New genetic environments of the macrolide-lincosamide-streptogramin resistance determinant *erm*(X) and their influence on potential horizontal transferability in bifidobacteria

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ABSTRACT

With the abuse of macrolide, lincosamide, and streptogramin (MLS), the traditionally safe bifidobacterial strains in the human intestine could serve as a reservoir of MLS resistance genes. In this study, the *erm*(X) gene was detected in 29 MLS-resistant strains and one MLS-susceptible strain among 92 bifidobacterial strains of human origin. This study is the first to report *erm*(X)-mediated MLS resistance in *Bifidobacterium pseudocatenulatum*, *Bifidobacterium breve* and *Bifidobacterium bifidum*. The insertion sequences (ISs) flanking antibiotic resistance (AR) genes (i.e., the genetic environment of AR genes) could contribute to the horizontal spreading of AR. However, the potential transferability of *erm*(X) in bifidobacteria has not been previously verified. Here, we retrieved four genetic environments (I–IV) of *erm*(X) from 30 *erm*(X)-positive bifidobacterial strains. This study is the first to identify the *erm*(X) gene in three new genetic environments (II, III and IV) in bifidobacteria. The *erm*(X) gene was individually flanked by IS*1249* or IS*3* in genetic environments I, II and IV and was simultaneously flanked by IS*1249* and IS*3* elements in genetic environment III. Only the transfer of *erm*(X) from genetic environment III simultaneously flanked by IS*1249* and IS*3* elements was successfully observed in filter mating experiments. These findings indicate a synergic effect of IS*1249* and IS*3* elements in the transfer of *erm*(X) in bifidobacteria, and further reveal that the various genetic environments of *erm*(X) result in significant differences in the transferability of *erm*(X) in bifidobacteria.

1. Introduction

Bifidobacteria are Gram-positive, bifid-shaped anaerobes that are common in the human intestine [1–3]. Numerous *Bifidobacterium* species are ingested as probiotics and have acquired a 'generally regarded as safe' (GRAS) status [4,5]. However, excess amounts of macrolide, lincosamide, and streptogramin (MLS) are applied in the treatment of various infections, potentially leading to strong antimicrobial selection pressure and an increased rate of acquiring MLS resistance genes in the human gastrointestinal tract (GIT) [6,7]. The role of bifidobacteria in harboring MLS resistance genes in the intestine has become a matter of concern. *erm*(X)-mediated MLS resistance was recently reported in six *Bifidobacterium thermophilum* strains, one *Bifidobacterium animalis* subsp. *lactis* strain isolated from pig feces and three *Bifidobacterium longum* strains isolated from human feces [8,9]. However, it remains unknown whether MLS resistance genes other than *erm*(X) can be detected in bifidobacteria and whether MLS resistance genes can be detected in bifidobacterial species of human origin other than *B. longum*.

Antibiotic resistance (AR) genes within potentially mobile elements can spread horizontally between species and genera via conjugation mechanisms in open environments, such as the gastrointestinal tract [10,11]. In vitro filter mating experiments have demonstrated the horizontal transferability of the MLS resistance determinant *erm*(B) in *Lactobacillus fermentum* NWL24, *Lactobacillus brevis NWL*59, and *Lactobacillus plantarum* M345 [12–14], and a higher transfer frequency of the *erm*(B) gene from *Lactobacillus plantarum* M345 to *Enterococci faecalis* was detected after the addition of 0.50 μg/mL erythromycin to mating plates [14]. Although the MLS resistance gene *erm*(X) has been reported to be integrated into the bifidobacterial chromosome in one genetic environment as part of the Tn*5432* transposon [8,9], the transferability of this MLS resistance determinant in bifidobacteria with and without selective pressure from erythromycin treatment has not been experimentally verified. In addition, the influence of various sequences flanking AR genes on the horizontal transferability of the MLS resistance determinant from bifidobacteria to other bacteria remains unclear.

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Table 1
MIC susceptibility profiles of erythromycin and clindamycin, and corresponding genotypes for 19 *B. lactis* strains, 2 *B. adolescentis* B . **6**

With the abuse of MLS, the traditional safe bacterial strains in the human intestine, such as bifidobacteria, may serve as a reservoir of MLS resistance genes with the potential to transfer these genes to other pathogens, which is increasingly viewed as a threat to human health. Therefore, this study was performed to assess 92 bifidobacterial strains isolated from the feces of 14 healthy individuals, 1 type strain and 7 commercial strains via phenotypically and genotypically screening the profiles of acquired MLS resistance and to investigate the genetic environments and horizontal transferability of the MLS resistance determinants in different strains.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 100 individual strains of *Bifidobacterium* were investigated in this study: 1 type strain, 7 commercial strains, and 92 strains isolated from the feces of 14 healthy individuals. Among these 92 strains, 54 strains were isolated from the fecal samples of 6 adults between 25 and 35 years old, and 38 of these strains were isolated from the feces of 8 healthy children under 3 years old. The first letter in the names of the 92 strains ('J', 'L', 'F', 'W', 'N', 'Y', 'A', 'Z', 'D', 'X', 'H', 'a', 'B', or 'S') represents the origin among the 14 individuals. The strains included the following species: *Bifidobacterium longum* (n = 45), *Bifidobacterium breve* (n = 18), *Bifidobacterium lactis* (n = 19), *Bifidobacterium pseudocatenulatum* (n = 12), *Bifidobacterium bifidum* (n = 3), *Bifidobacterium adolescentis* (n = 2), and *Bifidobacterium infantis* (n = 1) (Tables 1, 2 and 3).

The strains were grown in de Man Rogosa Sharpe (MRS) medium supplemented with 0.05% (*w/v*) L-cysteine (MRSC) for 12–48 h at 37 °C aerobically (AnaeroGen™, Oxoid Ltd, Basingstoke, UK).

2.2. Antimicrobial susceptibility testing

The susceptibility of 100 strains to erythromycin and clindamycin was determined using Etest strips (bioMérieux, Marcy-l'Étoile, France). Briefly, the strains were grown aerobically in MRSC medium for 24 h at 37 °C. An inoculum was prepared by suspending colonies in MRSC broth to achieve the turbidity of a 1.0 McFarland standard (3×10^8 cells/mL). The cultures were uniformly applied to an agar plate using a sterile cotton swab. After drying for 15 or 20 min, Etest strips were placed on the agar plates and incubated aerobically at 37 °C for 48 h. The minimum inhibitory concentration (MIC) was defined by the intersection of the growth ellipse margin using an Etest strip. The strains were characterized as resistant or sensitive to a specific antibiotic by comparing the MIC values to the breakpoints for *Bifidobacterium* according to the European Food Safety Authority (EFSA) [15].

2.3. DNA isolation and PCR detection of MLS resistance determinants

Total genomic DNA was extracted from the bifidobacteria as previously described [16], with the addition of a cell disruption step using a Fast Prep instrument (Thermo Fisher Scientific, USA) prior to the extraction procedure. Screening for 20 MLS resistance genes was performed in 100 strains. The primers used for amplification of the *erm*(X) gene are shown in Table 4. The primers for the amplification of the other 19 MLS resistance genes, including 7 *erm* methylase genes [*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), *erm*(T), and *erm*(43)], 7 efflux genes [*msr*(A), *msr*(C), *mef*(A), *mef*(B), *mef*(C), *vga*(A), and *vga*(B)], and 5 inactivating enzyme genes [*vgb*(A), *vat*(A), *vat*(C), *vat*(E), and *lnu*(A)] were used as previously described [17–25]. The positive controls for PCR were *Bacteroides thetaiotaomicron* JCM5827 [*erm*(A), *erm*(B), *erm*(C), *erm*(F), and *erm*(G)], *Streptococcus pyogenes* CMCC32067 [*erm*(T)], *Staphylococcus lentus* N3 [*erm*(43)], *Pseudomonas straminea* 9946 [*msr*(A), and *msr*(C)], *Streptococcus pneumonia* CMCC 31968 [*mef*(A), *mef*(B), and *mef*(C)], *Enterococcus faecalis* PCM896 [*vga*(A), *vgb*(A), and *vat*(E)], *Enterococcus faecalis* GvF14 [*vga*(B), and *vat*(C)], and *Streptomyces aureus*13-364 [*vat*(A), and *lnu*(A)]. PCR was performed with TaKaRa Ex Taq DNA polymerase using the component concentrations recommended by the provider (TaKaRa, Dalian, China). All positive amplicons were purified using a PCR Purification Spin Kit (Qiagen, Germany) and subsequently sequenced at the BGI Company (Shanghai, China).

2.4. RNA extraction, cDNA synthesis, and real-time PCR for quantitative expression of the erm(X) gene

Real-time quantitative PCR was performed to measure the expression of the *erm*(X) genes among 15 strong MLS-resistant *B.*

Table 2
MIC susceptibility profiles of erythromycin and clindamycin and corresponding genotypes for 45 *B. longum* strains.

Species	Strain	Origin	MIC (μg/mL)		*erm*(X)	The other 19 MLS resistance genes
			ERY	CLI		
B. longum	Pronova BL88-Onlly[a]	Human	<0.016	<0.016	–	–
	A33	Child feces	<0.016	<0.016	–	–
	A42	Child feces	<0.016	<0.016	–	–
	W11	Adult feces	<0.016	<0.016	–	–
	W12	Adult feces	<0.016	<0.016	–	–
	W14	Adult feces	<0.016	<0.016	–	–
	W210	Adult feces	<0.016	<0.016	–	–
	Z1	Child feces	<0.016	<0.016	–	–
	W22	Adult feces	<0.016	<0.016	–	–
	N34	Adult feces	<0.016	<0.016	–	–
	N45	Adult feces	<0.016	<0.016	–	–
	N51	Adult feces	<0.016	<0.016	–	–
	Y27	Adult feces	<0.016	<0.016	–	–
	Y35	Adult feces	<0.016	<0.016	–	–
	Y33	Adult feces	<0.016	<0.016	–	–
	Z21	Child feces	<0.016	<0.016	–	–
	Z31	Child feces	<0.016	<0.016	–	–
	D41	Child feces	<0.016	<0.016	–	–
	D510	Child feces	<0.016	<0.016	–	–
	D512	Child feces	<0.016	<0.016	–	–
	D514	Child feces	<0.016	<0.016	–	–
	X41	Child feces	<0.016	<0.016	–	–
	X33	Child feces	<0.016	<0.016	–	–
	H1	Child feces	<0.016	<0.016	–	–
	H21	Child feces	<0.016	<0.016	–	–
	H32	Child feces	<0.016	<0.016	–	–
	H34	Child feces	<0.016	<0.016	–	–
	L2	Adult feces	<0.016	<0.016	–	–
	L8	Adult feces	<0.016	<0.016	–	–
	N7	Adult feces	>256	>256	+	–
	W211	Adult feces	>256	>256	+	–
	W21	Adult feces	>256	>256	+	–
	W24	Adult feces	>256	>256	+	–
	W29	Adult feces	>256	>256	+	–
	W212	Adult feces	>256	>256	+	–
	W41	Adult feces	>256	>256	+	–
	a44	Child feces	>256	>256	+	–
	A31	Child feces	>256	>256	+	–
	A44	Child feces	>256	>256	+	–
	A45	Child feces	>256	>256	+	–
	A47	Child feces	>256	>256	+	–
	F21	Adult feces	>256	>256	+	–
	F313	Adult feces	>256	>256	+	–
	F7	Adult feces	>256	>256	+	–
	Y2	Adult feces	6	>256	+	–

ERY, erythromycin; CLI, clindamycin.
[a] Commercial strain obtained from the Shanghai Jiao Da Onlly Co. (Shanghai, PR China).

longum strains and 1 moderate MLS-resistant *B. longum* strain Y2 as well as 6 strong MLS-resistant *B. pseudocatenulatum* strains and 1 MLS-susceptible *B. pseudocatenulatum* strain D52. Briefly, 16 *erm*(X)-positive *Bifidobacterium longum* strains and 7 *B. pseudocatenulatum* strains were grown anaerobically in MRSC at 37 °C for 16 h. Total RNA was extracted using the Easy Pure RNA Kit (TransGen Biotech, Beijing, China) and further analysed for quantity and quality using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was assessed through agarose gel electrophoresis. cDNA was synthesized from each sample (500 ng) using All-in-One First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) and amplified using Forget-Me-Not™ qPCR Master Mix (Biotium, USA) in the Mastercycler ep realplex system (Eppendorf, Hamburg, Germany) as follows: 1 cycle at 95 °C for 2 min and 45 cycles at 95 °C for 5 s, 55 °C for 5 s and 72 °C for 25 s. The primers used for RT-PCR amplification of the *erm*(X) gene are listed in Table 4. The fold-change in *erm*(X) gene expression in each strong MLS-resistant *B. longum* strain vs. the moderate MLS-resistant *B. longum* strain Y2, or each strong MLS-resistant *B. pseudocatenulatum* strain vs. the MLS-susceptible *B. pseudocatenulatum* strain D52 was determined according to the $2^{-\Delta\Delta Ct}$ method [26], using 16S rRNA as the reference gene [27].

2.5. Genome walking

The sequences flanking *erm*(X) in the 30 *erm*(X)-positive bifidobacterial strains were retrieved through nested PCR using a Genome Walking Kit (TaKaRa, Dalian, China). The walking PCR assays were performed in three steps using the AP forward primers supplied in the above kit and a group of three reverse primers (SP1, SP2, and SP3) designed according to the manufacturer's instructions. Six groups of reverse SP primers were designed to amplify the upstream and downstream flanking sequences of *erm*(X), IS*1249,* and IS*3* (Table 4).

The products obtained in the third cycle of nested PCR were purified using a PCR Purification Spin Kit (Qiagen, Germany) and subsequently sequenced at the BGI Company (Shanghai, China) using the specific reverse primer SP3.

Table 3
MIC susceptibility profiles of erythromycin and clindamycin and corresponding genotypes for 12 *B. pseudocatenulatum* strains, 18 *B.breve* strains, and 3 *B. bifidum* strains.

Species	Strain	Origin	MIC (μg/mL)		*erm*(X)	The other 19 MLS resistance genes
			ERY	CLI		
B. pseudocatenulatum	L37	Adult feces	<0.016	<0.016	–	–
	W13	Adult feces	<0.016	<0.016	–	–
	W28	Adult feces	<0.016	<0.016	–	–
	N2	Adult feces	<0.016	<0.016	–	–
	A35	Child feces	<0.016	<0.016	–	–
	D52	Child feces	<0.016	<0.016	+	–
	J56	Adult feces	>256	>256	+	–
	H23	Child feces	>256	>256	+	–
	Z25	Child feces	>256	>256	+	–
	a39	Child feces	>256	>256	+	–
	Y1	Adult feces	>256	>256	+	–
	F312	Adult feces	>256	>256	+	–
B. breve	ATCC 15700[a]	Human	<0.016	<0.016	–	–
	Pronova BB8[b]	Human	<0.016	<0.016	–	–
	BBW	Child feces	<0.016	<0.016	–	–
	BBM	Child feces	<0.016	<0.016	–	–
	BB2	Child feces	<0.016	<0.016	–	–
	BB	Child feces	<0.016	<0.016	–	–
	N1	Adult feces	<0.016	<0.016	–	–
	N24	Adult feces	<0.016	<0.016	–	–
	L211	Adult feces	<0.016	<0.016	–	–
	W46	Adult feces	<0.016	<0.016	–	–
	SQS3-56	Child feces	<0.016	<0.016	–	–
	SQS3-64	Child feces	<0.016	<0.016	–	–
	SQS5-51	Child feces	>256	>256	+	–
	SQS5-52	Child feces	>256	>256	+	–
	A27	Child feces	>256	>256	+	–
	A34	Child feces	>256	>256	+	–
	a313	Child feces	>256	>256	+	–
	a37	Child feces	>256	>256	+	–
B. bifidum	Pronova BB47[b]	Human	<0.016	<0.016	–	–
	Y24	Adult feces	<0.016	<0.016	–	–
	Y21	Adult feces	>256	>256	+	–

ERY, erythromycin; CLI, clindamycin.
[a] Type strain.
[b] Commercial strain obtained from the Shanghai Jiao Da Onlly Co. (Shanghai, PR China).

The types of genetic environments in the 30 *erm*(X)-positive bifidobacterial strains were determined based on overall consideration of the upstream and downstream IS elements flanking the *erm*(X) gene.

2.6. Filter mating experiments

Filter matings were performed to investigate the ability of the 30 *erm*(X)-positive bifidobacterial strains (donors) to transfer the *erm*(X) to *erm*(X)-negative *Enterococcus faecalis* StF-EFM (recipient), which exhibits erythromycin and clindamycin susceptibility (MIC = 0.38 μg/mL), according to Gevers and colleagues [28]. The donor and recipient strains were grown to mid-exponential phase in MRSC medium. Subsequently, 1 mL of donor and 1 mL of recipient culture were mixed. Control cultures of the recipient and donor strains alone consisted of 1 mL of the donor or recipient and 1 mL of phosphate-buffered saline (PBS). The mixture was filtered through a sterile filter (0.45 μm; MF-Millipore membrane filter, HAWP 02500, Millipore) using a filter holder (SX00 02500, Millipore) and a vacuum pump. The filters were incubated anaerobically on non-selective BHI agar (Oxoid) at 37 °C for 24 h and treated with 1 mL PBS, and rigorously shaken to wash the cells from the filters. The cells were collected through centrifugation and resuspended in 1 mL PBS. Ten-fold serial dilutions were plated onto donor-, recipient-, and transconjugant-selective agar plates, and incubated at 37 °C for 48 h. *Enterococcus faecalis* StF-EFM (recipient) can grow aerobically on Pfizer Enterococcus Selective (PSE) agar, whereas *Bifidobacterium* cannot grow on this medium; therefore, the transconjugants were

Table 4
Primers used in the present study.

Name	Sequence (5'-3')	Target	Reference
ermX_F	ATGTTGATTTCAGGTACCGC	*erm*(X)	[8]
ermX_R	AGTCACCTGGAAGAGATCG		
ermX_F[a]	AAATTCTCCGAAGGGCAGGG	176-bp *erm*(X)	This study
ermX_R[a]	GATGGTGCGGTGAAGAGGAA		
ermX_U_SP1	GTTTCTTGTGTGAGTTTGGCAGC	Upstream region of *erm*(X)	This study
ermX_U_SP2	CCGCTTCCTGGTCCTATCTCAAT		
ermX_U_SP3	GATGAACTTGTGGTCGGTGAGAA		
ermX_D_SP1	CACCTCACCACTGCCATTCTTC	Downstream region of *erm*(X)	This study
ermX_D_SP2	GTGGTCCCCATGGTTCACATTTCA		
ermX_D_SP3	GTTGACGGGGGGATCTTAGTGA		
IS1249_U_SP1	GCTAACACCGTTTCGTTTGGCTA	Upstream region of IS*1249*	This study
IS1249_U_SP2	TTTCGACGTTTGACGCTGGAGG		
IS1249_U_SP3	GGTGCCGTTGCGTTTCATTTCA		
IS1249_D_SP1	GTGTCCCAGGTTTTGTTCCGTTTG	Downstream region of IS*1249*	This study
IS1249_D_SP2	ACAGTGGGATTCACACCCTCAA		
IS1249_D_SP3	CGGATTTTCACGGATTGACCTGT		
IS3_U_SP1	ACAGTCCTGGGGCTAAATCGGTCT	Upstream region of IS*3*	This study
IS3_U_SP2	CATCACTGCTGAACTCAACGACCG		
IS3_U_SP3	AGCCTGTGCTGCCAATCGTGTGTT		
IS3_D_SP1	AGTTCAGCAGTGATGCGACGAG	Downstream region of IS*3*	This study
IS3_D_SP2	ATACGAAGACCGATTTAGCCCC		
IS3_D_SP3	GGTCGTCTCTTCCGCAAAATAC		

[a] Primers for the *erm*(X) gene used for real-time quantitative PCR.

detected aerobically on selective PSE agar supplemented with erythromycin (16 μg/mL).

To examine the effects of the erythromycin concentration on the transfer frequency of *erm*(X) from the 30 donor strains to the recipient strain, filter matings were conducted, but were modified by adding three concentrations (0.10, 0.20 and 0.30 μg/mL) of erythromycin to the BHI mating agar plates.

The transfer frequency was expressed as the number of transconjugant colonies per recipient colony formed after the mating period.

2.7. Confirmation of the transconjugants

Presumptive transconjugants were picked randomly from selective agar plates. The susceptibility of the transconjugants to erythromycin and clindamycin was determined. To verify that these isolates were true transconjugants and not mutants, genomic DNA was extracted from the recipient strain *Enterococcus faecalis* StF-EFM and the transconjugants, and PCR assays were performed with universal primers specific for partial 16S rRNA gene sequences (900-bp fragment) [29] and the *erm*(X) gene [8].

2.8. Nucleotide sequence accession numbers

The nucleotide sequences from genetic environments I, II, III, IVa, and IVb were submitted to the GenBank database with the following accession numbers: KP994168-KP994173, KT313484 and KT313485. The partial 16S ribosomal RNA gene sequences of *Enterococcus faecalis* StF-EFM and the transconjugant were deposited in the GenBank database under accession numbers KX673822 and KX673823. The nucleotide sequence of the *erm*(X) gene from the transconjugant was deposited in the GenBank database under accession number KX685688.

2.9. Statistical analysis

The reported values are the means of three repetitions. Student's *t* test was used to compare the mRNA expression levels of *erm*(X) genes, and one-way analysis of variance was used to examine the potential influence of different erythromycin concentrations on transfer frequencies. Statistical significance was defined at $P < 0.05$.

3. Results

3.1. Antimicrobial susceptibility testing

All 19 *Bifidobacterium lactis* strains, 2 *Bifidobacterium adolescentis* strains and 1 *Bifidobacterium infantis* strain were susceptible to erythromycin and clindamycin (Table 1). The 16 *Bifidobacterium longum* strains shown in Table 2 and 6 *Bifidobacterium pseudocatenulatum* strains, 6 *Bifidobacterium breve* strains and 1 *Bifidobacterium bifidum* strain shown in Table 3 exhibited MIC values higher than the EFSA breakpoints for erythromycin and clindamycin. Among the 29 MLS-resistant strains, 28 strains displayed strong resistance (MICs > 256 μg/mL) and 1 *B. longum* strain Y2 displayed moderate erythromycin resistance (MIC = 6 μg/mL).

3.2. Detection of MLS resistance genes

The *erm*(X) was identified in 29 MLS-resistant strains and 1 MLS-susceptible *B. pseudocatenulatum* strain D52 (Table 2 and Table 3). Among the 30 *erm*(X)-positive strains, the *erm*(X) sequences were identical, presenting 99% identity with the 23S rRNA methyltransferase previously identified in *Corynebacterium glucuronolyticum* ATCC 51867 (GenBank accession number EEI28071).

Table 5
Fold-change in *erm*(X) gene expression in 15 strong MLS-resistant *B. longum* strains vs. the moderate MLS-resistant *B. longum* strain Y2 and 6 strong MLS-resistant *B. pseudocatenulatum* strains vs. the MLS-susceptible *B. pseudocatenulatum* strain D52, measured using real-time PCR.

Species	Strain	Fold-change in *erm*(X) gene expression
B. longum	N7	4.53 ± 0.48[a]
	W211	4.68 ± 0.53[a]
	W21	4.32 ± 0.46[a]
	W24	4.06 ± 0.43[a]
	W29	4.48 ± 0.25[a]
	W212	4.54 ± 0.44[a]
	W41	4.16 ± 0.25[a]
	a44	4.89 ± 0.43[a]
	A31	4.05 ± 0.26[a]
	A44	4.68 ± 0.32[a]
	A45	4.82 ± 0.29[a]
	A47	4.36 ± 0.34[a]
	F21	5.05 ± 0.40[a]
	F313	4.34 ± 0.36[a]
	F7	4.95 ± 0.39[a]
B. pseudocatenulatum	J56	8.09 ± 0.34[b]
	H23	8.13 ± 0.24[b]
	Z25	8.15 ± 0.32[b]
	a39	7.57 ± 0.33[b]
	Y1	8.73 ± 0.25[b]
	F312	7.50 ± 0.34[b]

The data are the mean ± standard deviation (SD) from three independent assays and show a significant difference compared with *B. longum* strain Y2 or *B. pseudocatenulatum* strain D52 ($P < 0.05$).

[a] The fold-change in *erm*(X) gene expression was obtained by comparing with the results for the moderate MLS-resistant *B. longum* strain Y2 according to the $2^{-\Delta\Delta Ct}$ method.

[b] The fold-change in *erm*(X) gene expression was obtained by comparing with the results for the MLS-susceptible *B. pseudocatenulatum* strain D52 according to the $2^{-\Delta\Delta Ct}$ method.

However, the other 19 MLS resistance determinants were not present in 100 bifidobacterial strains examined (Tables 1, 2 and 3).

3.3. Transcriptional expression of the erm(X) gene

As shown in Table 5, the expression of the *erm*(X) gene was 4- to 5-fold higher in the 15 strong MLS-resistant *B. longum* strains than that in moderate MLS-resistant *B. longum* strain Y2. The expression of the *erm*(X) gene was 7.5- to 8-fold higher in the 6 strong MLS-resistant *B. pseudocatenulatum* strains than in the MLS-susceptible *B. pseudocatenulatum* strain D52.

3.4. Analysis of the sequences flanking erm(X)

Retrieval of the sequences flanking *erm*(X) through genome walking in the 30 *erm*(X)-positive bifidobacterial strains revealed four different genetic environmental types (I–IV) of the *erm*(X) gene (Table 6 and Fig. 1).

In three strains exhibiting genetic environment I (Fig. 1a), the transposon Tn*5432*, including two direct-repeat IS*1249* elements, flanked the *erm*(X) gene. The duplicated sequences flanking the Tn*5432* insertion consisted of 8 nucleotides (GTCACCTA) in *B. breve* SQS5-51 and 6 nucleotides (TCCAGG) in two *B. longum* strains, W211 and N7. Three open reading frames (ORFs), encoding an adenosine triphosphate (ATP)-binding protein, keratinase, and a putative cell surface protein, were identified upstream of the IS*1249* in *B. breve* SQS5-51, *B. longum* W211, and *B. longum N7*, respectively. An ORF encoding a putative auxin efflux carrier protein was detected downstream of IS*1249* in *B. longum* strains W211 and N7.

In *B. pseudocatenulatum* strain D52, exhibiting genetic environment II (Fig. 1b), a 366-bp sequence encoding a probable voltage-gated channel protein was obtained 206 bp upstream of the *erm*(X)

Table 6
Genetic environmental types and transfer frequency of 30 *erm*(X)-positive bifidobacterial strains.

Strain[a]	

start codon, and one IS*1249* element was retrieved 732 bp downstream of the *erm*(X) gene.

In six strains exhibiting genetic environment III (Fig. 1c), three ORFs (*orf1*, *orf2*, and *orf3*) were obtained 206 bp upstream of the *erm*(X) start codon, and one IS*1249* element was retrieved 732 bp downstream of the *erm*(X) gene. The *orf*1 and the *orf*2 contained an 1196-bp sequence that showed 100% nucleotide identity with the sequence previously identified in *Bifidobacterium longum subsp. longum* BBMN68 (GenBank accession number CP002286.1). The functional domains of the 398-amino acid protein encoded by the 1196-bp sequence were identified as IS*3* elements using the NCBI conserved domain database (CDD) [30] and a searchable database of insertion sequences (ISs) (ISfinder) [31].

Among 20 strains exhibiting genetic environment IV (Fig. 1d), 19 strains exhibited genetic environment IVa, whereas one *B. longum* Y2 exhibited genetic environment IVb, with one nucleotide transition (C-A). Two direct-repeat IS*3* elements (1196-bp) were detected flanking the *erm*(X) gene in the 20 bifidobacterial strains.

3.5. Comparison of the promoter region of erm(X) in 30 erm(X)-positive bifidobacterial strains

In the 30 *erm*(X)-positive bifidobacterial strains shown in Fig. 2, the *ermLP* gene, coding for a short leader peptide (15 amino acids), was obtained 149 bp upstream of the *erm*(X) start codon, and the -10 regions of the *erm*(X) genes all exhibited the same 6-bp nucleotide sequence (TATAAT). The same -35 promoter region sequence found in *erm*(X) (TTGACC) was retrieved in the 28 strong MLS-resistant strains (Fig. 2a, c and d) and occurred within a 27-bp terminal inverted repeat in genetic environment I (Fig. 2a) or within terminal inverted repeats of 10 bp (5'-AGGCTCGCCCC-3') with a single-nucleotide deletion in genetic environments III and IVa (Fig. 2c, d). However, one nucleotide transition (C-A) occurred in the -35 region of *erm*(X) in the moderate MLS-resistant *B. longum* Y2 (Fig. 2e), and two nucleotide transitions (T-C and A-C) occurred in the -35 region of *erm*(X) in MLS-susceptible *B. pseudocatenulatum* D52 (Fig. 2b).

3.6. Mobility of the erm(X) gene

As shown in Table 6, filter matings were successful for six donor strains in which *erm*(X) was identified in genetic environment III (*B. longum* W21, *B. longum* W24, *B. longum* W29, *B. longum* W212, *B. longum* W41, and *B. pseudocatenulatum* J56) at a frequency of approximately 10^{-8} following the addition of 0.00, 0.20, and 0.30 μg/mL erythromycin to the mating plates, or at a frequency of approximately 10^{-7} following the addition of 0.10 μg/mL erythromycin to the mating plates. However, no transconjugants were observed on any of the selective agar plates in the presence of different erythromycin concentrations (frequency of $< 10^{-9}$ Fig. 2

4. Discussion

This study detected the presence of the MLS resistance gene in 29 MLS-resistant strains and one MLS-susceptible strain among a collection of 92 bifidobacterial strains originating from the feces of 14 healthy individuals. The selective pressure of such an intensive use of antibiotics has led sensitive bacteria to acquire specific AR genes to survive [32]. Therefore, the high frequency of the MLS resistance gene in intestinal bifidobacteria indicates serious MLS abuse in the human intestine. Bifidobacteria have a long and safe history of use as probiotics [4,5], and only *erm*(X)-mediated MLS resistance had been reported in *B. thermophilum* and *B. animalis* subsp. *lactis* of pig origin [8] and in *B. longum* of human origin [9]. However, the identification of acquired MLS resistance in four bifidobacterial species of human origin in the present study reflected the presence of the *erm*(X) gene, and this study provides the first report of *erm*(X)-mediated MLS resistance in *B. pseudocatenulatum*, *B. breve*, and *B. bifidum*. Moreover, none of the other 19 MLS resistance genes were identified in any of the bifidobacterial strains examined. These results indicate that MLS resistance in bifidobacteria might be generally mediated by *erm*(X), which is more widely spread in strains and species than previously recognized.

There is a general consensus that -35 promoter elements play a significant role in activating gene expression [33], and a recent report showed that site-directed mutagenesis in the -35 promoter regions of AR genes led to reversion of the MIC value in *Pseudomonas aeruginosa* [34]. In the present study, among the 30 *erm*(X)-positive bifidobacterial strains examined, 28 strains displayed strong MLS resistance and exhibited the same -35 promoter region sequence, whereas one *B. longum* strain Y2 displayed moderate MLS resistance and exhibited one nucleotide transition (C-A) in the -35 promoter region, and one *B. pseudocatenulatum* strain D52 displayed MLS susceptibility with two nucleotide transitions (T-C and A-C) in the -35 promoter region. The present study also showed a significantly lower expression level of the *erm*(X) gene in the moderate MLS-resistant *B. longum* strain Y2 than in the strong MLS-resistant *B. longum* strains, and a lower expression level of the *erm*(X) gene was observed in the MLS-susceptible *B. pseudocatenulatum* strain D52 than in the strong MLS-resistant *B. pseudocatenulatum* strains. These results imply that the expression of the *erm*(X) might be affected by variations in the -35 promoter region, further establishing a connection between the genotype and phenotype of MLS resistance in bifidobacteria.

The sequences flanking acquired AR genes (i.e., the genetic environments of AR genes) could contribute to horizontal AR spreading in the intestine [6,10,11]. Furthermore, the transfer of AR genes to opportunistic pathogens, such as Enterococci, which cause serious diseases and nosocomial infections in humans due to the acquisition of AR determinants, has recently been highlighted [35,36]. However, until recently, no reports had verified the potential horizontal transferability of *erm*(X) in bifidobacteria. The present study retrieved four genetic environments (I–IV) of *erm*(X) in bifidobacteria and is the first to identify the *erm*(X) gene in three new genetic environments (II, III and IV) in bifidobacteria. No transfer of *erm*(X) was observed in strains exhibiting genetic environments I, II, and IV; however, the transfer of *erm*(X) in strains exhibiting genetic environment III to *E. faecalis* strain StF-EFM was observed in filter mating experiments and was further enhanced by adding 0.10 μg/mL erythromycin. These results indicate that the various genetic environments of *erm*(X) are associated with significant differences in the horizontal transferability of *erm*(X) in bifidobacteria, and an appropriate concentration of erythromycin could increase the transfer efficiency of *erm*(X) in bifidobacteria.

Insertion sequences (ISs) are mobile elements that are commonly distributed in bacterial genomes [37]. It is widely recognized that IS-carried transposase genes can enhance the transfer of neighboring antibiotic resistance genes across species and genera [38]. In bifidobacteria, only IS*1249* elements were previously demonstrated to flank the *erm*(X) gene. However, among 30 *erm*(X)-positive bifidobacterial strains examined in the present study, the *erm*(X) gene was individually flanked by IS*1249* in 4 strains (genetic environments I and II) and by IS*3* in 20 strains (genetic environment IV), whereas it was simultaneously flanked by IS*1249* and IS*3* elements in 6 strains (genetic environment III). Furthermore, it was found that the *erm*(X) gene flanked by IS*1249* or IS*3* alone (genetic environments I, II, and IV) could not be transferred through filter mating experiments; however, the transfer of *erm*(X) was successfully observed when the gene was simultaneously flanked by IS*1249* and IS*3* elements (genetic environment III). These findings reveal a synergic effect of the IS*1249* and IS*3* elements in the transfer of *erm*(X) in bifidobacteria.

5. Conclusions

In conclusion, this study identified the *erm*(X) gene in 29 MLS-resistant strains and one MLS-susceptible strain among 92 bifidobacterial strains of human origin and provides the first report of *erm*(X)-mediated MLS resistance in *B. pseudocatenulatum*, *B. breve*, and *B. bifidum*. Four genetic environments (I–IV) of *erm*(X) were retrieved in the 30 *erm*(X)-positive bifidobacterial strains, and this study is the first to identify the *erm*

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