ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Cloning and Study of New DNA Methyltransferase M.FatI Modifying Cytosine in a Recognition Site CATG.

Vladimir S Dedkov*, Danila A Gonchar, Murat A Abdurashitov, Svetlana G Udalyeva, Lyubov A Urumceva, Valery A Chernukhin, Galina V Mutylo, and Sergey Kh Degtyarev.

SibEnzyme Ltd., Ak. Timakov str. 2/12, Novosibirsk, 630117, Russia.

ABSTRACT

A fragment of *Flavobacterium aquatile* NL3 DNA carrying the gene of DNA methyltransferase M.FatI was cloned in pUC19 plasmid. DNA was sequenced and M.FatI gene was analyzed. A recombinant strain *Esherichia coli* was grown up and the enzyme was purified. M.FatI specificity was determined by a blocking of some restriction endonucleases and computer modeling. It's well known that M.NlaIII produces 5'-C(m6A)TG-3', whereas FatI MTase modifies the cytosine residue with formation 5'-(m5C)ATG-3'. The sensitivity of restriction endonucleases to FatI-methylation has been studied.

Keywords: gene cloning, enzyme isolation, bacterial DNA methyltransferase, enzyme specificity, restriction endonuclease, methylation sensitivity

Corresponding author

ISSN: 0975-8585

INTRODUCTION

DNA methylation of animals and plants is involved in gene expressions, embryonic development, differentiation, carcinogenesis, protection against viruses, in aging. DNA methylation of bacteria functions in DNA replication, reparation, protection against phages in restriction-modification (RM) systems. DNA methyltransferases (MTases) transfer the methyl group from S-adenosyl-L-methionine (SAM) to cytosine or adenine in double-stranded DNA. The modified bases are 5-methylcytosine (5mC or m5C), N4-methylcytosine (N4mC or m4C) or N6-methyladenine (N6mA or m6A). The MTases differ in their recognition sequence and the base to be modified within this site [1].

The most different MTases are found in bacteria. These enzymes partly belong to restriction-modification (RM) systems. About 350 prototypes of RM systems and thousands of their analogs are known at present time [2]. Each RM system consists of restriction endonuclease (ENase) and MTase. Both cognate enzymes recognize the same site. ENase cuts a recognition site thus protecting bacterial cell from a foreign DNA invasion. MTase methylates the recognition site in host DNA and protects it from cleavage with a cognate ENase [3], [4]. ENases of various bacteria recognizing the same site are called isoschizomers. New MTases are a perspective tool for a study of DNA-protein interactions. One of these studies is a determination of ENases sensitivity to a new type of methylation.

The subject of this work is DNA MTase from *Flavobacterium aquatile* NL3. The ENase FatI, a neoshizomer of NlaIII [5], cuts the site C(m6A)TG [2] which M.NlaIII forms [6]. Therefore FatI MTase must have unique specificity, differing from M.NlaIII, to protect cognate DNA from FatI ENase. The purpose of this work is FatI MTase gene cloning and study of recombinant enzyme properties and substrate specificity. We have performed a comparative study of sensitivity of different ENases to DNA methylation by M.FatI.

MATERIALS AND METHODS

Enzymes, DNA and reagents

Restriction endonucleases, alkaline phosphatase (calf intestinal) and T4 DNA ligase as well as pUC19, phage λ (dam-, dcm-) and T7 DNAs, ATP, reaction buffers were obtained from Sibenzyme Ltd, Russia. Lysozyme was from Helicon, Russia. Bovine serum albumin (fraction V) (BSA) was from Americanbio.com, USA. S-adenosyl-L-methionine (SAM) – was from New England Biolabs Inc., USA.

Strains and culture medium

Escherichia coli K-12 strain RR1 (ΔmcrC-mrr recA+) was from New England Biolabs Inc, USA. *Flavobacterium aquatile* NL3 with RM-system FatI was from Sibenzyme Ltd, Russia. L-broth contained 10 g/L Tryptone (Organotechnie, France), 5 g/L Yeast extract (same firm), 5 g/L NaCl, 0.5 g/L $MgCl_2$, 1 mg/L thiamin. For plates L-broth was supplemented with 15 g/L agar (C.T. Roeper GmbH, Germany). Ampicillin was added up to 100 μ g/mL, streptomycin – up to 25 μg/mL.

Transformation and colony screening procedures

F. aquatile NL3 was grown in flasks containing 300 mL of L-broth at 25°C with 130 rpm for 40 h. 10 μg *F. aquatile* NL3 DNA, isolated as described [7], was uncompletely digested with 20 units of Kzo9I (^GATC) in the reaction mixtures of 200 μL for 1 h at 37°C. Digested DNA was cleared by phenol and precipitated with ethanol. Hybrid plasmid was constructed by ligating 10 μL of Kzo9I-digested *F. aquatile* NL3 DNA with 3.0 μg of phosphatase-treated BamHI-digested pUC19 vector by using 2,000 units of T4 DNA ligase. The reaction was allowed to proceed for 16 h at 4°C in 20 μL of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP. The ligated DNA was precipitated with ethanol and dissolved in 12 μL of water.

2.5 μg DNA of the ligation mixture was added to approximately 3.0 x 10^9 RR1 cells in 50 μL that had been treated for the electroporation in "Easyject Prima" according to the manufacturer's instructions (EquiBio, UK). After the processing by an impulse the mixture was added to 1.0 mL of L-broth and incubated for 1 h at 37°C. For calculation of transformants 10 μL of the culture were plated on L-agar with ampicillin (250 clones

were grown after 16 h at 37°C). The genomic library (1.0 mL) was grown in 100 mL of L-broth with ampicillin for 16 h at 37°C with shaking.

Total DNA of hybrid plasmids was isolated from 100 mL culture of 25000 transformants with use QIAGEN Plasmid Maxi Kit (Germany) according to the manufacturer's instructions. 10 μg DNA was digested with 10 units of FatI for 4 h at 55°C, precipitated with ethanol and dissolved in 15 μL of water. According to the protocols [7] the digested DNA was incubated with 3.0 x 10^7 RR1 cells that had been treated with $CaCl_2$. Cells were plated on L-agar containing ampicillin and 28 clones were obtained after 16 h at 37°C. Each clone was grown in L-broth with ampicillin and its plasmid DNA was isolated with use QIAGEN Plasmid Miniprep Kit (Germany). DNAs from clones were cat with FatI and analyzed by electrophoresis in 1% agarose gel. DNA of plasmid pM.FatI-3 steady to FatI was used for RR1 retransformation. The obtained clone was named *E. coli* N3 (pM.FatI).

DNA sequencing

The sequencing of DNA was carried out on the ABI 3130xI Genetic Analyzer device (Applied Biosystems, USA).

Purification of DNA methyltransferase M.FatI from *Escherichia coli* N3 (pM.FatI)

E. coli N3 (pM.FatI) cells were grown till stationary phase in L-broth with ampicillin in 20 L Vessel (New Brunswick Scientific, USA) at 30˚C with aeration for 8 h. Cells were harvested at 8000 x g and stored at -20˚C.
The enzyme purification was carried out at 4˚C with use Buffer A (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 7 mM β-mercaptoethanol) and Buffer B (10 mM K-phosphate pH 7.2, 0.1 mM EDTA, 7 mM β-mercaptoethanol). 100 g of harvested cells, suspended in 500 mL of Buffer A with 0.2 M NaCl, 50 g/L glycerol, 5 g/L Triton® X-100 (non-ionic detergent, Sigma) and 0.1 mM PMSF (proteases inhibitor), were disrupted by sonication. Cell debris was removed by centrifugation at 20,000 x g for 1 h. The supernatant was loaded onto a Phosphocellulose P11 (Whatman, UK) of 300 mL bed volume and eluted with 3 L of a 0.2 to 0.6 M NaCl gradient in Buffer A for 15 h. 200 fractions with 15 ml were collected. Fractions were assayed for M.FatI activity as described below. Fractions 74-96 containing peak of activity were combined, and, after a dialysis against 3 L of Buffer A with 0.05 M NaCl for 4 h, loaded onto a Heparin-Sepharose (Bio-Rad, USA) of 50 mL bed volume column. Protein was eluted with 1 L of 0.05-0.5 M NaCl gradient in Buffer A. 100 fractions were collected. 62–66 containing the activity were pooled, loaded onto a Sephacryl S-200 (Bio-Rad, USA) of 1 L bed volume column and eluted with 1 L of 0.8 M NaCl in Buffer A. Fractions 64–71 of 100 were were loaded onto a Hydroxyapatite (Bio-Rad, USA) of 50 mL bed volume column and eluted with 1 L of a 0.01-0.2 M K-phosphate gradient, pH 7.2 in Buffer B. Fractions 61–68 of 100 containing activity were combined, concentrated by a dialysis against 1 L of Buffer A with 500 g/L glycerol, 0.05 M of NaCl and stored at -20˚C.

DNA methyltransferase assay

The assay based on a protection of methylated DNA from cognate restriction endonuclease. M.FatI sample was incubated in 50 μL with 50 μg/mL λ DNA (dam–, dcm-), 1 mM SAM, 33 mM Tris-acetate, pH 7.9, 1 mM EDTA, 66 mM potassium acetate 1 mM DTT, 0.1 mg/mL BSA in a well of 96 Well Microplate (Medpolymer, Russia) at 25˚C for 1 h. Then, Microplate was warmed up on a bath at 65˚C for 10 min to inactivate the enzyme. To cleave not modified DNA the mixture was supplemented with 5 μL of 1000 u/mL FatI restriction endonuclease diluted with 10 mM Tris-HCl pH 7.6, 50 mM KCl, 0.1 mM EDTA, 200 μg/mL BSA, 1 mM DTT and 100 mM magnesium acetate. After incubation at 55˚C 1 h the mixture was analyzed by electrophoresis in 8 g/L agarose gel as described [7]. One unit of M. FatI activity methylated □□ μg of λ DNA in 1 h blocking the activity of FatI restriction endonuclease.

DNA methylation with M.FatI

Reaction mixture, in a 2 mL Eppendorf tube, containing 0.4 mg/mL λ or T7 DNA, 10 mM SAM, 33 mM Tris-acetate pH 7.9, 1 mM EDTA, 66 mM potassium acetate 1 mM DTT, 0.1 mg/mL BSA and 100 u/mL M.FatI enzyme in a total volume of 1.0 mL, was incubated at 25˚C for 20 h. Then, 0.1 mL of 100 g/L SDS (Sigma) and 0.2 mL of 3 M KCl were added. The mixture was warmed at 65˚C for 10 min to dissolve SDS-precipitate, cooled at 0˚C for 15 min for precipitate formation. The supernatant, pooled after centrifugation at 10,000 x g for 3

min, was mixed with 0.6 volume of isopropanol. DNA pellet was rinsed with 800 g/L ethanol, dried on air and dissolved in 0.7 mL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) to the concentration 0.5 mg/mL.

DNA cleavage with restriction endonucleases and electrophoresis in agarose gel

Native or M.FatI-methylated λ or T7 DNA were cleaved in 50 µL of 50 µg/mL DNA, 0.1 mg/mL BSA, appropriate SE buffer, 125 u/mL restriction endonuclease in a well of 96 Well Microplate (Medpolymer, Russia) for 2 h at the temperature recommended by the manufacturer (Sibenzyme Ltd). Reaction was stopped by addition 10 µL of 0.25 M Na-EDTA, pH 8.5, 500 g/L sucrose and 5 g/L bromphenol blue. The Microplate was warmed up on a bath at 65˚C for 10 min and cooled on ice.

The electrophoresis was carried out in 8 g/L of LE agarose (Segenetic) in TAE buffer with 0.5 µg/mL ethidium bromide (Sigma) at 5 V/cm for 2.5 h as described [7]. The fluorescence of DNA was revealed on the Herolab GmbH device.

Determination of DNA methyltransferase specificity

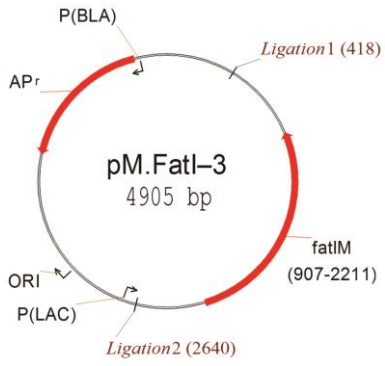
M.FatI recognition sequence and its methylated basis were defined using methylation sensitivity of restriction endonucleases [2] accoding to [8]. As described above λ DNA (dam-, dcm-), as native and M.FatI-methylated, were cut by restriction endonucleases FaeI (CATG^), FatI (^CATG), RsaI (GT^AC), BstC8I (GCN^NGC) и Zsp2I (ATGCA^T). Then DNA fragments were analyzed by the electrophoresis in 0.8% agarose gel. Beforehand blocking of restriction endonucleases by the methylation was modeled to reveal an overlapping of methylation and cleavage. Methylation of (mC)ATG was simulated as editing λ DNA sequences by replacements CATG on NATN and designated (C=>N)AT(G=>N). Then, simulated cleavage of native and edited DNA by restriction endonucleases and electrophoresis was performed with Vector NTI program. In the program a restriction endonuclease didn't cut recognition site if any of bases was replaced by N. Thus the methylated site was probed by restriction endonucleases. M.FatI specificity was determined by an analysis of experimental and simulated results.

RESULTS AND DISCUSION

Selection of clones carrying the M.FatI gene and DNA sequencing.

MTase clones were selected by the resistance of recombinant DNA to the cognate restriction endonuclease according to [9]. A genomic library of *F. aquatile* NL3 DNA was obtained in pUC19/BamHI vector DNA as 25000 *E. coli* RR1 transformants. A total DNA of hybrid plasmids was digested with FatI and used for additional RR1 cells transformation. Plasmid DNAs from 28 clones were cut with FatI and analyzed by electrophoresis in 10 g/L agarose gel. DNA of pM.FatI-3 plasmid steady to FatI was used for RR1 retransformation. The obtained clone was named *E. coli* N3 (pM.FatI). Final plasmid DNA, pM.FatI-3, was sequenced. A restriction map of this recombinant plasmid with ORF of M.FatI gene is shown in Figure 1.
Figure 1 Dedkov VS et al.

Figure 1: Restriction map of pM.FatI-3 plasmid DNA: Ligation, sites (bp); 418 – 2640 bp, F. aquatile NL3 DNA fragment; 2640 – 418 bp, pUC19 DNA; ORI, replication start; P(BLA), β-lactamase promoter; Apr, ampicillin resistance; P(LAC), β-galactosidase promoter; FatIM, gene of FatI MTase. The map was simulated with Vector NTI program.



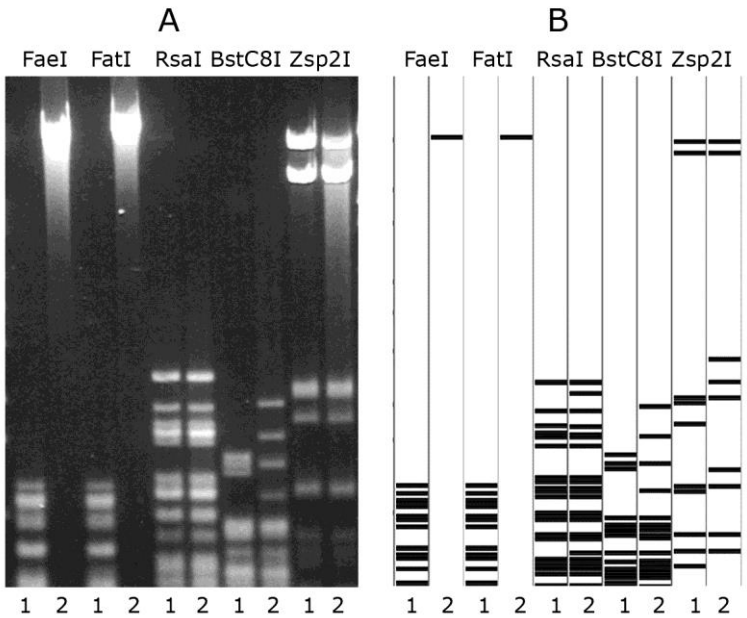
The nucleotide and amino aside sequences of M.FatI were deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov/nuccore/LN869918.1). With the aid of Protein Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) the putative conserved motifs of M.FatI were detected in AdoMet_MTases superfamily with Cytosine-C5 specific DNA methyltransferases domain hit. This result shows that M.FatI probably forms (m5C)ATG and differs from M.NlaIII which forms C(m6A)TG [6]. Nevertheless, it was necessary to purify the enzyme preparation in order to determine M.FatI specificity.

M.FatI purification and characterization.

To isolate M.FatI enzyme preparation the cells of *E.coli* N3 (pM.FatI) were grown in L-broth with ampicillin. The enzyme was purified from cellular extract by subsequent chromatography on Phosphocellulose P11, Heparin-Sepharose, Sephacryl S-200 and Hydroxyapatite as described at “Materials and methods”. As a result the enzyme preparation M.FatI was obtained with activity 10 u/µL.

The specificity of M.FatI was confirmed by a blocking of restriction endonucleases hydrolysis and modeling accoding to [8] as described in “Materials and Methods”. Figure 2 (A and B) shows that the methylation of λ DNA with M.FatI completely blocks DNA hydrolysis with ENases FaeI (CATG^) and neoschizomer FatI (^CATG). RsaI (GT^AC) which sensitive to m4C [2] [10] cleaved the sites GTACATG overlapping with M.FatI. Therefore FatI MTase didn't form (m4C)ATG. Nevertheless the MTase blocked BstC8I (GCN^NGC) which sensitive to m5C [2] [11] in the case of overlapping internal cytosines in the site GCATGC. Therefore M.FatI forms (m5C)ATG. As it was shown [2] [11] RsaI cut GTA(m5C) and Zsp2I cut ATG(m5C)AT, therefore they also cut sites modified by M.FatI (Figure 2).

Figure 2: Determination of M.FatI-methylation specificity by blocking of restriction endonucleases (A) and by modeling (B): Lanes: 1, unmethylated λ DNA; 2, λ DNA methylated with M.FatI (A) or edited (C=>N)AT(G=>N) (B). DNA was digested with FaeI (CATG^), FatI (^CATG), RsaI (GT^AC), BstC8I (GCN^NGC) and Zsp2I (ATGCA^T). Electrophoresis was performed in 8 g/L agarose, simulation with Vector NTI program.



Thus the results of gene analysis and the probing of the methylated site by restriction endonucleases show that M.FatI forms 5 '-(m5C)ATG-3’ on DNA and belongs to cytosine-(C5)-DNA methyltransferases (EC 2.1.1.73) [1]. M.FatI possesses a new specificity among known analogs [2] and it can quite be of interest to molecular and genetic works.

Methylation sensitivity of restriction endonucleases.

An essential characteristic of restriction endonucleases is methylation sensitivity of these enzymes. This feature is used for the studying of DNA methylation in particular of a carcinogenesis. A number of ENases were tested for methylation sensitivity with λ and T7 DNAs methylated by M.FatI on (m5C)ATG. The model and

ISSN: 0975-8585

experimental results are shown in Figure 3 and Figure 4 and are interpreted in Table 1. If some cases the methylation of particular sequences was simulated as described [8], [11].

Figure 3: Simulated and experimental cleavage of native and M.FatI-methylated λ DNA with restriction endonucleases: Lanes: 1, native nucleotide sequence and DNA; 2, sites of the sequence were edited (A) as (C=>N)AT(G=>N), or DNA methylated with M.FatI (B). Simulation was performed with Vector NTI program. Cleavage was carried out with each endonuclease activity sufficient for 5-fold excess of DNA. DNA bands in 8 g/L agarose were visualized by fluorescence with ethidium bromide.

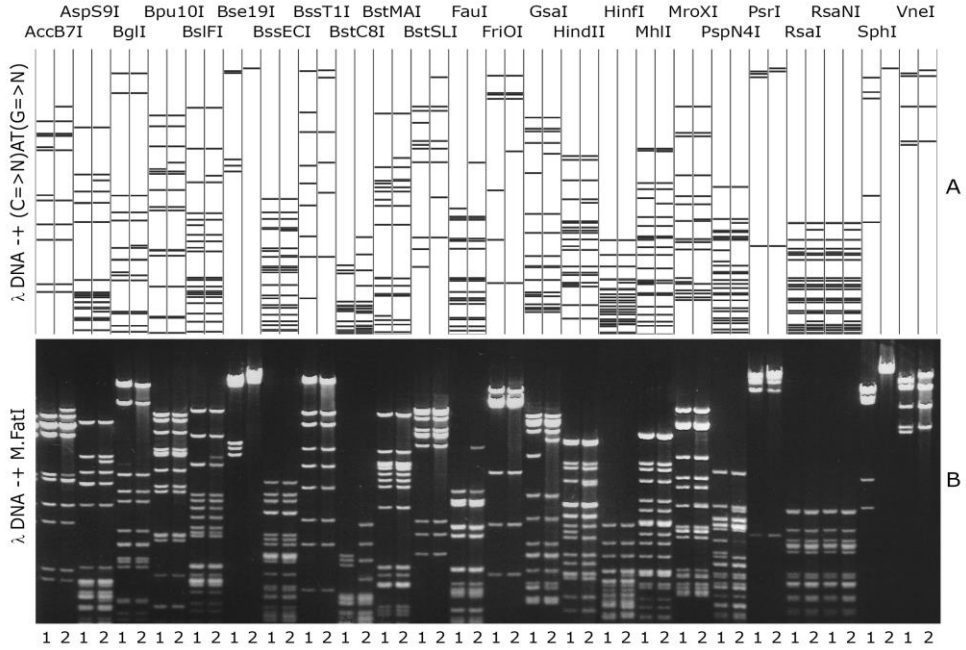
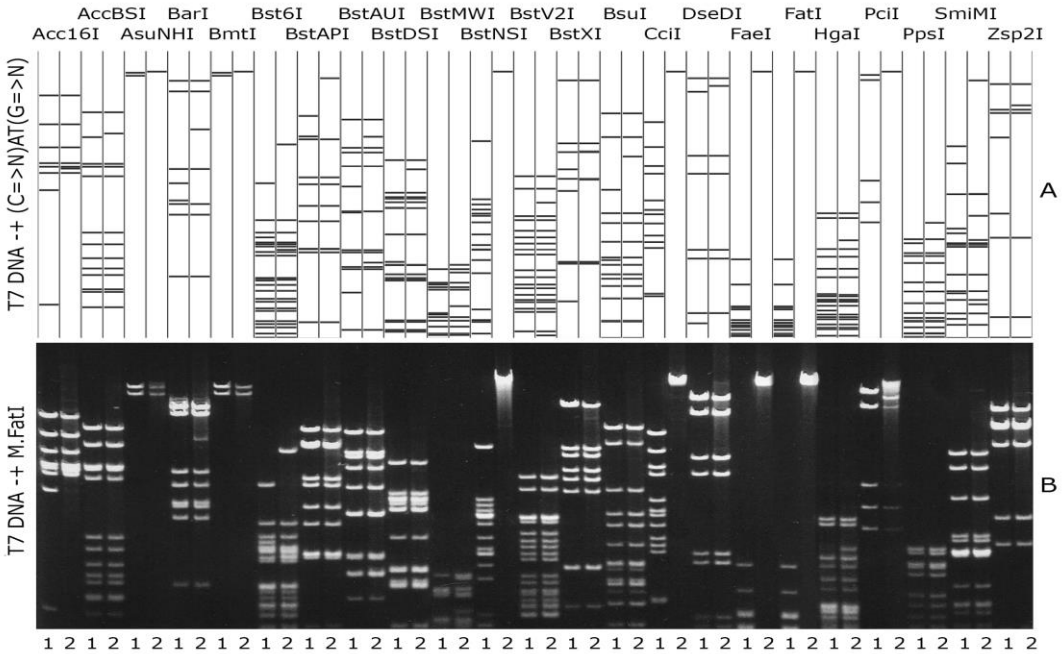


Figure 4: Simulated and experimental cleavage of native and M.FatI-methylated T7 DNA with restriction endonucleases: Lanes: 1, native nucleotide sequence and DNA; 2, sites of the sequence were edited (A) as (C=>N)AT(G=>N), or DNA methylated with M.FatI (B). Simulation was performed with Vector NTI program. Cleavage was carried out with each endonuclease activity sufficient for 5-fold excess of DNA. DNA bands in 8 g/L agarose were visualized by fluorescence with ethidium bromide.



ISSN: 0975-8585

Table 1: Methylation sensitivity of some restriction endonucleases.

ENase	Methylated site on λ DNA (5'-3'/3'-5')	Cleavage (%)*	ENase	Methylated site on T7 DNA (5'-3'/3'-5')	Cleavage (%)*
AccB7I	C(m5C)ANNNNNTGG GGTNNNNNACC	10	Acc16I	TGCG(m5C)A ACGCGT	0*
AspS9I	GGNC(m5C) CCNGG	0*	AccBSI	GAGCGG (m5C)TCGCC	100*
BglI	GC(m5C)NNNNNGGC CGGNNNNNCCG	100	AsuNHI	GCTAG(m5C) CGATCG	100*
Bpu10I	CCTNAG(m5C) GGANTCG	100	BarI	GAAGNNNNNNTAC (m5C)TTCNNNNNNATG	80*
BslFI	GGGAC (m5C)CCTG	80*	BmtI	GCTAG(m5C) CGATCG	100*
Bsp19I	C(m5C)ATGG GGTA(m5C)C	0*	Bst6I	CTCTT(m5C) GAGAAG	0*
BssECI	C(m5C)NNGG GGNN(m5C)C	100*	BstAPI	G(m5C)ANNNNNTGC CGTNNNNNACG	100*
BssT1I	C(m5C)WWGG GGWW(m5C)C	100	BstAUI	TGTA(m5C)A ACATGT	100*
BstC8I	G(m5C)NNGC CGNN(m5C)G	0	BstDSI	C(m5C)RYGG GGYR(m5C)C	100*
BstMAI	GTCT(m5C) CAGAG	100*	BstMWI	G(m5C)NNNNNNNGC CGNNNNNNNCG	0
BstSLI	GKGCM(m5C) CMCGKG	100*	BstNSI	R(m5C)ATGY YGTA(m5C)R	0*
FauI	CCCG(m5C) GGGCG	50	BstV2I	GAAGA(m5C) CTTCTG	100*
FriOI	GRGCY(m5C) CYCGRG	100*	BstXI	C(m5C)ANNNNNNTGG GGTNNNNNNACC	100
GsaI	CCCAG(m5C) GGGTCG	0*	BsuI	GTATCC (m5C)ATAGG	100*
HindII	GTYRA(m5C) CARYTG	0*	CciI	T(m5C)ATGA AGTA(m5C)T	0*
HinfI	GANT(m5C) CTNAG	50	DseDI	GACNNNNNNGT(m5C) CTGNNNNNNTAG	100*
MhlI	GDGCH(m5C) CHCGDG	100*	FaeI	(m5C)ATG GTA(m5C)	0*
MroXI	GAANNNNTT(m5C) CTTNNNNAAG	100*	FatI	(m5C)ATG GTA(m5C)	0*
PspN4I	GGNNC(m5C) CCNNGG	5*	HgaI	GACG(m5C) CTGCG	0
PsrI	GAACNNNNNNTAC (m5C)TTGNNNNNNATG	50*	PciI	A(m5C)ATGT TGTA(m5C)A	10*
RsaI	GTA(m5C) CATG	100	PpsI	GAGTC (m5C)TCAG	100*
RsaNI	GTA(m5C) CATG	100*	SmiMI	(m5C)AYNNNNRTG GTRNNNNYAC	100*
SphI	G(m5C)ATGC CGTA(m5C)G	0*	Zsp2I	ATG(m5C)AT TACGTA	100
VneI	GTGCA(m5C) CACGTG	0*			

The sensitivity of ENases was tested by cleavage λ and T7 DNA methylated by M.FatI (m5C)ATG. Single letter code: R = A or G, Y = T or C, M = A or C, K= G or T, W = A or T, S = G or C, D = A or G or T (not C), H = A or C or T (not G), B = C or G or T (not A), V = A or C or G (not T), N = A or C or G or T. * Data are obtained for the first time.

Some results shown in Table 1 confirm known data about methylation sensitivity of restriction endonucleases [2]. Other results were obtained for the first time (* noted cleavage %).

CONCLUSIONS

The gene of a new DNA methyltransferase FatI, from bacterial strain *Flavobacterium aquatile* NL3 was cloned in *Escherichia coli*. Recombinant M.FatI enzyme preparation was isolated and it was shown that MTase modified the cytosine residue in the recognition sequence CATG producing 5'-(m5C)ATG-3' M.FatI differs from

the similar enzymes [2], for example, M.NlaIII forming C(m6A)TG [6]. The new data on sensitivity of different restriction endonucleases to M.FatI-methylation have been obtained.

ACKNOWLEDGEMENTS

The work was carried out with financial support of Ministry of Education and Science of the Russian Federation under the Agreement No. 14.576.21.0077 of 06.11.2014 (the unique RFMEFI57614X0077 identifier) signed within the Federal Target Program "Research and Development in Priority Directions of Development of a Scientific and Technological Complex of Russia for 2014 — 2020".

REFERENCES

[1] Cheng X, Blumenthal RM (Eds.). S-Adenosylmethionine-Dependent Methyltransferases. Structures and Functions: Singapore, New Jersey, London, Hong Kong: World Scientific Publishing Co. Pte. Ltd.; 1999.
[2] REBASE (2015). Available at: http://rebase.neb.com
[3] Arber W, Dussoix D. J Mol Biol 1962; 5: 18–36.
[4] Boyer HW. Annu Rev Microbiol 1971; 25: 153–176.
[5] Dedkov VS, Kileva EV, Popichenko DV, Degtyarev SKh. Biotechnology in Russia 2002; 5: 1-5.
[6] Labbe D, Holtke HJ, Lau PCK. Mol Gen Genet 1990; 224(1): 101-110.
[7] Sambrook J, Fritsch EF and Maniatis T. Molecular Cloning. A laboratory manual, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. 1989.
[8] Dedkov VS. Mol Genet Microbiol Virol 2009; 24 (3): 114-120.
[9] Mann MB, Rao RN, Smith HO. Gene 1978; 3: 97-112.
[10] Dedkov VS, Mikhnenkoiva NA, Djanobilova ZK, Tarasova MV. A M. Biotechnology in Russia 2012; 2: 32-42.
[11] Dedkov VS. Mol Genet Microbiol Virol. 2012; 27: 40-47.