Mutation in 23S rRNA is associated with erythromycin resistance of human vaginal *Lactobacillus rhamnosus*

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INTRODUCTION

Due to the over-use of antibiotics, an increased interest? focus has been given to studies of the emergence and spread of the resistance to antimicrobials in bacteria. Albeit the studies of molecular mechanisms of the antibiotic resistance have been focused primarily on clinically relevant species (Farell et al, 2003), the interest for the antibiotic resistance in lactic acid bacteria (LAB) has been intensively expanded (Danielsen and Wind, 2003). The presence of antibiotic-resistant bacteria used in human consumption as well as bacteria inhabiting human organism has posed a potential threat to human health (Flórez *et al*, 2005; Levy, 2002). Among other microbes in human vagina, lactobacilli - including *Lactobacillus rhamnosus*, are believed to play an important role in microbial defense against vaginal colonization by exogenous pathogenic microorganisms (Redondo-López, 1990). Nevertheless, in some cases commensal lactobacilli can also represent a health problem (Cannon *et al*., 2005). Recently, many investigators propose that commensal bacteria which may act as reservoirs of antibiotic resistance genes (Levy and Salayers, 2002), present a serious threat due to the ability to further transfer resistance to other bacteria including pathogen ones (Blake *et al*., 2003). The major cause of macrolide resistance in *Helicobacter pylori* is the inability of the macrolides to bind to the components of the bacterial ribosome i.e., peptydiltransferase region of domain V of the 23srRNA (Weisblum, B, 1995). A chromosomal mutations identified as A-G transitions at two positions 2058 and 2059, that alters the erythromycin binding site in 23 S rRNA, have been shown to confer macrolide resistance in a number of clinical isolates including *H. pylori and Streptococcus pneumoniae* (Versalovic *et al*., 1996; Farell *et al.,* 2003).

The aim of this study was to obtain an understanding of the nature and molecular basis of erythromycin resistance of human vaginal *L. rhamnosus* strains, for which the commonest genes driving resistance to macrolides in Gram-positive organisms were not encountered

MATERIALS AND METHODS

Bacterial isolates and identification by 16S rDNA sequencing. Six *Lactobacillus* sp. strains (Table 1) were isolated from vaginal swab specimens of six healthy women collected during routine gynecological examination and identified at species level as *L. rhamnosus* based on the repetitive DNA element PCR using the $(GTG)_5$ primer as previously described (Begovic *et al*., 2007). According to the antibiotic susceptibility testing the isolates were considered resistant to erythromycin (Begovic *et al.*, 2007). In this study, additional identification of strains was obtained by sequencing of 16S rRNA gene segment of human vaginal lactobacilli obtained in PCR reactions with universal primers Y1 and Y2 (Young *et al.*, 1991). Y2 primer served as a sequencing primer. The sequences were analysed with BLAST (www.ncbi.nih.gov). List of straine is given in table 1. Bacteria were grown in MRS broth medium (Merck GmbH, Darmstadt, Germany) at 37°C (*L. casei* at 30°C) under aerobic conditions.

PCR protocol. Total genomic DNA of each isolate was prepared according to methods previously described (Hopwood *et al*. 1985). For the amplification of DNA probe for the hybridization experiment**,** primers 23SlcF2 (5'-CCGACCCGCACGAAAGGCG-3') and 23SlcR2 (5'-GCCCGACTTTCGTCCCTGC-3) amplifying a 429 bp fragment of the 23S rRNA gene from *Lactobacillus casei* ATCC 393 were used in PCR reaction. One microgram of genomic DNA was used in a PCR mixture (total volume of 50 μl) containing 1 x PCR

71 buffer with KCl (Fermentas UAB, Vilnius, Lithuania), 1.5 mM $MgCl_2$, 0.2 mM concentration
72 of each nucleotide (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), 1.5 U of
73 recombinant *Taq* DNA Polymerase (Fermentas UAB, Vilnius, Lithuania) and 15 pmol of
74 each primer. The cycling program was: 1 cycle at 95°C for 5 min, 30 cycles of 94°C for 30 s,
75 55°C for 30 s and 72°C for 30 s and the final elongation step at 72°C for 7 min.
76 For amplification of a 1.2 kbp fragment of the 23S rRNA gene for restriction fragment length
77 polymorphism (RFLP) analysis two recently described universal primers 1104f and 2241r
78 were used, following the PCR conditions as reported (Hunt et al., 2006). Amplicons were
79 obtained from all strains. They were purified using the Gen Elute PCR Clean Up kit (Sigma
80 Chemical Co., St. Louis, Mo., USA) and subjected to RFLP and sequencing.
81
82 **Southern blot hybridization.** A whole genome DNA from *L. rhamnosus* BGHV 719 and *L.*
83 *casei* ATCC393 were digested with *Kpn*I, *Sal*I, *Pst*I *Sma*I, *Sph*I, *EcoR*V and *Xba*I
84 (Fermentas) and hybridization procedure was performed as previosly described in Sambrook
85 and Russell (2001). Non-radioactive labeling of the DNA probe amplified in PCR reaction
86 and detection of the signal was performed with DIG DNA Labeling and Detection Kit (Roche
87 Diagnostic GmbH, Germany).
88 **E-Test**
89 For the E-test (AB Biodisk, Solna, Sweeden) determination of MICs, strains were grown on
90 the LSM mixed medium formulation plates (LSM medium suplementad with 0.3g/l cysteine)
91 (Klare *et al*. 2005). Plates were incubated aerobically at 37°C. The MICs for erythromycin
92 were determine after 24 and 48 hours of incubation on the basis of Clinical Laboratory
93 Standards (NCCLS) criteria.
94

PCR-RFLP analysis. This method enables rapid mutation detection by restriction digestion of PCR product without the sequencing. The transition mutation from A to G at position 2058 in the erythromycin-resistant 23S rRNA sequence introduces a recognition site for the restriction enzyme *Bbs*I. Thus, 10 µl of the amplicons were digested with this enzyme and *Hin*dIII, for 1 h at 37ºC. Digestions were electrophoresed in 1% agarosa gels with TBE buffer following standard conditions (Saambrock and Russell, 2001). *Bbs*

Sequence analysis of 23S rRNA gene. Purified amplicons of the 23S rRNA genes were sequenced by cycle extension in an ABI 370 DNA sequencer (Applied Biosystems, Foster City, Ca., USA) using the oligonucleotide 1104f as a primer, and the resulting sequences compared to others held in public databases using the BLAST program.

RESULTS

Lactobacillus identification. According to the results of 16S DNA sequencing and BLAST analysis, all but one isolate were confirmed to belong to the *L. rhamnosus* species. The isolate BGHV747 was identified as *L. fermentum*.

MIC determination. Based on previously published results of antimicrobial susceptibility testing (Begovic *et al.,* 2007), a total of five *L. rhamnosus* and one *L. fermentum* isolates, categorized as highly resistant to erythromycin, were selected for the additional antibiotic susceptibility testing using E-test method. Results of the MIC determination are summarized in Table 1. Overall, *L. rhamnosus* strains were confirmed as highly resistant to erythromycin (MIC >256 µg/ml) although some differences in time of reaction between isolates was observed. For *L. rhamnosus* BGHV1, BGHV29, BGHV389 and *L. fermentum* BGHV747,

erythromycin resistance was evident after 24 hours. However, for two strains, BGHV20 and BGHV719 both resistant to erythromycin, a slower, time delayed growth on halos of inhibition was detected after 48 hours.

rRNA operon number. For the initial determination of rRNA gene copy number in *L. rhamousus* BGHV719, Southern blot analysis using a probe specific for 23S rRNA gene from *L. casei* ATCC 393 was performed. According to the analysis of published genome sequence (Makarova *et al*., 2006), *L. casei* genome encompasses five copies of 23S rRNA genes. Genomic DNA of both species was restricted with seven restriction enzymes; digestion of *L. rhamnosus* genome DNA with *Sph*I revealed five bands while digestion with *Sal*I, *Sma*I, *EcoR*V or *Xba*I gave less than five bands following the digestion and Southern blot analysis with a probe specific for 23S rRNA gene of *L. casei* (data not shown). Restriction with *Kpn*I revealed at least six bands in *L. rhamnosus* BGHV719 and exactly five bands for *L. casei*, idicating that although phylogeneticly close (Skerman *et al*., 1980), these two species differ in a number of 23S rRNA gene copies (Fig 1). Additional two bands of large size observed for *L. rhamnosus* are not taken into analysis since a weak signal was also seen for the control strain.

A2058 transition mutation. Isolates with A2058 transition mutation A→G created an additional *Bbs*I site that became evident after the *Bbs*I restriction digestion of 23S rRNA R amplicon (Fig2). Unlike *L. rhamnosus* isolates, *Bbs*I restriction digestion of *L. fermentum* R amplicon did not create the additional restriction band. It also appeared that the intensity of the additional band in *Bbs*I digestion differs between the isolates although the amount of the R amplicon used in the digestion was approximately the same for all the isolates. The

intensity of bands created in the control *Hind*III digestion was identical for all strains. The strongest band signal was observed for *L. rhamnosus* BGHV1 which according to the 16S rDNA sequencing (Fig 3) posseses the greatest number of mutated operons. A weaker intensity of the band was detected for *L. rhamnosus* BGHV20, BGHV29 and BGHV719 isolates that harbour approximately same number of mutated and non mutated copies of 23S rDNA according to the 16S rDNA sequencing (Fig 3).

Sequencing. To confirm the presence of the transition mutation in erythromycin resistant strains DNA sequencing of 23S rRNA gene amplicon was conducted. The analysis of obtained sequences revealed the presence of point mutation in V domain of 23S rRNA gene known to be associated with macrolide resistance (Sigmund *et al*., 1988). A transition mutation A→G at the position cognate with *E. coli* 23S rRNA position A2058 was discovered in all *L. rhamnosus* isolates (Fig. 3). However, this mutation has not been detected for erythromycin resistant *L. fermentum* BGHV747. According to sequencing results none of the *L. rhamnosus* isolates was resistant homozygous i.e., contained only mutant copies of 23S rRNA genes. In addition, the intensity of heterozigousity of the sequences at the position A2058 revealed differences between the isolates. Sequencing of the *L. rhamnosus* isolates BGHV29 and BGHV389 yielded a mixed base A or G at the position A2058 with stronger signal for A nucleotide. In contrast, for the isolates BGHV20 and BGHV719 stronger signal was observed for G2058 mutation. The sequence of the amplicon from *L. rhamnosus* BGHV1 showed a significant difference between the signals for A2058 (a weak signal) and G2058 mutation (strong signal). These results revealed that not all human vaginal *L. rhamnosus* 23S rRNA genes from this study carry G2058 mutation. We can also speculate that the differences in a number of mutated 23S rRNA genes exist between the isolates. Since the

whole genome of *L. rhamnosus* and *L. fermentum* has not been sequenced yet, the positions of nucleotide analogous to *E. coli* A2058, A2059 and A2060 remain to be determined for this species. In *L. casei* ATCC 334, according to the published genome sequence (Makarova *et al.*, 2006), the positions of corresponding nucleotides are A1673, A1674 and A 1675 respectively to positions in *E. coli*.

DISCUSSION

The aim of this study was to elucidate the molecular basis of erythromycin resistance in human vaginal *L. rhamnosus* strains isolated from healthy Serbian women. The results of the preceding study did not reveal the nature of erythromycin resistance since the isolates did not harbour *erm*(A), *erm*(B) or *erm*(C) genes (*Begovic et al*., 2007). These findings have led us to the presumption that other molecular mechanisms may be responsible for the observed erythromycin resistance in human vaginal isolates. The data presented in this study provide evidence that erythromycin resistance in *L. rhamnosus* strains is due to the presence of A→G transition mutation in 23S rRNA gene at the position A2058 of 23S rRNA gene (following *Escherichia coli* numbering). Additionaly, the number of mutated 23S rRNA genes seems to differ between the isolates we can speculate that different ways of erythromycin emergence occured in these isolates. On the other hand the nature of erythromycin resistance of *L. fermentum* still remains unclear and requires further analysis. This work represents the first description of the molecular basis of erythromycin resistance associated with 23S rRNA sequence change in *Lactobacillus* sp.

Mutations at A2058 confer a high-level erythromycin resistance due to a large reduction of the affinity of the drug interaction with V region of 23S rRNA gene (Douthwaite and Aagard,

191 1993). Our results are in concordance with these findings since MICs for erythromycin
192 determined in present study are high (MICs > 256 µg/ml). It appears that approximately 50%
193 of ribosomal targets of the resistant phenotype are present in all analyzed lactobacilli and
194 according to Sigmund *et al*., (1988) this is a percentage required for the high-level macrolide
195 resistance in bacteria. Thus, the resistance based on a single mutational event is more
196 probable to occur in microorganisms that contain a small number (one or two copies) of
197 rRNA genes per genome (Nash and Inderlied, 1995). A genome of *L. casei*, a species closely
198 related to *L. rhamnosus* (Skerman *et al*., 1980) contains five copies of 23S rRNA genes.
199 From the results of Southern blot hybridization experiments we can speculate that *L.*
200 *rhamnosus* genome possess at least six copies of this gene. Statistically, an emergence of
201 resistance by mechanism of mutation in 23S rRNA should occur rarely in species with
202 multiple rRNA operons. On the other hand, a long history of antibiotic use, and often abuse,
203 has intensified and accelerated the development of antibiotic resistance among bacteria
204 (Levy, 2002).
205 The aspect of the presence of antibiotic resistant commensal bacteria in human organism
206 needs to be studied more carefully. Although the link between antibiotic consumption and the
207 prevalence of resistance is difficult to establish (Austin *et al*. 1997), the selective pressure
208 imposed by the uncontrolled use of antibiotics is probably the mayor contributing factor to
209 the emergence of antibiotic resistance among human resident microlflora. Overall, two
210 important aspects of antibiotic resistance of non-pathogenic bacteria should be outlined. First,
211 the mayor concern regarding bacterial resistance to antibiotics is the possibility of horizontal
212 transfer of resistance determinants (Levy, 2002) particularly to pathogens, which has already
213 been demonstrated (Blake *et al*., 2003). The second aspects relates to lactobacilli as potential
214 pathogens and the therapeutic problem of fast and efficient antibiotic therapy, particularly for

immunocompromised and elderly people that are being endangered by commensal bacteria infections (Cannon *et al*., 2005). The frequency of different infections caused by opportunistic bacterial pathogens is in constant increase (Wright, 2007). The results from this study on lactobacilli carrying different number of mutated 23rs rRNA, isolated from five different subjects indicate an independent emergence of erythromycin resistance among these isolates. These findings call for a more detailed studies of microevolution of antibiotic resistance among non-pathogenic bacteria and the development of the strategies (Bonhoeffer *et al.*, 1997) to at least slow the rate at which antibiotic resistance increases among bacteria.

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REFERENCES

Blake, D. P., Hillman, K., Fenlon, D.R, and Low, J.C. Transfer of antibiotic resistance between commensal and pathogenic members of the *Enterobacterioaceae* under ileal conditions. (2003) *J Appl Microbiol* **95**, 428-436.

Cannon, J.P., Lee, T.A., Bolanos, J.T. and Danziger, L.H. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. (2005) *Eur J Clin Microbiol Infect Dis* **24**, 31-40.

Danielsen, M. and Wind, D. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. (2003) *Int J Food Microbiol* **82**, 1-11.

Douthwaite, S, and Aagard C. Erythromycin binding is reduced in ribosomes with conformational alternations in the 23S rRNA peptidil transferase loop. (1993) *J Mol Biol* **3**, 725-731.

Farell, D. J., Douthwaite, S., Morrisey, I., Bakker, S., Poehlsgaard, J., Jakobsen, L, and Felmingham, D. Macrolide resistance by ribosomal mutation in clinical isolates of *Streptococcus pneumoniae* from the PROTEKT 1999-2000 study. (2003) *Antimicrob Agents Chemother* **47**, 1777-1783.

Flórez, A. B., Delgado, S. and Mayo, B. Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. (2005) *Can J Microbiol* **51**, 51-58.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bryton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H. (1985) *Genetic manipulation of Streptomyces, a laboratory manual*. Norwich, UK, The John Ines Foundation.

Klare, I., Konstabel, C., Muller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H. and Witte, W. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. (2005) *Appl Environ Microbiol* **71**, 8982-8986.

Levy, S.B. Factors impacting on the problem of antibiotic resistance. *J Antimicrob Chemother* (2002) **49**, 25-30.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B, and Mills, D. Comparative genomics of the lactic acid bacteria. (2006) *Proc Natl Acad Sci USA* **103**,15611-15616.

Nash, K. A, and Inderlied, C.B. Genetic basis of mscrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease. (1995) *Antimicrob Agnets Chemother* **39**, 2625-2630

National Committe for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard M7-A3. NCCLS. Villanova. PA. (1997).

285 Redondo-López, V., Cook, R.L, and Sobel, J.D. Emerging role of lactobacilli in the control
286 and maintanance of the vaginal bacterial microflora. (1990) *Rev Infect Dis* **12,** 856-872.
287
288 Skerman, V.B.D., McGowan, V, and Sneath, P.H.A. Approved lists of bacteria names. *Int J*
289 *Sys Bacteriol* (1980) **30**, 225-420.
290
291 Sambrook, J., and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual. Cold
292 Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
293
294 Sigmund., C.D., Ettayebi, M.,Borden, A, and Morgan, E.A. Antibiotic resistance mutations in
295 ribosomal RNA genes of *Escherichia coli (*1988). *Meth Enzym* **164**:673-690.
296
297 Weisblum, B. Erythromycin resistance by ribosome modification. (1995) *Antimicrob Agnets*
298 *Chemother* **39**: 577-585.
299
300 Wright, G.D. The antibiotic resistome: the nexus of chemical and genetic diversity. (2007)
301 *Nature Rev* **5**,175-186.
302
303 Young, J.P.W., Downer, H.L, and Eardly, B,D. Phylogeny of the prototrophic *Rhizobium*
304 strain BTAil by polymerase chain reaction-based sequencing of a 16S rRNA segment. (1991)
305 *J Bacteriol* **173**, 2271-2277.
306
307

308 **Table 1. Minimum inhibitory concentration (MIC) of erythromycin for the** human

309 vaginal lactobacilli utilized in this work in LSM medium.

Isolate	**Species according to 16S rDNA sequencing**	**MIC of erythomycin (µg/ml)**	
		24h	**48h**
BGHV20	*L. rhamnosus*	0.25 (>256)*	0.25 (>256)*
BGHV1	*L. rhamnosus*	>256	>256
BGHV747	*L. fermentum*	0.50	0.50
BGHV29	*L. rhamnosus*	0.064	0.125 (>256)*
BGHV389	*L. rhamnosus*	0.19	0.19 (>256)*
BGHV719	*L. rhamnosus*	0.25 (>256)*	0.25 (>256)*

310 * Strains showed a clear halo of growth inhibition (retardation);

311 although enough growth on the halos was evident at 48 h

312

Fig. 1. Hybridization profiles of *L. casei* ATCC334 (lane 1) and *L. rhamnosus* BGHV719 (lane 2). A fragment of 429 bp. from *L. casei* ATCC334 served as a probe for chromosomal DNA completely digested wit *KpnI* enzyme. The arrows indicate six copies of 23S rRNA in *L. rhamnosus* BGHV719.

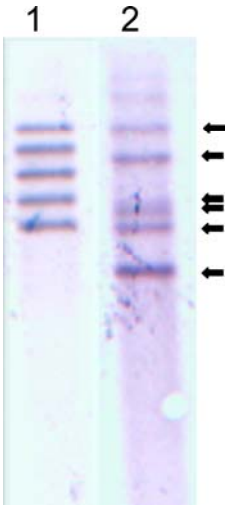
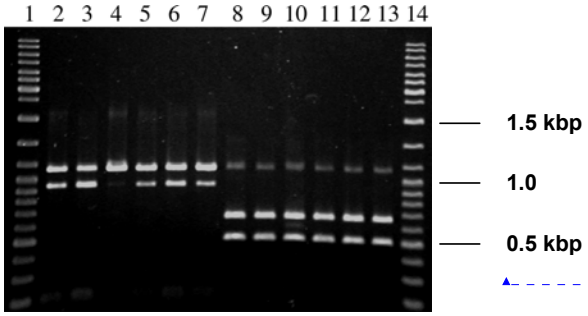


Fig. 2. Restriction profiles of R amplicon treated with *Bbs*I and *Hind*III from erythromycin resistant vaginal isolates of lactobacilli. *Bbs*I (lanes 1-6) or *Hind*III (lanes 7-12) digestion **of.1.2 kbp** fragment of the 23S rDNA gene generated by PCR amplification using primers 1104f and 2241r (Hunt et al., 2006) as described in Materials and Methods. Lanes: 1 and 14, 100 bp molecular weight ladder **(**Fermentas GMBH, St.Leon-Rot, Germany**)**; 2 and 8, *L.rhamnosus* BGVH20; 3 and 9, *L.rhamnosus* BGVH1; 4 and 10, *L. fermentum* BGVH747; 5 and 11, *L.rhamnosus* BGVH29; 6 and 12, *L.rhamnosus* BGVH389; 7 and 13, *L.rhamnosus* BGVH719.



Con formato

Fig. 3. Dendograms of 23S rDNA sequences obtained by PCR with 1104f and 2241r(primers. The arrows point to a nucleotide position in 23S rDNA gene from human vaginal lactobacilli equivalent to that of *E. coli* A2058 (T2058).