

Constitutive and Inducible Expression of the rRNA Methylase Gene *erm(B)* in *Campylobacter*

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Macrolides are the antimicrobials of choice for treating human campylobacteriosis. The recent emergence of *erm(B)* in *Campylobacter* bacteria threatens the utility of this class of antibiotics. Here we report the constitutive and inducible expression of *erm(B)* in *Campylobacter* isolates derived from diarrheal patients and food-producing animals. Constitutive expression of *erm(B)* was associated with insertion and deletion in the regulatory region of the gene, providing the first documentation of the differential expression of *erm(B)* in *Campylobacter* bacteria.

Campylobacter bacteria are among the most common causes of bacterium-mediated diarrheal disease worldwide (1). Additionally, *Campylobacter* infection can lead to extraintestinal complications such as polyarthralgia (i.e., reactive arthritis) and Guillain-Barré syndrome (2). In general, the occurrence of human *Campylobacter* infection has been attributed largely to the consumption of contaminated food animal products, resulting from the high prevalence of *Campylobacter* in these animals (3).

Erythromycin, a 14-membered macrolide antibiotic, is recommended for use as the first-line treatment of campylobacteriosis (4), while 16-membered macrolides (e.g., tylosin and spiramycin) are some of the most common growth-promoting agents in food animal production worldwide (5). Macrolide resistance among *Campylobacter jejuni* and *Campylobacter coli* strains is generally much more severe in developing countries than in developed countries (5–9). In addition, there is a much higher frequency of macrolide resistance in *C. coli* than that in *C. jejuni*, both in isolates derived from humans and in those from food animals (6, 10, 11). Very recently, the rRNA methylase Erm(B) has emerged as an important mechanism of acquired macrolide-lincosamide (ML) resistance in *C. jejuni* and *C. coli* (8, 12, 13), the two most common human disease-causing *Campylobacter* species. In Gram-positive bacteria, the expression of *erm* genes can be either constitutive or inducible (14). The mechanism of *erm(B)* induction has not been thoroughly investigated (14); however, the induction of *erm(C)* has been well studied and it is believed to be due to structural alterations, including sequence deletions, duplications/insertions, and point mutations in the *erm(C)* regulatory region, which acts as a translational attenuator (15). To our knowledge, no information is available concerning the mechanism of *erm(B)* induction in *Campylobacter* bacteria.

This study included 29 *Campylobacter* strains with a chromosome-borne *erm(B)* gene (28 of *C. coli*, 1 of *C. jejuni*), 27 of which were collected during 2007 to 2012 from food-producing animals (chickens, swine, and ducks) and diarrheal patients in different provinces and cities in China (8) and 2 of which (DZB40 and 86c) were collected in 2013 from swine slaughterhouses in Shandong and Guangdong, respectively. Details of the origins and identification of the above-mentioned 27 *Campylobacter* strains have been described previously (8).

Campylobacter isolates were evaluated for susceptibility to 14-, 15-, and 16-membered macrolides, as well as clindamycin, by the agar dilution method with incubation at 42°C for 24 h under microaerophilic conditions according to CLSI document VET01-A4 (16). *C. jejuni* ATCC 33560 was used as a quality control strain. To determine MICs following induction, isolates were grown on Mueller-Hinton agar containing either erythromycin at 0.5 mg/liter or clindamycin at 0.25 mg/liter. Three independent experiments were conducted to confirm the reproducibility of the MIC patterns.

The *erm(B)*-positive *Campylobacter* strains were classified as having a constitutive ML antibiotic resistance phenotype if resistance was independent of preincubation with the inducer, while inducible resistance was dependent on preincubation (Table 1). Inducible-*erm(B)*-positive *C. coli* strains DZB40 and 86c were susceptible to all of the antibiotics tested, except for tylosin, prior to induction. Following induction with erythromycin and clindamycin independently, the two strains attained resistance to erythromycin and azithromycin and also showed increased MICs of tylosin (Table 1). This observation is consistent with previous findings by other investigators (17, 18) and further suggests that some bacteria with inducible *erm* are susceptible to macrolides and lincosamides before induction, although isolates carrying *erm(B)* usually exhibit ML resistance. Notably, the two inducible-*erm(B)*-carrying strains did not show increased clindamycin MICs after induction. This is similar to previous findings on *erm(C)* induction (19, 20), which showed that a strain expressing inducible

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TABLE 1 MICs of ML antibiotics for *Campylobacter* strains determined with and without induction

Strain ^a	Host ^b	Region ^c	Type ^d	MIC (mg/liter) ^e									MIC (mg/liter)		
				Erythromycin			Azithromycin			Tylosin			Clindamycin		
				N	I-e	I-c	N	I-e	I-c	N	I-e	I-c	N	I-e	I-c
C179b	C	GD	C	512	512	512	128	128	128	>1,024	>1,024	>1,024	512	512	512
ZP-GX-1	C	SD	C	256	256	256	256	256	256	>1,024	>1,024	>1,024	512	512	512
ZC113	S	SD	C	512	512	512	256	256	256	>1,024	>1,024	>1,024	1,024	1,024	1,024
DZB4	S	SD	C	256	256	256	128	128	128	>1,024	>1,024	>1,024	1,024	1,024	1,024
DH67	D	SD	C	512	512	512	128	128	128	>1,024	>1,024	>1,024	1,024	1,024	1,024
SH-CCD11C073	H	SH	C	512	512	512	128	128	128	>1,024	>1,024	>1,024	1,024	1,024	1,024
HN-CCD07046	H	HN	C	256	256	256	256	256	256	>1,024	>1,024	>1,024	1,024	1,024	1,024
DZB40	S	SD	I	2	32	16	<0.25	8	4	8	128	32	1	1	1
86c	S	GD	I	4	32	16	<0.25	8	4	16	128	16	1	1	1

^a All strains are *C. coli*, except C179b (*C. jejuni*). The first seven strains (except DZB40 and 86c) are representatives of the 27 *Campylobacter* strains collected from 2007 to 2012, which have been described previously (8). The other 20 *Campylobacter* strains were 158, LH-GX-14, ZP-GX-12, ZP-GX-14, ZP-GX-20, ZP-GX-26, ZP-GX-28, ZP-GX-35, ZP-GX-36, ZP-GX-37, ZP-GX-5, ZP-GX-8, SH-CCD11C226, SH-CCD11C287, SH-CCD11C365, SH-CCD11C416, SH-CCD11C419, SH-CCD11C490, SH-CCD11C518, and SH-CCD11C682.

^b C, chicken; S, swine; D, duck; H, human.

^c GD, Guangdong Province; SD, Shandong Province; SH, Shanghai Municipality; HN, Henan Province.

^d C, constitutive; I, inducible.

^e The erythromycin, azithromycin, tylosin, and clindamycin MIC breakpoints were all ≥ 8 mg/liter. N, not induced; I-e, induced with erythromycin; I-c, induced with clindamycin.

erm(C) developed resistance only to 14- and 15-membered macrolides and remained susceptible to lincosamides. In this study, erythromycin and clindamycin were able to act as inducers of *erm(B)* gene expression in *Campylobacter* to various degrees. The MICs for the other 27 *Campylobacter* strains were almost identical; hence, the MICs for 7 epidemiologically distinct *Campylobacter* strains, as representatives of the 27 *Campylobacter* strains, are shown in Table 1. These 27 strains, including the only *C. jejuni* strain (C179b), were highly resistant to ML antibiotics without prior induction, indicating constitutive expression of *erm(B)*.

Potential attenuators located within the regulatory region of constitutively expressed *erm(B)* in *Campylobacter* isolates were amplified by PCR as described previously and then sequenced three times in both directions from independent replicons (21). The inducibly expressed *erm(B)* regulatory region in Tn917 of *Enterococcus faecalis* (GenBank accession no. M11180) was used as the reference sequence. PCR amplification of the *erm(B)* regulatory regions, from the -10 signal site to the 5' end of *erm(B)*, of all 27 *Campylobacter* strains revealed the presence of two different amplification products. The *erm(B)* regulatory region of DZB4 yielded an amplification product of 693 bp (GenBank accession number KC876749), whereas the *erm(B)* regulatory regions of the remaining 26 *Campylobacter* strains produced a smaller 61-bp product (e.g., C179b, accession number KF864551) (Fig. 1). A comparison with the equivalent region of the inducibly expressed *erm(B)* gene of Tn917 is presented in Fig. 1. On the basis of this comparison, the only difference was a 210-bp deletion in the regulatory region of C179b, compared with that of Tn917, that removed the entire *erm(B)* attenuator. Several open reading frame (ORFs) are present within the 210-bp deleted region, including an ORF corresponding to a 27-amino-acid (aa) leader peptide that is known to play a crucial role in the induction process as a partner in mRNA secondary-structure formation (15). Moreover, the deletion of IR3 to IR7 renders IR8 accessible to ribosomes, independent of the presence or absence of an inducer. The simultaneous absence of the leader peptide and several inverted repeats (IRs)

from these 26 *Campylobacter* strains explains the observed constitutive ML resistance.

C. coli isolate DZB4 contained a 635-bp substitution of the three bases (GTT) at position 17 that are present in Tn917 (Fig. 1). Sequence analysis revealed the presence of a novel 136-aa ORF, *fosX^{CC}*, in the 635-bp substitution, which was recently reported to mediate resistance to fosfomycin in *Campylobacter* (22). As both the leader peptide and IRs found in Tn917 were missing from the *erm(B)* regulatory region of DZB4, the large substitution likely had the same effect on *erm(B)* expression as the 210-bp deletion that was detected in the other 26 *Campylobacter* strains. This kind of large insertion in the *erm* regulatory region, causing a shift from inducible to constitutive *erm* gene expression, was also observed in the *erm(A)* regulatory region of *Streptococcus agalactiae* (17). The possibility of the insertion of another antibiotic resistance gene into the regulatory region, ensuring the transmission of the multiple-drug resistance element into the genome of *Campylobacter*, is a cause for concern. However, the 635-bp substitution carrying the fosfomycin resistance gene *fosX^{CC}* in DZB4 has not yet been identified in any other *Campylobacter* strains.

In the two inducible *erm(B)*-positive *C. coli* strains DZB40 and 86c, a 27-aa leader peptide and four pairs of IRs (IR1 to IR8) were detected in the *erm(B)* regulatory region, which could form stable RNA secondary structures and modulate the expression of *erm(B)*. It has been reported that inducible *erm* expression was a consequence of differences in mRNA base composition (14, 15). Additionally, induction could cause *erm* expression by destabilizing mRNA secondary structures in the inducible *erm* strains (14, 15). The molecular and structural bases for the induction of *erm(B)* in *Campylobacter* are unknown and remain to be determined in future studies. Interestingly, both DZB40 and 86c were susceptible to most of the antibiotics tested prior to induction. After induction, the MICs of macrolides substantially increased but did not reach the levels of strains with constitutively expressed *erm(B)* (Table 1). The coding sequence (CDS) of *erm(B)* in the two isolates was intact and did not show any frame shift, substitu-

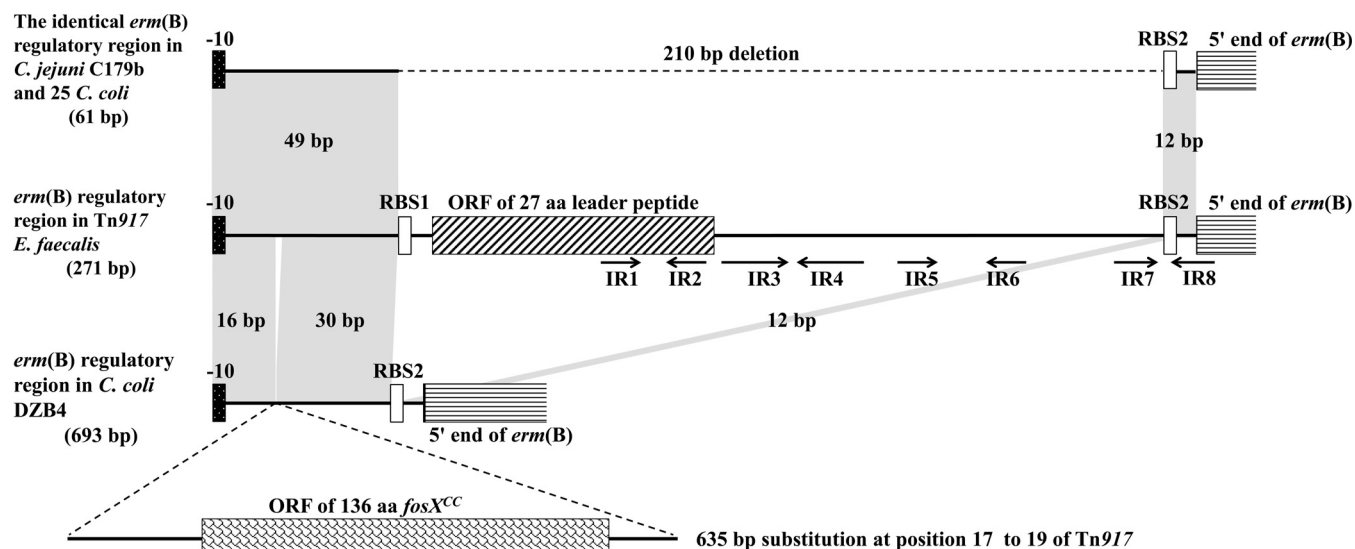


FIG 1 Comparison of the regulatory regions of constitutively expressed *erm(B)* from *C. coli* DZB4, *C. jejuni* C179b, and 25 other *C. coli* strains (shown in Table 1) with the corresponding region of inducibly expressed *erm(B)* of Tn917. RBS1 and RBS2 represent the ribosome binding sites of the leader peptide and *erm(B)*, respectively. The ORFs corresponding to the 27-aa leader peptide, the 5' terminus of *erm(B)*, and the fosfomycin resistance gene *fosX^{CC}* are shown as boxes. The arrows indicate IR1 to IR8, which are responsible for the stem-loop structures of mRNA in Tn917. Regions of 100% homology are marked by gray shading. The 210-bp deletion in C179b and the 635-bp substitution in DZB4 are indicated.

tion, or sequence deletion that might affect the MICs. Thus, the lower MICs could not be explained by alteration of the CDS. It is possible that inducible *erm(B)* is not expressed at a level as high as that of constitutive *erm(B)* and thus confers only an intermediate level of resistance.

In conclusion, constitutive *erm(B)* gene expression in *Campylobacter* was more prevalent, probably because of the use of erythromycin and tylosin for therapeutic purposes and growth promotion in food-producing animals. The data obtained in this study suggest that constitutively expressed *erm(B)* of *Campylobacter* might have evolved from inducibly expressed *erm(B)* (e.g., Tn917) following deletion or recombination in the regulatory region. Moreover, the 210-bp deletion in the constitutively expressed *erm(B)* regulatory region was consistently detected in the chromosomal DNA of 26 epidemiologically distinct *C. coli* and *C. jejuni* strains isolated from humans and food-producing animals, suggesting the presence of a common mechanism for constitutive expression. Additionally, *erm(B)*-positive *Campylobacter* strains that are susceptible to ML antibiotics, such as the inducible *erm(B)*-positive *Campylobacter* strain investigated in this study, pose a hidden risk to public health, as they would not be detected by diagnostic methods that rely only on the resistance phenotype. Furthermore, inducible *erm(B)*-positive *Campylobacter* bacteria in the intestines of humans and food-producing animals treated for extensive periods with the appropriate inducer antibiotics might increase the reservoir of resistant *Campylobacter* bacteria.

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