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MECHANISMS OF RESISTANCE

Correlation between *gyrA* and CmeR Box Polymorphism and Fluoroquinolone Resistance in *Campylobacter jejuni* Isolates in China

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ABSTRACT Sequence analysis of 79 ciprofloxacin-resistant *Campylobacter jejuni* isolates collected in China showed resistance-related sequence variations in *gyrA* and CmeR-Box. All the isolates contain an identical Thr-86-Ile substitution in GyrA. Several novel CmeR-Box variations, including point substitutions, deletion, and insertion, were identified. The point insertion or deletion led to dramatically reduced binding of CmeR to the *cmeABC* promoter, which significantly increases the expression of *cmeABC* and contributes to the high fluoroquinolone resistance.

KEYWORDS *Campylobacter jejuni*, DNA gyrase, *cmeABC*, fluoroquinolone resistance

Campylobacter jejuni is the major foodborne pathogen that causes human bacterial gastroenteritis (1, 2). Chicken is a major reservoir of *C. jejuni* (3), and contaminated chicken products are recognized as the main source of *C. jejuni*-related human infections (4). Moreover, the extensive use of antibiotics in the poultry industry has led to the increasing prevalence of antibiotic-resistant *C. jejuni* strains. Some isolates are especially resistant to clinically used antibiotics, such as fluoroquinolones (FQ), which have been commonly used to treat acute bacterial diarrhea (3, 5, 6). Therefore, surveillance of the FQ susceptibility of *C. jejuni* is important for not only the purpose of animal breeding but also public health (7, 8). In this study, in order to uncover the underlying genetic mechanisms of FQ resistance in *C. jejuni*, we undertook an investigation of the genetic polymorphism of *gyrA* and the promoter region of the *cmeABC* operon and examined the correlation between the promoter polymorphism and the expression of *cmeABC*, as well as the fluoroquinolone resistance.

From 2014 to 2016, 79 ciprofloxacin-resistant *C. jejuni* strains in China were isolated from poultry-related samples, including intestinal tracts, anal swabs, feces, and poultry meat. To investigate the molecular basis of high FQ resistance in the *C. jejuni* isolates, we analyzed the full gene sequences encoding the DNA gyrase subunits (*gyrA* and *gyrB*) (9, 10) and the topoisomerase IV subunits (*parC* and *parE*) (11, 12), which are known targets of FQ, the regulatory protein CmeR, and the sequence of the promoter region of *cmeABC*, which encodes an efflux pump (13, 14), by DNA sequencing as previously described (15, 16). The MICs of ciprofloxacin against the 39 isolates which represent different sequence variants were tested according to CLSI guidelines (17, 28). *C. jejuni* strain ATCC 33560 was used as the quality control. Statistical significance was analyzed using Student's *t* test.

As shown in Table 1, a series of point substitutions were identified in the *gyrA* gene from the *C. jejuni* isolates, and among these substitutions, two nonsynonymous se-

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TABLE 1 Sequence polymorphisms of *gyrA* in 79 ciprofloxacin-resistant *C. jejuni* isolates

Type of variation	No. of strains	Percentage	Substitutions in different points[a]								
			Gly-78 (GGT)	His-81 (CAC)	Thr-86 (ACA)	Gly-110 (GGC)	Ser-119 (AGT)	Ala-120 (GCC)	Val-149 (GTT)	Ser-157 (AGC)	Val-161 (GTT)
1	62	78.5	—	(−T)	Ile(-T-)	—	(−C)	(−T)	—	(−T)	(−C)
2	3	3.8	—	(−T)	Ile(-T-)	(−T)	(−C)	(−T)	—	(−T)	(−C)
3	6	7.6	—	(−T)	Ile(-T-)	—	(−C)	(−T)	Ile(A−)	(−T)	(−C)
4	1	1.3	(−C)	—	Ile(-T-)	—	(−C)	(−T)	—	(−T)	(−C)
5	1	1.3	(−C)	—	Ile(-T-)	—	(−C)	—	—	(−T)	—
6	1	1.3	—	(−T)	Ile(-T-)	—	(−C)	—	—	(−T)	—
7	5	6.3	—	—	Ile(-T-)	—	—	—	—	—	—

[a]—, no change.

quence substitutions were found. Each of the isolates had a Thr-86-Ile substitution, which was reported as the most common cause of FQ resistance in *C. jejuni* strains (10, 18). Val-149-Ile substitution was found in 6 isolates, and the MICs of ciprofloxacin against these isolates ranged from 4 to 128 μg/ml. As codon 149 is not in the FQ resistance-determining region of *gyrA* (codons 69 to 120) (10), we take it as a circumstantial sequence variation. Another two reported substitutions, Asp-90-Asn and Ala-70-Thr, which might cause medium-level FQ resistance (19), were not found in the *gyrA* gene of our isolates. All of the point substitutions in *gyrB* were synonymous substitutions which did not cause any changes in amino acid sequence. The Arg-139-Gln variation of *parC* was reported in *C. jejuni* by Gibreel et al. (20). However, we failed to amplify the *parC* and *parE* genes by PCR in our isolates, and this result was consistent with those of other studies (21).

The *cmeABC* operon encodes an important drug efflux pump in *C. jejuni* (22), and it is negatively regulated by CmeR, which binds to a 16-base inverted repeat (IR) sequence (TGTAATA[or T]TTTATTACA; the repeat sequences are underlined) named CmeR-Box located in the promoter region of the *cmeABC* operon (16, 23). In contrast with previous studies (16, 24), we did not find mutations inactivating CmeR (16). Instead, several nucleotide variations were found in the CmeR-Box of our isolates, making 12 sequence variants (listed in Table 2). Compared with the conserved CmeR-Box (TGTAATA[or T]TTTATTACA), up to 48.1% of our isolates contained one point substitution, and 2.5% contained two point substitutions in the IR sequence. The most frequent substitution site was the second nucleotide of the IR sequence (26.6% G to A, 13.9% C to T in the reverse repeat). In addition, 15.2% of the isolates contained a point deletion, and one isolate contained a point insertion between the inverted sequences.

To analyze the correlation between the sequence variations in the CmeR-Box and ciprofloxacin resistance levels, we tested the MICs of the isolates containing each different sequence variation. In instances where there were more than 3 isolates per sequence variation, 3 to 6 isolates were selected randomly for testing. As shown in Table 2, the MICs of isolates with point substitutions, insertion, or deletion were significantly higher than those of the isolates with a conserved CmeR-Box ($P < 0.05$). In particular, the MICs of isolates with the point insertion or deletion between the two halves of the inverted sequences showed the highest MIC values ($P < 0.05$). We further examined the expression level of *cmeA* in these isolates by real-time reverse transcriptase PCR. The mRNA was extracted from 2 ml of bacteria in exponential phase (optical density at 600 nm [OD_{600}] = 0.5), and the relative expression based on *C. jejuni* NCTC 11168 was calculated using the comparative threshold ($2^{-\Delta\Delta CT}$) method (25). As shown in Table 2, the expression of *cmeA* in the isolates with point substitution, insertion, or deletion was higher than that in the isolates with the conserved CmeR-Box, and the *cmeA* in the isolates with a point insertion or deletion showed the highest expression levels ($P < 0.05$). These results are similar to those for ciprofloxacin resistance in these isolates.

The electrophoretic mobility shift assay (EMSA) was then carried out to further assess the binding of CmeR to the CmeR-Box sequence variants, as previously described

TABLE 2 Sequence variations in CmeR-Box of ciprofloxacin-resistant *C. jejuni* isolates

Type of variation	Sequence variant	Sequence of CmeR-Box[a]	No. of strains[b]	Percentage	MICs (μg/ml) (related expression levels of *cmeA*)[c]	
					Of tested strains	Average
Conserved sequences	1	TGTAATTTTTATTACA	6 (5)	7.6	4 (0.82 ± 0.07), 4 (0.86 ± 0.11), 4 (0.79 ± 0.08), 8 (1.11 ± 0.14), 8 (0.93 ± 0.11)	5.6 (0.90 ± 0.09)
	2	TGTAATATTTATTACA	20 (6)	25.3	4 (1.04 ± 0.09), 4 (1.07 ± 0.04), 4 (1.21 ± 0.07), 8 (1.12 ± 0.11), 8 (1.37 ± 0.19), 8 (1.17 ± 0.21)	6 (1.16 ± 0.09)
Point substitution	3	TATAATTTTTATTACA	17 (5)	21.5	4 (1.32 ± 0.26), 16 (1.28 ± 0.17), 32 (1.37 ± 0.22), 64 (2.38 ± 0.19), 64 (2.11 ± 0.22)	36 (1.69 ± 0.44)
	4	TGTAGTTTTTATTACA	6 (3)	7.6%	32 (2.43 ± 0.31), 32 (2.55 ± 0.18), 64 (3.43 ± 0.25)	42.7 (2.80 ± 0.42)
	5	TGTAATTTTTATTATA	5 (3)	6.3	16 (3.21 ± 0.33), 64 (2.11 ± 0.31), 64 (2.73 ± 0.33)	48 (2.68 ± 0.38)
	6	TATAATATTTATTACA	4 (3)	5.1	64 (3.14 ± 0.48), 64 (2.81 ± 0.32), 128 (4.22 ± 0.29)	85.3 (3.39 ± 0.56)
	7	TATAATATTTATTACA	1 (1)	1.3	64 (2.12 ± 0.11)	64 (2.12)
	8	TGTAATATTTATTGCA	1 (1)	1.3	32 (1.70 ± 0.24)	32 (1.70)
	9	TGTAATATTTATTATA	4 (3)	5.1	8 (1.81 ± 0.22), 8 (2.28 ± 0.35), 16 (2.11 ± 0.31)	10.7 (2.07 ± 0.17)
	10	TGTAATATCTATTATA	2 (2)	2.5	32 (3.49 ± 0.51), 32 (5.08 ± 0.83)	32 (4.29 ± 0.79)
Point deletion	11	TGTAAT-TTTATTACA	12 (6)	15.2	128 (13.32 ± 0.41), 128 (16.39 ± 0.93), 128 (15.67 ± 1.11), 128 (16.80 ± 0.68), 128 (9.71 ± 0.77), 128 (10.58 ± 0.38)	128 (13.75 ± 2.54)
Point insertion	12	TGTAATATTTTATTACA	1 (1)	1.3	128 (8.08 ± 1.63)	128 (8.08)

[a]The positions underlined indicate sequence variations in CmeR-Box.
[b]The number of isolates randomly chosen for MIC and *cemA* expression level test are in parentheses.
[c]Relative expression based on *C. jejuni* NCTC 11168 was calculated using the $2^{-\Delta\Delta CT}$ method. 16S rDNA was used as the internal reference.

(22). As shown in Fig. 1, the CmeR-Box with the point insertion or deletion showed significantly decreased binding by CmeR. This result suggests that the *cmeABC* operon with the point insertion or deletion in its CmeR-Box was highly derepressed. The functional CmeR is a dimeric two-domain molecule, and the dimer could bind the CmeR-Box to repress expression of the *cmeABC* operon. The N-terminal domain of each monomer binds to one half of the IR in the CmeR-Box (26, 27). We suppose that the point insertion or deletion identified here may change the distance between two

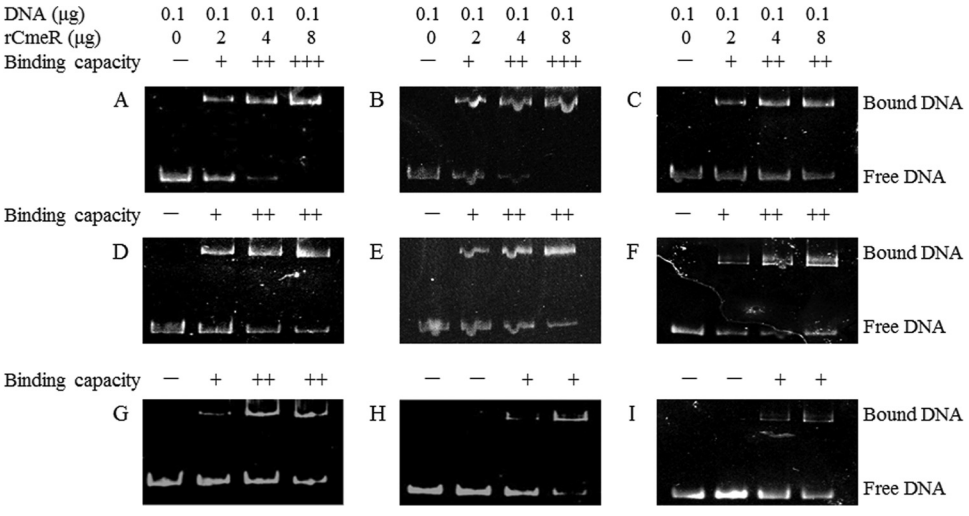

FIG 1 Binding of CmeR to the variant *cmeABC* promoter DNA. The serial numbers of CmeR-Box sequence variants are listed in Table 2. The tested promoters included conserved CmeR-Box (variation types 1 [A] and 2 [B]), point substitutions (variation types 3 [C], 4 [D], 7 [E], 8 [F], and 10 [G]), point deletion (variation type 11 [H]), and point insertion (variation type 12 [I]). Considering that the CmeR-Box is an inverted repeat, we chose variation type 3 to represent point substitution at the second position of the forward or inverted repeat in our test. +++, all of the DNA was bound; ++, some of the DNA was bound; +, little DNA was bound; −, none of the DNA was bound.

consecutive binding sequences, which leads to a dramatically decreased binding affinity of the CmeR dimer to the CmeR-Box.

In summary, all of our ciprofloxacin-resistant *C. jejuni* isolates possessed a Thr-86-Ile substitution in GyrA. Most of the resistant strains contained extra variations in the CmeR-Box, including point substitutions, point insertion, or point deletion, among which the strains with the point insertion or deletion had high ciprofloxacin resistance levels. In this work, we identified several new sequence variations of the CmeR-Box. The point insertion or deletion in the CmeR-Box led to reduced binding by CmeR, which might significantly increase the expression of *cmeABC* and enhance the ciprofloxacin resistance level in the *C. jejuni* isolates. This study helps us to further understand the roles of the CmeR-CmeABC efflux pump system in fluoroquinolone resistance in *C. jejuni*.

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