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Emergence of Multidrug-Resistant *Campylobacter* Species Isolates with a Horizontally Acquired rRNA Methylase

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Antibiotic-resistant *Campylobacter* constitutes a serious threat to public health, and resistance to macrolides is of particular concern, as this class of antibiotics is the drug of choice for clinical therapy of campylobacteriosis. Very recently, a horizontally transferrable macrolide resistance mediated by the rRNA methylase gene *erm*(B) was reported in a *Campylobacter coli* isolate, but little is known about the dissemination of *erm*(B) among *Campylobacter* isolates and the association of *erm*(B)-carrying isolates with clinical disease. To address this question and facilitate the control of antibiotic-resistant *Campylobacter*, we determined the distribution of *erm*(B) in 1,554 *C. coli* and *Campylobacter jejuni* isolates derived from food-producing animals and clinically confirmed human diarrheal cases. The results revealed that 58 of the examined isolates harbored *erm*(B) and exhibited high-level resistance to macrolides, and most were recent isolates, derived in 2011-2012. In addition, the *erm*(B)-positive isolates were all resistant to fluoroquinolones, another clinically important antibiotic used for treating campylobacteriosis. The *erm*(B) gene is found to be associated with chromosomal multidrug resistance genomic islands (MDRGIs) of Gram-positive origin or with plasmids of various sizes. All MDRGIs were transferrable to macrolide-susceptible *C. jejuni* by natural transformation under laboratory conditions. Molecular typing of the *erm*(B)-carrying isolates by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) identified diverse genotypes and outbreak-associated diarrheal isolates. Molecular typing also suggested zoonotic transmission of *erm*(B)-positive *Campylobacter*. These findings reveal an emerging and alarming trend of dissemination of *erm*(B) and MDRGIs in *Campylobacter* and underscore the need for heightened efforts to control their further spread.

Campylobacter is the leading bacterial cause of food-borne illnesses worldwide and primarily causes gastroenteritis (1). For clinical treatment of campylobacteriosis, fluoroquinolones and macrolides are often prescribed (2); however, the resistance of *Campylobacter* to clinically important antibiotics is increasingly reported. Indeed, the 2013 CDC report identified antibiotic-resistant *Campylobacter* as a serious antibiotic resistance threat in the U.S. (http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf). Due to the increasing prevalence of fluoroquinolone-resistant *Campylobacter* worldwide (3–5), macrolides are now considered the drug of choice for therapeutic purposes (2, 6). Thus, development and spread of macrolide resistance in *Campylobacter* will significantly limit the options for clinical treatment.

Historically, macrolide resistance in *Campylobacter* has been at a low level of incidence and is mainly mediated by point mutations in the 23S rRNA or in the *rplD* and *rplV* genes encoding 50S ribosomal subunit proteins L4 and L22. The mutations occur at low frequencies and incur fitness costs in *Campylobacter* (6–9), partly explaining the low prevalence of macrolide resistance in *Campylobacter*. rRNA methylases, which represent major mechanisms for macrolide resistance in other bacterial organisms (10), were not identified in *Campylobacter* until recently. A recent work by our group reported the identification of a Gram-positive *erm*(B) gene in a single porcine *Campylobacter coli* isolate (11), which carried no mutations in the 23S rRNA or in the *rplD* and *rplV* genes. *erm*(B) encodes a rRNA methylase and mediates high-level macrolide resistance (MIC = 512 μg/ml) in *Campylobacter*, and it can be transferred between *Campylobacter jejuni* and *Campylobacter coli* via natural transformation (11). This was the first identification of a horizontally transferrable macrolide resistance mechanism in thermophilic *Campylobacter* spp.

The identification of a horizontally transferrable *erm*(B) in *Campylobacter* is alarming, as the gene confers high-level resistance to macrolides and can be disseminated by horizontal gene transfer. However, nothing is known about the distribution of *erm*(B) in *Campylobacter* and the association of *erm*(B)-carrying *Campylobacter* strains with clinical disease. To answer this question and facilitate the control of antibiotic-resistant *Campylobacter*, we investigated the incidence of *erm*(B) in *Campylobacter* isolates derived from both human diarrheal cases and food-producing animals, determined the gene environments surrounding *erm*(B), and characterized the *erm*(B)-positive isolates by molecular typing methods.

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MATERIALS AND METHODS

***Campylobacter* strains, growth conditions, and antimicrobial susceptibility testing.** A total of 1,554 *Campylobacter* isolates (1,157 *C. coli* and 397 *C. jejuni* isolates) were analyzed in this study. Detailed information on isolates is listed in Table S1 in the supplemental material. These include 75 human isolates, 789 swine isolates, 433 chicken isolates, 227 duck isolates, and 30 isolates from chicken carcasses. The previously identified *erm*(B)-carrying *C. coli* ZC113 of swine origin was included as a reference in this study (11). The 75 human *C. coli* isolates were from the retrospective collection of Chinese Centers for Disease Control and Prevention (CDC, Beijing, China) and were obtained during 2001–2011 from clinically confirmed gastroenteritis in four different provinces or cities. Due to the lack of a surveillance system for the *Campylobacter* spp. in patients with diarrhea or gastrointestinal disease in hospitals in China, clinical *Campylobacter* isolates were available from only a few hospitals, which had a collaborative relationship with China CDC. All patients have not been administered macrolides, but whether these patients had taken macrolides by themselves at home was unknown. The swine isolates were cultured from feces, and the chicken and duck isolates were cultured from cloacal swabs during 2008–2012 from Shandong, Ningxia, and Guangdong provinces under the routine program of surveillance for antimicrobial resistance in bacteria of animal origin (12, 13). Although information on antimicrobial usage for the herds/flocks from which the isolates were obtained was not available, the antibiotic usage records for some of the farms in which the isolates was collected indicated that macrolides, such as tylosin, spiramycin, and erythromycin, had been commonly used for curing or preventing bacterial infections (14). In addition, 30 *Campylobacter* isolates were cultured from whole chicken carcasses, which were collected after processing but before distribution in two slaughterhouses located in geographically separated regions of Shandong province in May 2011. All of the above-mentioned isolates were obtained based on one isolate per animal. In laboratory, all these *Campylobacter* isolates were grown on Mueller-Hinton agar (MHA; Sigma-Aldrich, MO, USA) at 42°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2). The *Campylobacter*-specific growth supplements and selective agents (Oxoid, Hampshire, United Kingdom) were added to the media when needed. The isolates were further confirmed by PCR as *C. coli* or *C. jejuni* as previously described (15). The standard agar dilution method recommended by Clinical and Laboratory Standards Institute (CLSI; 2008) was used to determine the MICs of various antibiotics in the *Campylobacter* isolates (16). *C. jejuni* ATCC 33560 was used as the quality control strain.

PCR identification of *erm*(B) in various *Campylobacter* isolates, and determination of its location and genetic environments. The *erm*(B) was identified in various *Campylobacter* isolates by PCR and sequencing. An 421-bp amplicon of *erm*(B) was produced by using the primers erm(B)-F (5′-GGGCATTTAACGACGAAACTGG) and erm(B)-R (5′-CTGTGGTATGGCGGGTAAGT), which were designed according to the conserved regions of the *erm*(B) genes found in *C. coli* ZC113, *Enterococcus faecium* (accession no. JN899585), *Streptococcus pneumoniae* (X52632), and *Lactobacillus reuteri* (AY082384). A modified random primer-walking strategy as previously described was performed using primers which were complementary to locations inside *erm*(B) for the sequence of entire open reading frame (ORF) of this gene (17). To determine whether *erm*(B) was localized in plasmids or on the chromosome, we analyzed the *erm*(B)-positive *Campylobacter* isolates by S1 nuclease pulsed-field gel electrophoresis (PFGE) and Southern blotting. Whole cells of the isolates were embedded in agarose gel plugs and digested with S1 nuclease (TaKaRa, Dalian, China), and the DNA was separated by PFGE as described previously (18). For Southern blotting, the 421-bp PCR product of *erm*(B) was used as the probe for hybridization. The probe was labeled with a digoxigenin (DIG) High Prime I DNA labeling and detection starter kit (Roche Diagnostics, Mannheim, Germany). Hybridization was performed at 44°C for 14 h. Membranes were washed twice at room temperature (22 to 25°C) with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS for 5 min and twice at 68°C with 0.5× SSC and 0.1% SDS for 15 min. DIG was detected with specific antibodies using a DIG High Prime I DNA labeling and detection starter kit according to the protocol of the supplier. The genetic environments of *erm*(B) in 26 *C. coli* isolates that harbored a chromosomally carried *erm*(B) were determined by using a long-range PCR with primers cadf-F2 and pfo-R2 or a modified random primer-walking sequencing strategy as described previously (15, 17).

Genotyping. Multilocus sequence typing (MLST) for *Campylobacter* was performed following the method described previously (19). The allelic profiles and the sequence types were generated by blasting the *Campylobacter* sequences in the MLST database (http://www.pubmlst.org/campylobacter). PFGE was performed using a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA, USA), according to the rapid protocol for *Campylobacter* (20), and *Salmonella* H9812 was used as the reference marker (digested with XbaI), while all *Campylobacter* isolates were digested with SmaI. The dendrograms were constructed from the PFGE data by the unweighted pair group method with arithmetic average (UPGMA) with the Dice coefficient using InfoQuest FP software, version 4.5 (Bio-Rad Laboratories, USA).

Plasmid typing. Plasmid typing was performed for the *C. coli* isolates that harbored a plasmid-borne *erm*(B) gene (Table 1). Since no *Campylobacter*-specific plasmid typing methods are available (21, 22), the incompatibility groups were characterized using the replicon typing methods for *Enterobacteriaceae* as previously described (23, 24).

Transfer of macrolide resistance by natural transformation or electrotransformation. Macrolide resistance in *C. coli* isolates was transferred to the macrolide-susceptible *C. jejuni* strain NCTC 11168 or 81-176 by natural transformation, which was performed according to the method described by Wang and Taylor (25). We also determined if these *erm*(B) gene-carrying plasmids in *C. coli* isolates could be transferred to *C. jejuni* NCTC 11168 or 81-176 and *C. coli* ATCC 33559 using both the natural transformation and electroporation methods (26). The transformants were selected on MH agar plates containing erythromycin (10 μg/ml). The transformation without donor DNA was used as a negative control. All copies of the 23S rRNA gene of the transformants were sequenced to confirm lack of mutations in this gene in the transformants (27). Moreover, to confirm the transfer of the *erm*(B) gene and other resistance genes to the transformants, long-range PCR assays were conducted in the transformants using primers cadf-F2 and pfo-R2 to amplify the region between *cadF* and *cj1476c* genes (15), and primers rdxA-F (5′-GGTATTTTGGCTCGTGAGCTTG) and ACE-R (5′-GCATATGAAGACAAGGGAGCT) to amplify the region between the *nfsB* and Δ*aac* genes.

Nucleotide sequence accession numbers. The *erm*(B)-carrying segments in various isolates have been deposited in GenBank, and their accession numbers are KC876748 (ZP-GX-1), KC876749 (DZB4), KC876750 (HN-CCD07046), KC876751 (SH-CCD11C073), and KC876752 (SH-CCD11C365).

RESULTS AND DISCUSSION

Presence of *erm*(B) in *Campylobacter* isolates. In total, 1,554 *Campylobacter* isolates (1,157 *C. coli* and 397 *C. jejuni* isolates) were examined in this study, including 75 isolates from human diarrheal cases and the rest from swine, chicken, and duck species (see Table S1 in the supplemental material). Among the isolates examined, 58 were positive for *erm*(B), including 57 *C. coli* and 1 *C. jejuni* isolates (Table 1). Ten of the *erm*(B)-positive isolates were from humans, 32 from swine, 14 from chicken, and 2 from duck, and the percentage of *erm*(B)-positive *Campylobacter* isolates in each of the sources is indicated in Table S2 in the supplemental material. The MIC results indicated that all 58 isolates were resistant to erythromycin, clindamycin, ciprofloxacin, and tetracycline, and 39 of them (67%) were also resistant to gentamicin (Table 1). Most of the 58 isolates showed an erythromycin MIC of ≥512 μg/ml. Twenty-two of the 58 (38%) *erm*(B)-positive *Campylobacter* strains harbored the A2075G (equivalent to A2059G in

TABLE 1 Characteristics of the 58 *erm*(B)-positive *Campylobacter* isolates identified in this study

Isolate[a]	Host[b]	Province/city[c]	Sample	Yr of isolation	Mutation in 23S rRNA[g]	Location of *erm*(B)[d]	MDRGI type[e]	MIC (μg/ml)[f]					
								CIP (4)	ERY (32)	CLI (8)	GEN (8)	TET (16)	CHL (32)
ZC113 (reference strain)	S	SD	Feces	2008	—	C	I	**32**	**512**	**256**	**128**	**128**	4
C179b	C	GD	Feces	2012	—	C	—	**32**	**>512**	**256**	1	**256**	4
YC74	S	NX	Feces	2009	A2075G	P		**64**	**128**	**16**	**>256**	**128**	4
ZP-GX-1	C	SD	Carcass	2011	—	C	III	**16**	**128**	**>256**	**256**	**512**	8
ZP-GX-5	C	SD	Carcass	2011	—	C	III	**32**	**512**	**128**	**128**	**512**	8
ZP-GX-8	C	SD	Carcass	2011	—	C	III	**32**	**512**	**128**	**128**	**512**	8
ZP-GX-12	C	SD	Carcass	2011	—	C	III	**32**	**512**	**128**	**128**	**512**	8
ZP-GX-14	C	SD	Carcass	2011	—	C	III	**64**	**512**	**128**	**128**	**512**	8
ZP-GX-20	C	SD	Carcass	2011	—	C	III	**32**	**512**	**128**	**128**	**512**	8
ZP-GX-26	C	SD	Carcass	2011	—	C	III	**64**	**512**	**128**	**128**	**256**	8
ZP-GX-28	C	SD	Carcass	2011	—	C	III	**16**	**128**	**>256**	**128**	**512**	8
ZP-GX-35	C	SD	Carcass	2011	—	C	III	**32**	**512**	**128**	**128**	**512**	8
ZP-GX-36	C	SD	Carcass	2011	—	C	III	**64**	**512**	**128**	**128**	**512**	8
ZP-GX-37	C	SD	Carcass	2011	—	C	III	**64**	**512**	**128**	**128**	**256**	8
LH-GX-14	C	SD	Carcass	2011	—	C	III	**64**	**256**	**128**	**128**	**256**	4
DH46a	D	SD	Feces	2012	—	C	—	**64**	**512**	**256**	**>256**	**128**	16
DH67	D	SD	Feces	2008	—	C	III	**64**	**512**	**256**	**256**	**128**	**64**
DZ5	S	SD	Feces	2012	A2075G	C	—	**32**	**512**	**32**	1	**512**	8
DZ50	S	SD	Feces	2008	A2075G	C, P	—	**32**	**512**	**8**	**256**	**256**	4
DZ14a	S	SD	Feces	2012	A2075G	P		**16**	**512**	**32**	**>256**	**128**	4
DZB1	S	SD	Feces	2012	A2075G	P		**32**	**512**	**16**	1	**512**	4
DZB4	S	SD	Feces	2012	—	C	II	**32**	**256**	**256**	**>256**	**128**	4
DZB5	S	SD	Feces	2012	A2075G	P		**32**	**512**	**32**	**>256**	**512**	8
DZB7	S	SD	Feces	2012	—	C	—	**64**	**64**	**16**	2	**64**	4
DZB12	S	SD	Feces	2012	—	C	I	**32**	**512**	**256**	**>256**	**128**	8
DZB41	S	SD	Feces	2012	A2075G	P		**16**	**512**	**32**	1	**512**	4
158	C	SD	Feces	2008	—	C	III	**32**	**32**	**>256**	**128**	**128**	2
TH34	S	GD	Feces	2012	A2075G	P		**32**	**128**	**32**	1	**128**	16
TH45	S	GD	Feces	2012	—	P		**32**	**>512**	**32**	**>256**	**512**	16
TH48	S	GD	Feces	2012	A2075G	P		**16**	**>512**	**64**	**256**	**128**	**32**
TH62	S	GD	Feces	2012	A2075G	P		**16**	**>512**	**32**	1	**512**	8
TH74	S	GD	Feces	2012	A2075G	P		**16**	**>512**	**64**	1	**512**	**32**
TH80	S	GD	Feces	2012	A2075G	P		**32**	**>512**	**32**	**>256**	**32**	**32**
TH96	S	GD	Feces	2012	A2075G	P		**8**	**>512**	**64**	4	**128**	**32**
TH117	S	GD	Feces	2012	A2075G	C	—	**32**	**>512**	**128**	**256**	**512**	8
TH119	S	GD	Feces	2012	A2075G	P		**16**	**>512**	**64**	1	**32**	4
52-3b	S	GD	Feces	2012	A2075G	P		**32**	**>512**	**32**	1	**128**	**32**
84a	S	GD	Feces	2012	A2075G	C	—	**16**	**512**	**32**	1	**128**	4
08-23	S	GD	Feces	2012	A2075G	C	—	**32**	**>512**	**32**	**>256**	**64**	16
08-120	S	GD	Feces	2012	A2075G	P		**8**	**>512**	**32**	2	**128**	4
10-5-18	S	GD	Feces	2012	A2075G	P		**8**	**>512**	**64**	**256**	**128**	**32**
10-7-1a	S	GD	Feces	2012	—	P		**8**	**>512**	**64**	1	**512**	4
10-7-1c	S	GD	Feces	2012	—	P		**8**	**>512**	**32**	1	**512**	4
10-13-6a	S	GD	Feces	2012	A2075G	P		**32**	**>512**	**32**	**256**	**128**	8
10-33-66	S	GD	Feces	2012	A2075G	P		**32**	**>512**	**32**	**256**	**128**	**64**
JW16	S	NX	Feces	2012	—	P		**32**	**32**	**32**	4	**64**	4
LWC2	S	NX	Feces	2012	—	P		**8**	**512**	**32**	1	**128**	2
LWD21	S	NX	Feces	2012	—	P		**16**	**32**	**16**	0.5	**32**	4
LWD84	S	NX	Feces	2012	—	P		**32**	**64**	**8**	2	**128**	4
HN-CCD07046	H	HN	Stool	2007	—	C	IV	**128**	**128**	**256**	**256**	**128**	**32**
SH-CCD11C073	H	SH	Stool	2011	—	C	V	**32**	**128**	**256**	**128**	**512**	**32**
SH-CCD11C226	H	SH	Stool	2011	—	C	III	**64**	**512**	**256**	**>256**	**128**	**64**
SH-CCD11C287	H	SH	Stool	2011	—	C	VI	**256**	**512**	**256**	**256**	**128**	**64**
SH-CCD11C365	H	SH	Stool	2011	—	C	VI	**256**	**512**	**256**	**128**	**512**	16
SH-CCD11C416	H	SH	Stool	2011	—	C	VI	**64**	**128**	**256**	**128**	**512**	16
SH-CCD11C419	H	SH	Stool	2011	—	C	III	**64**	**512**	**256**	**128**	**128**	**32**
SH-CCD11C490	H	SH	Stool	2011	—	C	VI	**128**	**128**	**256**	**256**	**128**	**64**
SH-CCD11C518	H	SH	Stool	2011	—	C	VI	**256**	**512**	**256**	**256**	**512**	**64**
SH-CCD11C682	H	SH	Stool	2011	—	C	VI	**64**	**128**	**256**	**128**	**128**	16

[a] All but one are *C. coli*, and C179b is *C. jejuni*. The *erm*(B)-carrying isolate *C. coli* ZC113 identified in the previous study (11) is included as a reference.
[b] S, swine; C, chicken; D, duck; H, human.
[c] SD, Shandong; NX, Ningxia; GD, Guangdong; HN, Henan; SH, Shanghai.
[d] *erm*(B) is on the chromosome (C), the plasmid (P), or both (C, P).
[e] —, the genetic environment of *erm*(B) in the chromosome could not be determined by the primer walking strategy used in this study.
[f] CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TET, tetracycline; CHL, chloramphenicol. Numbers in parentheses are MIC breakpoints. The MICs over the breakpoint are in bold.
[g] —, no mutations detected in 23S rRNA gene.

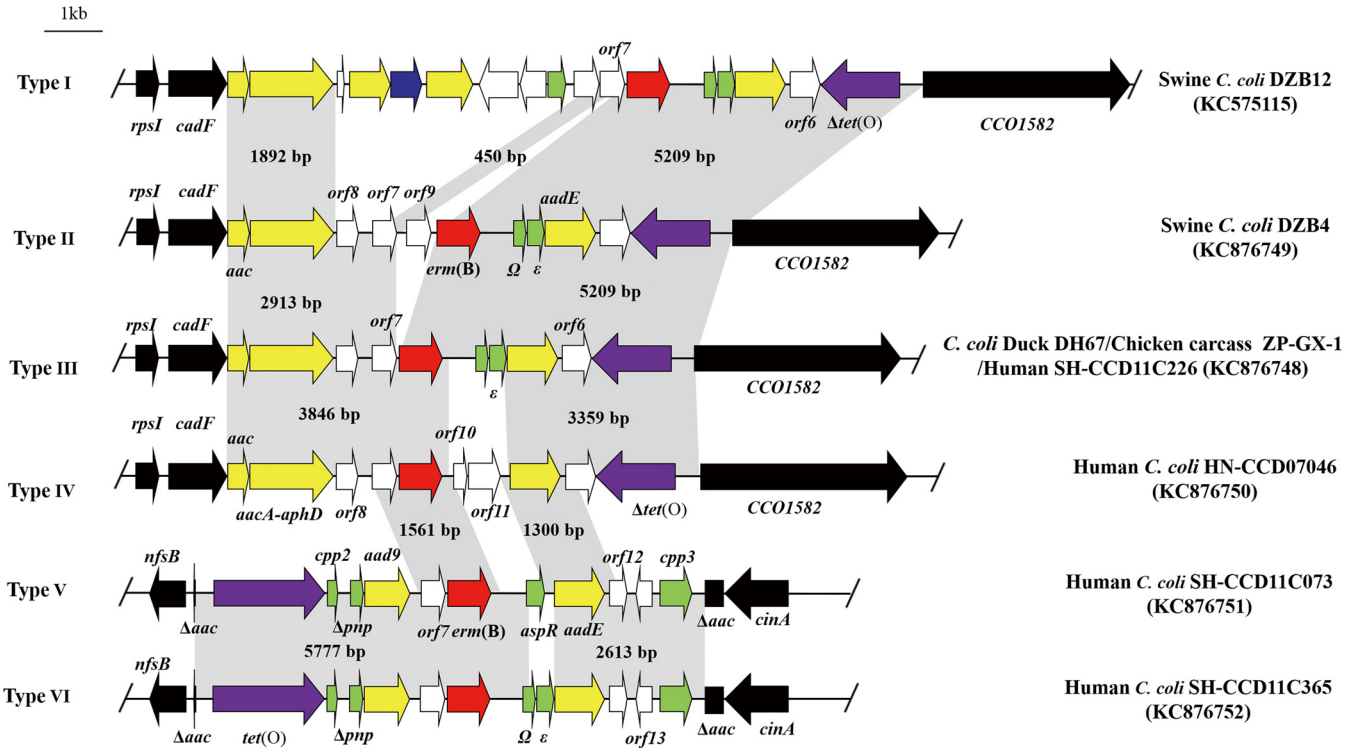


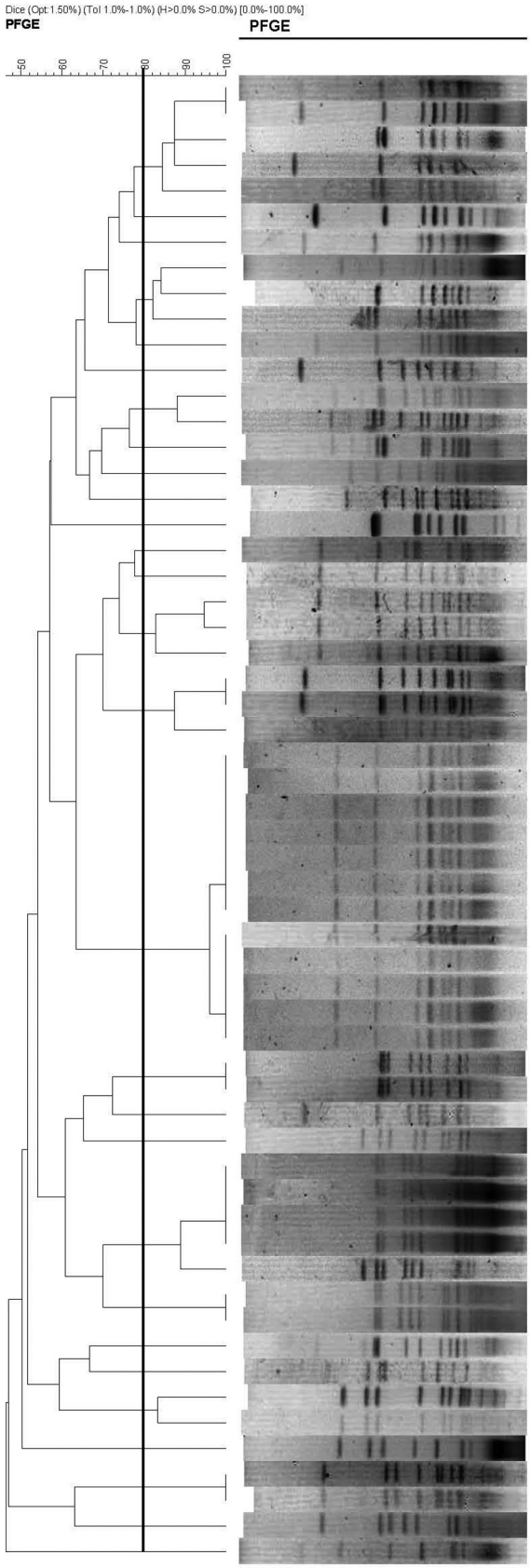
FIG 1 Chromosomal organization and comparison of six different types (I to VI) of MDRGIs in *erm*(B)-positive *C. coli* isolates. *erm*(B) is in red, aminoglycoside resistance genes are in yellow, the streptothricin resistance gene (*sat4*) is in blue, the tetracycline resistance gene [*tet*(O)] is in purple, genes with predicted functions are in green, and genes coding hypothetical proteins are in white. The *tet*(O) gene is intact in types V and VI but is truncated in other types. The border genes of the MDRGIs are depicted by black box arrows. The gray shading indicates regions sharing more than 98% DNA identity. A representative strain for each type of MDRGIs is indicated on the right of the panel.

E. coli) mutation in 23S rRNA, and these isolates exhibited no significant difference ($P > 0.05$) in the MICs of erythromycin compared with the other 36 isolates that had no mutation in the target gene (Table 1). Sequencing of the PCR products revealed three variants of *erm*(B) among the 58 isolates with lengths of 738 bp ($n = 34$), 753 bp ($n = 21$), and 765 bp ($n = 3$). PFGE and Southern blotting revealed that 33 (57%) isolates, including the *C. jejuni* isolate C179b, carried *erm*(B) on the chromosome, while 24 (41%) isolates harbored *erm*(B) on plasmids of various sizes (Table 1; representative results are shown in Fig. S1 in the supplemental material).

Notably, most (53/58) of the *erm*(B)-positive *Campylobacter* isolates were obtained in recent years (2011-2012) (see Table S1 in the supplemental material). Among the 720 isolates collected from 2007 to 2009, 5 (0.7%) were positive with *erm*(B), while 53 (6.4%) of the 829 isolates collected during 2011-2012 were positive for *erm*(B). This finding suggests the recent emergence and a rising trend for *erm*(B)-carrying *Campylobacter* isolates in China.

Genetic environments of the *erm*(B) gene on the chromosome of *C. coli* isolates. We further determined the genetic environments of *erm*(B) in 26 *C. coli* isolates that harbored a chromosomally carried *erm*(B) gene. In all cases, *erm*(B) was associated with multidrug resistance genomic islands (MDRGIs), which were inserted between *cadF* and *CCO1582* (19 isolates) or in the *aac* gene, located between *nfsB* and *cinA* (7 isolates) (Fig. 1). The MDRGIs were classified into six types (designated I to VI) according to their gene contents and insertion sites on chromosome, and the previously identified MDRGI in ZC113 was designated type I (Table 1; Fig. 1). Types I to IV were bordered by *cadF* and *CCO1582*, while types V and VI were inserted in the *aac* gene. The GC contents of the MDRGIs ranged from 34.3% to 37.3%, higher than that (31.4%) of the genome of *C. coli* RM2228. Among them, type III was the most common and was observed in 16 isolates originated from both human and other animal species. Types IV, V, and VI were detected only in the human isolates, while types I and II were observed only in the swine isolates (Table 1).

Most of the ORFs in the MDRGIs are either identical or >90% identical to their orthologs in Gram-positive bacteria (see Table S3 in the supplemental material), implying that all *erm*(B)-carrying MDRGIs in *Campylobacter* were derived from Gram-positive origins. The *orf7* gene encodes a conserved hypothetical protein that shares 95.8 to 97.1% amino acid (aa) identity to a hypothetical ORF in *Clostridium difficile* NAP08 (ZP_06892641) and is located immediately upstream of *erm*(B) in all but type II MDRGIs (Fig. 2). For type II, *orf9* is inserted between *orf7* and *erm*(B). The deduced amino acid sequence of *orf9* showed 64.7% amino acid (aa) identity (86/133) to the fosfomycin resistance protein FosX of *Listeria monocytogenes* (YP_005962817). The two ORFs (Ω and ε) immediately downstream of *erm*(B) were conserved in types I, II, III, and VI. The Ω gene encodes 71 aa and shares 97.2% (69/71) aa identity to the Ω gene in *Streptococcus agalactiae* (ZP_22245542). The ε gene encodes 95 aa and shows 98.8% (89/90) aa identity to the corresponding region of ε in *Streptococcus pyogenes* (YP_232758). Regarding the other resistance genes, the aminoglycoside resistance gene *aadE*, located downstream of *erm*(B), was highly conserved (99.9% nucleotide identity) in all MDRGIs,



	Province /City	Host	PFGE type	MLST type	CC
DZB12	SD	S	A1	5923	828
ZC113	SD	S	A1	1625	828
DZ50	SD	S	A2	1625	828
SH-CCD11C419	SH	H	A3	1625	828
DH67	SD	D	A4	5948	-
HN-CCD07046	HN	H	B	5517	828
SH-CCD11C226	SH	H	C	899	828
DH46a	SD	D	D1	1586	828
DZB4	SD	S	D2	5512	828
SH-CCD11C073	SH	H	D3	872	828
JW16	NX	S	E	5943	-
SH-CCD11C682	SH	H	F	828	828
08-23	GD	S	G1	828	828
DZB7	SD	S	G2	5946	828
84a	GD	S	H	1058	828
LWC2	NX	S	I	2737	-
YC74	NX	S	J	828	828
DZ5	SD	S	K	1579	828
08-120	GD	S	L	854	828
TH117	GD	S	M	1130	828
TH45	GD	S	N1	1096	828
TH48	GD	S	N2	1595	828
TH34	GD	S	N3	2732	828
DZB1	SD	S	O1	1107	828
DZB5	SD	S	O1	832	828
TH62	GD	S	O2	5290	828
ZP-GX-14	SD	CC	P1	860	828
ZP-GX-8	SD	CC	P1	860	828
ZP-GX-28	SD	CC	P1	860	828
ZP-GX-35	SD	CC	P1	860	828
ZP-GX-36	SD	CC	P1	860	828
ZP-GX-37	SD	CC	P1	860	828
ZP-GX-5	SD	CC	P1	860	828
LH-GX-14	SD	CC	P2	860	828
ZP-GX-1	SD	CC	P2	860	828
ZP-GX-12	SD	CC	P2	860	828
ZP-GX-20	SD	CC	P2	860	828
ZP-GX-26	SD	CC	P2	860	828
10-13-6a	GD	S	Q	6074	-
10-33-66	GD	S	Q	6074	-
TH119	GD	S	R	854	828
DZ14a	SD	S	S	1579	828
SH-CCD11C287	SH	H	T1	1145	828
SH-CCD11C416	SH	H	T1	1145	828
SH-CCD11C490	SH	H	T1	1145	828
SH-CCD11C518	SH	H	T1	1145	828
SH-CCD11C365	SH	H	T2	1145	828
10-5-18	GD	S	U	2642	828
LWD84	NX	S	U	854	828
DZB41	SD	S	V	854	828
TH74	GD	S	W	6075	-
52-3b	GD	S	X1	1096	828
TH96	GD	S	X2	1096	828
158	SD	C	Y	899	828
10-7-1a	GD	S	Z	6073	-
10-7-1c	GD	S	Z	6073	-
LWD21	NX	S	AB	4085	828
TH80	GD	S	AC	1104	828

FIG 2 PFGE and MLST typing of 58 *erm*(B)-positive *C. coli* isolates. STs 5923, 5943, 5946, 5948, 6073, 6074, and 6075 were newly designated in this study. SmaI was used for PFGE. Provinces and cities include Shandong (SD), Ningxia (NX), Guangdong (GD), Henan (HN), and Shanghai (SH). Sources and host species include swine (S), chicken (C), chicken carcasses (CC), duck (D), and human (H). −, a clonal complex cannot be assigned.

while the *aac* and *aacA-aphD* genes in types I to IV exhibited nucleotide identity of 99.9%. The *aad9* gene in types V and VI also showed 99.8% nucleotide identity to the *aad9* gene on pCG8245 in *C. jejuni* (AY701528) (28). The tetracycline resistance gene *tet*(O) was intact in types V and VI but was truncated in the other types of MDRGIs. Detailed information on the ORFs in types I to VI is presented in Table S3 in the supplemental material. During the investigation of the genetic environment of *erm*(B) in C179b and in other seven *C. coli* isolates, in which the *erm*(B) gene was chromosome borne but was not inserted between *cadF* and *CCO1582* or in the *aac* gene located between *nfsB* and *cinA*, the primer-walking strategy used in this study was not successful despite several attempts. Thus, the flanking regions of *erm*(B) in these isolates are unknown. Whole-genome sequence analysis will be needed to determine the exact location of the *erm*(B) insertion in these isolates.

Although the identified MDGRIs vary in size, gene content, and insertion sites on the chromosome (Fig. 1), they shared a common origin of Gram-positive bacteria. The *erm*(B) gene and its neighboring genes in the MDRGIs are either identical or almost identical to their orthologs in Gram-positive bacteria, including *Streptococcus*, *Enterococcus*, *Clostridium*, *Listeria*, and *Lactobacillus* (see Table S3 in the supplemental material), implying that all *erm*(B)-carrying MDRGIs in *Campylobacter* were derived from Gram-positive origins. How *Campylobacter* acquired the MDGRIs from Gram-positive bacteria is unknown and remains to be determined.

Molecular typing and phylogenetic analysis of the *erm*(B)-carrying *C. coli* isolates. PFGE and MLST were used to type the 57 *erm*(B)-positive *C. coli* isolates plus the previously reported *erm*(B)-carrying *C. coli* isolate ZC113. MLST generated 30 sequence types (STs), seven of which were newly designated in this study (Fig. 2). All 30 STs except for eight singletons were clustered into one clonal complex, CC828. Using a cutoff of 80% pattern similarity, the *erm*(B)-positive *C. coli* isolates were clustered into 28 PFGE subtypes (Fig. 2). The PFGE results generally corresponded to the MLST typing data, as most isolates of the same STs also appeared in the same PFGE subtypes. For instance, 4 isolates of human origin had the same ST (1145) and PFGE type (T1). Likewise, the 12 isolates from chicken carcasses, all belonged to ST 860 and could be clustered together at >95% (P1 and P2) according to the PFGE types (Fig. 2). Among the 75 human *C. coli* isolates examined in this study, 10 of them harbored the *erm*(B) gene and all of them were on chromosomal MDRGIs. These 10 isolates were from hospitals in Shanghai ($n = 9$, collected in 2011) and Henan province ($n = 1$, collected in 2007). Six of the 10 isolates were from pediatric patients (50 days to 6 years of age), while the others were from adults (24 to 54 years of age). Notably, 5 of the human isolates (4 from children and 1 from an adult) shared the same PFGE type and MLST type (Fig. 2), and were all from Shanghai, suggesting that this particular genotype was associated with an outbreak in the area. It should be acknowledged that nine out of the 10 human *erm*(B)-positive isolates were from Shanghai, suggesting that the true extent of *erm*(B) gene dissemination among humans in China cannot be determined in this study. Interestingly, a human isolate (SH-CCD11C419) from Shanghai and swine isolates (ZC113 and DZ50) from Shandong were clustered together by PFGE and MLST, all of which belong to ST1625 and PFGE subtype (A) using a cutoff of 80% genetic similarity for PFGE (Fig. 2), suggesting that they are clonal and suggesting the possibility of zoonotic transmission of this clone.

TABLE 2 Antimicrobial susceptibility of *Campylobacter* transformants carrying the six types of MDRGIs

Strain[a]	MIC (μg/ml)[b]				
	CIP	ERY	CLI	GEN	TET
NCTC 11168	<0.25	1	0.125	0.5	0.5
81–176	<0.25	0.5	0.25	0.25	128
11168-type I (ZC113)	<0.25	512	256	>64	0.5
81–176-type II (DZB4)	<0.25	64	128	64	128
11168-type III (ZP-GX-8)	<0.25	128	512	>64	0.25
11168-type IV (HN-CCD07046)	<0.25	128	256	>64	0.25
81–176-type V (SH-CCD11C073)	<0.25	128	128	1	128
81–176-type VI (SH-CCD11C365)	<0.25	256	128	1	128

[a] The strains in parentheses indicate the donors carrying MDRGIs.
[b] CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TET, tetracycline.

Transfer of *erm*(B)-carrying MDRGIs between *C. coli* and *C. jejuni*. Our previous study showed that the type I MDRGI from ZC113 could be transferred to *C. jejuni* NCTC 11168 via natural transformation (11). In this study, we examined five other types of MDRGIs and found that they were also transferable from *C. coli* isolates to *C. jejuni* NCTC 11168 or 81-176 by natural transformation. Transfer of the MDRGIs to the transformants was confirmed by long-range PCR. Six different amplicons of expected sizes, including 12,210 bp, 8,927 bp, 8,281 bp, 8,384 bp, 9,660 bp, and 9,684 bp, were obtained from transformants 11168-type I, 81-176-type II, 11168-type III, 11168-type IV, 81-176-type V, and 81-176-type VI, respectively (Table 2). The sequencing results confirmed that the MDRGIs in the transformants were identical to those in the six types of donors. All transformants exhibited high-level resistance to erythromycin and clindamycin (Table 2), consistent with the known function of *erm*(B) in conferring resistance to macrolides, lincosamides, and streptogramin B (10, 29). Although the transformation was done under laboratory conditions, this finding is alarming, as *Campylobacter* is naturally transformable, and transfer of *erm*(B) between *C. coli* and *C. jejuni* could conceivably occur in natural environments, which would pose a great concern for public health, as a large portion of human campylobacteriosis is associated with *C. jejuni* (30, 31).

We also tried to transfer some of the *erm*(B)-carrying plasmids by natural transformation and electroporation. However, both methods failed to move the plasmids to recipient *Campylobacter* strains (*C. coli* ATCC 33559 and *C. jejuni* NCTC 11168 and 81-176). This could be due to the difficulty of genetic manipulation of *Campylobacter*, as transformation of *Campylobacter* with plasmid DNA is less efficient than that with chromosomal DNA (32). Additionally, most of the *erm*(B)-carrying plasmids were above 70 kb in size (data not shown), which may limit the efficiency of transformation. At present, it is unknown if these plasmids can be transferred by conjugation. Despite the difficulty in transferring the plasmids, we performed plasmid typing with the 24 *C. coli* isolates harboring a plasmid-borne *erm*(B). The PCR and sequencing results revealed that 14 of them were typeable and belonged to four incompatibility (Inc) groups, including IncA/C (isolates JW16, 10-13-6a, 10-33-66, TH119, and TH74), IncW (isolates LWC2, LWD21, TH34, and DZ50), IncY (isolates DZB5, 10-5-18, and TH96), and IncFIA (isolates 10-7-1a and 10-7-1c).

Ten of the 24 isolates were not typeable with the methods used in this study. However, it should be noted that it is unknown whether these identified plasmid types belonged to the *erm*(B)-carrying plasmids or to other plasmids, as a given *C. coli* isolate may contain more than one plasmid. The detailed genetic features of the *erm*(B)-carrying plasmids and their transfer mechanisms remain to be determined in future studies.

The identification of multiple *erm*(B)-positive isolates in this study reinforced the findings in our recent report (11) and indicates that *erm*(B) has emerged and is disseminating in *Campylobacter*. This finding is significant, as macrolides are the key antibiotic for clinical therapy of *Campylobacter* infections and resistance mediated by rRNA methylase was not identified until recently (11). Previously identified macrolide resistance mechanisms in *Campylobacter* involved 23S rRNA mutations in conjunction with the function of the CmeABC efflux pump (33, 34). The 23S rRNA mutations are well known for conferring high-level macrolide resistance in *Campylobacter* (7), but resistance-conferring mutations in 23S rRNA occur at low frequencies and incur a significant fitness cost in the absence of antibiotic selection pressure (35), contributing to the low prevalence of macrolide-resistant *Campylobacter*. The contribution of *erm*(B) to macrolide resistance in *Campylobacter* was shown in our recent report (11) and in this study using the transformants, in which 23S rRNA mutations were absent and *erm*(B) alone elevated the MICs up to 512 μg/ml (Table 2). The data from Table 2 and part of the data in Table 1 (for isolates with no 23S rRNA mutations) clearly show the significance of *erm*(B) in conferring high-level macrolide resistance in *Campylobacter*. Interestingly, the presence of both 23S rRNA mutation and *erm*(B) did not appear to show an additive effect on resistance, as macrolide MICs of the isolates harboring both mechanisms did not differ significantly ($P > 0.05$) from those of the isolates having only *erm*(B) (Table 1). The emergence of *erm*(B) in *Campylobacter* will likely change the landscape of macrolide resistance in *Campylobacter*, as this resistance determinant is horizontally transferable and may not cause a fitness burden in the organism. This possibility warrants further investigation.

In summary, this study reveals the presence of a horizontally transferable *erm*(B) in multiple *Campylobacter* isolates from various sources. In 59% of the cases, *erm*(B) was carried on chromosomal MDRGIs, along with other antibiotic resistance determinants. The MDRGIs thus confer resistance to multiple classes of antibiotics, including macrolides, lincosamides, and aminoglycosides. Additionally, all *erm*(B)-carrying isolates exhibited resistance to fluoroquinolones and tetracycline (Table 1). Thus, the *erm*(B)-positive isolates, regardless of the location of *erm*(B) (on the chromosome or a plasmid) or their origin of isolation (from human patients or farm animals), are resistant to all antibiotics that are important for clinical treatment of campylobacteriosis. The multidrug resistance phenotype of the *erm*(B)-positive isolates underlines the need for enhanced efforts to curb their further spread, as there will be limited options for clinical treatment of human patients infected by these isolates.

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