NOTE

Genetic mechanisms of variation in erythromycin resistance in *Lactobacillus rhamnosus* strains

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Lactobacillus rhamnosus Oxy and Pen are probiotic strains capable of colonizing the human and animal gastrointestinal tract, where they are considered to exert an array of beneficial effects.¹ A growing interest has recently been observed in probiotics for the treatment of antibiotic-associated diarrhea (AAD), which is a common complication of antibiotic use. Ruszczyński *et al.*² have shown the efficiency of *Lb. rhamnosus* Oxy and Pen administration in the prevention of AAD in children receiving antibiotic treatment. To allow the use of probiotic strains along with antibiotic therapy, these strains should be intrinsically resistant to a broad group of antibiotics. Intrinsically antibiotic-resistant probiotic strains may benefit patients whose normal intestinal microbiota have become unbalanced or greatly reduced in numbers because of the administration of various antimicrobial agents.³ There have been only a few reports on the antibiotic resistance of *Lb. rhamnosus* strains isolated from food and those of human origin.^{4–6}

Macrolides have a broad spectrum of antimicrobial activity. Antibiotics from this family target the 23S rRNA nucleotide within the 50S ribosomal subunit tunnel, thereby blocking peptide chain elongation.⁷ Bacterial resistance to macrolides, lincosamides and streptogramins (MLS phenotype) is often due to efflux systems, methylases or inactivating enzymes, for which more than 40 genes have been reported.⁸ It can also be caused by mutations in genes encoding ribosomal proteins and in the 23S rRNA gene. These chromosomal mutations alter the erythromycin binding site in the 23S rRNA molecule.⁹ MLS resistance in *Lactobacillus* is frequently associated with the presence of *erm* genes.^{10,11} The genes *erm*(A), *erm*(B), *erm*(C) and *erm*(F) are widely distributed among Gram-positive and Gram-negative bacteria.¹² These genes have been found in plasmids and on the chromosome of *Lactobacilli* spp.^{13,14}

The objective of the present study was to determine the spectrum of antibiotic resistance of *Lb. rhamnosus* strains Oxy and Pen, and to elucidate the genetic mechanisms underlying their resistance to macrolides.

Strains of *Lb. rhamnosus* Pen and Oxy were obtained from Biomed Serum and Vaccine Production Plant Ltd in Lublin, Poland.

They were deposited at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, with the numbers 2593 (*Lb. rhamnosus* Pen) and 2595 (*Lb. rhamnosus* Oxy). Bacteria were stored at -80°C in MRS medium with the addition of 20% (vol/vol) glycerol. The strain was revitalized in MRS broth at 37°C for 24 h. After two passages, the bacterial culture was used to prepare a 24-h inoculum in MRS (BTL) medium under the same conditions.

Susceptibilities to 15 antibiotics were determined by the broth microdilution method using the LAB susceptibility test medium.¹⁵ Minimum inhibitory concentrations (MICs) were determined. Cefdinir, cefotaxime, cefuroxime, cefepime, cloxacillin, ampicillin, penicillin, carbenicillin, chloramphenicol, meropenem, streptomycin, nalidixic acid, erythromycin, vancomycin and doxycycline were tested in the concentration range of $0.125\text{--}512\text{ }\mu\text{g ml}^{-1}$. The growth rate of the tested *Lb. rhamnosus* strains in the medium with erythromycin (final concentration of $2\text{--}512\text{ }\mu\text{g ml}^{-1}$) was monitored by measuring the OD (OD_{600}) using Bioscreen C (LabSystem, Helsinki, Finland). All experiments were carried out on honeycomb plates for 72 h at 37°C .

Total bacterial DNA was extracted according to the method described by Varmanen.¹⁶ The presence of *erm*(A), *erm*(B), *erm*(C) and *erm*(F) genes was analyzed by specific polymerase chain reaction (PCR), using purified chromosomal DNA as a template. The primers and reaction conditions were used as reported by Jensen *et al.*,¹² Hunt *et al.*¹⁷ and Flórez *et al.*^{4,14}

Amplicons of 23S rRNA (1200 bp) obtained from the tested strains were purified using the DNA Extraction Kit (Fermentas, St Leon-Rot, Germany) and subjected to restriction fragment length polymorphism (RFLP) analysis. The amplicons were digested with *Bbs*I and *Hind*III, which anticipate the mutation.

The nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and the capillary sequencing system, 3730 Genetic Analyzer (Applied Biosystems, USA). The *erm*(A), *erm*(C), and 23S rRNA gene sequences obtained in this study, as well as those from the databases, were analyzed by Clustal W and BLAST programs.

The nucleotide sequences of 23S rRNA determined in this work were deposited in the GenBank Database with the accession numbers JQ952565 and JQ952566, respectively.

For *Lb. rhamnosus* Oxy and Pen, the MIC of each of cefdinir, cefotaxime, cefuroxime, cloxacillin, penicillin, chloramphenicol, meropenem, nalidixic acid, vancomycin and doxycycline was $>512 \mu\text{g ml}^{-1}$. The MICs of cefepim were $256 \mu\text{g ml}^{-1}$ for both strains. The MICs of other antibiotics differed between the tested strains. The MIC of streptomycin was $64 \mu\text{g ml}^{-1}$ for *Lb. rhamnosus* Oxy and $512 \mu\text{g ml}^{-1}$ for *Lb. rhamnosus* Pen. The respective MICs of carbenicillin and ampicillin were $32 \mu\text{g ml}^{-1}$ and $16 \mu\text{g ml}^{-1}$ for *Lb. rhamnosus* Oxy and $>512 \mu\text{g ml}^{-1}$ for *Lb. rhamnosus* Pen. The greatest difference was observed for erythromycin: $2 \mu\text{g ml}^{-1}$ for *Lb. rhamnosus* Oxy and $>512 \mu\text{g ml}^{-1}$ for *Lb. rhamnosus* Pen.

In microcultures, the growth rate of *Lb. rhamnosus* Oxy showed that erythromycin had a bacteriostatic effect. It was observed that $2 \mu\text{g ml}^{-1}$ of the antibiotic inhibited the growth of the tested strains for up to 20 h (Figure 1). After this time, the culture entered the logarithmic growth phase, and was then the same as control culture in the stationary phase. With an increase in erythromycin concentration, the growth inhibition period became longer and reached 30 h at the concentration of $512 \mu\text{g ml}^{-1}$. *Lb. rhamnosus* Pen in microcultures showed the same growth rate in the medium with various erythromycin concentrations as in the control medium (Figure 2).

The results of PCR amplification for the selected *erm* genes in the tested strains showed that *Lb. rhamnosus* Pen contained *erm*(A), *erm*(B) and *erm*(C) genes, whereas *Lb. rhamnosus* Oxy contained only *erm*(A) and *erm*(C) genes (Figure 3). RFLP analysis revealed that after digestion of 23S rRNA amplicons with *Bbs*I, fragments of expected size (1100 and 900 bp) were observed only in the erythromycin-resistant strain *Lb. rhamnosus* Pen (Figure 4). Amplicons from both strains were also digested with *Hind*III, giving rise to two identical fragments of 620 and 500 bp for only *Lb. rhamnosus* Pen.

Analysis of 23S rRNA gene sequences showed a high similarity (97%) between the tested strains. Similarity was also observed for the

erm(A) and *erm*(C) gene sequences (85% and 63%, respectively). Table 1 shows the prevalence of *erm*(A) and *erm*(C) genes among bacterial species, and the similarity level was calculated using the ClustalW2 program.

Lb. rhamnosus Oxy and Pen colonize the intestinal mucosa and normalize gastrointestinal microflora, especially after the use of antibiotics. In a recent study, these strains have been characterized as having no plasmids, and the size of their chromosomal DNA has been estimated to be about 3 Mbp (results not shown). Some authors have observed a high resistance to macrolides in *Lb. rhamnosus* strains of human origin.⁴ In the present study, both of the investigated strains proved to be entirely resistant to most of

Figure 2 Growth curves of *Lb. rhamnosus* Pen in the presence of different concentrations of erythromycin.

Figure 1 Growth curves of *Lb. rhamnosus* Oxy in the presence of different concentrations of erythromycin.

Figure 3 Electrophoretic separation of PCR products obtained by using primer pairs ErmBf and ErmBr (tracks: 1—Oxy strain, 2—Pen strain, M—marker size).

Figure 4 Electrophoretic separation of 23S rRNA amplification product digested by the restriction enzyme *Bbs*I: M—marker; strain Oxy: 1—23S rRNA amplification product without enzyme digestion, 2—after enzymatic digestion.

Table 1 Similarity between *erm*(A) and *erm*(C) gene sequences from *Lb. rhamnosus* Oxy and Pen and *erm*(A) and *erm*(C) gene sequences from GeneBank databases

Gene	Organism	Similarity (%)	Gene Bank No.
<i>erm</i> (A) <i>Lb. rhamnosus</i> Oxy	<i>Lb. rhamnosus</i> Pen	85	
	<i>Corynebacterium diptheriae</i>	81	X51472
	<i>Haemophilus influenzae</i>	81	L42023
	<i>Arthrobacter</i> spp.	73	M11276
<i>erm</i> (A) <i>Lb. rhamnosus</i> Pen	<i>Lb. rhamnosus</i> Oxy	85	
	<i>C. diptheriae</i>	84	X51472
	<i>H. influenzae</i>	81	L42023
	<i>Arthrobacter</i> spp.	81	M11276
<i>erm</i> (C) <i>Lb. rhamnosus</i> Oxy	<i>Staphylococcus chromogenes</i>	86	U82607
	<i>S. saprophiticus</i>	76	Q2YHL5
	<i>S. hyicus</i>	76	Y09003
	<i>Lb. reuterii</i>	76	Q7BV37
	<i>S. epidermidis</i>	75	M12730
	<i>S. aureus</i>	74	M19652
	<i>S. simulans</i>	74	AF019140
	<i>S. equorum</i>	74	X82668
	<i>S. hominis</i>	74	Y09001
	<i>Lb. rhamnosus</i> Pen	63	
<i>erm</i> (C) <i>Lb. rhamnosus</i> Pen	<i>S. chromogenes</i>	84	U82607
	<i>S. epidermidis</i>	65	M12730
	<i>Lb. reuterii</i>	63	Q7BV37
	<i>S. aureus</i>	63	M19652
	<i>S. luteus</i>	63	U5DU97
	<i>S. subtilis</i>	63	M13761
	<i>S. haemolyticus</i>	63	Y09002
	<i>S. simulans</i>	63	AF019140
	<i>S. equorum</i>	63	X82668
	<i>S. hominis</i>	74	Y09001
	<i>Lb. rhamnosus</i> Oxy	63	

the tested antibiotics, but a large difference was revealed in their sensitivity to erythromycin. Genes *erm* (A, B, C and F) confer macrolide resistance in *Lactobacilli*.¹⁸ Our study showed that the highly resistant *Lb. rhamnosus* Pen had three *erm* genes (A, B and C), whereas the sensitive *Lb. rhamnosus* Oxy had only two *erm* genes, *erm*(A) and *erm*(C). Catalouk and Gogebakan¹⁹ have reported that among the 64 strains of *Lb. rhamnosus* they tested, only 43 strains possessed the *erm*(B) gene. For *Lb. rhamnosus* Pen, after amplification, we obtained a product of 748 bp, which was similar to the 753 and 738 bp reported for *Lb. fermentum* and *Enterococcus faecalis*, respectively.¹² Moreover, in this study, a mutation in the 23S rRNA gene was observed in the erythromycin-resistant *Lb. rhamnosus* Pen. Genetic analysis showed that the difference in erythromycin resistance between the tested strains was due to the presence of *erm* genes coding for protein methyltransferases, which catalyze either monomethylation (type I) or dimethylation (type II) reactions at the exocyclic N⁶ position of a specific adenine residue in 23S rRNA.^{9,20} In addition, erythromycin resistance observed in *Lb. rhamnosus* Pen strain was caused by target-site modification: a mutation in domain II of 23S rRNA in the most frequent antibiotic binding site.²⁰ Our results suggest that the presence of the *erm*(A) and *erm*(C) genes is sufficient to remove the bacteriostatic effect of erythromycin on *Lb. rhamnosus* Oxy. Other mechanisms of macrolide resistance in *Lactobacilli* are related to the presence of *msr* genes, which are responsible for active removal of erythromycin from the cell (efflux pump) and *ere* genes encoding the enzyme erythromycin esterase, which inactivates the antibiotic by hydrolyzing its lactone ring.⁸

The sequence analysis of 23S rRNA genes from *Lb. rhamnosus* Oxy and Pen revealed a 97% similarity. This region is highly conservative, and is often used for taxonomic identification and phylogenetic studies.⁹ The analysis of *erm*(A) and *erm*(C) showed similarities of 85% and 63%, respectively. The highest similarity of the *erm*(C) sequence was observed for *Staphylococcus* spp. This observation is confirmed by a phylogenetic study by Kim *et al.*,²⁰ in which it was found that horizontal *erm* gene transfer occurs not only within closely related genera and species, but also among phylogenetically remote bacteria. The base composition of *erm* genes provides some information on the acquisition of resistance genes from different organisms. The lower G + C content in *erm* genes than in the chromosomal DNA found in pathogens implies horizontal gene transfer. A comparable G + C content in both *erm* and chromosomal DNA implies the presence of intrinsic *erm* genes.²⁰ In our study, the frequency of G and C nucleotides in the *erm*(A) and *erm*(C) genes of both examined strains was between 40 and 47%. Kankainen *et al.*²¹ reported a G + C content of 47% for several strains of *Lb. rhamnosus*. Thus, it is probable that *Lb. rhamnosus* Oxy and Pen have intrinsic *erm* genes in the chromosomal DNA. The study by Kankainen *et al.*²¹ revealed a high similarity of general genomic features in *Lb. rhamnosus* strains.

In the present study, a difference in erythromycin resistance was detected between two *Lb. rhamnosus* strains. The highly resistant strain (Pen) was determined to carry *erm* genes (A, B and C) and to have a mutation in the 23S rRNA gene, whereas the sensitive strain (Oxy) had neither the *erm*(B) gene nor the mutation. Antibiotic resistance of LAB could be regarded as a beneficial property. In our strains, potentially nontransferable resistance genes were identified. Both phenotypic tests and molecular tools are needed to scientifically guarantee the presence of acquired resistance genes in probiotic bacteria, as pointed out in this article. In our opinion, such studies should primarily focus on *erm*-positive *Lactobacilli* because of the clinical importance of macrolides.

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