1 **Acquired macrolide resistance in the human intestinal strain *Lactobacillus***
2 ***rhamnosus* E41 associated with a transition mutation in the 23S rRNA gene**

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4 Ana Belén Flórez, Víctor Ladero, Mohammed Salim Ammor, Miguel Ángel Álvarez,
5 and Baltasar Mayo*

6

7 Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n, 33300-
8 Villaviciosa, Asturias, Spain

9

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15 *Corresponding author:

16 Baltasar Mayo

17 Instituto de Productos Lácteos de Asturias (CSIC)

18 Carretera de Infiesto s/n

19 33300-Villaviciosa

20 Spain

21

22 Tel.: 34+985 89 21 31

23 fax: 34+985 89 22 33

24 E-mail address: baltasar.mayo@ipla.csic.es

ABSTRACT

RFLP and DNA sequencing of PCR products showed that a *Lactobacillus rhamnosus* strain of human origin resistant to macrolides, from which no resistance determinants have been detected by specific PCR and microarray screening, contained a heterozygous A-to-G transition mutation at position 2058 (*Escherichia coli* numbering) of its 23S rRNA gene.

The resistance gene reservoir hypothesis suggests that beneficial and commensal bacterial populations in food and the gastrointestinal tract (GIT) of animals and humans may play a role in the transfer of antibiotic resistance (Teuber et al., 1999; Salyers et al., 2004). To reduce the spread of such resistance, the appropriate use of antibiotics is important (MARAN, 2002; DANMAP, 2003), as is the screening for antibiotic resistance in bacteria intended to be used in food systems (Teuber et al., 1999; European Commission, 2005). Distinguishing between intrinsic and acquired resistance is essential, and with respect to the latter it is important to determine whether it is caused by genomic mutation or added genes; the last of these poses the greatest risk of horizontal transmission (Maiden, 1998; Normak and Normak, 2002).

Lactobacillus species are members of the lactic acid bacteria (LAB) group, and are capable of colonising habitats as diverse as fresh and fermented plant materials, meat products, fish, dairy, sourdoughs, fermented beverages, and the human and animal GIT (Kandler and Weiss, 1986). The use of selected species of lactobacilli as starter organisms in industrial food and feed fermentations has a long tradition (Cogan, 1996; Bernardau et al., 2006). Moreover, the lactobacilli form one of the subdominant bacterial populations of the human and animal GIT (Vaughan et al., 2002), where they are thought to exert an array of beneficial effects, including the inhibition of pathogens,

50 the alleviation of lactose intolerance, the boosting of the immune response, and the
51 lowering of cholesterol levels. Benefit is also derived from their possible anti-
52 carcinogenic and anti-mutagenic activities, etc. (Ouweland et al., 2002; Sanders, 2003).

53 Erythromycin and other macrolides are the best alternatives for the treatment of
54 penicillin-allergic patients. Bacterial resistance to macrolides, lincosamides and
55 streptogramins (MLS phenotype) is often due to efflux systems, methylases, and
56 inactivating enzymes, for which more than 40 genes have been reported (Roberts et al.,
57 1999a). However, it can also be due to mutations in ribosomal proteins L4 and L22 or in
58 the 23S rRNA molecule (Leclercq, 2002). In fact, chromosomal mutations altering the
59 erythromycin binding site in the V domain of the 23S rRNA gene (at cognate position
60 A2058 [*Escherichia coli* numbering]) has been shown in a number of clinical isolates,
61 including *Mycoplasma* spp. (Meier et al., 1994; Lucier et al., 1995), *Helicobacter pylori*
62 (Versalovic et al., 1996), *Propionibacterium* spp. (Ross et al., 1997), and *Bordetella*
63 *pertussis* (Bartkus et al., 2003). Here we report on the identification of a 23S rRNA
64 mutation in a *Lactobacillus rhamnosus* strain conferring resistance to erythromycin,
65 clindamycin and some other macrolides, in which PCR and microarray screening
66 techniques have failed to identify any macrolide resistance genes. To our knowledge,
67 this is the first report of such a mutation in an LAB species.

68

69 **Minimum inhibitory concentration (MIC) of macrolides for *L. rhamnosus* E41.**

70 During a recent survey, two *L. rhamnosus* isolates were identified as resistant to both
71 erythromycin (MIC 1024 $\mu\text{g ml}^{-1}$) and clindamycin (MIC 256 $\mu\text{g ml}^{-1}$) in a microbroth
72 assay (Delgado et al., 2005). Resistance to these antibiotics is confirmed in the present
73 work by the Etest method (AB Biodisk, Solna, Sweden). In contrast, the MICs of
74 erythromycin and clindamycin in susceptible *L. rhamnosus* isolates (E51, G92, G94,

75 E52, E57, F46) and the industrial probiotic strain *L. rhamnosus* LMG 18243 (strain
76 GG) were 1 and 0.5 µg ml⁻¹ respectively. Typing of the isolates by phenotypic (API50
77 CHL, bioMérieux, Montalieu-Vercieu, France) and genetic (random amplification of
78 polymorphic DNA) methods identified them as the same strain.

79

80 **Identification of erythromycin resistance determinants in *L. rhamnosus* E41.**

81 Genes *ermA*, *erm(B)*, *erm(C)*, *erm(F)* and *mef(A)*, which confer macrolide resistance,
82 are widely distributed among Gram positive and Gram negative organisms (Jensen et
83 al., 1999). Some have already been characterized in plasmids (Tannock et al., 1994;
84 Fons et al., 1997; Gfeller et al., 2003) and on the chromosome (Flórez et al., 2006) of
85 lactobacillus species. In the present study, the presence of these five genes was
86 therefore analysed by specific PCR using purified total DNA from E41 as a template.
87 The primers and PCR conditions used were those reported by Roberts et al. (1999b).
88 No amplification was obtained with any of the specific primer couples. Analysis of two
89 original *L. rhamnosus* isolates for resistance genes using DNA microarrays with more
90 than 300 oligonucleotide probes, of which 42 corresponded to genes involved in MLS
91 phenotype, also returned negative results (Ammor et al., 2006).

92

93 **PCR amplification and analysis of 23S rRNA gene sequences.** After ruling out the
94 presence of added genes as responsible for the MLS phenotype in strain E41, a search
95 for mutations in its ribosomal components was performed. The *L. rhamnosus* sequences
96 for the genes encoding the L4 and L22 proteins, and that of 23S RNA, are not available
97 in public databases. Therefore to amplify a segment of the 23S RNA gene, including
98 the critical 2048 residue (*E. coli* 23S rRNA numbering) involved in macrolide
99 resistance, we made use of the recently described universal primers 1104f and 2241r

100 (Hunt et al., 2006). For comparison, the same segment of the 23S rRNA from seven
101 erythromycin- and clindamycin-susceptible strains, including *L. rhamnosus* GG, was
102 amplified under identical conditions. An amplicon of around 1200 bp was obtained
103 from all strains. This was purified using the Gen Elute PCR Clean Up kit (Sigma
104 Chemical Co., St. Louis, Mo., USA) and subjected to restriction fragment length
105 polymorphism (RFLP) analysis and sequencing.

106 The transition mutation from A to G at position 2058 in the erythromycin-resistant
107 23S rRNA sequence introduces a recognition site for the restriction enzyme *Bbs*I. Thus,
108 amplicons were all digested with this enzyme - which anticipates the mutation - before
109 analysis of the sequences. Fragments of the expected size were only observed in the
110 amplicon obtained from the erythromycin-resistant strain (Fig. 1, line 1). However, part
111 of the amplicon appeared to be undigested, suggesting either partial digestion with *Bbs*I
112 had occurred, or the simultaneous presence of wild-type and mutant copies of the 23S
113 rRNA gene. As a control, amplicons were also digested with *Hind*III, the cleavage site
114 of which is in the middle of the amplified region in the 23S rRNA sequences of many
115 lactobacillus species. With this enzyme, all the amplicons were digested, giving rise to
116 identical fragments. This supports the idea of heterozygosity for the 23S rRNA genes in
117 the *L. rhamnosus* E41 studied.

118 To support these results, all amplicons were sequenced in an ABI PRISM 370
119 sequencer (Applied Biosystems, Foster City, Ca., USA), and the sequences obtained
120 were compared to one another and to those in databases. In the sequence corresponding
121 to the resistant strain, a transition mutation (A to G) was observed at the corresponding
122 position of 2058; this was not seen in the susceptible strains (Fig. 2). The
123 chromatograms showed a fluorescence signal for an adenine residue in E41 as well as
124 in the wild-type susceptible strains, indicating its heterozygous status. Although no

125 information is available on the number of rRNA operons in *L. rhamnosus*, it is expected
126 to range from four to seven, as in other *Lactobacillus* species (Klaenhammer et al.,
127 2002). The sequences obtained in this work showed the highest homology (97%) to a
128 partial sequence of the *L. casei* 23S rRNA gene (accession no. AF098107), and
129 significant homology (93%) to the 23S rRNA sequences from other lactobacilli and
130 enterococci. Comparing the wild-type and mutant sequencing signals in the resistant
131 strain with the signal of the restriction fragments digested with *BbsI*, it is tempting to
132 speculate that more than one copy of the mutated sequence is present in the *L.*
133 *rhamnosus* L42 23S gene.

134

135 In conclusion, this paper reports a chromosomal mutation of the 23S rRNA gene as the
136 most plausible cause of macrolide resistance in *L. rhamnosus* E41. Analysis of other
137 ribosomal components thought to be involved in macrolide resistance should exclude
138 other possibilities. *Lactobacillus rhamnosus* E41 shows promising probiotic properties
139 (Delgado and Mayo, unpublished); it would be especially useful to people undergoing
140 long-term macrolide treatment.

141

142 **Nucleotide sequence accession numbers.** The wild-type and mutant 23S rRNA gene
143 sequences of *L. rhamnosus* were assigned GenBank accession nos. EF030190 and
144 EF030191, respectively.

145

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Figure 1

Figure 2

Figure Legends

Figure 1. PCR restriction fragment length polymorphism (RFLP) analysis of 23S gene amplicons from the macrolide resistant strain *Lactobacillus rhamnosus* E41 (line 1) and a series of *L. rhamnosus* susceptible strains [E51, G92, G94, E52, E57, F46 and LMG 18243 (strain GG); lines 2 through 8, respectively] digested with the restriction enzymes *Bbs*I (left) and *Hind*III (right). M, molecular weight marker.

Figure 2. Representative sequence chromatograms of 23S rRNA genes for the wild type macrolide-susceptible *Lactobacillus rhamnosus* LMG 18243 (strain GG) (left) and that of the macrolide-resistance *L. rhamnosus* E41 (right). Arrows indicate the nucleotide at position 2058 (of the *Escherichia coli* numbering). Note the heterozygous nature (G/A) of strain E41 at this position.