *Campylobacter* spp. were between 3×10^{-9} and $\leq 10^{-10}$ as measured by a single-step selection. There were no significant differences ($P = 0.7135$) between the frequencies of mutant emergence measured with 4 $\mu\text{g/ml}$ or 8 $\mu\text{g/ml}$ of erythromycin (Table 1). In addition, there were no significant differences in the mutant emergence frequencies between *C. coli* and *C. jejuni* regardless of the concentrations of erythromycin used on the plates. All of the examined mutants had Ery MICs of 8 to 256 $\mu\text{g/ml}$ (Table 2). These findings indicate that the emergence of spontaneous Ery^r mutants in *Campylobacter* under in vitro culture conditions is at a low frequency.

Effect of tylosin treatment on the emergence of Ery^r *Campylobacter* in chickens. To determine if tylosin treatment influences the development of macrolide resistance in *Campylobacter*, broiler chickens were first infected with *C. jejuni* or *C. coli* and then treated (single or multiple times) with tylosin administered in drinking water at the dose (0.53 g/liter) recommended for therapeutic use in commercial poultry production in the United States. In the first experiment, the chickens inoculated with *C. jejuni* (S3B or 700819) were quickly colonized and shed the organism at a level between 10^5 and 10^7 CFU/g feces before the initiation of tylosin treatment (Fig. 1A). Two days after the initiation of the tylosin treatment, the number of colonized chickens decreased drastically, with *Campylobacter* detectable only in a few chickens. The few chickens that remained colonized on days 2 and 4 after initiation of the tylosin treatment shed significantly lower numbers of organisms in the feces than those in the nontreated groups (Fig. 1A). However, the number of colonized chickens and the level of colonization recovered and reached the levels for the nontreated groups after cessation of the treatment (Fig. 1A). Nontreated chickens remained colonized and shed *Campylobacter* at approximately 10^5 to 10^6 CFU/g feces throughout the study (Fig. 1A). During the entire experiment, no *Campylobacter* colonies were observed on plates containing 8 $\mu\text{g/ml}$ Ery. In addition, representative colonies (10 isolates/sample/group) collected from the chickens were tested by the agar dilution method for MICs of erythromycin. All of the isolates had the same Ery MIC (2 $\mu\text{g/ml}$) as that of the parent strains, S3B and 700819, further confirming that the single tylosin treatment did not select for Ery^r *Campylobacter*. In the second experiment, the chickens were infected with a mixture of two

strains of *C. coli* (AW-II-35 and AW-II-37) and then treated with tylosin. As shown in Fig. 1B, the treatment did not eradicate *C. coli* from the chickens despite a significant reduction in the level of colonization during the treatment ($P < 0.01$). On day 3 after the initiation of tylosin treatment, only 20% of the birds shed detectable levels of *Campylobacter* in feces; however, 100% of the birds became shedders again on the next sampling day. No Ery^r mutants were detected in any of the

TABLE 2. Ery MICs and mutations in various *Campylobacter* strains and mutants

Strain	Background	Ery MIC ^a ($\mu\text{g/ml}$)	Mutation in 23S rRNA	Ery MIC of the <i>cmeB</i> mutant ($\mu\text{g/ml}$) ^b
Wild-type strains				
700819	<i>C. jejuni</i>	2	None	0.125
S3B	<i>C. jejuni</i>	2	None	1
RN14B	<i>C. coli</i>	2	None	0.125
MR32D	<i>C. coli</i>	2	None	ND ^d
AW-II-35	<i>C. coli</i>	0.125	None	ND
AW-II-37	<i>C. coli</i>	0.125	None	ND
In vitro-selected mutants				
S3BE2-2	S3B	256	None	0.125
S3BE4-1	S3B	32	None	ND
S3BE4-2	S3B	32	None	ND
S3BE4-3	S3B	128	None	ND
S3BE8-1	S3B	256	None	ND
RN14BE4-2	RN14B	8	A2074G ^c	0.125
RN14BE4-3	RN14B	32	None	0.125
RN14BE8-1	RN14B	32	None	ND
RN14BE8-2	RN14B	32	None	ND
RN14BE8-3	RN14B	32	None	ND
MR32DE4-1	MR32D	8	None	ND
MR32DE8-1	MR32D	32	None	ND
In vivo-selected mutants				
1	700819	8	None	0.250
2	700819	8	None	0.125
3	700819	32	None	0.250
4	700819	>512	A2074G	32
5	700819	>512	A2074G	32
6	700819	>512	A2074G	32
7	700819	8	None	0.250
8	700819	16	None	0.250
9	700819	32	None	0.250
10	700819	8	None	0.125
11	700819	8	None	0.250
12	700819	8	None	0.250

^a Determined by the agar dilution method.

^b Each *cmeB* mutant was generated from the corresponding isolate by insertional mutagenesis.

^c Present in one of the three copies of the 23S rRNA gene.

^d ND, no mutagenesis of *cmeB* was done in the isolate.

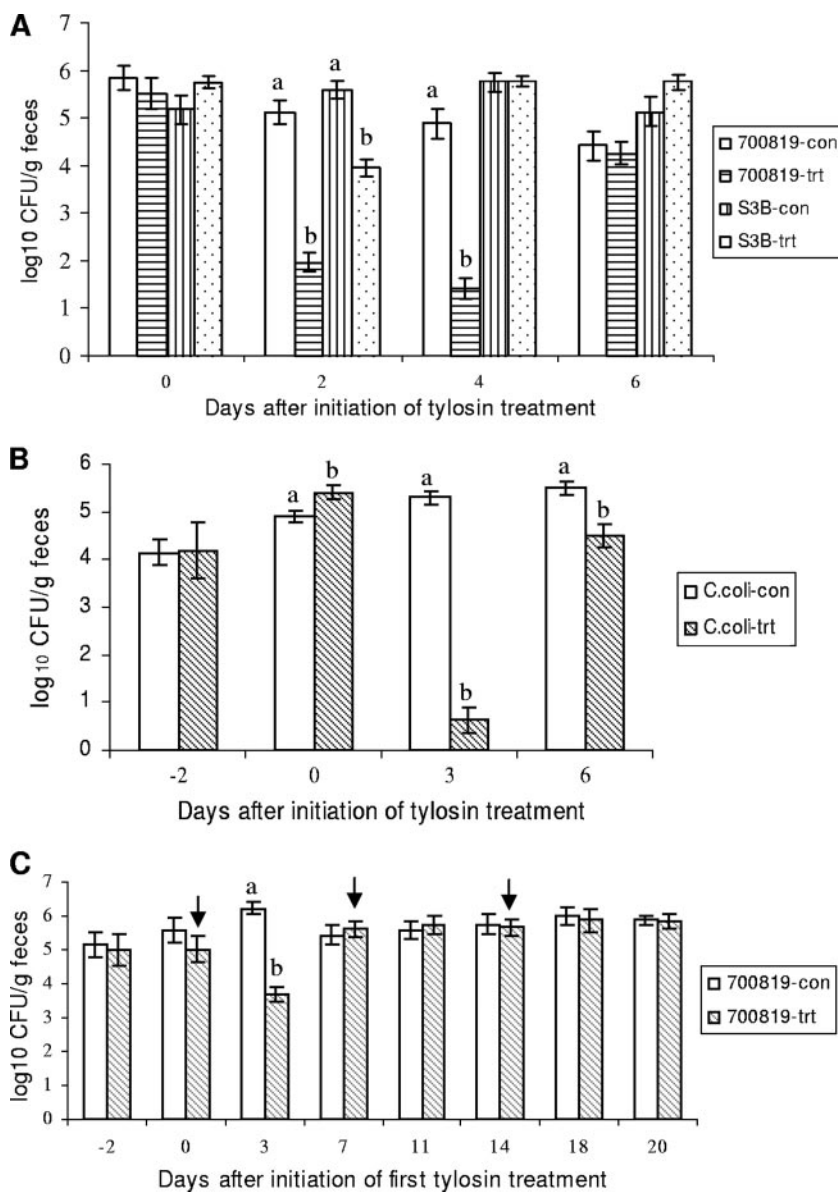


FIG. 1. Effect of tylosin treatment on the colonization of *Campylobacter* in chickens. (A) Chickens were inoculated with *C. jejuni* 700819 and S3B, respectively. Two (700819-trt and S3B-trt) of the groups received a single treatment with tylosin, while the other two groups (700819-con and S3B-con) were nontreated controls. (B) Two groups of chickens were inoculated with a mixture of *C. coli* strains AW-II-35 and AW-II-37. One group (*C. coli*-trt) received a single treatment with tylosin, while the other (*C. coli*-con) served as a nontreated control group. (C) Chickens were infected with *C. jejuni* 700819. One group (700819-con) served as the nontreated control, while the other group (700819-trt) received three tylosin treatments at a weekly interval. The arrows in panel C indicate the starting day of each treatment. In all three experiments (A to C), tylosin was given in drinking water (0.53 g/liter) for three consecutive days for each treatment. Each bar represents the arithmetic mean \pm standard deviation of the mean log₁₀ CFU/g feces in each group. The CFU in individual chickens were converted to log₁₀ units before calculating the arithmetic means. For the two groups inoculated with the same strain, the means on the same sampling day labeled with different letters are significantly different ($P < 0.01$).

treated chickens by either the differential plating method or the MIC test. The findings from the *C. coli* experiment were consistent with the results from the first experiment with *C. jejuni* and indicated that a single tylosin treatment did not select for macrolide-resistant *Campylobacter* in chickens. In the third experiment, the chickens inoculated with *C. jejuni* 700819 received three tylosin treatments at a weekly interval. As shown in Fig. 1C, the colonization level of 700819 dropped significantly ($P < 0.01$) during the first treatment but returned

to the level of the nontreated controls afterward and persisted during the subsequent treatments. Despite the three treatments, no Ery^r mutants were detected in any of the treated chickens by both differential plating and MIC testing. Together, these results indicated that short-term treatment of *Campylobacter*-infected chickens with tylosin at a high dose did not result in the selection of Ery^r *Campylobacter* regardless of the number of treatments or the species of *Campylobacter* treated.

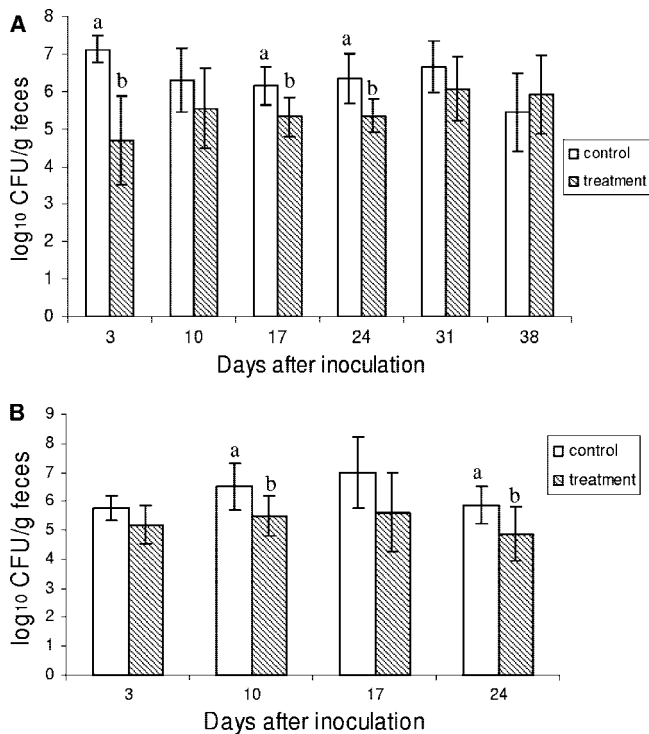


FIG. 2. Levels of *Campylobacter* colonization in chickens given tylosin at a growth-promoting dose in feed. In both experiments (A and B), the chickens were inoculated with *C. jejuni* 700819. One group received nonmedicated feed (control), while the other (treatment) received medicated feed containing tylosin (50 mg tylosin/kg feed). In both experiments, the chickens in the treatment groups were given the medicated feed during the entire experiment (41 days). For panel A, the birds were inoculated with *C. jejuni* at 3 days of age, while for panel B the chickens were inoculated at 17 days of age. Each bar represents the mean \log_{10} CFU/g feces \pm standard deviation in each group. Different letters above the bars of each sampling day denote significant differences ($P < 0.01$).

Emergence of Ery^r *Campylobacter* upon long-term exposure to tylosin at a low dose. To determine if long-term use of tylosin as a growth promoter affects the development of Ery resistance in *C. jejuni*, strain 700819 was inoculated into chickens that received either nonmedicated feed or feed supplemented with tylosin at a dose (50 mg/kg feed) used for growth promotion. In the first experiment, the chickens were inoculated with *Campylobacter* at 3 days of age. All chickens, including the ones with the medicated feed, were colonized by *C. jejuni* on day after inoculation (DAI) 3 (Fig. 2A). The shedding level of *Campylobacter* in feces was consistently higher (up to 2.4 \log_{10} units) in the chickens given nonmedicated feed than in those given the tylosin-containing feed, except that on DAI 38 the shedding levels of *C. jejuni* for the nonmedicated group were slightly lower (but the difference was not statistically significant: $P = 0.3$) than those for the medicated group (Fig. 2A). No Ery^r *C. jejuni* mutants were detected in the nonmedicated group throughout the study. However, in the group fed with tylosin, Ery^r mutants were detected in the chickens on DAI 31 and 38 (Fig. 3A). In the second experiment, the chickens were inoculated with *C. jejuni* 700819 at 17 days of age. Similar to the first experiment, the level of *Campylobacter*

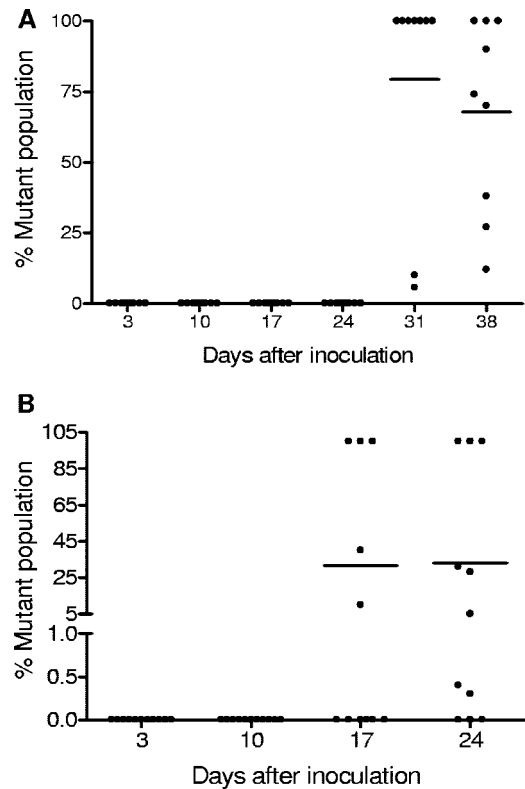


FIG. 3. Development of Ery^r *C. jejuni* in individual chickens fed with tylosin (50 mg tylosin/kg feed) in two different experiments (A and B). See the legend of Fig. 2 for the details of the treatment. Each solid circle represents the percentage of the mutant population in each chicken, which was determined by differential plating using plates containing 8 $\mu\text{g/ml}$ of Ery. The mean of each group is indicated by a horizontal bar.

colonization in the medicated group was consistently lower (the differences were significant on DAI 10 and 24: $P < 0.01$) than that in the nonmedicated group (Fig. 2B). In the medicated group, the mutant colonies growing on the Ery plates were detected on DAI 17 and 24 (Fig. 3B).

Differential plating, in which the Ery^r *Campylobacter* mutants and the total *Campylobacter* colonies in each swab sample were simultaneously enumerated by using two different types of plates, revealed the dynamics of emergence of Ery^r *Campylobacter* in the chickens. In the first experiment, no birds showed Ery^r *Campylobacter* mutants at DAI 3, 10, 17, and 24 but, on DAI 31, the mutant populations had become dominant in seven of the nine chickens given the tylosin-medicated feed (Fig. 3A). On DAI 38, the mutant population varied in individual chickens, ranging from 12 to 100% (Fig. 3A). From the samples collected from the tylosin-treated chickens on DAI 31 and 38, 27 *Campylobacter* isolates from individual chickens were randomly selected for MIC testing. Among the 27 isolates examined, 10 isolates displayed an Ery MIC of $>512 \mu\text{g/ml}$, 2 isolates showed an Ery MIC of 32 $\mu\text{g/ml}$, 3 isolates had an Ery MIC of 8 $\mu\text{g/ml}$, and the rest had Ery MICs of $<8 \mu\text{g/ml}$. In the second experiment, *Campylobacter* mutants were not detected on Ery-containing (8 $\mu\text{g/ml}$) plates on DAI 3 and 10 but appeared on DAI 17 and 24 in the group fed with tylosin (Fig. 3B). Thirty-five isolates collected on DAI 17 and 24 from the

tylosin-treated group were examined for Ery MICs, among which 4 showed an Ery MIC of 16 $\mu\text{g/ml}$, 13 had an Ery MIC of 8 $\mu\text{g/ml}$, and the rest had an Ery MIC of $<8 \mu\text{g/ml}$. In both experiments, no Ery^r *Campylobacter* mutants from the non-treated chickens were detected by differential plating and the MIC tests (data not shown). In addition, representative Ery^r isolates derived from the tylosin-fed chickens were analyzed by PCR (for the *cmp* gene) and PFGE, which revealed that the *cmp* gene sequence and the KpnI restriction patterns of the mutants were identical to those of strain 700819 (data not shown), indicating that the mutants were derived from the parent strain.

Resistance mechanisms associated with Ery^r mutants. Both in vitro- and in vivo-selected mutants with different Ery MICs were chosen for 23S rRNA gene sequence analysis and *cmeB* mutation (Table 2). None of the in vitro-selected mutants with Ery MICs ranging from 8 to 256 $\mu\text{g/ml}$ carried the resistance-associated mutations in the 23S rRNA gene, except for the *C. coli* derivative RN14BE4-2 (Ery MIC = 8 $\mu\text{g/ml}$), which harbored the A2074G point mutation in the 23S rRNA gene (Table 2). According to the sequence result, it appeared that the A2074G mutation was present in only one of the three copies of the 23S rRNA gene in RN14BE4-2, because the sequence chromatogram showed double peaks in the same position where the G peak was 50% shorter than the A peak. For the in vivo-selected mutants, all of the 10 highly resistant mutants (Ery MIC $> 512 \mu\text{g/ml}$) displayed the specific A2074G point mutation in the 23S rRNA gene but none of the 18 examined mutants with Ery MICs from 8 to 32 $\mu\text{g/ml}$ showed point mutations in the gene (partly shown in Table 2). Inactivation of *cmeB* in the *Campylobacter* mutants dramatically reduced the resistance to Ery (Table 2). For the mutants without any mutations in the 23S rRNA gene, inactivation of *cmeB* reversed the acquired Ery resistance to a level that was even below the wild-type MIC (Table 2), indicating that the CmeABC efflux pump plays an essential role in maintaining the acquired Ery resistance in these mutants. For those in vivo-selected isolates with the A2074G point mutation, inactivation of *cmeB* reduced the resistance from >512 to 32 $\mu\text{g/ml}$. To determine if *cmeABC* was overexpressed in the Ery^r mutants, we compared the protein levels of CmeABC in several Ery^r mutants and their wild-type strain using immunoblotting, and no obvious differences in CmeABC expression were observed (data not shown). In addition, MIC tests indicated that the Ery^r mutants did not show cross-resistance to non-macrolide antibiotics, including enrofloxacin, oxytetracycline, florfenicol, and penicillin (data not shown).

DISCUSSION

Results from this study revealed several distinct features associated with Ery resistance development in *Campylobacter*. Firstly, the in vitro plating results (Table 1) using single-step selection and the in vivo findings from the treatment with tylosin suggest that both *C. jejuni* and *C. coli* have extremely low rates of spontaneous mutation to Ery^r (Table 1), which is in contrast to the emergence of FQ resistance in *Campylobacter* (21, 47). Secondly, it appears that the acquisition of Ery resistance in *Campylobacter* involves a stepwise process and requires prolonged exposure to the selection pressure.

This conclusion is supported by the fact that Ery^r mutants emerged in the chickens fed with tylosin for an extended period (Fig. 3) but were not detected in the chickens treated with tylosin-medicated water for short periods. The conclusion is also supported by the finding that the Ery^r mutants obtained by a single-step selection in vitro had an Ery MIC of $\leq 256 \mu\text{g/ml}$ and that the majority of them lacked the resistance-associated mutations in the 23S rRNA gene (Table 2), which suggests that emergence of high-level Ery resistance (MIC > 512) may require multiple mutation steps. The low frequency of spontaneous mutation of *Campylobacter* to macrolide resistance and the need for prolonged exposure for resistance development may explain the relatively low prevalence of Ery^r *Campylobacter* compared with FQ-resistant *Campylobacter*.

An interesting finding of this study is that treatment of chickens with a high dose of tylosin in drinking water did not select for Ery^r mutants. For the water medication, the chickens were given a high dose of tylosin for three consecutive days during each treatment. Neither single nor multiple short-term water medications selected for Ery^r mutants in the chickens, even though the treatments transiently reduced the number of *Campylobacter* in the intestinal tracts of chickens (Fig. 1). In fact, all of the tested isolates from the water medication study had the same Ery MICs as the parent strains, indicating that there were no changes in the susceptibility of *Campylobacter* to Ery before and after the water medications. These findings indicate that, under the experimental conditions used in this study, no Ery^r *Campylobacter* was selected by the water medication. However, these results may not be used to predict resistance development on poultry farms, where each flock contains a large number of birds and can be infected by multiple strains of *Campylobacter*, which may respond differently to water medication with tylosin. Nevertheless, findings from this study suggest that the probability of selection for Ery resistance in *Campylobacter* is low when tylosin is given to chickens for a short period of time.

This study clearly showed that use of tylosin as a growth promoter resulted in the emergence of Ery^r *C. jejuni* in the chickens under laboratory conditions (Fig. 3). During the experiments, the chickens were fed with a low dose of tylosin for the entire period (41 days). Ery^r mutants were not detected during the first 2 to 4 weeks of exposure and were observed only after an extended period of exposure to the antibiotic (Fig. 3). The lack of Ery^r mutants during the early weeks is consistent with the result of the water medication experiments, in which single or multiple short-term treatments did not select for Ery^r *Campylobacter*. Together, these results suggest that continuous exposure to a macrolide for an extended period is required for Ery resistance development in *Campylobacter*. Thus, the different outcomes in resistance development between the two types of treatments (therapy or growth promotion) are likely due to the difference in the lengths of exposure, instead of the routes of administration or the doses of tylosin used in the treatments. However, we cannot exclude the possibility that the drug doses and exposure time interact with each other in the selection of Ery resistance in *Campylobacter*. This possibility remains to be determined in future studies. In this study, we evaluated only the response of *C. jejuni* to the growth-promoting dose of tylosin because *C. jejuni* represents the most important *Campylobacter* species associated with hu-

man food-borne disease. We speculate that *C. coli* would also behave similarly when exposed to tylosin at a low dose for a prolonged period of time. This possibility remains to be determined in future studies.

Various antibiotics, such as macrolides and tetracycline, have been used as growth promoters to improve average daily weight gain and feed efficiency in food-producing animals (18, 36). Use of antibiotics as growth promoters in animal feed is considered a great selection pressure for antibiotic resistance since bacteria are exposed to subtherapeutic levels of drugs over a long period of time. The association between use of a growth promoter and resistance development has been documented in several circumstances (45). For example, a strong link between use of avoparcin and macrolides as growth promoters in poultry and pigs and resistance development in fecal enterococci was demonstrated in Denmark, Finland, and Norway (2, 9). Another study also reported a clear effect of tylosin used as a growth promoter on the development of Ery^r enterococci and staphylococci in pigs (1). The same study also revealed that the effect of tylosin on Ery resistance was immediate in enterococci but was gradual in staphylococci, suggesting that different bacterial pathogens may respond differently to tylosin in terms of resistance development (1). In Denmark, ending the use of antimicrobial growth promoters (including tylosin) significantly reduced the prevalence of antibiotic-resistant enterococci in chickens and pigs and Ery^r *Campylobacter* in pigs (46), providing compelling evidence for the role of antimicrobial growth promoters in selecting resistant bacteria. Our finding that long-term use of tylosin at a subtherapeutic concentration selects for Ery^r *Campylobacter* in chickens (Fig. 3) is consistent with these previous observations. However, this study was conducted in a laboratory environment using a small number of chickens, which may not fully represent the production conditions on poultry farms. Thus, the findings from this study should be interpreted cautiously, considering the complexity of antibiotic resistance development on animal farms, which is influenced by multiple factors including antimicrobial usage, animal species, production environments, and the genetic backgrounds of bacterial species as well as management practices.

Previous studies by us and other researchers (22, 31, 35) showed that *C. jejuni* is highly mutable in response to FQ treatment and that FQ-resistant mutants occurred in FQ-treated chickens rapidly after the initiation of treatment. The feature of FQ resistance development in response to antibiotic treatment is in clear contrast to the emergence of Ery^r *Campylobacter* in poultry, and this difference may be due to the dissimilarity in the mode of action and resistance mechanisms. Macrolide antibiotics target bacterial ribosomes and inhibit protein synthesis, while FQs target DNA gyrase and inhibit DNA replication and transcription. For FQ resistance, a single-step mutation in GyrA is sufficient for conferring high-level resistance, while a stepwise process may be required for the development of Ery resistance. It is also possible that the gyrase gene in *Campylobacter* is intrinsically more mutable than the 23S rRNA gene, leading to a higher frequency of emergence of FQ-resistant mutants than of Ery^r mutants.

Previous studies indicate a general trend toward greater prevalence of macrolide-resistant *C. coli* than of macrolide-resistant *C. jejuni* in both animals and humans (7, 8, 30, 41).

However, our results revealed that there appear to be no significant differences in the frequencies of emergence of Ery^r mutants between *C. jejuni* and *C. coli*, which was shown by both in vitro and in vivo experiments, and suggested that *C. coli* may not be intrinsically more mutable than *C. jejuni* with regard to developing macrolide resistance. The difference in the observed prevalence of macrolide resistance between *C. jejuni* and *C. coli* isolated from food-producing animals may be related to their particular association with certain species of host animals and the production practices associated with the host animals. For example, *C. coli* is the predominant *Campylobacter* species in pigs and turkeys (3, 26, 29, 44) and macrolide antibiotics are used more often in these two species than in chickens. In contrast, *C. jejuni* is predominantly associated with chickens, for which macrolides are less frequently used for growth promotion (12). In addition, the production cycle for broiler chickens is significantly shorter than that for turkeys or pigs, implying that broiler chickens are less exposed to antibiotics than turkeys and pigs. It should also be pointed out that cross-resistance between lincosamides (e.g., clindamycin and lincomycin) and macrolides has been reported with *Campylobacter* (29, 42). Thus, use of antibiotics of the lincosamide class in animal production may also affect the emergence and prevalence of Ery^r *Campylobacter*.

Interestingly, all of the in vivo-selected mutants that displayed high-level Ery resistance (MIC > 512 µg/ml) contained the A2074G mutation in all three copies of the 23S rRNA gene, which is different from the previous finding that the predominant mutation detected in *Campylobacter* mutants with high-level Ery^r is A2075G (20). Gibreel et al. (19) has suggested that the reason that the A2074G mutation occurs at a very low frequency among different Ery^r *C. jejuni* isolates is likely the negative effect of the mutation on the growth rate of *Campylobacter*. However, the growth rate of the in vivo-selected Ery^r mutants with the A2074G mutation from this study appeared to be similar to that of the parent strain (ATCC 700819), as indicated by the sizes of the colonies over a period of 72 h (data not shown). Given the evidence that none of the sequenced Ery^r *C. jejuni* isolates exhibited the A2075G mutation, it is likely that emergence of a specific point mutation that confers high-level Ery^r is dependent on the genetic features of a strain, the environment in which the mutation is selected, and/or specific macrolide agent used for selection.

Recently the involvement of the CmeABC efflux system in macrolide resistance has been examined by several research laboratories (11, 19, 33). It is a general notion that CmeABC is linked to low and intermediate resistance to erythromycin, while the 23S rRNA mutations can confer a high level of erythromycin resistance (33, 38). Another recent study concluded that 23S rRNA mutations and CmeABC work together in *C. coli* to confer high-level macrolide resistance (11). In this study, the Ery^r mutants (except for RN14BE4-2) obtained by a single-step selection did not have mutations at position 2074 or 2075 in the 23S rRNA gene and had erythromycin MICs between 8 µg/ml and 256 µg/ml (Table 2). Thus, the 23S rRNA mutations were not the major contributor to the acquired resistance in these strains. At present we don't know if there are any mutations in ribosomal proteins L4 (G74D) and L22 of the mutants as reported by Cagliero et al. (10). Regardless of the presence of target mutations, inactivation of *cmeB* greatly re-

duced the resistance to Ery (Table 2), indicating that CmeABC is a significant player in maintaining the acquired resistance to Ery. Interestingly, the contribution of CmeABC to the increased Ery resistance is not due to overexpression of this efflux pump because immunoblotting demonstrated that both the parent strain (S3B) and its mutants produced similar amounts of the efflux proteins. In many of the mutants, inactivation of CmeB led to the decrease of Ery MIC to a level that was even below that of the wild-type strains (Table 2). This can be explained by the known role of CmeABC in the intrinsic resistance to various antibiotics (28). Thus, the loss-of-function mutation in CmeB is expected to reduce both the intrinsic and acquired resistance of *Campylobacter* to macrolides. The new findings from this work further confirm that CmeABC is a significant player in conferring Ery resistance in *Campylobacter*.

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