

Restriction endonuclease Bis I from *Bacillus subtilis* T30 recognizes methylated sequence 5'-G(5mC)↓NGC-3'

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Bacillus subtilis strain T30, producing a novel restriction endonuclease Bis I, has been isolated and characterized. The enzyme recognizes methylated DNA sequence 5'-G(5mC)↓NGC-3' and cleaves it as it is shown by arrow. Due to cleavage of only modified DNAs Bis I may find a practical application in genetic engineering experiments as well as in determination of eukaryotic DNA methylation status.

INTRODUCTION

Bacterial restriction-modification (RM) system consists of DNA-methyltransferase (MTase, M.) and restriction endonuclease (ENase) which recognize the same short (mostly palindromic) DNA sequence. MTase modifies a recognition sequence in a host DNA preventing it from a cleavage by a corresponding ENase. ENase destroys any forensic DNA penetrating to the cell by cleavage inside or nearby the recognition sequence. Thus, RM system protects bacteria from infection. Since discovery first restriction endonucleases in the late 60's of last century more than two hundred of various recognition sequences of restriction enzymes (prototypes) have been discovered and characterized [1]. The most of them are II-type restriction endonucleases, which have found a wide application in molecular biology and modern biotechnology. Among all these II-type enzymes there is only one prototype Dpn I which recognizes and cleaves methylated DNA and doesn't cleave unmethylated DNA. However, the methylated nucleotide within Dpn I recognition sequence is adenine [2]. McrBC ENase recognizes methylated PuC sequences but requires GTP for action and cleaves DNA not at definite position [3]. Besides, there are some repair enzymes, which recognize hemimethylated DNA sequences but cleave only one strand [4]. Here we report a novel restriction endonuclease Bis I, that recognizes and cleaves cytosine-modified sequence 5'-G(5mC)↓NGC-3'. Bis I belongs to type II restriction endonucleases because it doesn't require cofactors except Mg²⁺ ions. According to recently proposed nomenclature [5] Bis I is a second (after Dpn I) member of subtype IIM enzymes, which cleaves only methylated DNA.

MATERIALS AND METHODS

Strain growth and purification of Bis I. The cultural, morphological, physiological and biochemical studies of strain T30 were performed according to methods described in [6]. The properties of the strain and results of its 16S rRNA analysis allowed us to identify it as *Bacillus subtilis* T30 [7].

The strain was grown at 30 °C in 20 l fermenter (LKB, Sweden) with aeration (10 l/min). Nutritious medium included 1% tryptone (Organotechnie, France), 0,5% yeast extract (Organotechnie, France), 0,5% NaCl and 0,05% MgCl₂ (pH 7,5). At a late logarithmic stage of growth cells were precipitated by centrifugation. Precipitate of approx. 100 g of wet cells was stored at -20 °C.

The enzyme purification was performed based on column chromatography techniques. 15 g of biomass were suspended in 50 ml of buffer A (10 mM Tris-HCl, pH 7.5, 0,1 mM EDTA, 7 mM β-mercaptoethanol) containing 0,1 M NaCl, 0,3 mg/ml lysozyme, 0,1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was incubated for 3 h with permanent mixing and then cells were disrupted by ultrasonic treatment. The cell debris was removed by centrifugation at 15000 rpm for 30 min.

Supernatant was loaded onto phosphocellulose P-11 («Whatman», England) column (40 ml). The column was washed with 80 ml of buffer A containing 0,1 M NaCl. 0,15-0,65 M NaCl gradient in buffer A (400 ml) was used for enzyme's elution and 8 ml fractions were collected. The elution fractions with Bis I activity were pooled and dialysed against 20 volumes of buffer A with 0,1 M NaCl.

Dialysed fractions were loaded onto 7 ml column with heparine-sepharose («Bio-Rad», USA). Elution was performed using 0,2-0,65 M NaCl gradient in buffer A (140 ml) and 2,8 ml fractions were collected. The fractions with Bis I activity were pooled together and dialysed against 20 volumes of buffer A with 0,1 M NaCl.

These active fractions were loaded onto 4 ml column with phosphocellulose P-11. The enzyme was eluted with 0,2-0,65 M NaCl gradient in buffer A (80 ml). 2 ml fractions were collected and analyzed and those of them with the most active enzymatic activity were pooled and concentrated by dialysis against 20 volumes of 50% glycerol, 10 mM Tris-HCl, pH 7.55, 0,1 mM EDTA, 7 mM β-mercaptoethanol, 0,1 M NaCl. Purified preparation of Bis I was stored at -20 °C.

Enzyme assay. Substrate for detection of enzymatic activity was a plasmid pFsp4HI1, which contains gene for Fsp4H I DNA MTase. This MTase modifies cytosine residue within 5'-GCNGC-3' sequence. The activity of Bis I was measured in 50 µl of a reaction mixture containing DNA in 10 mM Tris-HCl, pH 9.0, 10 mM MgCl₂, 150 mM KCl, 1mM DTT. After incubation for one hour at 37 °C the reaction was terminated by addition of 10 µl stop solution. The DNA fragments were resolved by electrophoresis in 1% and 2% agarose gels.

Determination of the recognition sequence and the cleavage position. The recognition sequence was determined by comparison of Bis I digestion pattern of pFsp4HI1 with theoretically predicted pattern of cleavage of this substrate by GCNGC-recognizing ENase. The recognition sequence was confirmed by Bis I cleavage of 1) pUC19 DNA methylated with Fsp4H I MTase and 2) synthetic oligonucleotides duplexes.

The cleavage position was determined by digestion of [³²P]-labeled oligonucleotides containing the recognition sequence with subsequent comparison of the fragments lengths. Oligonucleotides fragments separation was performed by electrophoresis in 20% PAG (Tris-borate buffer with 7M urea).

RESULTS AND DISCUSSION

Bacterial strain T30 has the following properties. Colonies on agarized Luria-Bertrani medium are grey, round, surface dull; become opaque. Cells are motile, rod-shaped (0,6-0,8x1,5-2,5 μm), may form chains. Endospores are ellipsoidal, paracentral, do not cause sporangium to swell. Microorganism is not able to grow anaerobically. It is catalase-positive. Hydrolysis of starch and casein was observed, tyrosine was not hydrolysed. Strain produces acid from D-glucose, D-xylose, D-mannitol and L-arabinose.

Growth in a nutritious medium allows to harvest approx. 5 g/l of cells. Bis I was isolated from frozen cells by several chromatographic steps as described in "Materials and methods". The yield of purified enzyme was 200 u/ml.

Bis I doesn't cleave any standard substrates including lambda (dam+, dcm+ and dam-, dcm-), T7 or Ad2 DNAs. Bis I cleaves pFsp4HI1 DNA which carries gene of Fsp4H I DNA-methyltransferase (recognition sequence GCNGC (Fig. 1B). The pattern of the observed DNA fragments corresponds to the predicted one for pFsp4HI1 cleavage by ENase recognizing 5'-GCNGC-3' site (data of the right column). We also tested some other plasmids carrying genes of M.Hha I (recognition sequence GCGC), M.Fau IA and M.Fau IB (recognition sequences CCCGC and GCGGG, respectively). No cleavage of these DNAs was observed. These data indicate that the enzyme recognizes and cleaves nucleotide sequence 5'-GCNGC-3', methylated by Fsp4H I MTase.

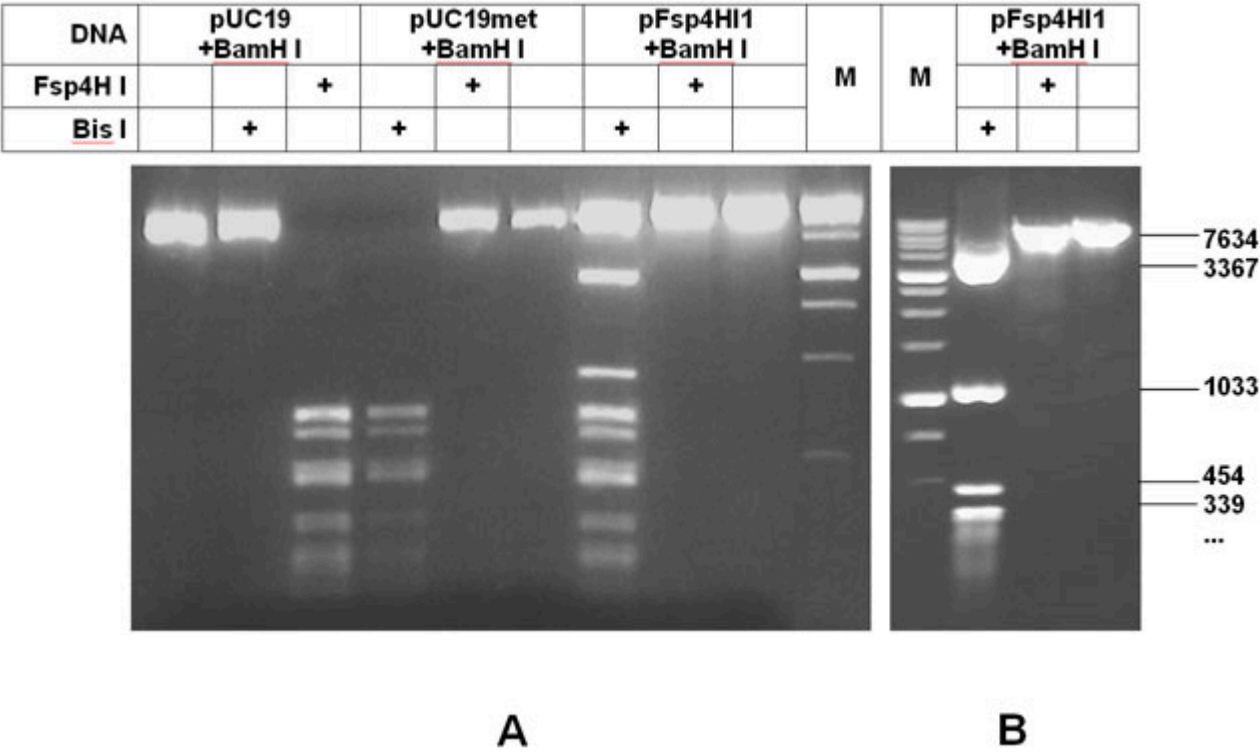


Fig. 1. Bis I and Fsp4H I cleavage of plasmid DNAs, linearized with BamH I. **A** - electrophoresis in 2% agarose gel, **B**- electrophoresis in 1% agarose gel. pUC19met - pUC19 methylated with M.Fsp4H I. M -

DNA fragment size marker 1Kb (SibEnzyme). Theoretical lengths of fragments produced by pFsp4HI1 cleavage at 5'-GCNGC-3' sites are shown at right.

To confirm this recognition sequence of Bis I we completely methylated pUC19 DNA by Fsp4H I MTase and then cleaved this modified DNA by Bis I. Fig. 1A shows that native pUC19 DNA is cleaved by Fsp4H I ENase (recognition site 5'-GCNGC-3') and isn't cleaved by Bis I. At the same time methylated pUC19 DNA is cleaved by Bis I and is not cleaved by Fsp4H I ENase. The digestion patterns of DNA cleavages are the same. Thus, all experimental data indicate that Bis I recognition site is methylated sequence 5'-GCNGC-3'.

For final verification and cleavage position determination we used synthetic oligonucleotide duplexes containing or not containing modified cytosine residues (5'-GCNGC-3' sites are underlined):

L7: 5' GAGTTTAGCGGCTATCGATCT 3'

L8: 5' AGATCGATAGGCCGCTAAACTC 3'

L9: 5' GAGTTTAG(5mC)GGCTATCGATCT 3'

L10: 5' AGATCGATAG(5mC)CGCTAAACTC 3'

L11: 5' GAGTTTAG(5mC)AGCTATCGATCT 3'

L12: 5' AGATCGATAG(5mC)TGCTAAACTC 3'

The results of cleavage of these oligonucleotide duplexes are presented on Fig.2. As it is clear from the photo, Bis I indeed cleaves only methylated sequences 5'-G(5mC)NGC-3'.

Duplex	L7*/L8			L9*/L10			L11*/L12		
Fsp4H I		+			+			+	
Bis I			+			+			+

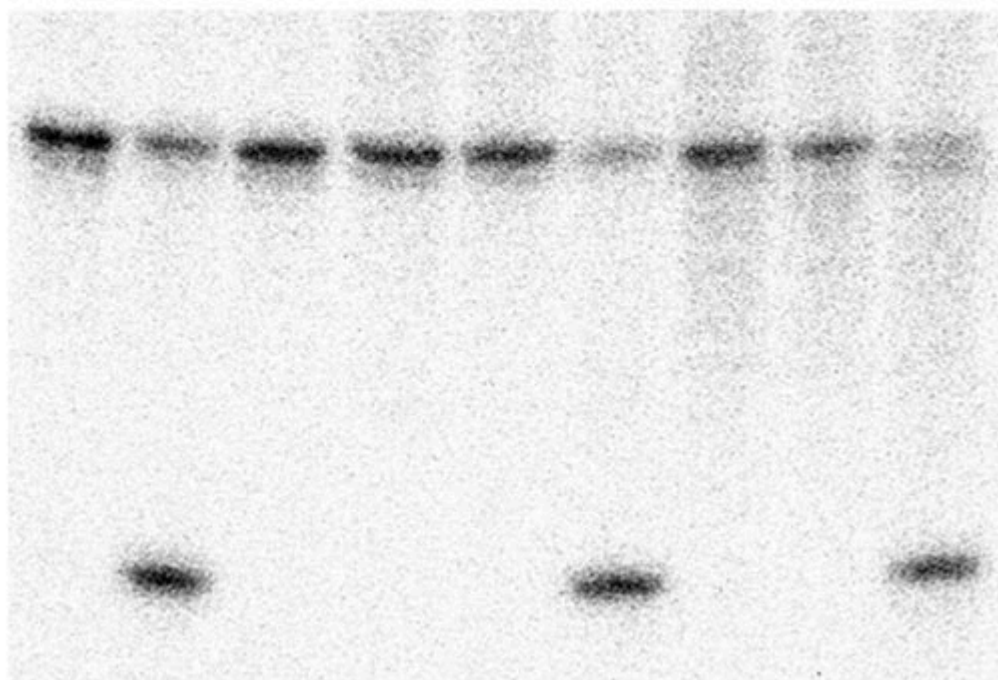


Fig. 2. Cleavage of oligonucleotide duplexes with Bis I and Fsp4H. Labeled chains in duplexes are marked with *.

Duplex	L7*/L8				L9*/L10			
Bis I		+			+			
ExoIII			+			+		
Fsp4H I				+			+	

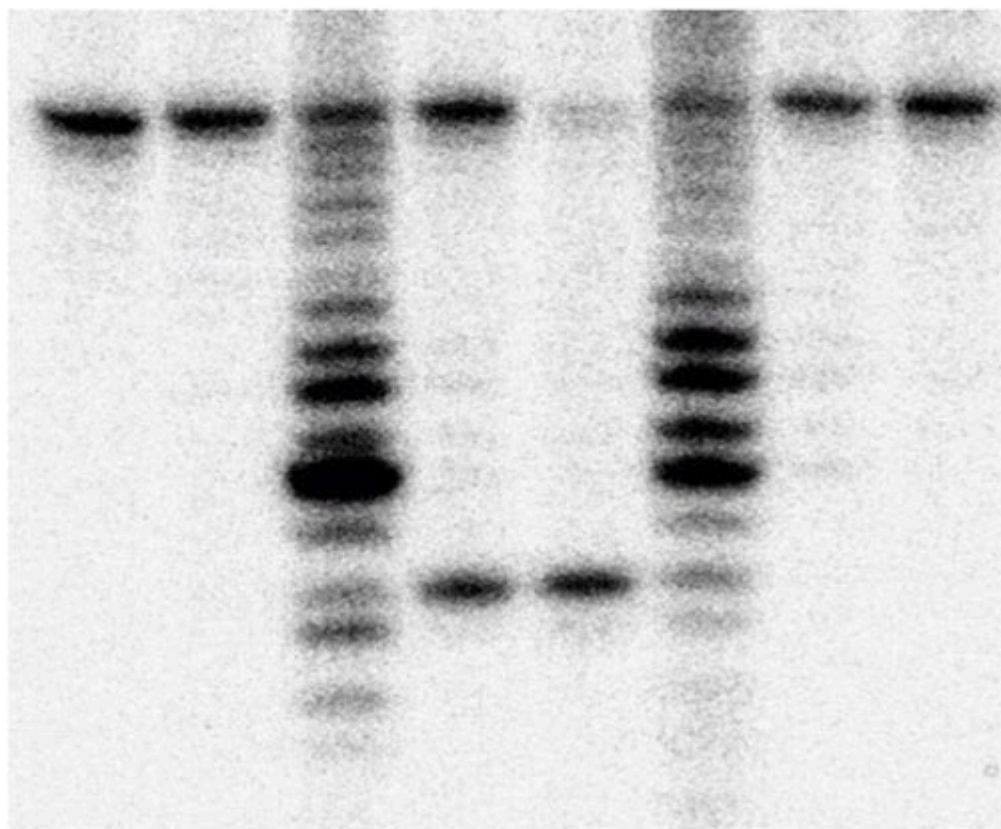
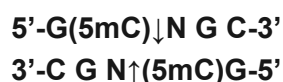


Fig. 3. Bis I cleavage position determination using oligonucleotide duplexes. Labeled chains in duplexes are marked with *. ExoIII - exonuclease III from *E. coli* (oligonucleotide fragments size marker).

On the Fig.3 results of L7/L8 and L9/L10 oligonucleotide duplexes cleavage by Bis I and Fsp4H I ENases are shown. Comparison of oligonucleotides cleavage by Bis I and Fsp4H I clearly indicates that a phosphodiester bond to be hydrolyzed by Bis I is the same as in Fsp4H I cleavage of nonmethylated oligonucleotide. Hence, Bis I recognizes and cleaves the following sequence as indicated by the arrows:



Detailed study of synthetic oligonucleotides cleavage will be presented in the next publications.

Thus, in this report we have described a new site-specific endonuclease Bis I which has been isolated and purified from *B. subtilis* T30. It should be noted that this is a first site-specific restriction enzyme, which recognizes and cleaves only cytosine-methylated palindromic nucleotide sequence. Probably Bis I is not a part of classic two-component RM system because there is no need in corresponding DNA MTase. The indirect evidence of MTase absence is resistance of *B. subtilis* T30 chromosomal DNA to cleavage by Bis I (data not shown). We believe, that Bis I and similar (if any) restriction enzymes may be considered to be a next step in evolution of relations between bacteria and bacterial virus (bacteriophage). It's well known that some phages of Bacillus species encode multispecific DNA-methyltransferases which are able to methylate several different sites of phage DNA and thus prevent phage DNA from a cleavage by bacterial restriction enzymes of a corresponding RM system [8]. We believe that origin of Bis I is connected with a next step of evolution, when bacteria produces a restriction enzyme which cleaves not all methylated DNA but the definite phage's DNA methylated sites. In this way bacterial cell is protected against a particular methylated phage DNA infection.

It's interesting to note that Bis I and other similar enzymes may find an application in determination of 5mC-methylation in eukaryotic and human DNA. This modification is widely distributed among high organisms and plays an important role in regulation of cell processes. However, a detailed role of methylation is not studied yet [9].

SibEnzyme Ltd.: Patent RU 2 270 859 C1

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