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Development, Stability, and Molecular Mechanisms of Macrolide Resistance in *Campylobacter jejuni*▽

Dave Bryson Caldwell, Ying Wang,§ and Jun Lin*

Department of Animal Science, The University of Tennessee, Knoxville, Tennessee 37996

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Previous studies of macrolide resistance in *Campylobacter* were primarily focused on strains from various origins or used in vitro systems. In this study, we conducted both in vitro and in vivo experiments to examine the development, stability, and genetic basis of macrolide resistance in *Campylobacter jejuni* using erythromycin-resistant (Ery^r) mutants derived from the same parent strain. Chickens inoculated with low-level Ery^r mutants (MIC = 32 or 64 μg/ml) at 15 days old did not shed highly Ery^r mutants (MIC > 512 μg/ml) after prolonged exposure to a low dose of tylosin. The low-level Ery resistance was not stable in vitro or in vivo in the absence of macrolide selection pressure. However, high-level Ery resistance displayed remarkable stability in vitro and in vivo. Ribosomal sequence analysis of 69 selected Ery^r mutants showed that specific point mutations (A2074G or A2074C) occurred in all highly Ery^r mutants. No mutations in ribosomal protein L4 were observed in any of the in vitro-selected Ery^r mutants. However, three specific mutations in L4, G74D, G57D, and G57V, were widely found among in vivo-selected Ery^r mutants. Insertion of three amino acids, TSH, at position 98 in ribosomal protein L22 was observed only in mutants selected in vitro. Inactivation of the CmeABC efflux pump dramatically reduced Ery MICs in Ery^r mutants. Together, these findings suggest that multiple factors contribute to the emergence of highly Ery^r *Campylobacter* in chicken, reveal resistance level-dependent stability of macrolide resistance in *C. jejuni*, and indicate that *C. jejuni* utilizes complex and different mechanisms to develop Ery resistance in vitro and in vivo.

Campylobacter jejuni is recognized as a leading bacterial cause of human gastrointestinal enteritis worldwide (1). *Campylobacter* species colonize the intestinal tract of wild and domestic animals (10). Most human *Campylobacter* infections are associated with consumption of undercooked poultry meat, as well as unpasteurized milk and contaminated water (10). To treat human campylobacteriosis, fluoroquinolones (FQs) and macrolide antibiotics, such as erythromycin (Ery), are the major drugs of choice (9, 12). However, during the past decades, FQ antibiotics have been losing their effect on *Campylobacter* because this pathogen has become highly resistant to FQs (9, 12). Therefore, macrolide antibiotics have been considered the best choice for treating human *Campylobacter* infections (1). Unfortunately, many studies have revealed a general trend of rising Ery resistance in *Campylobacter* (5, 9, 12). To develop effective measures to control and prevent the spread of macrolide-resistant *Campylobacter* spp., in vivo development and stability of macrolide resistance in *Campylobacter* are critical to our understanding but are still largely unknown.

Macrolide usage in food-producing animals is considered to be a major factor influencing the emergence of erythromycin-resistant (Ery^r) *Campylobacter* (12). Recently, we examined the direct impact of macrolide usage on Ery resistance development in chickens (16). When tylosin, a macrolide antibiotic, was given to chickens in feed at a growth-promoting dose (50 mg/kg feed), Ery^r mutants emerged in the birds after prolonged exposure to the antibiotic (16). Specifically, the chickens inoculated with an Ery-sensitive (Ery^s) *C. jejuni* strain at 17 days old shed Ery^r mutants 17 and 24 days after inoculation, with all Ery^r mutants displaying low-level Ery resistance (MIC = 8 to 16 μg/ml). When chickens were inoculated with the same Ery^s *C. jejuni* strain at 3 days old, Ery^r mutants suddenly emerged after 31 days of exposure to tylosin, with the majority of Ery^r mutants displaying high-level Ery resistance (MIC > 512 μg/ml); notably, no low-level Ery^r mutants were detected even 1 week prior to the emergence of large populations of highly Ery^r mutants (16). This interesting finding raises a question regarding in vivo development of Ery resistance in *C. jejuni*: is acquisition of low-level Ery resistance required for developing high-level Ery resistance in vivo? In other words, does extended exposure of low-level Ery^r mutants to macrolide antibiotics result in the emergence of highly Ery^r *C. jejuni* mutants? Examination of this issue is important for us to understand the dynamics of Ery resistance development in *C. jejuni* in response to macrolide usage in farm animals.

The stability of the resistant phenotype is also a key parameter influencing the development and transmission of antibiotic resistance (3). In many bacterial species, antibiotic resistance confers a reduction in bacterial fitness, and thus an antibiotic resistance phenotype is not stable in the absence of antibiotic selection pressure (3). However, distinct from that in other bacteria, FQ resistance in *Campylobacter* spp. is stable; FQ-resistant mutants do not show a fitness cost in vivo and are even ecologically competitive in the colonization of chickens, even in the absence of antibiotic selection pressure (17). With respect to Ery resistance in *Campylobacter*, the in vitro and particularly in vivo stabilities of the resistance phenotype are

* Corresponding author. Mailing address: Department of Animal Science, 2505 River Drive, Knoxville, TN 37996-4574. Phone: (865) 974-5598. Fax: (865) 974-7297. E-mail: jlin6@utk.edu.

§ Present address: Department of Microbiology and Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN 37996.

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TABLE 1. Experimental design of chicken studies

Expt and group	No. per group	Tylosin usage in feed[a]	*C. jejuni* strain	MIC of strain (μg/ml)		Chicken age (days) at challenge	Chicken age (days) at swab collection[b]
				Ery	Tylosin		
A							
1	9	−	DC2	32	64	15	17, 22, 29, 36, 43, 50
2	10	+	DC2	32	64	15	17, 22, 29, 36, 43, 50
B							
1	10	+	DC2	32	64	15	20, 34, 48
2	10	+	DC26	64	128	15	20, 34, 48
C							
1	12	−	DC2	32	64	3	12, 22, 38, 47
2	13	−	DC6	>512	>512	3	12, 22, 38, 47

[a] Chickens in each group received either antibiotic-free complete feed (−) or feed supplemented with tylosin (50 mg tylosin/kg feed) (+). The birds in the medicated group were given the tylosin-containing feed throughout the experiment.
[b] Cloacal swabs were collected from each chicken. Each swab was subjected to differential plating as described in Materials and Methods.

still not clear. It is also unknown if the stability of Ery resistance is affected by the resistance level that is associated with a specific ribosomal modification(s) in *C. jejuni*.

Significant progress has been made in elucidating molecular mechanisms of macrolide resistance in *Campylobacter* (6, 12). Modifications of the ribosomal target sites (e.g., the 23S rRNA gene and ribosomal proteins L4 and L22) and active efflux via the CmeABC multidrug efflux pump are the major mechanisms that confer macrolide resistance to *Campylobacter* (6, 12). High-level macrolide resistance in *Campylobacter* is attributed mainly to mutations in domain V of the 23S rRNA gene at positions 2074 and 2075 (e.g., A2074G and A2075G), while modifications of L4 and L22 contribute to low-level Ery resistance in *C. jejuni* (6, 12). Despite growing evidence of the mechanisms of macrolide resistance in *Campylobacter*, previous studies either focused on the comparison of isolates from various origins or examined macrolide-resistant mutants selected in vitro, greatly limiting the interpretation of association of specific molecular basis with acquired Ery resistance and limiting the elucidation of in vivo development of macrolide resistance in *Campylobacter*. Examination of in vivo- as well as in vitro-selected, macrolide-resistant mutants that are derived from the same sensitive parent strain would greatly improve our understanding of the mechanisms and development of macrolide resistance in *Campylobacter*.

To address the concerns described above and move toward the goal of preventing and controlling macrolide resistance in *Campylobacter*, we pursued the following three specific objectives in this study: (i) to determine if long-term exposure of low-level Ery^r *C. jejuni* (MIC = 32 or 64 μg/ml) to low doses of tylosin selects for high-level Ery^r mutants (MIC > 512 μg/ml) in chickens, (ii) to examine in vivo and in vitro stabilities of different levels of Ery resistance in *C. jejuni*, and (iii) to determine molecular mechanisms of macrolide resistance in Ery^r *C. jejuni* isolates derived from the same parent *C. jejuni* strain and correlate specific mechanisms to dynamic development and stability of Ery resistance in *C. jejuni*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. jejuni* NCTC 11168, an Ery^s strain (MIC = 1 μg/ml), was purchased from ATCC (catalogue no. 700819) and was used as a parent strain in this study selecting for macrolide-resistant mutants in vitro. NCTC 11168 was also used in a recent chicken study to select Ery^r mutants in vivo (16), from which a panel of individual Ery^r mutants (strains DC1 to DC20) isolated from chickens (Table 1; see Table 3) were used in this study. A new in vivo-selected Ery^r mutant, named DC26 (Ery MIC = 64 μg/ml), was derived from DC2 and isolated from one bird on day 43 in this study (experiment A) (Table 1). DC26 was further used for selecting Ery^r mutants with higher Ery resistance in this study (experiment B) (Table 1). The *Campylobacter* strains were grown routinely on Mueller-Hinton agar (MHA) plates or in Mueller-Hinton broth (MHB) at 42°C under microaerobic conditions. *Campylobacter*-specific growth supplements and selective agents (SR084E and SR117E; Oxoid) were added to the media when needed. When required, the MH media were also supplemented with various amounts of Ery.

Long-term exposure of low-level Ery^r *Campylobacter* mutants to a subtherapeutic dose of tylosin in chickens. Two independent experiments (experiments A and B) were conducted, and the experimental design is detailed in Table 1. For both trials, day-old broiler chicks (a gift from the commercial company Hubbard Hatchery, Pikeville, TN) were randomly assigned to two treatment groups. All birds were placed in sanitized wire cages with unlimited access to feed and water. All feed was prepared by the feed mill at the Johnson Animal Research and Teaching Unit. Medicated feed containing tylosin phosphate (Elanco Animal Health) was prepared, in accordance with the label for preparation of medicated feed used for growth promotion of chickens, to obtain a final tylosin concentration of 50 mg/kg. Prior to inoculation with *C. jejuni*, all birds were confirmed to be free of *Campylobacter* by culture of cloacal swabs. At 15 days old, all birds in each group were inoculated with fresh *C. jejuni* DC2 or DC26 culture (10^7 CFU/bird) via oral gavage. Because a distinct characteristic of *C. jejuni* colonization in poultry is that this organism is not detected in chickens less than 2 to 3 weeks old under commercial broiler production conditions, inoculation of birds with *C. jejuni* at 15 days old in these two trials should allow us to measure the impact of using tylosin as a growth promoter in the development of Ery resistance in *Campylobacter* under conditions similar to those of commercial production. After the birds were inoculated, cloacal swabs were collected from each chicken at different time points until the end of each experiment (Table 1). Samples from each bird were diluted serially and spread onto three different types of MHA plates to recover total *Campylobacter* populations (normal selective plates), Ery^r populations (selective plates containing 8 μg/ml of Ery), and highly Ery^r populations (selective plates containing 128 μg/ml of Ery). *Campylobacter* colonies were counted following 48 h of incubation at 42°C under microaerobic conditions. Individual colonies were collected from selective plates for each chicken and were used for MIC testing as described below. Multiple isolates with different Ery MICs were analyzed by PCR to confirm their genetic identities. The PCR analysis was done using primers specific for the *cmp* gene encoding the major outer membrane protein, as described previously by Huang et al. (13), and revealed no difference between input strain and output isolates.

In vivo stability of Ery resistance using the chicken model system. The in vivo stability of Ery resistance was evaluated using the chicken model system, and the experimental design is detailed in Table 1 (experiment C). The bird source and maintenance are the same as those for experiments A and B as described above. However, chickens in both groups received nonmedicated feed throughout the trial. To maximize exposure time to antibiotic-free feed, all birds were inoculated with approximately 10^7 CFU fresh *C. jejuni* culture via oral gavage at 3 days old.

Prior to inoculation with *C. jejuni*, all birds were confirmed to be free of *Campylobacter* by cultured cloacal swabs. The mutants DC2 and DC6, with different levels of Ery resistance, were used for evaluation of in vivo Ery resistance stability in this study (Table 1). After the birds were inoculated, cloacal swabs were collected at different time points. Isolation of *Campylobacter* spp. and differential plating for enumerating the proportion of the mutant colonies were conducted as described above. Representative isolates from each chicken were selected for the Ery MIC test.

Detection limit and statistical analysis. In all chicken experiments, the detection limit of the plating methods was approximately 100 CFU/g of feces. Student's *t* test was used to examine the significant difference in *Campylobacter* colonization levels (log transformed CFU/g feces) at each sampling point between the two treatment groups. A P value of <0.01 was considered significant.

In vitro stability of Ery resistance. The same Ery^r mutants DC2 and DC6 were also used for the in vitro stability test. Briefly, the two mutants were inoculated in antibiotic-free MHB and grown in microaerophilic conditions at 42°C. The *Campylobacter* culture was subcultured every 2 to 3 days in 4 ml fresh MHB (1:400 dilution) for 81 days in the absence of any antibiotics. Following passages 10, 20, and 33, the cultures were serially diluted (10-fold dilutions) in MHB and plated on both MHA plates and MHA plates supplemented with Ery at a final concentration of 8 μg/ml (for DC2 and DC6) or 128 μg/ml (for DC6). Plates were incubated at 42°C under microaerophilic conditions for 48 h. Total numbers of colonies on each type of plate were counted and compared. In addition, following passage 33 differential plating, 10 colonies for each mutant were randomly chosen from MHA plates and were subjected to the Ery MIC test as described below.

Antibiotic susceptibility test. MICs of antibiotics were tested using the agar dilution method as recommended by the CLSI (7). For quality control, *C. jejuni* ATCC 33560 and a quality control range for an Ery MIC of 0.25 to 2 μg/ml were used in this study. According to the breakpoints recommended by the CLSI (7), Ery MICs of ≤8 μg/ml and ≥32 μg/ml are considered susceptible and resistant, respectively. In this report, unless specifically defined, high-level Ery resistance or highly Ery^r mutants corresponded to an Ery MIC of >512 μg/ml in *C. jejuni*. Ery was purchased from Sigma Chemical Co., St. Louis, MO.

Selection of macrolide-resistant mutants in vitro. Erythromycin or tylosin tartrate (MP Biomedicals) was used as the selective agent for selecting spontaneous macrolide-resistant mutants in vitro. Briefly, Ery^s NCTC 11168 culture was grown in antibiotic-free MHA plates to the late logarithmic phase. The cultures were plated on increasing concentrations of Ery and tylosin (fourfold to 16-fold the initial MIC of NCTC 11168). Following 3 to 5 days of incubation under microaerophilic conditions at 42°C, a single macrolide-resistant colony on selective plates was selected, cultured in MHB, and stored at −80°C. The Ery^r and tylosin-resistant mutants obtained from the first round of selection were grown in MHB containing sublethal concentrations of Ery (i.e., 4 μg/ml for JL276 and 8 μg/ml for JL290) or tylosin (2 μg/ml for JL295) (see Table 4), respectively, and then plated on selective plates again to select mutants with a higher level of resistance. If needed, the procedure was repeated up to four times to obtain highly macrolide-resistant mutants. All in vitro-selected mutants were subjected to the MIC test using the standard agar dilution method as described above. In addition, genomic DNA prepared from each mutant was used for both PCR amplification and sequence analysis of the 23S rRNA gene and the ribosomal protein L4 and L22 genes as described below.

Sequence analysis of the 23S rRNA gene and ribosomal protein L4 and L22 genes. Domain V of the 23S rRNA gene sequence of *C. jejuni* was amplified by PCR with gene-specific primers (5′-GTAAACGGCGGCCGTAACTA-3′ and 5′-GACCGAACTGTCTCACGACG-3′) (14). Ribosomal protein L4 gene-specific primers 5′-GTAGTTAAAGGTGCAGTACCA-3′ and 5′-GCGAAGTTTG AATAACTACG-3′ and L22 gene-specific primers 5′-GAATTTGCTCCAACA CGC-3′ and 5′-ACCATCTTGATTCCCAGTTTC-3′ were used to amplify the complete L4 gene *rplD* and the L22 gene *rplV*, respectively (6). 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 45 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 being sequenced. DNA sequences were determined in the DNA facilities at the University of Tennessee. Sequence analysis was performed using the DNAStar package.

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 (16).

RESULTS

Colonization of Ery^r mutants in response to tylosin treatment. In both experiments A and B, all chickens were successfully colonized with *C. jejuni* as early as day 17 (for experiment A) or day 20 (for experiment B). In experiment A, the shedding level of *Campylobacter jejuni* in feces was consistently higher (up to 1.8 $\log_{10}$ units) in the chickens given nonmedicated feed than in those given tylosin-containing feed, except that on day 43 and day 50 the shedding levels of *C. jejuni* for the nonmedicated group were slightly lower ($P > 0.05$) than those from the medicated group (Fig. 1A). In experiment B, chickens were assigned to two groups that were inoculated with DC2 or DC26 and all chickens were treated with medicated feed throughout the study. As shown in Fig. 1B, both groups of chickens displayed similar shedding levels of *Campylobacter jejuni* throughout the study, although DC2 seemed colonize slightly better than DC26 on day 20.

Effect of prolonged low-dose tylosin treatment on the emergence of high-level Ery^r *C. jejuni* in chickens. In experiment A, in the absence of tylosin selection pressure (group 1, the control), the Ery MICs of isolates gradually decreased and returned to the wild-type level of susceptibility (Table 2), suggesting that low-level Ery resistance cannot persist for a long time in vivo in the absence of tylosin selection pressure. Specifically, when the chickens were 29 days old (14 days after inoculation), all isolates were susceptible to Ery, with Ery MICs of 4 μg/ml, which was also confirmed by the differential plating method (data not shown). Supplementation of tylosin in feed maintained the Ery resistance phenotype of DC2 throughout the study (Table 2). In addition, Ery^r mutants with increased MICs (64 μg/ml) emerged at different sampling days after continuous exposure to low doses of tylosin (Table 2). The twofold MIC increase in these Ery^r mutants is not caused by methodological error and has been confirmed by an independent MIC test. However, as long as 35 days of extended exposure of DC2 to low doses of tylosin did not select highly Ery^r mutants in experiment A (Table 2).

To further confirm the findings from experiment A, DC2 and its new in vivo derivative DC26 were used to challenge chickens receiving tylosin-containing feed. As shown in Table 2, regardless of the mutants used for inoculation, none of the chickens shed high-level Ery^r *C. jejuni* (MIC > 256 μg/ml) during 33 days of continuous exposure to low doses of tylosin, which was also confirmed by differential plating (data not shown). However, Ery^r mutants with increased resistance emerged in both groups of chickens. For example, a mutant with an Ery MIC of 256 μg/ml was obtained on day 48 in group 1 (Table 2). In group 2, which was inoculated with the DC26 mutant, the mutants with an Ery MIC of 128 μg/ml were selected on each sampling day (Table 2). Representative isolates obtained from experiments A and B are given in Table 3 and were used for examination of molecular mechanisms of Ery^r in *C. jejuni* as described below.

In vivo stability of Ery resistance in *C. jejuni*. All chickens were successfully colonized by *C. jejuni* DC2 or DC6 by as early as day 12 (9 days after inoculation) (Fig. 1C). An MIC test of all randomly selected isolates from each individual chicken at each time point showed that in vivo stability of Ery resistance was affected by the resistance level (Fig. 2A). For low-level

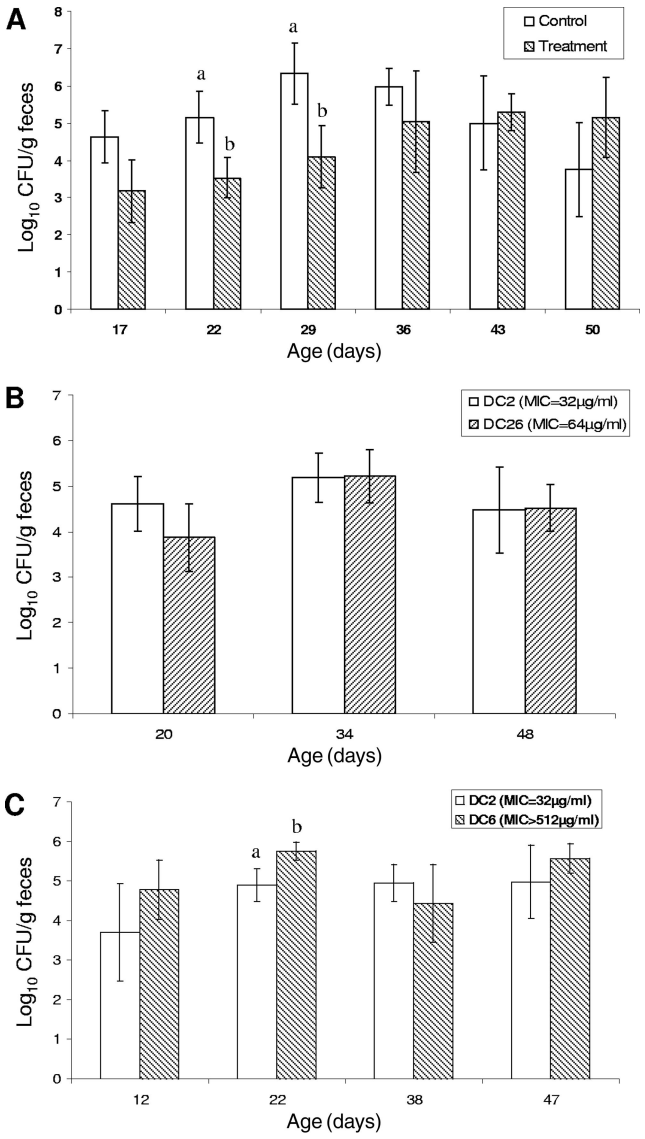

FIG. 1. Levels of *Campylobacter* colonization in chickens. (A) Shedding levels of *C. jejuni* DC2 in chickens receiving nonmedicated feed and feed supplemented with the growth promoter tylosin (50 mg tylosin/kg feed), corresponding to experiment A (Table 1). (B) Shedding levels of *C. jejuni* DC2 or DC26 in chickens receiving feed supplemented with the growth promoter tylosin (50 mg tylosin/kg feed), corresponding to experiment B (Table 1). (C) Shedding levels of low-level Ery^r DC2 or high-level Ery^r DC6 in chickens receiving nonmedicated feed, corresponding to experiment C (Table 1). Each bar represents the mean $\log_{10}$ CFU/g feces ± the standard deviation in each group. Different letters above the bars on each sampling day denote a significant difference ($P < 0.01$).

Ery^r mutant DC2, by day 22, 25% of the isolates displayed an Ery MIC of 4 μg/ml and the majority of isolates showed reduced Ery MICs (16 μg/ml). In fact, the instability of low-level Ery resistance was also observed in chickens as early as 9 days after inoculation (day 12); 2 of 12 isolates from 12-day-old chickens already displayed reduced Ery MICs (16 μg/ml). With further growth of DC2 in chickens without tylosin selection pressure, all isolates recovered on day 38 and day 47 were susceptible to Ery, with MICs comparable to those of the wild-type Ery^s strain (Fig. 2A). In contrast, all isolates from chickens inoculated with DC6 consistently displayed high levels of Ery resistance, regardless of the sampling date (Fig. 2A). These MIC results were also consistent with differential plating results, which showed that approximately 100% of *C. jejuni* populations from chickens inoculated with DC6 grew on selective MHA plates containing 128 μg/ml of Ery for the entire study but that no Ery^r *C. jejuni* mutant was detected in DC2-inoculated chickens by day 38 using selective MHA plates containing 8 μg/ml Ery (data not shown).

In vitro stability of Ery resistance in *C. jejuni*. As shown in Fig. 2B, more than 60% of DC2 populations could still be selected on MHA plates containing 8 μg/ml of Ery after 10 passages in the absence of antibiotic selection pressure, while all DC6 populations grew well on MHA plates containing 128 μg/ml of Ery. By passages 20 and 33, only 13% and 7% of DC2 populations were selected on MHA plates containing 8 μg/ml Ery, respectively (Fig. 2B). However, DC6 still displayed high stability after passages 20 and 33; approximately 100% of the population could still be recovered on MHA plates containing 128 μg/ml of Ery (Fig. 2B). These differential plating results were also confirmed by Ery MIC tests of representative isolates selected on MHA plates after passage 33 (10 isolates per strain). The majority of DC2 isolates (6 of 10) showed an Ery MIC of 2 μg/ml, which is comparable with the MIC of the Ery^s parent strain, and four other isolates displayed an Ery MIC of 8 μg/ml. However, 10 randomly selected DC6 isolates all displayed an Ery MIC of >512 μg/ml, indicating that high levels of Ery resistance are extremely stable in *C. jejuni* in vitro.

In vitro selection of macrolide-resistant mutants. The representative macrolide-resistant mutants selected in vitro are listed in Table 4. Four low-level Ery^r mutants (JL276 to JL279) were obtained from the initial selection by using Ery as the selective agent. The second round of selection using JL276 resulted in Ery^r mutants with increased MICs, such as JL283 to JL286. The highly Ery^r mutants (JL287 to JL289) were successfully obtained during the third round of selection using the JL286 strain. These highly Ery^r mutants displayed colony sizes on MHA containing 128 μg/ml of Ery similar to those on the MHA plates. In an independent selection experiment, although we also obtained low-level Ery^r mutants (JL290 to JL294) in the first round of selection and intermediate-level Ery^r mutants (JL299 and JL300) in the second round using JL290, we failed to obtain highly Ery^r mutants using JL299 as the parent strain even if the stepwise selection was repeated two more times. Highly Ery^r mutants also failed to be obtained from JL290 in other independent experiments, from which strains JL305 to JL308 were selected for analysis (Table 4). When tylosin was used as the selective agent, low-level Ery^r mutants (JL295 to JL298) were obtained in the initial selection step. The Ery^r mutants with much higher MICs (JL301 to JL304) were obtained from the second round of selection using JL295 grown in the presence of a sublethal concentration of tylosin. However, we failed to obtain highly Ery^r mutants using JL302 for selection in two independent experiments.

Molecular mechanisms of Ery resistance. (i) Analysis of 23S rRNA gene sequences. Thirty-nine in vivo-selected and 30 in vitro-selected Ery^r *C. jejuni* mutants (all NCTC 11168 derivatives) were subjected to PCR amplification of the 23S rRNA gene and *rplD* and *rplV* genes encoding ribosomal proteins L4

TABLE 2. Dynamic changes in Ery MICs of *C. jejuni* isolates from chickens receiving feed supplemented with tylosin (50 mg tylosin/kg feed) or nonmedicated feed

Expt and group[a]	Chicken age (days)	Ery MIC (μg/ml)[b]
A		
1 (control)	17	16 (9)
	22	4 (1), 16 (6), 32 (2)
	29	4 (9)
	36	1 (4), 2 (2), 4 (3)
	43	1 (6), 2 (2), 4 (1)
	50	1 (4), 2 (3), 4 (2)
2 (medicated)	17	16 (5), 32 (3), 64 (1)
	22	16 (9), 32 (1)
	29	32 (9), 64 (1)
	36	32 (8), 64 (2)
	43	16 (2), 32 (6), 64 (1)
	50	16 (1), 32 (9)
B		
1 (DC2)	20	32 (9), 64 (1)
	34	16 (2), 32 (7), 64 (1)
	48	32 (9), 256 (1)
2 (DC26)	20	64 (7), 128 (3)
	34	64 (8), 128 (2)
	48	64 (7), 128 (3)

[a] Experiment and group numbers are the same as those shown in Table 1. In experiment A, all chickens were inoculated with DC2 (Ery MIC = 32 μg/ml). In experiment B, two different groups of chickens that all received medicated feed were inoculated with DC2 (Ery MIC = 32 μg/ml) and its in vivo derivative DC26 (Ery MIC = 64 μg/ml).

[b] Numbers in parentheses indicate the total number of isolates corresponding to each MIC.

TABLE 3. Mutations in the 23S rRNA gene and modifications in ribosomal proteins L4 and L22 in Ery[r] *C. jejuni* mutants selected in vivo using parent strain *C. jejuni* NCTC 11168

Strain	Ery MIC (μg/ml)[b]	Mutation in the 23S rRNA gene[c]	Change in protein L4	Change in protein L22	Source or reference
NCTC 11168[a]	1	–	–	–	ATCC 700819
DC1	8	–	–	–	16
DC2	32	–	G74D	–	16
DC3	>512	A2074G	–	–	16
DC4	8	–	–	–	16
DC5	8	–	G74D	–	16
DC6	>512	A2074G	–	–	16
DC7	8	–	G74D	–	16
DC8	32	–	G74D	–	16
DC9	>512	A2074G	–	–	16
DC10	16	–	G57D	–	16
DC11	64	–	–	–	16
DC12	64	–	G74D	–	16
DC13	32	–	G74D	–	16
DC14	32	–	G57D	–	16
DC15	32	–	G74D	–	16
DC16	32	–	G74D	–	16
DC17	8	–	G57D	–	16
DC18	16	–	G74D	–	16
DC19	16	–	G57D	–	16
DC20	16	–	G74D	–	16
DC21	32	–	G74D	–	Expt A; this study
DC22	32	–	G74D	–	Expt A; this study
DC23	64	–	G74D	–	Expt A; this study
DC24	32	–	G74D	–	Expt A; this study
DC25	64	–	G74D	–	Expt A; this study
DC26	64	–	G74D	–	Expt A; this study
DC27	64	–	G74D	–	Expt A; this study
DC28	8	–	G74D	–	Expt C; this study
DC29	4	–	–	–	Expt C; this study
DC30	1	–	–	–	Expt C; this study
DC31	>512	A2074G	–	–	Expt C; this study
DC32	>512	A2074G	–	–	Expt C; this study
DC33	>512	A2074G	–	–	Expt C; this study
DC34	128	–	G74D	–	Expt B; this study
DC35	128	–	G74D	–	Expt B; this study
DC36	128	–	G74D	–	Expt B; this study
DC37	128	–	G74D	–	Expt B; this study
DC38	128	–	G74D	–	Expt B; this study
DC39	256	–	G74D, G57V	–	Expt B; this study

[a] *C. jejuni* strain NCTC 11168 shows a strong ability to colonize in chicken intestine. It was purchased from ATCC (catalog no. 700819).

[b] Determined by the standard agar dilution method.

[c] –, no mutation/modification.

and L22, respectively. The sequences of these PCR products were aligned to the corresponding sequence of parent strain NCTC 11168 to identify the specific mutations that occurred in the ribosome.

With respect to the 23S rRNA gene, all highly Ery[r] mutants isolated from chickens (DC3, -6, -9, -31, -32, and -33) displayed the same A2074G point mutation in the 23S rRNA gene but none of the rest of the 33 isolates with Ery MICs up to 256 μg/ml showed point mutations in the 23S rRNA gene (Table 3), which is consistent with our previous examinations of some in vivo-selected isolates (16). Isolates DC31, -32, and -33 are probably identical to DC6, the strain used for inoculation in experiment C in this study (Table 1). DC3, -6, and -9 were isolated from different birds in a previous study (16). Interestingly, all three in vitro-selected high-level Ery[r] mutants (JL287 to -289) displayed different point mutations in the 23S rRNA gene, with an A→C point mutation at position 2074 (Table 4). According to sequence results, it appeared that the A2074C mutation was present in two of the three copies of the 23S rRNA gene in mutants selected in vitro, because the sequence chromatogram showed double peaks in the same position, where the C peak was two times higher than the A peak. Notably, Ery was used as a selective agent to select these three in vitro Ery[r] mutants. To determine if different selective agents lead to different 23S rRNA gene mutations, we also used tylosin as a selective agent to select Ery[r] mutants in vitro. However, we failed to obtain highly Ery[r] mutants by using tylosin as the selective agent, and none of the tylosin-selected mutants displayed point mutations in the 23S rRNA gene, even if the Ery MIC was as high as 512 μg/ml (Table 4).

(ii) Analysis of ribosomal protein L4 gene sequences. Changes in ribosomal protein L4 (encoded by the *rplD* gene) were not detected in mutants selected in vitro (Table 4) but were observed in the majority of in vivo-selected Ery[r] mutants with an Ery MIC of ≤256 μg/ml (Table 3). It is important to mention that all isolates obtained from experiment A, DC39 from experiment B, and DC28 to DC30 from experiment C (Table 3) are directly related to DC2, the strain used for inoculation. Three specific changes in L4 were observed in these in vivo-selected isolates. Many mutants harbored a G74D

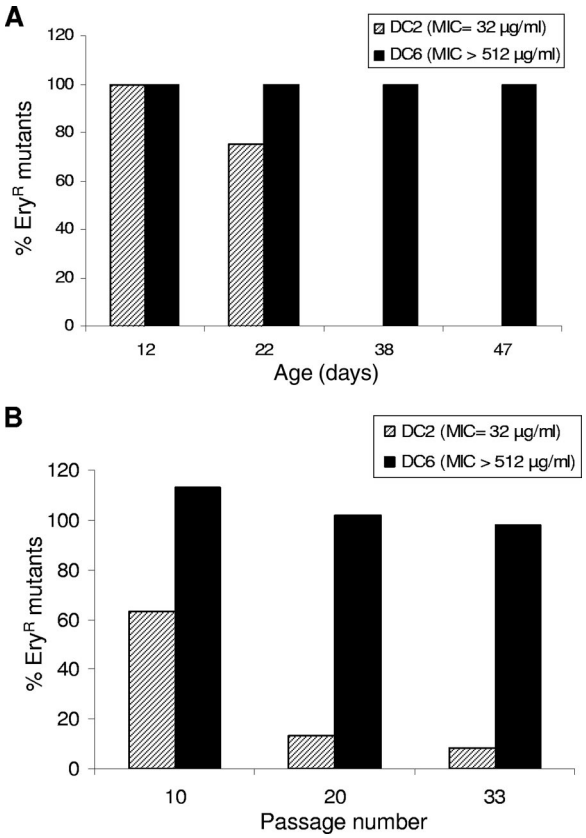

FIG. 2. Stability of Ery resistance of low-level Ery^r *C. jejuni* DC2 (MIC = 32 μg/ml) and high-level Ery^r *C. jejuni* DC6 (MIC > 512 μg/ml). (A) Stability of Ery resistance in vivo. Chickens in each group were inoculated with DC2 or DC6 via oral gavage at 3 days old. Chickens received nonmedicated feed throughout the study. The percentage of the Ery^r population for DC2 was calculated based on the MIC using a breakpoint of >8 μg/ml, and the percentage of the Ery^r population for DC6 was calculated based on the MIC using a breakpoint of >512 μg/ml. (B) Stability of Ery resistance in vitro. The percentage of the Ery^r population for DC2 was calculated based on differential plating using plates containing 8 μg/ml of Ery, and the percentage of the Ery^r population for DC6 was calculated based on differential plating using plates containing 128 μg/ml of Ery.

mutation in L4 that was also recently reported by Cagliero et al. (6). A G-to-A transition was found at nucleotide 170 of the *rplD* gene sequence in four in vivo-selected isolates (DC10, DC14, DC17, and DC19), which led to a Gly-to-Asp modification at position 57 of the L4 protein (Table 3). Another novel mutation is a G-to-T transition at nucleotide 170 of the *rplD* gene, which led to the Gly-to-Val modification at position 57 of the L4 protein in mutant DC39 (Table 3). DC39 showed a higher Ery MIC (256 μg/ml) than that of its parent strain, DC2. Notably, DC39 still carried a G74D mutation in L4 from DC2, which may work together with the new G57V mutation, conferring a higher level of Ery resistance.

(iii) Analysis of ribosomal protein L22 gene sequences. Modification in ribosomal protein L22 (encoded by the *rplV* gene) was observed only in the Ery^r mutants selected in vitro but not in the mutants selected in vivo (Tables 3 and 4). Specifically, a nine-base (ACTTCACAT) tandem duplication at position 292 in the *rplV* gene, which led to the insertion of three amino acids (TSH) at position 98 (Ins_{98}TSH) in protein L22, was observed in in vitro-selected isolates whose Ery MICs ranged from 32 to 512 μg/ml (Table 4). The choice of a specific selective agent (Ery or tylosin) did not affect the development of Ins_{98}TSH modification in the L22 protein (Table 4).

(iv) Inactivation of CmeABC multidrug efflux pump. To determine the contribution of the CmeABC efflux pump to the acquired Ery resistance, a CmeABC mutation was transferred to selected Ery^r mutants, and the Ery MIC of each isogenic CmeB mutant was measured by the standard agar dilution method. Regardless of the presence of a specific mutation in the 23S rRNA gene (e.g., DC32, JL287, and JL288), in the L4 protein (e.g., DC22 and DC27), or in the L22 protein (e.g., JL290, JL301, and JL303) or the absence of any mutation (JL283 and JL284), inactivation of *cmeB* dramatically reduced the Ery MIC (8- to 1,024-fold) compared to that of its parent strain (data not shown). This observation confirmed previous findings that CmeABC works synergistically with other mech-

TABLE 4. Mutations in the 23S rRNA gene and modifications in ribosomal proteins L4 and L22 in Ery^r *C. jejuni* mutants selected in vitro using parent strain *C. jejuni* NCTC 11168

Strain	Ery MIC (μg/ml)[b]	Mutation in the 23S rRNA gene[c]	Change in protein L4	Change in protein L22	Selective agent[e]
NCTC 11168[a]	1	–	–	–	NA
JL276	16	–	–	–	Ery
JL277	8	–	–	–	Ery
JL278	8	–	–	–	Ery
JL279	8	–	–	–	Ery
JL283	64	–	–	–	Ery
JL284	128	–	–	–	Ery
JL285	128	–	–	–	Ery
JL286	128	–	–	–	Ery
JL287	>512	A2074C[d]	–	–	Ery
JL288	>512	A2074C[d]	–	–	Ery
JL289	>512	A2074C[d]	–	–	Ery
JL290	32	–	–	Ins_{98}TSH	Ery
JL291	32	–	–	Ins_{98}TSH	Ery
JL292	8	–	–	–	Ery
JL293	8	–	–	–	Ery
JL294	8	–	–	–	Ery
JL295	8	–	–	–	Tyl
JL296	8	–	–	–	Tyl
JL297	8	–	–	–	Tyl
JL298	8	–	–	–	Tyl
JL299	64	–	–	Ins_{98}TSH	Ery
JL300	128	–	–	Ins_{98}TSH	Ery
JL301	512	–	–	Ins_{98}TSH	Tyl
JL302	512	–	–	Ins_{98}TSH	Tyl
JL303	256	–	–	Ins_{98}TSH	Tyl
JL304	256	–	–	Ins_{98}TSH	Tyl
JL305	256	–	–	Ins_{98}TSH	Ery
JL306	64	–	–	Ins_{98}TSH	Ery
JL307	256	–	–	Ins_{98}TSH	Ery
JL308	64	–	–	Ins_{98}TSH	Ery

[a] *C. jejuni* NCTC 11168 shows a strong ability to colonize in chicken intestine. It was purchased from ATCC (catalog no. 700819).
[b] Determined by the standard agar dilution method.
[c] –, no mutation/modification.
[d] The A2074C mutation was present in two of the three copies of the 23S rRNA gene.
[e] Erythromycin (Ery; Sigma) or tylosin tartrate (Tyl; MP Biomedicals) was used as the selective agent for selecting spontaneous macrolide-resistant mutants in vitro. NA, not applicable.

anisms to maintain high-level and low-level Ery resistance in *C. jejuni* (6, 16).

DISCUSSION

The results of this study reveal several unique features with respect to macrolide resistance development, stability, and associated molecular mechanisms in *C. jejuni*. First, long-term exposure of low-level Ery^r *C. jejuni* to the growth promoter tylosin did not select for high-level Ery^r *C. jejuni* mutants with resistance-associated mutations in the 23S rRNA gene (Tables 2 and 3), suggesting that multiple factors contribute to the emergence of highly Ery^r *C. jejuni* isolates. Second, in contrast to low-level Ery resistance, high-level Ery resistance displayed remarkable stability in vitro and in vivo (Fig. 2). Third, using a panel of Ery^r mutants derived from the same parent strain, sequencing analysis of the 23S rRNA, L4, and L22 genes revealed that molecular mechanisms contributing to Ery resistance in *C. jejuni* differ between high-level and low-level resistance isolates and between isolates selected in vivo and in vitro (Tables 3 and 4). Although stepwise mutations may contribute to the acquisition of higher-level macrolide resistance, emergence of highly Ery^r mutants with specific mutations in the 23S rRNA gene may be unique and may not need specific changes in ribosomal protein L4 or L22. Finally, we also observed two novel mutations in ribosomal protein L4 (G57D and G57V) from Ery^r mutants selected in vivo. Together, these findings indicated that *C. jejuni* utilizes complex and different mechanisms to develop Ery resistance in vitro and in vivo.

This study clearly showed that use of tylosin as a growth promoter maintained the persistence of a low-level Ery resistance phenotype and also resulted in the emergence of Ery^r *C. jejuni* mutants with a slightly but consistently increased resistance (Table 2). However, a highly Ery^r mutant did not emerge in the birds after prolonged exposure of low-level Ery^r mutants to tylosin (Table 2), suggesting that the length of exposure is not the sole factor contributing to emergence of high-level Ery^r *C. jejuni*. It seems that the emergence of highly Ery^r *C. jejuni* mutants harboring a specific mutation in the 23S rRNA gene may not need stepwise acquisition of other mutations, particularly those that occurred in ribosomal proteins L4 and L22. This finding is supported by the following evidence. First, highly Ery^r mutants suddenly emerged in tylosin-treated chickens without preemergence of low-level Ery^r mutants 1 week prior to the detection of these highly Ery^r mutants (16), indicating that *C. jejuni* may bypass the accumulation of specific mutations contributing to low-level Ery resistance for the development of high-level Ery resistance. Second, natural transformation studies using either the 23S rRNA gene PCR product (11) or genomic DNA (15) successfully introduced specific 23S rRNA gene mutations (e.g., A2074C and A2075G) into a sensitive *Campylobacter* strain and yielded Ery^r transformants with high frequency. The resulting transformants exhibited high-level Ery resistance and had a point mutation in the 23S rRNA gene that was identical to that of the corresponding donor mutant. These findings strongly indicated that a specific point mutation in the 23S rRNA gene alone (occurring in at least two of the three operons) is sufficient for conferring high-level Ery resistance. Thus, acquisition of mutations conferring low-level Ery resistance, such as mutations in L4 and L22, may not be needed for the development of high-level Ery resistance in *C. jejuni*. Third, we have obtained a panel of Ery^r mutants with modifications in ribosomal protein L4 or L22 (Tables 3 and 4). However, none of the highly Ery^r *C. jejuni* isolates displayed modifications in L4 or L22 in addition to a specific mutation in the 23S rRNA gene (Tables 3 and 4). On the other hand, long-term exposure of low-level Ery^r *C. jejuni* harboring modifications in L4 (DC2 and DC26) failed to select highly Ery^r *C. jejuni* isolates in vivo (Table 3). Furthermore, we failed to obtain a highly Ery^r *C. jejuni* mutant that has a specific mutation in the 23S rRNA gene in vitro using Ery^r mutants with mutations in L22 (e.g., JL290 and JL302) in this study. These findings suggested that acquisition of specific modifications in L4 or L22 may impede development of high-level Ery resistance in *C. jejuni*. This speculation will be determined in future studies. Together, multiple factors in conjunction with continuous macrolide antibiotic selection pressure may determine the rate of occurrence of highly Ery^r *C. jejuni*. For example, the genetic features of the bacterial strain (e.g., modification in L4, as discussed), the specific tissue concentration of the macrolide antibiotic during different growth phases, the anatomy and physiology of specific animal species, the choice of a specific macrolide antibiotic, and different production environments and management practices may affect the rate of emergence of high-level Ery^r *Campylobacter* on animal farms. These speculations remain to be determined.

Campylobacter jejuni has displayed unique features with respect to the fitness and stability of antibiotic resistance (17, 19). Chromosomal mutation-mediated or plasmid acquisition-mediated antibiotic resistance generally incurs a fitness cost in bacteria (2, 4). However, Luo et al. (17) demonstrated that FQ resistance in *Campylobacter* is very stable and that FQ-resistant mutants do not show a fitness cost in vivo. It has been observed that high-level Ery resistance associated with A2075G and A2074C mutations in the 23S rRNA gene in *Campylobacter* was stable in vitro in the absence of macrolide selection pressure (11, 15). In this study, both in vitro and in vivo experiments provided compelling evidence that low-level Ery resistance is not stable, while high-level Ery^r mutants displayed remarkable stability, in *C. jejuni*. When testing the in vivo stability of Ery resistance, isolates DC28 to DC30 (Table 3) were randomly selected from individual chickens inoculated with low-level Ery^r DC2. Compared to those of DC2, the Ery MICs of these isolates were dramatically reduced, regardless of the presence or absence of a specific G74D mutation in L4 (Table 3), indicating that the G74D mutation together with another uncharacterized mutation(s) contributes to low-level resistance in DC2 and that none of these mutations is stable in vivo. The highly Ery^r *C. jejuni* mutant DC6 used in this study contained the A2074G mutation in all three copies of the 23S rRNA gene. The remarkable stability of the A2074G mutation in DC6 (Fig. 2A) and the similar colonization abilities of DC6 and DC2 (Fig. 1C) indicated that high-level Ery resistance in *C. jejuni* is more problematic from a risk management perspective. At this stage, it is still unknown if DC6 shows a fitness cost in vivo and if DC6 is ecologically more competitive in the colonization of chickens than its Ery^s parent strain in the absence of antibiotic selection pressure. This issue remains to be determined in future studies.

Previous investigations of the mechanisms of macrolide re-

sistance in *Campylobacter* either focused on the comparison of isolates from various origins or examined macrolide-resistant mutants selected in vitro (6, 8, 11, 15, 18). Distinct from these studies, in this study we examined a panel of in vitro- and in vivo-selected Ery^r *C. jejuni* mutants that all derived from the same parent strain, *C. jejuni* NCTC 11168, and revealed several unique findings of the molecular mechanisms and development of Ery resistance in *Campylobacter*. First, with respect to high-level Ery resistance, all mutants selected in vivo displayed an A2074G mutation while all mutants selected in vitro displayed an A2074C mutation in the 23S rRNA gene. This difference is likely caused by chance due to low frequencies of occurrence of these mutations. However, we cannot rule out the role of the specific macrolide agent used for selection. In this study, tylosin was supplemented in feed for the selection of Ery^r mutants developed in chickens, and Ery was successfully used for selection of high-level Ery^r mutants in vitro (JL287 to -289). However, we failed to obtain high-level Ery^r mutants with specific point mutations in the 23S rRNA gene by using tylosin as a selective agent in vitro in this study. Second, with respect to low- and intermediate-level Ery resistance, the majority of mutants displayed modifications in L4 or L22, with L4 modifications occurring only in Ery^r mutants selected in vivo and L22 modification found only in mutants selected in vitro (Tables 3 and 4). Although this finding suggests that the selection environment seems to determine the specific type of ribosomal modification, Cagliero et al. (6) recently reported that a specific G74D mutation in ribosomal protein L4 was also observed in Ery^r *C. jejuni* mutants selected in vitro using Ery as the selective agent. Thus, modification in L4 can be found in Ery^r mutants selected in vivo or in vitro. Third, we identified two novel mutations, G57D and G57V, in ribosomal protein L4 in some in vivo-selected Ery^r *C. jejuni* mutants (Table 3). Notably, G57V developed after emergence of the G74D mutation in strain DC39 (Table 3), and these two mutations may work together to confer a higher level of Ery resistance in this mutant. Finally, as shown in this study, none of the highly Ery^r *C. jejuni* mutants contained specific modifications in L4 or L22 in addition to the mutation in the 23S rRNA gene. In addition, no highly Ery^r *C. jejuni* mutant was obtained when using a strain displaying a specific mutation in L4 or L22. Thus, mutations in L4 or L22 may impede emergence of highly Ery^r *C. jejuni* mutants. This possibility will be determined in future studies. Taken together, these findings indicated that *C. jejuni* utilizes complex and different mechanisms to develop Ery resistance in vitro and in vivo. Emergence of a specific mutation or modification that confers Ery resistance is dependent on the environment in which the mutation is selected, the genetic features of a strain, and/or the specific macrolide agent used for selection.

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