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Site-specific endonuclease BslI recognizes DNA sequence 5'-G(5mC)N[^]GC-3' and cleaves it producing 3' sticky ends

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A bacterial strain Bacillus simplex 23, a producer of a site-specific endonuclease BslI has been discovered. BslI recognizes the methylated DNA sequence 5'-G(5mC)N↓GC-3', like the earlier described site-specific endonuclease BsiI (recognition site 5'-G(5mC)↓NGC-3'), but differs in positions of DNA cleavage producing 3'-protruding ends. Due to its ability to cleave only methylated DNA, enzyme BslI can find an application in gene engineering works as well as in determining the methylation status of eucaryotic DNA.

INTRODUCTION

A few site-specific endonucleases are currently known which recognize and cleave methylated DNA only. These enzymes do not require cofactors besides Mg²⁺ ions and, therefore, can be considered to be IIM site-specific endonucleases [1]. These are restriction endonuclease *DpnI*, which recognizes and cleaves the nucleotide sequence 5'-G(6mA)↓TC-3' [2] and site-specific endonucleases *BsiI* and *GlaI* described in our works. The latter ones recognize and cleave DNA sequences 5'-G(5mC)↓NGC-3' [3] and 5'-G(5mC)↓G(5mC)-3' [4], [5], respectively.

The present paper describes a new enzyme *BslI*, which belong to IIM type of site-specific endonucleases and is a neoschizomer of recently discovered enzyme *BsiI* [3]. *BslI* recognizes the nucleotide sequence 5'-G(5mC)N↓GC-3' and cleaves it as shown by an arrow with the formation of 3'-protruding ends.

EXPERIMENTAL CONDITIONS

The producer strain growth

The bacterial strain *Bacillus simplex* 23 was isolated from soil according to screening procedure described earlier [6]. Morphological and physiological-biochemical properties of the strain were studied as described in [7]. The strain was identified according to the classifier [8] as well as by analyzing the nucleotide sequence of 16S ribosomal RNA [9].

The strain was grown in a fermenter at the temperature of 30°C in 20 L of nutrient medium containing 1% Trypton (Organotechnie, France), 0.5% yeast extract (Organotechnie, France), 0.5% NaCl and 0.05% MgCl₂, at pH 7.5 with aeration at 10 L/min and stirring at 200 rev/min. When the culture reached the late logarithmic growth stage, the cells were harvested by centrifugation. As a result, 100 g of biomass was obtained and frozen at -20°C.

Enzyme isolation

All procedures of enzyme isolation were performed at the temperature 4°C. Forty grams of frozen cells were suspended in 100 ml of Buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM β-mercaptoethanol) containing 0.2 M KCl, 0.3 mg/ml of lysozyme and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). The cells were disrupted by ultrasonic disintegrator Soniprep 150 (MSE, England) 10 times 1 min each at 1 min intervals to cool the suspension.

The crude lysate was clarified by centrifugation for 30 min at 12,000g on a J-2-21 centrifuge (Beckman, USA). Enzyme was isolated by chromatographic purification of the supernatant on the following resins: 30 ml of phosphocellulose P-11 (Whatman, England), 5 ml hydroxyapatite (Bio-Rad, USA), 7 ml of heparin-sepharose (Bio-Rad, USA).

First the supernatant was applied to a P-11 phosphocellulose column pre-equilibrated with Buffer A containing 0.2 M KCl. The column was washed with 60 ml of Buffer A containing 0.2 M KCl, then with a linear gradient of the KCl concentration (0.2 M - 1.5 M) in Buffer A. Fractions with endonuclease activity were combined and applied to a hydroxyapatite column pre-equilibrated with Buffer B (10 mM KH₂PO₄, pH 7.5, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.2 M KCl). The column was washed with 10 ml of Buffer B, then the protein was eluted by a linear gradient of the KH₂PO₄ concentration (0.01 M - 0.5 M) in Buffer B. Active fractions were combined, dialyzed against 20 volumes of Buffer A and applied to a heparin-sepharose column equilibrated with Buffer A containing 0.2 M KCl. The column was washed with 14 ml of Buffer A containing 0.2 M KCl. The enzyme was eluted by a linear gradient of the KCl concentration (0.2 M – 1 M) in Buffer A. Active fractions were combined and dialyzed against a concentrating buffer (50% glycerol, 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 0.2 M KCl). The enzyme preparation was stored at -20°C.

Enzyme activity determination

The plasmid pFsp4HI1 was used as a substrate to determine the activity of *B*/sl [9]. This plasmid contains Fsp4HI DNA-methyltransferase gene from *Flavobacterium* sp. 4H, which modifies the first cytosine base in the nucleotide sequence 5'-GCNGC-3'. Thus, plasmid pFsp4HI1 contains methylated sites 5'-G(5mC)NGC-3'/3'-CGN(5mC)G-5'.

B/sl displays the maximum of activity in a buffer of the following composition: 10 mM Tris-HCl, pH 8.5, 5

mM MgCl₂, 100 mM NaCl, and 1 mM β-mercaptoethanol supplemented with BSA (bovine serum albumin) to the final concentration of 0.1 mg/ml. This buffer was used in all experiments on DNA cleavage by *B*/sl.

The activity of the site-specific endonuclease *B*/sl in the course of purification was determined by incubating aliquots of fractions with pFsp4HI1 plasmid DNA. 2 µl- aliquots of the corresponding fractions from the chromatographic purification were added to 40 µl of the reaction mixture containing 2 µg of pFsp4HI1 plasmid DNA in the reaction buffer. The reaction mixture was incubated for 30 min at 30°C. The reaction products were separated in 1% agarose gel.

The minimal amount of the enzyme required for complete cleavage of 1 µg of pFsp4HI1 plasmid DNA in 20 µl of the reaction mixture at the temperature 30° C in 1 hour was considered to be 1 unit of enzymatic activity (u.)

Determination of the recognition site and positions of DNA hydrolysis

The patterns of specific cleavage of different DNAs were used to determine the specificity of enzyme *B*/sl. Different plasmid and phage DNAs as well as synthetic oligonucleotide DNA duplexes containing or non-containing methylated bases were used as substrates in determining the enzyme specificity. The recognition site of the site-specific endonuclease *B*/sl was determined by comparison of pFsp4HI1 plasmid DNA hydrolysis by enzymes *B*/sl and *B*/sl. DNA cleavage was performed under optimal conditions (30°C, reaction buffer – 10 mM TrisHCl, pH 8.5, 5 mM MgCl₂, 100 mM NaCl, 1 mM β-mercaptoethanol, 0.1 mg/ml BSA) for 1 hour. The cleavage products of plasmid DNA were separated by gel-electrophoresis in 1.5% agarose. The recognition site was confirmed in experiments on cleavage of synthetic oligonucleotide duplexes.

The positions of DNA hydrolysis by the site-specific endonuclease *B*/sl were determined by comparison of the fragments lengths after cleavage of a [³²P]-labeled synthetic oligonucleotide duplex with endonucleases *B*/sl and *B*/sl. The products of partial cleavage of the same duplex by endonuclease III from *E.coli* (ExoIII) were used as a fragment length marker. Hydrolysis products were separated by electrophoresis in 20% PAAG with 7 M urea in tris-borate buffer.

RESULTS AND DISCUSSION

The bacterial strain producing enzyme *B*/sl has the following characteristics. It forms white, smooth, bright, non-transparent, convex, round colonies 4 mm in diameter on Luria-Betrani medium agar. The cells are rod-like of 1x(3-5) µm, single or arranged in pairs or short chains. They form elliptic centrally located spores, which do not inflate sporangia. Cells are gram-positive, catalase-positive and oxidase-negative and do not grow under anaerobic conditions. They grow at the temperature of 10-40°C. Based on morphological and biochemical properties as well as the nucleotide sequence of 16S ribosomal RNA, the strain was identified as *Bacillus simplex* 23. Site-specific endonuclease produced by it was called *B*/sl.

The enzyme was isolated from the cellular extract by consecutive chromatographic procedures as described in “Experimental conditions”. The enzyme yield was 200 u./g of raw biomass, and the concentration was 1,000 u./ml.

Endonuclease *B*/sl does not hydrolyze standard viral and plasmid DNAs used to detect and to determine the activity of restriction endonucleases such as the DNAs of phages λ and T7, adenovirus-2, plasmids pUC19 and pBR322. Plasmids containing the genes of some bacterial methylases modifying cytosine bases in the DNA were also tested in DNA cleavage experiments: M.Fsp4HI methylates DNA with the formation of the sequence 5'-G(5mC)NGC-3' [10], M.HspAI modifies DNA with the formation of the sequence 5'-G(5mC)GC-3' [5], M.FauIA and M.FauIB methylate DNA with the formation of the sequences 5'-C(5mC)CGC-3' and 5'-G(5mC)GGG-3', respectively [11]. The results have shown that enzyme *B*/sl specifically cleaves only pFsp4HI1 plasmid DNA which carries 5'-G(5mC)NGC-3'/3'CGN(5mC)G-5' DNA sequences.

Figure 1 presents the results of pUC19 and pFsp4HI1 plasmids DNA hydrolysis by the site-specific endonucleases *B*/sl and *B*/sl. *B*/sl and *B*/sl don't cleave pUC19 and cut pFsp4HI1 plasmid DNA in the same way. The obtained data show that site-specific endonucleases *B*/sl and *B*/sl cleaves only methylated DNA and *B*/sl and *B*/sl have a similar recognition sequence.

Fig. 1. The comparison of the DNA fragment lengths after pFsp4HI1 (A) and pUC19 (B) DNA cleavage by endonucleases *B*/sl (run 1) and *B*/sl (run 2). Electrophoresis in 1/5% agarose gel. K - pFsp4HI1 plasmid DNA, M – 1 Kb molecular weight DNA marker (SybEnzyme) with the following DNA fragment lengths (kb): 10; 8; 6; 5; 4; 3; 2.5; 2; 1.5; 1; 0.75; 0.5; 0.25.

Experiments on the cleavage of synthetic oligonucleotide duplexes containing a methylated or non-methylated recognition site (the sequence 5'-GCNGC-3' is underlined) were performed to confirm these data and to determine the DNA cleavage positions:

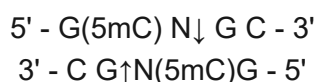
NN01: 5' GCTTGTACTTTAGCGGCATTGATTCTCACCACG 3'
 NN02: 5' CGTGGTGAGAATCAATGCCGCATAAAGTACAAGC 3'
 NN1: 5' GCTTGTACTTTAG(5mC)GGCATTGATTCTCACCACG 3'
 NN2: 5' CGTGGTGAGAATCAATG(5mC)CGCTAAAGTACAAGC 3'

As it is seen from Fig.2, endonuclease *B*/sl cleaves duplex NN1*/NN2 containing the methylated sequence 5'-GCNGC-3', but does not cleave duplex NN01*/NN02 containing the same non-methylated sequence. Thus, *B*/sl really cleaves only methylated nucleotide sequences 5'-G(5mC)NGC-3'.

Fig. 2. The cleavage of oligonucleotide duplexes NN01*/NN02 (a) and NN1*/NN2 (b) by endonucleases Fsp4HI (run 2) and Bsl (run 3). Run 1 – control DNA duplex without hydrolysis. Labeled oligonucleotides are shown with the sign *.

Fig. 3. The cleavage of oligonucleotide duplexes NN1*/NN2 (a) and NN2*/NN1 (b) by endonucleases *B*/sl (run 2) and *B*/sl (run 4). Run 1 – control duplex without hydrolysis. Run 3 – duplex hydrolyzed by exonuclease III from *E. coli*. Labeled oligonucleotides are shown with the sign *

The position of DNA cleavage by site-specific endonuclease *B*/sl was determined by comparing the lengths of the fragments produced in the cleavage of the oligonucleotide duplex NN1/NN2 by endonucleases *B*/sl and *B*isl (Fig. 3). It is seen from the Figure that the lengths of the products of DNA cleavage by these enzymes differ in 1 nucleotide, and a longer 5'-labeled oligonucleotide fragment is formed in DNA cleavage by endonuclease *B*/sl. Thus, *B*/sl cleaves the recognition sequence as shown by arrows:



The work on investigation of *B*/sl ability to cleave the recognition site with different distributions of methylated cytosine bases will be described separately.

Thus, the present work characterizes the site-specific endonuclease *B*/sl, which is a neoschizomer of enzyme *B*isl. Enzyme *B*/sl belongs to a rare group of site-specific endonucleases, which recognize and cleave only methylated DNA. Endonucleases *B*isl and *B*/sl recognize the nucleotide sequence 5'-G(5mC)NGC-3'/3'CGN(5mC)G-5', but *B*/sl, unlike *B*isl, cleaves it with the formation of 3'-protruding ends. So, *B*/sl is the first site-specific methyl-dependent endonuclease that cleaves DNA with the formation of 3'-protruding ends.

As both enzymes *B*isl and *B*/sl were found in bacterial strains referring to *Bacillus* genus, one can suppose that these enzyme might play some special role in *Bacillus* cells. Our previous suggestion that these enzymes provide the protection of bacteria against infection with phages containing methylated DNA is the most probable one [3]. It is known that a number of bacteriophages reproducing on *Bacillus* genus cells contain the genes of site-specific DNA-methyltransferases, which results in the methylation of the phages' DNAs in a number of nucleotide sequences, including the site 5'-GCNGC-3' [12]. In this case, the role of enzymes *B*isl and *B*/sl may consist in the cleavage of methylated DNA of bacteriophages penetrating the cell and, therefore, in the protection of the *Bacillus* cell against infection with the virus.

We believe that *B*isl and *B*/sl may be used for detection and analysis of methylated sites in eucaryote DNA, which usually contain a considerable number of 5-methyl cytosine bases and participate in the regulation of cellular processes [13].

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