Research Article

LraI from Lactococcus raffinolactis BGTRK10-1, an Isoschizomer of EcoRI, Exhibits Ion Concentration-Dependent Specific Star Activity

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Restriction enzymes are the main defence system against foreign DNA, in charge of preserving genome integrity. Lactococcus raffinolactis BGTRK10-1 expresses LraI Type II restriction-modification enzyme, whose activity is similar to that shown for EcoRI; LraI methyltransferase protects DNA from EcoRI cleavage. The gene encoding LraI endonuclease was cloned and overexpressed in E. coli. Purified enzyme showed the highest specific activity at lower temperatures (between 13°C and 37°C) and was stable after storage at −20°C in 50% glycerol. The concentration of monovalent ions in the reaction buffer required for optimal activity of LraI restriction enzyme was 100 mM or higher. The recognition and cleavage sequence for LraI restriction enzyme was determined as 5'-G/AATTC-3', indicating that LraI restriction enzyme is an isoschizomer of EcoRI. In the reaction buffer with a lower salt concentration, LraI exhibits star activity and specifically recognizes and cuts another alternative sequence 5'-A/AATTC-3', leaving the same sticky ends on fragments as EcoRI, which makes them clonable into a linearized vector. Phylogenetic analysis based on sequence alignment pointed out the common origin of LraI restriction-modification system with previously described EcoRI-like restriction-modification systems.

1. Introduction

Restriction endonucleases are generally accompanied by a cognate methyltransferase [1]. Both enzymes working together form a restriction-modification system (RM system). RM systems are important for the maintenance of the genome integrity of prokaryotic organisms. The range of biological processes that utilize RM system also includes involvement in DNA transposition [2] and recombination [3]. In addition, there is evidence that the genes for restriction and modification enzymes may act together as selfish elements [4, 5].

Restriction endonucleases exhibit high sequence specificity in substrate binding and use versatile DNA cleavage mechanisms and thus are excellent model systems for understanding DNA recognition and phosphodiester bond hydrolysis. Restriction endonucleases are classified according to their subunit composition, cofactor requirement, recognition site, cleavage site, and mode of action to define the different types (I, II, III, and IV). Restriction endonucleases Type II are essential tools for recombinant DNA technology. It seems unlikely that today’s modern molecular biology and the biotechnology industry would have developed without Type II restriction enzymes. Because of their great importance in gene analysis and cloning there is a constant need to discover new ones. According to data from the REBASE [6, http://rebase.neb.com] which summarizes all information known about every restriction enzyme and any associated protein, there are more than 3945 biochemically or genetically characterized restriction enzymes and, out of 3834 Type II restriction enzymes, 299 distinct specificities are known. By 2010, six hundred and forty-one restriction enzymes were commercially available, including 235 distinct specificities [7].
Because of the large number of sequenced genomes, rate of discovery of new putative restriction and modification genes is rising rapidly. In contrast, the number of restriction enzymes that are biochemically characterized has actually dropped down to the level that was three decades ago.

Restriction endonucleases Type II are homodimeric or tetrameric enzymes that cleave DNA at defined sites of 4–8 bp in length and require Mg$^{2+}$ ions for catalysis [8]. For many of restriction endonucleases Type II, it was found that modified conditions (lower ionic strength, higher pH, presence of different metallic cofactors, and organic solvents) could decrease their substrate specificity [9–13]. Under nonoptimal restriction conditions, these endonucleases can usually cleave degenerate sequences, which differ from standard recognition sites at only one nucleotide. This alteration in digestion specificity causing cleavage of DNA at novel, similar but not identical sequences is defined as enzyme star activity. Modification causing cleavage of DNA at novel, similar but not identical sequences is defined as enzyme star activity. Mod-2ification these sequence homology between EcoRI and RsrI revealed only a low level of overall similarity wherefore resistance to the EcoRI restriction enzyme was overexpressed and purified to identification. However, RsrI recognizes the sequence GAATTC and cleave at the same position (G/AATTC) and are sharing 50% amino acid sequence identity [15]. Interestingly, MunI recognizes the sequence CAATTG, which differs from the recognition sequence of EcoRI (and RsrI) only in the external base pairs. Comparison of the MunI amino acid sequence with that of EcoRI and RsrI revealed only a low level of overall similarity wherefore sequence homology between EcoRI and RsrI has a stronger significant [16].

This work describes for the first time the occurrence of EcoRI-like restriction-modification genes in lactococci. The objective was to clone, purify, and biochemically and genetically characterize novel lactococcal LraI restriction enzyme. LraI restriction enzyme was overexpressed and purified to the homogeneity from E. coli using pMAL expression and purification system. Results demonstrate that LraI restriction enzyme, although an isoschizomer of EcoRI, shows different characteristics. One of characteristics that could be further exploited is star activity of LraI that is limited to one variant of the recognition site, which after cleavage leaves identical cohesive ends as EcoRI and LraI restriction enzymes, so that the fragments obtained after digestion could be cloned without additional processing.

2. Material and Methods

2.1. Bacterial Strains and Culture Conditions. Lactococcus raffinolactis BGTRK10-1 was isolated from autochthonous sweet kajmak produced from sheep milk without the use of starter cultures in a household of the Vlašić mountain region, central Bosnia and Herzegovina [17] (Table 1). Preliminary strain classification was done according to its fermentation ability using API 50CHL (Api System SA; Bio-Merieux, Montelieu-Vercieu, France), temperature of growth (30° C, 37° C, and 45° C), growth in the presence of salt (4% and 6.5%), and pH tolerance. Final taxonomic classification of BGTRK10-1 was performed by sequencing of amplified 16S rDNA using primers previously described [18]. The strain was grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (0.5% w/v) (GM17) at 30° C. Escherichia coli DH5α, HB101, and ER2523 strains were grown aerobically in Luria-Bertani (LB) broth at 37° C, unless otherwise specified. Solid medium was made by adding L75% (w/v) agar (Torlak, Belgrade, Serbia), to the liquid media. Antibiotics were used at the following concentrations: erythromycin 300 μg/ml and ampicillin 100 μg/ml for selection and maintaining of transformants. The 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Fermentas, Vilnius, Lithuania) was added to LB medium plates for blue/white colour screening of colonies with cloned fragments at final concentration of 40 μg/ml.

2.2. Construction of Cosmid Library of L. raffinolactis BGTRK10-1. Total DNA isolated from the L. raffinolactis BGTRK10-1 was partially digested with XbaI restriction enzyme. Incubation was carried out during 1 h and was stopped in different time intervals by adding EDTA. Optimal digestion conditions were 30–40 kb, was purified and ligated overnight at 16° C with the pZYMcos vector [19] predigested with XbaI restriction enzyme and dephosphorylated. Ligation for formed concatamers of high molecular weight was checked on agarose gel and encapsulated into phage particles using packaging kit (Agilent Technologies). Encapsulated cosmids were transfected into E. coli HB101 magnesium cells and selection of clones was done on LA plates containing erythromycin 300 μg/ml. Constructed cosmid library in E. coli was stored in LB containing 15% (v/v) glycerol at −80°C.

2.3. DNA Manipulations. Total DNA from L. raffinolactis BGTRK10-1 was isolated by modified method described by Hopwood et al. [20]; the logarithmic phase cells were pretreated with lysozyme (4 mg/ml, for 15 min at 37°C) prior to treatment with SDS. For plasmid isolation from E. coli the QIAprep Spin Miniprep kit was used according to the manufacturer’s recommendations (Qiagen, Hilden, Germany). Standard heat-shock transformation was used for plasmid transfer into E. coli [21]. Digestion with restriction enzymes was conducted according to the supplier’s instructions (Thermo Fisher Scientific). The DNA fragments from agarose gels were purified using QIAquick Gel extraction kit as described by the manufacturer (Qiagen, Hilden, Germany). DNA was ligated with T4 DNA ligase (Agilent Technologies, USA) according to the manufacturer’s recommendations. Platinum™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Waltham, MA, USA) was used to amplify DNA fragments by PCR in GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with QiAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA sequencing was done by the Macro- gen Sequencing Service (Macrogen Europe, The Netherlands).
Table 1: Bacterial strains and plasmids used in this study.

| Strains or plasmids                  | Relevant characteristics                                                                 | Source or reference |
|--------------------------------------|------------------------------------------------------------------------------------------|---------------------|
| **Lactococcus raffinolactis**        |                                                                                          |                     |
| BGTRK10-1                            | Natural isolate from autochthonous sweet kajmak                                             | [17]                |
| **Escherichia coli**                 |                                                                                          |                     |
| DH5α                                 |                                                                                          | [21]                |
| HB101                                |                                                                                          | [24]                |
| ER2523                               |                                                                                          | New England Biolabs, Ltd. UK |
| ER2523/pAZIL-LraRM                   | Competent cells obtained by transformation of ER2523 cells with pAZIL-LraRM               | This study          |
| **Plasmids**                         |                                                                                          |                     |
| pAZIL                                | 7109 bp, Em', shuttle cloning vector                                                       | [19]                |
| pAZIL-LraRM                          | PCR fragments of Lra operon from BGTRK10-1 cloned into pAZIL vector predigested with Smal | This study          |
| pAZIL-LraI+672pBS                    | DNA fragment of 672 bp obtained after digestion of pBluescript SK+ with LraI+ activity cloned into pAZIL vector predigested with LraI | This study          |
| pAZILcos                             | 8194 bp, Em', shuttle cosmid vector                                                       | [19]                |
| pAZILcosLra                          | Cosmid selected from total Xbal cosmid library of BGTRK10-1                              | This study          |
| pBluescript SK+                      | 2958 bp, Amp', cloning vector                                                             | Stratagene          |
| pBSLraCla                            | CiaI fragment of 4239 bp obtained from pAZILcosLra cloned into pBluescript SK+ vector     | This study          |
| pMAL-c5X                             | 5677 bp, pMBI origin, lacI, maleE, bla, Factor Xa cleavage site;                           | New England Biolabs, Ltd. UK |
| pMAL-c5XRal-29                       | PCR fragment of LraI restriction endonuclease from pAZIL-LraRM cloned into pMAL-c5X vector predigested with XmnI and HindIII restriction enzymes | This study          |
| pMAL-c5XRal-3I                       | PCR fragment of LraI restriction endonuclease from pAZIL-LraRM cloned into pMAL-c5X vector predigested with XmnI and HindIII restriction enzymes | This study          |
| pMAL-c5XRal-42                       | PCR fragment of LraI restriction endonuclease from pAZIL-LraRM cloned into pMAL-c5X vector predigested with XmnI and HindIII restriction enzymes | This study          |

The primers used in PCR for amplification of LraI operon (lraIR and lraIM genes) were as follows: LraRM-Fw (5'-GTATAAGAAAAGAATCGC-3') and LraRM-Rev (5'-GCAGGCTATTTTCCCCTG-3'), while following primers were used for overexpression of LraI restriction enzyme (lraIR gene) in pMALc5X vector: LraI-Fw (5'-ATGGGCAAACATCAGTCG-3') and LraI-Rev (5'-CTCTAACGCTTTTCAATTAATCCTTTCCTTGAC-3'; HindIII restriction site is underlined). Total DNA (1 ng) was mixed with 17.9 μL of distilled water, 2.5 μL of 10x PCR buffer (Thermo Fisher Scientific), 1 μL dNTP mix (10 mM), 1.5 μL of MgCl2 (25 mM), 1 μL (10 pmol) of each primer, and 0.1 μL of Platinum™ Taq DNA Polymerase High Fidelity. Performed using the GeneAmp 2700 PCR Cycler (Applied Biosystems), the PCR programs consisted of initial denaturation (5 min at 96°C), 30 cycles of denaturation (30 s at 96°C), annealing (30 s at 40°C) and polymerization (2 or 1 min at 68°C), and an additional extension step of 5 min at 68°C. PCR fragments of LraI operon amplified using Platinum™ Taq DNA Polymerase High Fidelity were cloned into pAZIL vector predigested with Smal.

2.4. Recombinant LraIR Restriction Endonuclease Overexpression in E. coli and Purification. PCR fragment consisted of lraIR gene (from ATG to stop codon) obtained by LraFw/LraI-Rev primers and Platinum™ Taq DNA Polymerase High Fidelity was purified, digested with HindIII and cloned into pMAL-c5X vector digested with XmnI and HindIII restriction enzymes and transformed into ER2523 competent cells (New England Biolabs, Ltd. UK) previously transformed with pAZIL-LraRM construct (Table 1). Transformants were selected on LA Petri dishes containing 2% glucose, ampicillin 100 μg/ml, and erythromycin 300 μg/ml at 23°C. Confirmation of fragment presence in adequate orientation was obtained by restriction enzyme analysis (with SacI and HindIII digestion) and sequencing. Expression of recombinant protein was carried out at 23°C by induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Purification (cell lysis, affinity chromatography, and cleavage of fusion protein with Xa protease) was performed according to manufacturer instruction (pMAL Protein Fusion & Purification System; New England Biolabs, Ltd., UK). Purified recombinant LraI restriction endonuclease was stored at −20°C in CM
buffer (20 mM Tris-HCl pH7.4, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT) containing 50% glycerol.

2.5. Endonuclease Assays. Endonuclease activity was assayed by incubating various amounts of purified LraI enzyme in buffer recommended for use with EcoRI (50 mM Tris-HCl, pH 7.5, 10 mM magnesium chloride, 100 mM sodium chloride, 0.02% Triton X-100, supplemented with 100 μg/ml BSA; Thermo Fisher Scientific) containing 1 μg of plasmid DNA per 50 μl reaction mixture for 1 h at 37°C. One unit of enzyme activity was defined as purified LraI enzyme, which was able to completely cut 1 μg of plasmid DNA for 1 h.

Influence of reaction buffer composition on LraI enzyme activity was assayed in different commercial buffers (Buffer B (blue; 10 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, 100 μg/ml BSA), Buffer G (green; 10 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, 50 mM sodium chloride, 100 μg/ml BSA), Buffer O (orange; 50 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, 100 mM sodium chloride, 100 μg/ml BSA), Buffer R (red; 10 mM Tris-HCl pH 8.5, 10 mM magnesium chloride, 100 mM potassium chloride, 100 μg/ml BSA), Buffer Tango (yellow; 33 mM Tris–acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 100 μg/ml BSA), and buffer recommended for use with EcoRI; Thermo Fisher Scientific) used for reaction with 1 U of purified LraI enzyme and 1 μg of plasmid DNA for 1 h at 37°C. To measure the activity of purified LraI enzyme at different temperatures 1 U of purified LraI enzyme was incubated with 1 μg of plasmid DNA for 1 h at different temperatures (13°C, 23°C, 30°C, 37°C, 45°C, 60°C, and 80°C). In all endonuclease activity assays commercial EcoRI restriction enzyme (Thermo Fisher Scientific) was used as control. Reactions were stopped by addition 1/10 volume of stop solution (50 mM EDTA pH 8, 50% glycerol, 0.02% orange G) and products were analyzed by electrophoresis in 1% agarose gels.

2.6. Determination of LraI Cleavage Site. To determine the precise positions and nucleotide sequence of cleavage sites within double stranded DNA for the LraI restriction enzyme, plasmid SK+ was used as template. Plasmid was digested with recombinant enzyme LraI; complete digestion was confirmed by agarose gel electrophoresis and digest was sequenced with M13F and M13R primers (Macrogen Europe, The Netherlands). Simultaneously as control, the whole experiment was conducted with commercial EcoRI restriction enzyme (Thermo Fisher Scientific).

2.7. Bioinformatic Analysis of LraI Homologs. Sequence searches on the NCBI nucleotide and protein databases were conducted with BLAST [22] using lraIR/LraIR and lraLM/ LraLM sequences. The phylogenetic inferences between restriction and methylase enzymes were obtained by MEGA version 6.0 (http://www.megasoftware.net/). The first 30 protein reference sequences of EcoRI-like endonuclease or methyltransferase enzymes chosen according to results of BLASTP search and LraI restrictase and LraI methylase sequences separately were trimmed and aligned using Clustal W [23] with default parameters. The phylogenetic trees were constructed by the maximum-likelihood (ML) method using a Tamura-Nei model. Bootstrapping of 1000 replicates was used to infer confidence levels of ML trees.

The nucleotide sequences of DNA fragments carrying genes encoding LraI restriction-modification system and 16S rRNA from L. raffinolactis BGTRK10-1 were submitted to ENA GenBank under accession numbers LT222052 and LT854837, respectively.

3. Results and Discussion

3.1. Identification of LraI (EcoRI-Like) Methylase Activity in L. raffinolactis BGTRK10-1. The mesophilic lactic acid bacterium L. raffinolactis is prevalent in dairy foods, such as raw milks, natural dairy starter cultures, and a great variety of cheeses. L. raffinolactis BGTRK10-1 is a natural isolate from autochthonous young sweet kajmak produced in the Vlašić mountain region of central Bosnia and Herzegovina [17]. Strain BGTRK10-1 was selected because of its strong autoaggregation phenotype. In order to construct cosmid library of strain BGTRK10-1 to clone aggregation ability coding gene(s), total DNA of the strain was digested with several restriction enzymes (including EcoRI). It has been observed that the EcoRI did not cut isolated DNA, in several attempts, unlike the other used restriction enzymes. It was suspected that the strain possesses RM system (named LraI L. raffinolactis) that recognizes the same DNA sequence as EcoRI RM system.

Hence, the methylase activity of the strain L. raffinolactis BGTRK10-1, which protects its DNA from the digestion by EcoRI restriction enzyme, was quite accidentally discovered during routine laboratory work. Restriction endonucleases, commonly known as restriction enzymes, are ubiquitously present in prokaryotes. The main function of restriction enzymes is the protection against foreign genetic material, especially against bacteriophage DNA. Several restriction-modification systems have been identified in lactococci. Most of them are plasmid encoded and function as phage-resistance mechanism, which is very important for the strains used in the dairy industry in terms of preventing phage infection and cell lysis [25–29].

3.2. Selection of Clone Carrying LraI RM Operon from Cosmid Library. Cosmid DNA was isolated from total XbaI cosmid library in E. coli HB101 and 1 μg of DNA mix from total cosmid clones was subjected to digestion with EcoRI restriction enzyme and after that directly transformed into DH5α competent cells. Cosmid DNA isolated from obtained transformants was rechecked for resistance to EcoRI restriction enzyme digestion. One cosmid, named pAZILCosLra, providing resistance to EcoRI restriction enzyme digestion was selected for further analyses: subcloning and DNA sequencing (Table 1).

3.3. LraI Operon for RM System Provides Resistance to EcoRI Restriction Enzyme Digestion. To localize the minimum genetic unit on the cosmid pAZILCosLra that is responsible for the resistance to digestion with EcoRI restriction enzyme, the cosmid pAZILCosLra was digested with several restriction enzymes (XbaI-generated four fragments, HindIII-three
fragments, Clal-four fragments, and EcoRV-three fragments) and then subcloned into pBluescript SK+ vector digested with corresponding restriction enzymes. Only one construct, pBSLraCla (obtained with Clal), was able to reestablish the resistance to EcoRI restriction enzyme digestion (Table 1). The Clal DNA fragment of 4239 bp carrying complete information for resistance to EcoRI digestion was completely sequenced by primer walking. Four complete (EcoRI-like endonuclease, EcoRI-like methylase, hypothetical protein, and site specific integrase), one truncated (pentapeptide repeat containing protein), and one partial (N(5)-(carboxyethyl) ornithine synthase) open reading frame (ORFs) were revealed on Clal DNA fragment (Figure 1). Position of LraI RM operon in genome of strain BGTRK10-1 indicates the possibility that the operon was acquired by horizontal gene transfer; the conserved lactococcal gene for pentapeptide repeat containing protein is interrupted in the middle by LraI RM operon and immediately after methylease gene is located gene for site specific integrase. This event that occurred in the distant past is indicated by the fact that additional mutations were accumulated within the first part of the gene for pentapeptide repeat containing protein, most probably due to its non-functionality. The distance between the restrictase and the methylease genes is 10 nucleotides without promoter and ribosomal binding site and, in other EcoRI-like operons, strongly indicates polycistronic RNA transcription from upstream promoter and translation from consensus RBS (AGGAGA) 4 nucleotides distant from ATG codon of restrictase gene.

To confirm the functionality of LraI restriction-modification operon, a region that includes both lraIR and lraIM genes was amplified using LraRM-Fw and LraRM-Rev primers (for details see Section 2.2) and cloned into pAZIL vectors giving construct pAZIL-LraRM. Construct carrying only these two genes was completely sequenced while resistance to EcoRI restriction enzyme digestion was confirmed in vitro.

3.4. Cloning, Overexpression, and Purification of LraI Restriction Endonuclease. Plasmid clone pAZIL-LraRM was used as matrix for amplification of the open reading frame encoding LraI restriction endonuclease with primers LraI-Fw and LraI-Rev. Since HindIII restriction site has been integrated into LraI-Rev primer, obtained amplified fragment was first treated with HindIII to provide directed cloning of PCR fragment into expression vector pMAL-c5X, which was digested with XmnI and HindIII restriction enzymes. Ligation mix was transformed into ER2523 cells which were previously transformed with a pAZIL-LraRM vector expressing LraI methylase, in order to protect transformed cells from the nuclease activity of LraI towards their own. Transformants of ER2523/pAZIL-LraRM with pMAL-c5Xlral were successfully obtained when selection was carried out at 23°C on LA selective plates (erythromycin 300 μg/ml and ampicillin 100 μg/ml) containing 2% glucose in order to minimise expression of enzymes. Three clones (named pMAL-c5Xlral-29, pMAL-c5Xlral-31, and pMAL-c5Xlral-42, Table 1) were selected for restriction enzyme analysis, complete sequencing, and overexpression of enzyme. LraI restriction nuclease was successfully overexpressed in all three clones by over-night induction with 0.1 mM IPTG at 23°C and purified using amylose resins and cleaved by Xa protease (which cleaves fusion protein between maltose binding protein and clone providing release of exactly the same protein as natural). The overexpression of LraI restriction enzyme under aforementioned conditions (overnight induction with 0.1 mM IPTG at 23°C) represents the result that is similar to results observed by other researchers [30]. The possible explanation for this could be the expression of restriction enzymes is toxic at higher temperatures.

Purified LraI restriction enzymes from all three clones were stored at −20°C in CM buffer with 50% glycerol.

3.5. Functional Analysis, Determination of Ionic Strength, and Temperature Optimum of the Purified LraI Restriction Endonuclease Activity. Considering that LraI RM system provided complete protection against digestion of EcoRI endonuclease, it was assumed that it recognizes and cleaves the identical nucleotide sequence. Once plasmid pBluescript SK+ contains one EcoRI restriction site in polycloning region it was used for functional analysis of purified LraI restriction enzyme. Specific activity (1 IU) of purified LraI restriction enzyme was determined in EcoRI reaction buffer (Thermo Fisher Scientific) at 37°C. Different levels of LraI restriction enzyme expression were observed in selected clones, but specific activities (U/μg of purified proteins) were almost the same (1 U/50 ± 5 ng) among the clones pMAL-c5Xlral-29, pMAL-c5Xlral-31, and pMAL-c5Xlral-42 (Figure 2).

To determine optimal temperature for LraI activity, purified enzyme was incubated with pBluescript SK+ vector at temperatures ranging from 13°C to 80°C. LraI enzyme showed the highest activity at lower temperatures (between 13°C and 37°C), while at 45°C and higher temperatures it partially cut DNA (Figure 3). However, this is in agreement with the optimal growth temperature of strain BGTRK10-1 (30°C). Briefly, since the first description of EcoRI restriction enzyme in 1970 [31], more than 500 isochizomers have been reported or predicted with very high levels of identity (50–70%) pointing to the widespread distribution among species of different Phyla and indicating possible common origin. It seems that some specific characteristics, such as optimum working temperature, diverged depending on the optimum growth temperature of the enzyme producing bacteria, which is why we think that LraI exhibits better activity at lower temperatures. It was found that commercial EcoRI (used as control) showed high activity at 45°C in contrast to LraI enzyme pointing to the difference between these two enzymes.

In addition, stability of LraI enzyme was tested after different period of storage at −20°C; LraI enzyme did not lose activity after storage for more than six months at −20°C in CM buffer with 50% glycerol.

To establish the optimal salt concentration in the reaction buffer for LraI enzyme activity different commercial buffers, Buffer B, Buffer G, Buffer O, Buffer R, Buffer Tango, and buffer recommended for use with EcoRI (Thermo Fisher Scientific) were used. LraI enzyme exhibited high and specific activity in buffers with 100 mM and higher salt concentrations (Figure 4, buffer recommended for use with EcoRI, Buffer 2x Tango.
Figure 1: Schematic presentation of Clal DNA fragment of 4239 bp carrying complete information for providing resistance to EcoRI digestion. Clal DNA fragment containing following ORFs: truncated gene for pentapeptide repeat containing protein (Δorf1), EcoRI-like endonuclease (IraIR), EcoRI-like methylase (IraIM), hypothetical protein (hyp1), site specific integrase (intSS), and partial gene for N(5)-(carboxyethyl) ornithine synthase (parceo).

Figure 2: LraI activity assay. Digestion of pBluescript SK+ by purified LraI restriction enzyme LraI29, LraI31, and LraI42 from clones pMAL-cX5LraI-29, pMAL-cX5LraI-31, and pMAL-cX5LraI-42, respectively.

Figure 3: Determination of temperature optimum of the purified LraI restriction enzyme activity. Commercial EcoRI restriction enzyme was used in control reactions. It is interesting that in Buffer B and Buffer G commercial EcoRI restriction enzyme exhibited weaker activity, partial digestion.

3.6. LraI Is an Isoschizomer of EcoRI. Sequencing of double stranded cleaved DNA by the LraI enzyme has shown that the LraI enzyme recognizes the identical nucleotide sequence (5′-G/AATTC-3′), as expected, and cuts it at the same position (between G and A) like EcoRI enzyme (Figure 5) leaving identical sticky ends. The same cleavage results were obtained with LraI enzymes purified from all three clones which supported our conclusion that LraI enzyme is an isoschizomer of EcoRI.

3.7. Determination of the Cleavage Site of LraI Activity. One of the important characteristics of restriction enzymes is their high sequence specificity in order to adequately provide the function of protecting the genome integrity. In addition, it was established that restriction enzymes in nonoptimal conditions could exhibit a modified specificity so that the same restriction enzyme could recognize and cleave DNA at additional positions to canonical one [32]. For EcoRI restriction enzyme, it was detected that in at low ionic strength...
sequences GAATTG, at position 647; CAATTC, at position 850; TAATTC, at position 2824 were found), but were not cleaved, we conclude that LraI* activity specifically recognizes and cleaves only one variant of degenerate recognition sequence (5’-A/AATTC-3’). To further test the conclusion obtained on pBluescript SK+, the plasmid pAZIL sequence was analyzed and subjected to digestion by LraI enzyme under conditions that induce star activity. The obtained digestion results were completely in correlation with the predicted expectations (nine positions/fragments) (Figure 6(b)), so we could conclude that LraI* activity is limited to only one variant of the recognition sequence giving identical cohesive ends as in optimal conditions making the resulting fragments after LraI* activity cloneable without further processing into LraI or EcoRI treated vectors.

LraI restriction enzyme star activity meets all the given requirements: it recognizes only one variant of the sequence which can enable more precise restriction mapping and cloning, provides the same cohesive ends compatible with EcoRI (contained by most cloning vectors), and is completely controlled by low ion concentration and/or high pH. Both factors which induce star activity of LraI restriction enzyme, low ion strength (Buffer 1x Tango) and buffer with higher pH (Buffer R, pH 8.5), also influence on EcoRI, but, in contrast, LraI* recognizes only one additional sequence expressing more specific star activity.

3.8. Phylogenetic Similarity of LraI Restriction-Modification Enzymes with Others Belonging to EcoRI-Like Group. A search for number of restriction enzymes recognizing 5’-GAATTC-3’ sequence present in REBASE revealed 526 putative EcoRI-like proteins. Protein BLAST analysis showed that 196 restriction enzymes in NCBI database (from various microorganisms) share more than 50% identity on at least 50% protein coverage with LraI enzyme. Highest identity was observed with restriction endonucleases from streptococci (Streptococcus suis 68%, Streptococcus dysgalactiae 64%, and Streptococcus mutans 64%). It is interesting that similar but higher identity was observed for LraI methylase, again with streptococci (Streptococcus pseudopneumoniae 74%, Streptococcus suis 73%, Streptococcus mutans 73%, and Streptococcus dysgalactiae 68%). Similar percentage of identity was observed also at nucleotide level (about 70%) for both LraI restriction enzyme. The fact that most EcoRI isoschizomers, unlike other restriction enzymes, share a high level of identity, indicates their common origin [7].

Phylogenetic trees were constructed for both LraI restriction enzyme (Figure 7(a)) and methylase (Figure 7(b)) enzymes. Phylogenetic analysis showed that LraI homologs (restriction endonucleases and methylases) can be divided into two main branches, one (that could be additionally subdivided, comprising close homologs (Gram-negative bacteria and Cyanophyta) and the other comprising homologs from species belonging to Firmicutes phylum. The position of genus Fibrobacter (phylum: Fibrobacteres) which consists of only two species is interesting; its restriction enzyme belongs to one branch, while methylase protein belongs to the other (Figure 7).
Figure 6: Determination of the cleavage site of LraI* activity. (a) Digestion of pBluescript SK+ vector by LraI 29 (1 and 4), LraI 31 (2 and 5), and LraI 42 (3 and 6) in buffer recommended for use with EcoRI (1, 2, and 3) and in 1x Tango Buffer (4, 5, and 6); 7: undigested pBluescript SK+ vector by LraI 29 (1 and 4), LraI 31 (2 and 5), and LraI 42 (3 and 6) in buffer recommended for use with EcoRI (1, 2, and 3) and in 1x Tango Buffer (4, 5, and 6); 7: undigested pAZIL vector; L: ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific).

Figure 7: Phylogenetic similarity of LraI RM system with other belonging to EcoRI-like group constructed using a Tamura-Nei model. (a) Phylogenetic tree for LraI restriction; (b) phylogenetic tree for LraI methylase enzymes.
4. Conclusions

We identified a potent Type II restriction endonuclease in L. raffinolactis BGTRK10-1, named LraI. The recognition and cleavage sequence for LraI restriction enzyme was determined as $5'\text{-G/AATTTC-3'}$, indicating that LraI restriction enzyme is an isoschizomer of EcoRI but with different characteristics. One of characteristics that has been thoroughly studied is star activity of LraI restriction enzyme that is limited to one variant of the recognition site and cuts another alternative sequence $5'\text{-A/AATTTC-3'}$ leaving the same sticky ends on fragments as EcoRI, making the fragments obtained after digestion easy to clone without additional processing.

Conflicts of Interest

The authors declare no conflicts of interest.

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