

ISOLATION AND CHARACTERIZATION OF *BliAI*, AN ISOSCHIZOMER OF *ClAI* FROM *BACILLUS LICHENIFORMIS*

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ABSTRACT

The restriction endonuclease *BliAI*, an isoschizomer of *ClAI*, which recognizes the sequence 5'-AT↓CGAT-3', was purified from a natural isolate identified as *Bacillus licheniformis*. The restriction endonuclease was isolated from cell extracts using single-step purification by phosphocellulose column chromatography. The restriction endonuclease is active at 37°C and over a wide range of pH and salt concentration. The molecular weight of the purified restriction enzyme is consistent with a value of 39 kDa.

Key words: type II restriction endonuclease, *BliAI*, *ClAI*, isoschizomers, *Bacillus licheniformis*

INTRODUCTION

Type II restriction-modification (R-M) systems are pairs of enzymes: one, a restriction endonuclease (RE); the other, a methyltransferase, with opposing intracellular activities (21,30). The RE recognizes specific sequences on DNA and cleaves it at a particular site, whereas the cognate methyltransferase modifies DNA within the same recognition sequence, thereby preventing cleavage by the RE. By possessing these two opposing enzymes, bacteria may protect their own DNA and still degrade foreign DNA, thereby limiting the spread of invading DNA molecules within the bacterial population (2,8,13,29). In addition, DNA methylation may be involved in the regulation of chromosomal DNA replication (14,27) and gene expression (5,10), transposon movement (20), or DNA mismatch repair (15).

Restriction endonucleases play a crucial role in the control of phage propagation and lateral gene spreading in natural environments. Type II restriction endonucleases activities of *Bacillus licheniformis* strains have been detected in many environments and analyzed by conventional techniques (22).

The present study relates the characterization of an isoschizomer of *ClAI* from *B. licheniformis*, *BliAI*, which recognizes the 6-base pair interrupted palindrome 5'-AT↓CGAT-3', and cleaves double-stranded DNA after the first T in the sequence, producing 2-nucleotide long 5'-protruding ends.

MATERIALS AND METHODS

Bacterial strain isolation and cultivation

B. licheniformis bacteria were isolated from samples of the water collected in the Amazon river on the outskirts of the city of Manaus (60° 1'W, 3° 7'S). At the time of the sampling the temperature was 36°C. Bacterial isolation (colony isolation), growth, harvesting, washing and storage have been described elsewhere (6,7).

Identification of bacterial isolates

Bacterial identification was performed by small-subunit rRNA gene (SSUrDNA) DNA sequencing (17). The bacterial genomic DNA was purified using a QIAGEN Genomic Tip 20/G (QIAGEN-GmbH), yielding 13 µg from 2 mL. The SSU rDNA was amplified

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by 30 cycles of PCR using primers 530F (5' TGA CTG ACT GAG TGC CAG CMG CCG CGG 3') and 1492R (5' TGA CTG ACT GAG AGC TCT ACC TTG TTA CGM YTT 3') (Invitrogen - USA) (4). The PCR mixture (50 μ L) contained 1 μ L of template DNA, 50 mM Tris pH 8.4, 500 μ g/mL of BSA, 1.5 mM MgCl₂, 250 μ M dNTPs, 400 nM of each primer, and 2.5U of Taq DNA Polymerase (CenBiot Enzimas - BR). All reagents were combined and heated at 94°C for 2 min. Thirty-five cycles of PCR were performed in a PTC 200 thermocycler (MJ Research, USA) as follows: 94°C for 2 min, 50°C for 30 seconds, and 72°C for 2 min; followed by a final elongation at 72°C for 2 min. The PCR products were directly sequenced with a DYEnamic ET Dye Terminator Cycle Sequencing Kit in the MegaBACE 1000 System (Amersham Pharmacia Biotech – UK), using 530F and 1492R primers. PCR amplification and amplicon DNA sequencing were repeated five times in order to confirm the results. The sequences were analyzed by multiple sequence alignments using the computer program Clustal X (11). The consensus sequence derived from the multiple alignment was used to search the GenBank database using BLAST (1).

Purification procedure

Cells from a log-phase culture were disrupted by three passes through a French press. The purification was based on a protocol described elsewhere (9) with minor modifications. Cell debris was first removed by centrifugation. Streptomycin sulfate was then slowly added to the resulting supernatant to a final concentration of 1.8% in order to remove DNA, which was then centrifuged at 28,000 \times *g* for 1h at 4°C. The supernatant was dialyzed against PC Buffer (100 mM K₂HPO₄/KH₂PO₄ pH 7.5, containing 0.1 mM EDTA, 10 mM β -mercaptoethanol (β -ME), 150 mM NaCl, and 0.02 mM phenyl-methyl-sulfonyl-fluoride (PMSF) and clarified at 28,000 \times *g* for 20 min at 4°C. The clear supernatant was loaded on a 2 \times 12 cm phosphocellulose column (P11, Whatman Inc, USA) previously equilibrated with PC buffer, washed with three column volumes with same buffer and eluted with a NaCl linear gradient (0 – 600mM). Active fractions were identified by digestion of bacteriophage λ DNA in “One for all” buffer (Pharmacia, UK)

Fractions showing restriction activity were pooled out and dialyzed into long-term storage buffer (50% glycerol /PC buffer containing 50 mM NaCl), and stored at -20°C.

Molecular weight determination

The molecular weight of the enzyme was estimated by gel filtration chromatography using an FPLC Superdex-75 HR 10/30 column (Amersham Pharmacia Biotech) previously calibrated with the following molecular weight markers: 65 kDa, bovine serum albumin; 45 kDa, ovalbumin; 36 kDa, glyceraldehyde 3-phosphate dehydrogenase; 29 kDa, bovine carbonic anhydrase; 24 kDa trypsinogen; 12.4 kDa, horse heart cytochrome and 6.5 kDa aprotinin.

Enzyme characterization, quality control tests, and restriction cleavage analysis

The optimum salt concentration, pH and temperature were determined for the purified enzyme preparation with a set of SuRE/Cut buffers for restriction enzymes, namely A, B, H, L and M (Boehringer Mannheim), using a standard protocol (3) in the presence of 0.5 μ g of λ DNA and 1U of enzyme. One unit of enzyme activity is defined as the amount of restriction endonuclease required to totally digest 1 μ g of λ DNA in 1 h at 37°C in a reaction volume of 50 μ l. The purity of the endonuclease preparation with respect to “non-specific-nucleases” was evaluated by “over-digestion” tests. Restriction cleavage analysis was performed by the digestion of DNAs of bacteriophages λ and ϕ X174 and plasmids pBR322 and pCI-neo, using the same set of SuRE/Cut buffers as described above. DNA fragments were analyzed by gel electrophoresis (24). A commercial enzyme *Cla*I (Invitrogen) was used as control.

Determination of the cleavage site of *Bli*AI using pCRBlunt+*mecA* DNA

The DNA of a pCRBlunt plasmid plus a stretch of 245 nucleotides was inserted in the specific site located at the multiple cloning site. The stretch of the *mecA* gene from *Staphylococcus aureus* used was the nucleotide between 1106 and 1351 (23). The purified restriction enzyme in the presence of Mg²⁺ at 37°C in SuRE/Cut buffer H for 1 hour, was used in order to determine the cleavage site of this restriction enzyme, and sequencing of the digested DNA solution was performed by the chain termination method (25) using ³³P-dNTPs and the Thermo Sequenase radiolabeled terminator cycle sequencing Kit (Amersham Pharmacia Biotech – UK). The primers utilized were as follows: -T7 Promoter Primer, 5'-TAA TAC CAC TCA CTA TAG GG-3' (Novagen Cat n° 69348-3) and M13 reverse, 5'-CAG GAA ACA GCT ATG AC-3' (Invitrogen Cat n° 13432013). The same protocols for DNA cleavage and sequencing were performed using commercially available *Cla*I (Invitrogen).

RESULTS AND DISCUSSION

Sequencing of SSUrDNA and sequence alignments to other 16S rRNA coding DNA sequences revealed 99% identity with *B. liqueniformis* 16S ribosomal RNA gene, partial sequence GenBank accession D31739. *Bacillus licheniformis* [gi:497790].

The molecular weight of the purified restriction enzyme was estimated by gel filtration and the elution profile was consistent with a value of 39 kDa for the native protein. The optimum pH, temperature, and salt concentration were estimated to be, respectively, 7.5 at 37°C in SuRE/Cut Buffer H (50 mM Tris-HCl, containing, 10 mM MgCl₂, 100 mM NaCl, and 1 mM dithioerythritol). Similarly, at pH 8.0 in the above buffer, activity

was essentially the same as well in SuRE/Cut Buffer M at pH 7.5 at 37°C.

One unit of *BliAI* is defined as the amount of enzyme required to cleave 1 µg of λ DNA in a suitable buffer in one hour at 37°C in a 50-µL reaction.

The restriction fragment patterns produced by the purified enzyme and commercially available *ClaI* analyzed by the digestion of DNAs of bacteriophages λ and φX174 and plasmids pBR322 and pCI-neo were compared to allow the determination of the recognition sequence of the *B.licheniformis* RE. It was found that for λ DNA there were 16 restriction fragments, and for the substrates φX174, pBR322 and pCI-neo there were zero, 1 and 2 restriction fragments, respectively (Fig. 1). The cleavage restriction patterns are identical to the one seen with *ClaI*, suggesting that the unknown restriction enzyme recognizes the same DNA sequence as *ClaI* does.

The primers designed to anneal specifically either to a DNA sequence starting from nucleotide 205 or from nucleotide 400 of the original plasmid pCR Blunt, allow the synthesis of single-stranded DNA of different lengths. This stretch of DNA sequence, part of the *mecA* gene from *S. aureus*, was chosen

because it possesses only one *ClaI* digestion site. The nucleotide sequence determination by the dideoxy chain termination method of the amplified fragments, produced by pCR Blunt+mecA plasmid DNA digestion with the *B.licheniformis* enzyme and *ClaI*, clearly showed that 5'-AT↓CGAT-3 is the recognition sequence (Fig. 2). The DNA sequencing results confirmed the restriction cleavage analysis

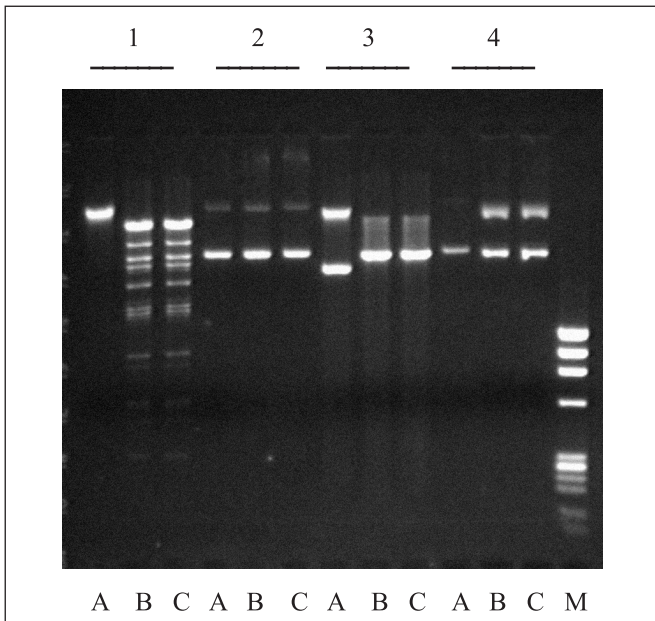


Figure 1. Agarose gel electrophoresis of restriction fragments produced by cleavage of four DNA substrates. The cleaved DNAs were separated by electrophoresis on 0.8% agarose gel and visualized under UV after ethidium bromide staining. (1) λ phage DNA, (2) φX174 phage DNA, (3) pBR322 DNA and (4) pCI-neo DNA. (A) undigested DNA, (B) *BliAI* and (C) *ClaI* (invitrogen-USA) (M) 1 µg φX174/*HaeIII* markers (G1761-Promega).

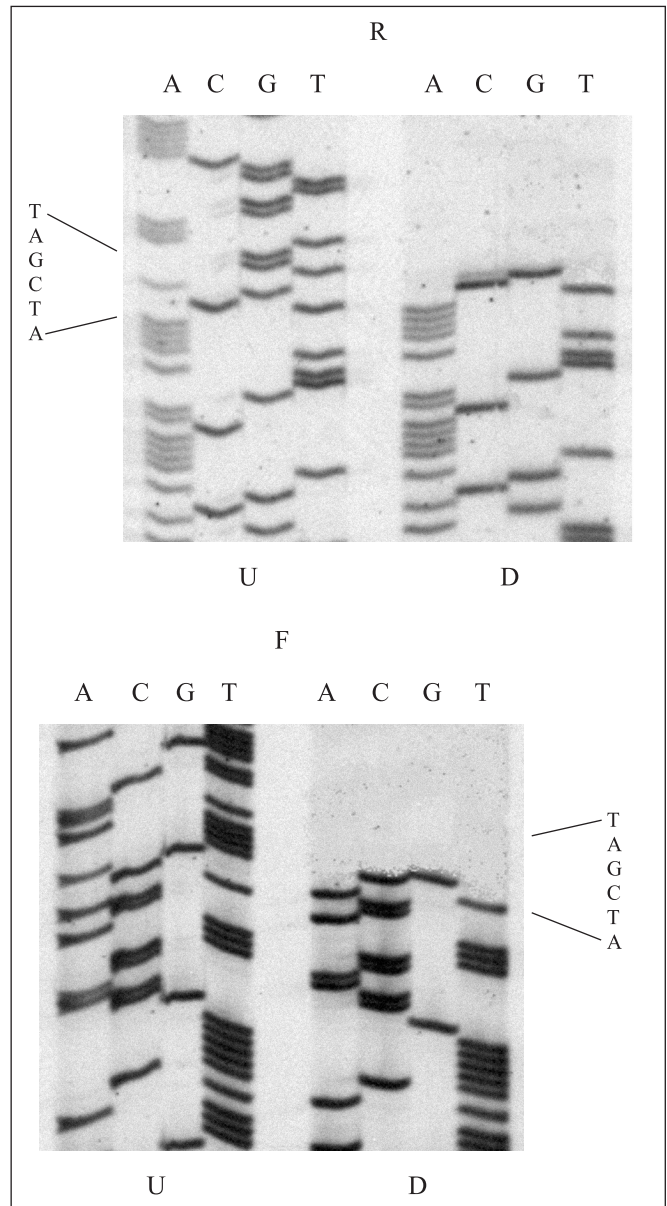


Figure 2. DNA sequencing gel showing the labeled product, approximately 20 bases either side of the putative cut site of *BliAI*. R and F are the amplification products using, respectively, the reverse and forward primers. U and D are the same amplification products undigested and digested, respectively.

and strongly suggest that the *B. licheniformis* restriction enzyme is an isoschizomer of *ClaI*.

The restriction endonuclease *ClaI* was first described in 1981 (26). The first communication of an isoschizomer of *ClaI* from *B. licheniformis* *Bli41I* was described eight years later (19), at the same time that another isoschizomer of *ClaI* from *B. licheniformis* *Bli86I* was described (12). Soon after other isoschizomers of this same enzyme were also described, *BliRI* in 1992 (16) and *Bli576I* and *Bli585I* in 1995 (18). However, since 1995, no *ClaI* isoschizomers from *B. licheniformis* have been described.

To date, 102 isoschizomers of this restriction endonuclease have been found in many bacterial genera, 44 of them in the genus *Bacillus*, five in the species *B. licheniformis*. They are listed on the web server REBASE (<http://rebase.neb.com>) including nine commercially available from 17 different suppliers.

Apart from the five *ClaI* isoschizomers found in the species *B. licheniformis*, there are eight more type II RE isoschizomers described in this bacterial species, none as a prototype.

The type-II restriction enzyme reported here was named *BliAI*, *Bli* as recommended by the official nomenclature (28), and the intervening letter "A" for Amazon as suggested by Dr. Richard Roberts, since this bacteria was found in the Amazon River.

The *BliAI* enzyme has been deposited on the official REBASE web site (www/http.rebase.neb.com) under enzyme number # 6509.

RESUMO

Isolamento e caracterização de *BliAI*, um isoesquisômero de *ClaI* de *Bacillus licheniformis*

A endonuclease de restrição *BliAI*, um isoesquisômero de *ClaI*, que reconhece a seqüência 5'-AT↓CGAT-3', foi purificada de um isolado natural identificado como *Bacillus licheniformis*. A endonuclease de restrição em questão foi isolada a partir de um extrato celular em um único passo cromatográfico utilizando uma coluna contendo a resina fosfocelulose. A endonuclease de restrição é ativa à 37°C e em uma ampla escala de pH e concentração de sais. O peso molecular da enzima purificada corresponde a um valor de kDa 39.

Palavras-chave: Endonuclease de restrição do tipo II, isoesquisômero, *Bacillus licheniformis*

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