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Determination of the recognition sequence of the type II restriction endonuclease, *Lla*CI, from *Lactococcus lactis* W15

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Abstract

A new type II restriction endonuclease, called *Lla*CI, was partially purified from *Lactococcus lactis* subsp. *cremoris* W15. The characterisation of the *Lla*CI endonuclease showed it to be an isoschizomer of *Hin*dIII, recognising the sequence 5-′A↓AGCTT-3′. The cleavage site is indicated by the arrow.

Keywords: *Lactococcus lactis*; Lactic acid bacteria; *Lla*CI; Type II restriction endonuclease; *Hin*dIII isoschizomer; Recognition sequence

1. Introduction

Lactococcus lactis is widely used in starter cultures for the manufacture of dairy products causing characteristic texture and flavour changes and exercising a preservative effect on the fermented product. During the fermentation processes, the lactococci are often challenged with a variety of bacteriophages, the most common belonging to the 936, P335, and c2 species [1]. As the fermentation cannot be performed aseptically the starter culture must be phage resistant. This demand may be fulfilled by constructing lactococcal strains with improved phage resistance, however, this requires knowledge of naturally occurring phage defence mechanisms in *L. lactis*. The phage defence mechanisms have been classified into the following four groups based on their mode of action, interference with phage adsorption, inhibition of phage DNA injection, restriction-modification (R/M) systems, and abortive infection systems [2]. R/M systems appear to be the most frequently encountered resistance mechanisms in *L. lactis* [3].

Until now only the recognition sequences of the five following restriction endonucleases (R-ENases) endogenous to lactococci have been published, *Scr*FI recognising 5′-CC↓NGG-3′ where N is any nucleotide [4], *Lla*497I (before named *Lla*I) recognising 5′-CCWGG-3′ [5] where W is A or T, *Lla*AI [6] and *Lla*DCHI [7] recognising 5′-↓GATC-3′, and *Lla*BI recognising 5′-C↓TRYAG-3′ where R is A or G, and Y is C or T [6].

Since type II R/M systems are easier to detect, and presumably also to clone, than other types of R/M system, we screened *L. lactis* strains, isolated from a mixed Cheddar cheese starter culture [8], for the presence of type II R-ENase activity. We found

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that *L. lactis* W15 expressed type II R-ENase activity, denoted *Lla*CI [9]. In this paper, the purification of the R-ENase *Lla*CI and determination of its recognition/cleavage site is presented.

2. Materials and methods

2.1. Bacteria and growth conditions

L. lactis subsp. *cremoris* strain W15 was grown in GM17 at 30°C without shaking [10]. *E. coli* strain XL1-Blue was grown in LB media at 37°C. The concentration of ampicillin was 50 μg ml^{-1}. Plasmid DNA was prepared by the alkaline lysis method, or for sequencing by the CsCl gradient method [11]. The vector pBluescript SKII+ (Strategene, La Jolla, USA) was used for subcloning.

2.2. Purification of the LlaCI R-ENase

An overnight culture (50 ml) of *L. lactis* W15 was harvested by centrifugation, washed once in buffer A (20 mM phosphate pH 7.8, 5% glycerol, 5 mM DTE), and resuspended in 5 ml cold buffer A. The cells were ruptured by passing them through a French Press. Cell debris was removed by centrifugation (224 000 × *g*; 2 h; 4°C). The R-ENase was partially purified by a one-step FPLC chromatographic procedure, using a 1.0 × 12 cm CM-Sepharose Fast Flow (Pharmacia Biotech Europa GmbH, Freiburg, Germany) column. After sample application, the column was washed with buffer A, and eluted with 100 ml of a KCl linear gradient (0–0.5 M KCl) in buffer A.

2.3. Assay for LlaCI activity

Unless otherwise specified the enzyme activity was performed at 37°C for 1 h with a 5 μl sample in the reaction mixture (15 μl) containing NEBuffer 2 (10 mM Tris-Acetate pH 7.9, 10 mM MgCl, 50 mM NaCl, 1 mM DTT) from New England Biolabs GmbH (Schwalbach, Germany), and 0.4 μg non-methylated lambda DNA. Electrophoresis was performed on a 0.8% agarose gel. One unit was defined as the amount of enzyme required to digest 1 μg of lambda DNA completely in 60 min at 37°C.

2.4. DNA methods

Digestion by R-ENases, subcloning of fragments, and transformation of *E. coli*, was carried out by standard procedures [11]. The nucleotide sequence was determined by the dideoxynucleotide chain termination method [12] using double stranded plasmid template DNA, the Sequencing Kit, (S^{35})-dATP, and standard primers complementary to the region of the plasmid upstream of the fragments.

3. Results

3.1. Purification of the LlaCI R-ENase

The R-ENase was purified from 50 ml overnight

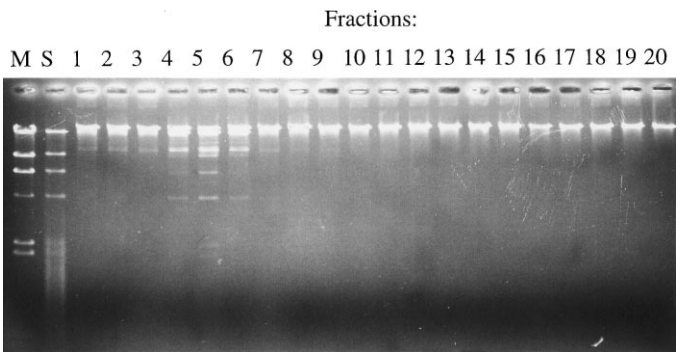

Fig. 1. *Lla*CI R-ENase activity of column fractions after CM-Sepharose chromatography. The R-ENase assays were performed as described in Section 2 with 0.4 μg lambda DNA. S, R-ENase activity of sample prior to column chromatography; 1–20, R-EN activity of fractions; M, *Hin*dIII-digested lambda DNA markers (0.4 μg).

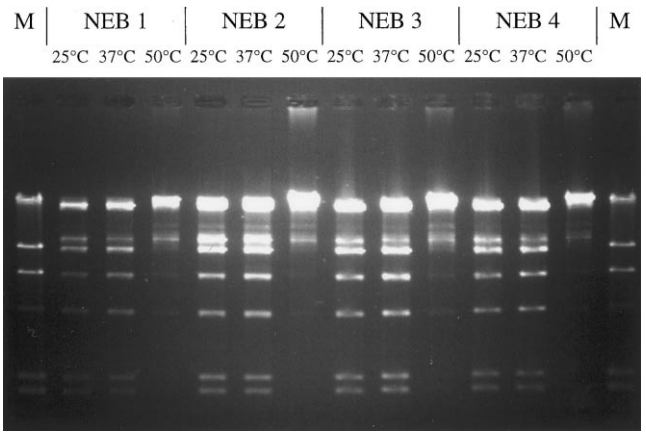

Fig. 2. *Lla*CI R-ENase assay performed with different NEBuffers at 25°C, 37°C, and 50°C. The R-ENase assays were performed as described in Section 2 with 0.4 μg lambda DNA. M, *Hin*dIII-digested lambda DNA markers (0.4 μg).

culture. After cell disruption by a French Press and cation exchange chromatography the fractions were assayed for R-ENase activity as described in Section 2. The results in Fig. 1 show that the type II R-ENase, which cleaved lambda DNA, was eluted from the column in fractions 4–6 at between 80 mM and 150 mM (KCl). No non-specific nucleases were discovered. The total yield of *Lla*CI R-ENase in fractions 4–6 was estimated to be 14 000 U.

3.2. Optimal conditions for activity and storage of the LlaCI R-ENase

The *Lla*CI R-ENase assay was conducted in different commercial buffers from New England Biolabs (NEBuffer 1, 2, 3 and 4) at 25°C, 37°C and 50°C. The results in Fig. 2 show that the *Lla*CI activity was about the same at 25°C and 37°C and very low at 50°C. Of the different commercial buffers examined, both NEBuffer 2 and 3 gave a high R-ENase activity. The results in Fig. 3 show the stability of the *Lla*CI R-ENase when stored in varying amounts of glycerol (final concentration: 5%, 14%, 21%, 32%, and 45%) at −20°C or at 4°C for 21 days. The results show that the stability of the *Lla*CI R-ENase was higher after storage at −20°C than at +4°C. Addition of glycerol also improved the stability of the *Lla*CI R-ENase. Fig. 3 shows that the highest stability was obtained with the addition of 32% glycerol.

3.3. Determination of the recognition sequence

In order to determine the recognition sequence of the R-ENase *Lla*CI, plasmid pSA3 [13] was digested with partially purified *Lla*CI R-ENase. The obtained fragments were treated with Mung Bean nuclease to create blunt ends [11] or filled out with the Klenow fragment of the *E. coli* DNA polymerase. The blunt-ended pSA3 *Lla*CI-digested fragments were ligated

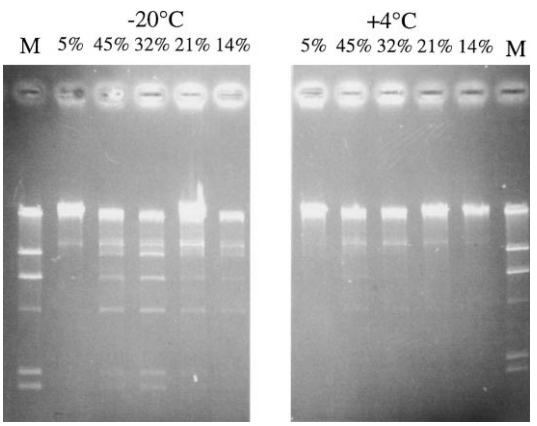

Fig. 3. *Lla*CI R-ENase activity after storage at −20°C and +4°C in varying amounts of glycerol. The R-ENase assays were performed as described in Section 2 with 0.4 μg lambda DNA. A sample of fraction 5 eluted from the CM-Sepharose column was stored for 21 days at −20°C and +4°C with the indicated final concentration of glycerol. M, 0.4 μg *Hin*dIII-digested lambda DNA markers.

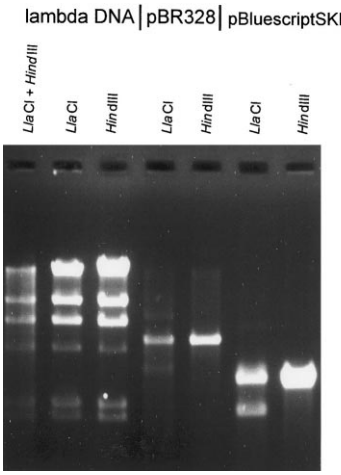

Fig. 4. Agarose gel showing the restriction patterns obtained by cleaving different DNA substrates with *Lla*CI and *Hin*dIII. The R-ENase assays were performed as described in Section 2 with lambda, pBR328, and pBluescript SKII+ DNA as substrates.

into pBluescript IISK+ vector, digested with *Eco*RV, and clones containing inserts were isolated. The sequencing of these clones revealed the recognition sequence 5′-A↓AGCTT-3′, where the cleavage site is indicated with an arrow. To confirm this result the plasmid pSA3 was digested with *Lla*CI and the fragments obtained were cloned without any modification into pBluescript IISK+ digested with *Hin*dIII.

The endonuclease activity was compared with the commercial *Hin*dIII endonuclease. As shown in Fig. 4 the cleavage patterns of the R-ENases *Lla*CI and *Hin*dIII on lambda, pBR 328 and pBluescript IISK+ DNA were identical, and double digestions of lambda DNA with both *Lla*CI and *Hin*dIII resulted in the same fragments as produced in single digestion with *Lla*CI or *Hin*dIII. The results show that *Lla*CI and *Hin*dIII are isoschizomers.

4. Discussion

In this paper we have determined the specificity of a new type II R/M system. It recognises the sequence 5′-A↓AGCTT-3′, the same sequence as the commercial R-ENase *Hin*dIII. Phages, earlier isolated from whey samples collected at a dairy plant using the Cheddar cheese starter culture (where *L. lactis* W15 was isolated from), have been characterised with respect to their number of cleavage sites for different restriction endonucleases [14]. We found that the phages belonging to the 936 species have fewer *Hin*dIII sites (from 0–2) than expected (10 sites) in their genome. Since the efficiency of plating decreases logarithmically as the number of sites in the phage DNA increases [15] phages may escape restriction by omitting sites for restriction endonucleases. The reduced number of *Hin*dIII/*Lla*CI sites in the isometric headed 936, may indicate that these phages are often exposed to R/M systems with *Lla*CI specificity. However, we only found two, out of the 62 *L. lactis* strains, isolated from the mixed Cheddar cheese starter culture, that expressed *Lla*CI R-ENase activity [9].

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