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International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

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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ARTICLE INFO

Article history:

Received 26 August 2016

Accepted 30 April 2017

Keywords:

Bifidobacteria

MLS resistance

erm(X)

Genetic environment

Horizontal transfer

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 IS1249 or IS3 in genetic environments I, II and IV and was simultaneously flanked by IS1249 and IS3 elements in genetic environment III. Only the transfer of *erm(X)* from genetic environment III simultaneously flanked by IS1249 and IS3 elements was successfully observed in filter mating experiments. These findings indicate a synergic effect of IS1249 and IS3 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.

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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* NWL59, 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 Tn5432 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 1MIC susceptibility profiles of erythromycin and clindamycin, and corresponding genotypes for 19 *B. lactis* strains, 2 *B. adolescentis* strains, and 1 *B. infantis* strain.

Species	Strain	Origin	MIC ($\mu\text{g/mL}$)		<i>erm</i> (X)	The other 19 MLS resistance genes
			ERY	CLI		
<i>B. lactis</i>	Pronova BL99 ^a	Human	<0.016	<0.016	–	–
	Pronova BL25 ^a	Human	<0.016	<0.016	–	–
	Pronova BI516 ^a	Human	<0.016	<0.016	–	–
	J310	Adult feces	<0.016	<0.016	–	–
	J311	Adult feces	<0.016	<0.016	–	–
	J316	Adult feces	<0.016	<0.016	–	–
	J317	Adult feces	<0.016	<0.016	–	–
	L35	Adult feces	<0.016	<0.016	–	–
	L36	Adult feces	<0.016	<0.016	–	–
	L38	Adult feces	<0.016	<0.016	–	–
	L310	Adult feces	<0.016	<0.016	–	–
	L311	Adult feces	<0.016	<0.016	–	–
	L312	Adult feces	<0.016	<0.016	–	–
	F5	Adult feces	<0.016	<0.016	–	–
	F9	Adult feces	<0.016	<0.016	–	–
	F10	Adult feces	<0.016	<0.016	–	–
	F11	Adult feces	<0.016	<0.016	–	–
	F12	Adult feces	<0.016	<0.016	–	–
	F18	Adult feces	<0.016	<0.016	–	–
	<i>B. adolescentis</i>	W25	Adult feces	<0.016	<0.016	–
W42		Adult feces	<0.016	<0.016	–	–
<i>B. infantis</i>	Pronova BI211 ^a	Human	<0.016	<0.016	–	–

ERY, erythromycin; CLI, clindamycin.

^a Commercial strain obtained from the Shanghai Jiao Da Onlly Co. (Shanghai, PR China).

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 pneumoniae* 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 ($\mu\text{g/mL}$)		<i>erm(X)</i>	The other 19 MLS resistance genes
			ERY	CLI		
<i>B. longum</i>	Pronova BL88-Only ^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 $2^{-\Delta\Delta\text{Ct}}$ 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)*, IS1249, and IS3 (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 3MIC susceptibility profiles of erythromycin and clindamycin and corresponding genotypes for 12 *B. pseudocatenuatum* 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			
<i>B. pseudocatenuatum</i>	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	+	–	
	<i>B. breve</i>	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	+	–	
<i>B. bifidum</i>	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	ATGTGATTTTCAGGTACCGC	<i>erm(X)</i>	[8]
ermX_R	AGTCACCTGGAAGAGATCG		
ermX_F ^a	AAATTCTCCGAAGGGCAGGG	176-bp <i>erm(X)</i>	This study
ermX_R ^a	GATGGTGCCTGAAGAGGAA		
ermX_U_SP1	GTTTCTGTGTGAGTTGGCAGC	Upstream region of <i>erm(X)</i>	This study
ermX_U_SP2	CCGCTTCTGGTCTATCTCAAT		
ermX_U_SP3	GATGAACTTGTGGTCGGTGAGAA	Downstream region of <i>erm(X)</i>	This study
ermX_D_SP1	CACCTCACCACCTGCCATCTTC		
ermX_D_SP2	GTGGTCCCATGTTTACATTTCA		
ermX_D_SP3	GTTGACGGGGGATCTTAGTGA	Upstream region of IS1249	This study
IS1249_U_SP1	GCTAACACCCGTTTCGTTGGCTA		
IS1249_U_SP2	TTTCGACGTTTTCAGCTGGAGG		
IS1249_U_SP3	GGTCCGTTGCGTTTCAITTTCA	Downstream region of IS1249	This study
IS1249_D_SP1	GTGTCCAGGTTTTGTTCCTTTG		
IS1249_D_SP2	ACAGTGGGATTACACCCCTCAA		
IS1249_D_SP3	CGGATTTTACCGGATTGACCTGT	Upstream region of IS3	This study
IS3_U_SP1	ACAGTCTGGGGTAAATCGGTCT		
IS3_U_SP2	CATCACTGCTGAACCTAACGACCG		
IS3_U_SP3	AGCCTGTGCTGCCAATCGTGTGT	Downstream region of IS3	This study
IS3_D_SP1	AGTTCAGCAGTGATGCGCAGG		
IS3_D_SP2	ATACGAAGACCGATTAGCCCC		
IS3_D_SP3	GGTCGTCTTCCGAAAATAC		

^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 <i>erm(X)</i> gene expression
<i>B. longum</i>	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
<i>B. pseudocatenulatum</i>	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 Tn5432, including two direct-repeat IS1249 elements, flanked the *erm(X)* gene. The duplicated sequences flanking the Tn5432 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 IS1249 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 IS1249 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	Genetic environmental type of <i>erm(X)</i> gene ^b	Transfer frequency ^c under different concentrations of erythromycin (µg/mL)			
		0.00	0.10	0.20	0.30
N7	I	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
W211	I	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
SQS5-51	I	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
D52	II	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
W21	III	3.76 × 10 ⁻⁸	1.15 × 10 ⁻⁷	3.87 × 10 ⁻⁸	3.84 × 10 ⁻⁸
W24	III	5.11 × 10 ⁻⁸	1.12 × 10 ⁻⁷	5.33 × 10 ⁻⁸	5.54 × 10 ⁻⁸
W29	III	9.22 × 10 ⁻⁸	1.65 × 10 ⁻⁷	9.16 × 10 ⁻⁸	9.07 × 10 ⁻⁸
W212	III	6.39 × 10 ⁻⁸	1.53 × 10 ⁻⁷	6.98 × 10 ⁻⁸	7.75 × 10 ⁻⁸
W41	III	5.13 × 10 ⁻⁸	1.52 × 10 ⁻⁷	5.09 × 10 ⁻⁸	5.26 × 10 ⁻⁸
J56	III	5.10 × 10 ⁻⁸	1.23 × 10 ⁻⁷	5.20 × 10 ⁻⁸	4.99 × 10 ⁻⁸
a44	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
A31	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
A44	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
A45	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
A47	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
F21	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
F313	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
F7	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
H23	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
Z25	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
a39	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
Y1	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
F312	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
SQS5-52	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
A27	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
A34	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
a313	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
a37	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
Y21	IVa	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹
Y2	IVb	<1.07 × 10 ⁻⁹	<1.10 × 10 ⁻⁹	<1.40 × 10 ⁻⁹	<1.93 × 10 ⁻⁹

^a Strains that could transfer *erm(X)* to *E. faecalis* StF-EFM are highlighted in gray.
^b Different genetic environments represented in Fig. 1.
^c The transfer frequency is expressed as the number of transconjugant colonies per recipient colony formed after the mating period.

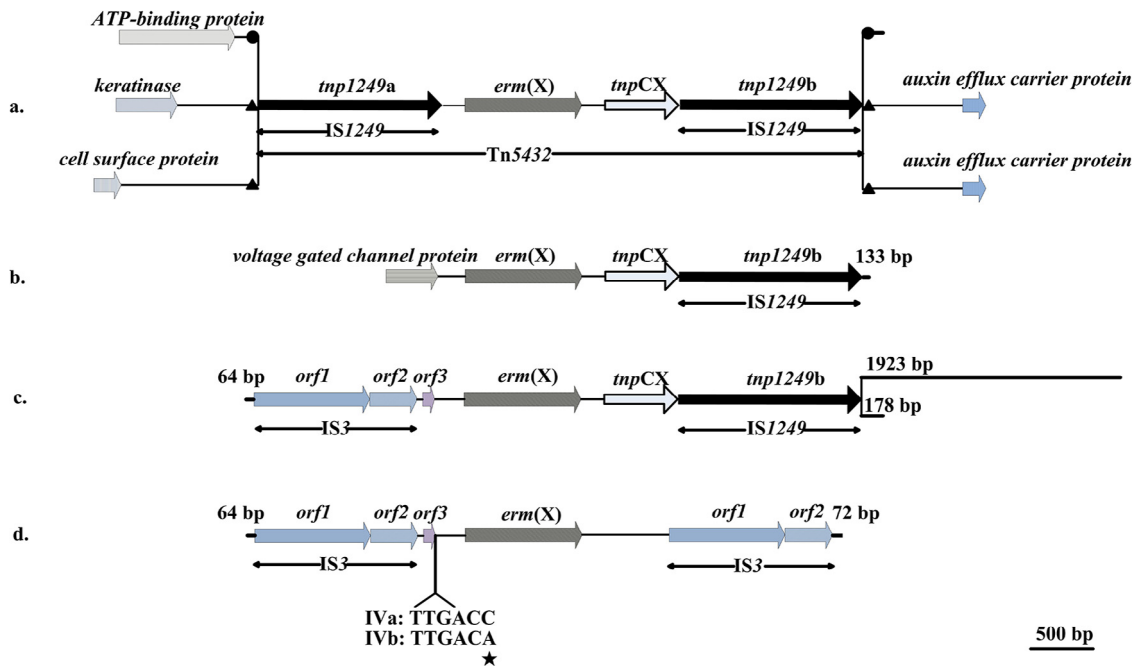


Fig. 1. Schematic map of four genetic environments (I–IV) of *erm(X)* in 30 bifidobacterial strains. The organization of the upstream and downstream sequences flanking *erm(X)* is shown. (a) Environment I. (b) Environment II. (c) Environment III. (d) Environment IV. The inserted sequences of Tn5432 in environment I are indicated as ‘●’ and ‘▲’. ‘●’ represents the 8 nucleotides (GTCACCTA) found in *B. breve* SQS5-51, and ‘▲’ represents the 6 nucleotides (TCCAGG) found in *B. longum* strains W211 and N7. A one-nucleotide transition between environments IVa and IVb is indicated with ‘★’.

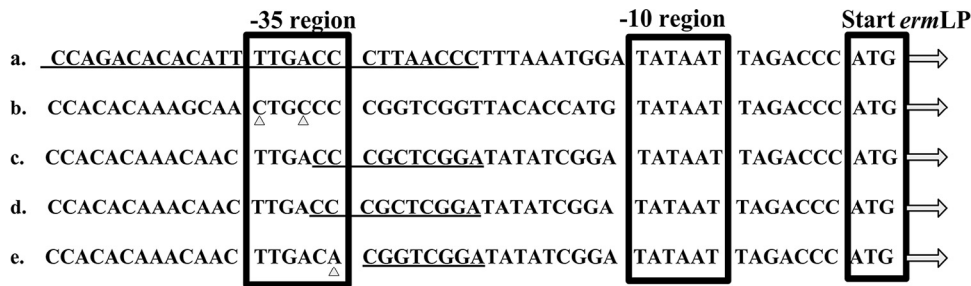


Fig. 2. Comparison of the promoter regions of *erm(X)* in 30 *erm(X)*-positive bifidobacterial strains. (a) Three strains exhibiting environment I. (b) *B. pseudocatenulatum* D52 exhibiting environment II. (c) Six strains exhibiting environment III. (d) Nineteen strains exhibiting environment IVa. (e) *B. longum* strain Y2 exhibiting environment IVb. The nucleotide sequences upstream of the putative leader peptide gene *ermLP* are shown. Arrows indicate the direction of *ermLP* transcription. The *ermLP* start codon and the probable *erm(X)* promoter elements (-10 region and -35 region) are boxed. Nucleotide sequences of terminal inverted repeats provided by IS1249 or other genes are underlined. 'Δ' represents the nucleotide transition in the -35 promoter region of the *erm(X)* gene.

start codon, and one IS1249 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 IS1249 element was retrieved 732 bp downstream of the *erm(X)* gene. The *orf1* and the *orf2* 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 IS3 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 IS3 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 $\mu\text{g}/\text{mL}$ erythromycin to the mating plates, or at a frequency of approximately 10^{-7} following the addition of 0.10 $\mu\text{g}/\text{mL}$ 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 $< 10^{-9}$ per recipient cell) in matings between the other 24 strains exhibiting the other three genetic environments (I, II and IV) and *Enterococcus faecalis* StF-EFM.

3.7. Effect of erythromycin on the transfer frequency

The presence of erythromycin resulted in significantly higher ($P < 0.05$) transfer frequencies of *erm(X)* from six donor strains exhibiting genetic environment III to *Enterococcus faecalis* StF-EFM on plates containing 0.10 $\mu\text{g}/\text{mL}$ erythromycin, but not on plates with higher erythromycin concentrations (0.20 and 0.30 $\mu\text{g}/\text{mL}$) (Fig. 3).

3.8. Verification of transconjugants

All transconjugants displayed strong MLS resistance with an MIC $> 256 \mu\text{g}/\text{mL}$. Total genomic DNA was extracted from the transconjugants and analysed via PCR. The amplified 16S rRNA sequences confirmed that all the transconjugants were derived from the recipient strain, *E. faecalis* StF-EFM. Furthermore, the *erm(X)* gene was not observed in the recipient strain, whereas all the transconjugants were confirmed to harbor the *erm(X)* gene, showing 100% identity to the sequences in the donor strains.

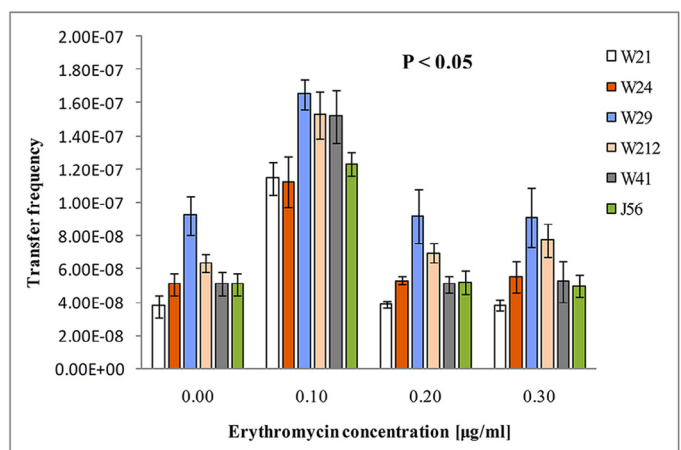


Fig. 3. Effect of different erythromycin concentrations on the transfer frequency in filter mating between six bifidobacterial strains (W21, W24, W29, W212, W41 and J56) exhibiting genetic environment III and *Enterococcus faecalis* StF-EFM. Each bar represents the average value for three independent experiments. Error bars represent standard deviations.

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 IS1249 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 IS1249 in 4 strains (genetic environments I and II) and by IS3 in 20 strains (genetic environment IV), whereas it was simultaneously flanked by IS1249 and IS3 elements in 6 strains (genetic environment III). Furthermore, it was found that the *erm(X)* gene flanked by IS1249 or IS3 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 IS1249 and IS3 elements (genetic environment III). These findings reveal a synergic effect of the IS1249 and IS3 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(X)* gene in three new genetic environments (II, III and IV) in bifidobacteria. Successful transfer was observed only for *erm(X)* exhibiting environment III in bifidobacteria in our filter mating experiments, which indicates that the various genetic environments of *erm(X)* result in significant differences in the transferability of *erm(X)* in bifidobacteria.

Funding: This work was supported by Shanghai Industry-University Joint Research Program [grant number HU CXY-2016-010].

Competing interests: None declared.

Ethical approval: Not required.

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