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Recombinant DNA-Methyltransferase M1.BspACI from *Bacillus psychrodurans* AC: Purification and Properties

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Abstract—A restriction–modification system from *Bacillus psychrodurans* AC (recognition sequence 5′-CCGC-3′) comprises two DNA methyltransferases: M1.BspACI and M2.BspACI. The *bspACIM1* gene was cloned in the pJW2 vector and expressed in *Escherichia coli* cells. High-purity M1.BspACI preparation has been obtained by chromatography on different carriers. M1.BspACI has a temperature optimum of 30°C and demonstrates maximum activity at pH 8.0. M1.BspACI modifies the first cytosine in the recognition sequence 5′-CCGC-3′. The kinetic parameters of M1.BspACI DNA methylation are as follows: K_m for phage λ DNA is 0.053 μM and K_m for S-adenosyl-L-methionine is 5.1 μM. The catalytic constant (k_{cat}) is 0.095 min^{-1}.

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Key words: DNA methyltransferase, *Bacillus psychrodurans*, enzyme kinetics

Site-specific DNA methyltransferases (methylases) are widespread in prokaryotes. These enzymes catalyze the transfer of a methyl group from the donor, S-adenosyl-L-methionine (SAM), to the adenine or cytosine base in a certain nucleotide sequence. As a rule, methylases together with restriction endonucleases form restriction–modification systems (RM systems), where the restriction endonuclease protects bacterial cells from penetration of foreign DNA and the methylase modifies the host DNA to protect it from hydrolysis by its own restrictase. RM systems with a non-palindromic recognition site (IIS type) contain at least two DNA methyltransferases, each modifying only one of the DNA chains [1]. The Michaelis constants for DNA established for this type of methylases usually significantly differ from K_m for methyltransferases recognizing symmetric nucleotide sequences [2]. This is presumably associated with different mechanisms of methylation reaction catalyzed by these two types of enzymes.

Abbreviations: HAP, hydroxyapatite; M, site-specific DNA methyltransferase, methylase; ORF, open reading frame; PCR, polymerase chain reaction; R, restriction endonuclease, restrictase; RM, restriction–modification; SAM, S-adenosyl-L-methionine.

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Previously we found the strain of *Bacillus psychrodurans* AC producing restriction endonuclease *BspAC*I, which recognizes the non-palindromic nucleotide sequence 5′-CCGC-3′ [3]. Isolation and the biochemical and substrate properties of recombinant DNA methyltransferase M1.BspACI are described in this paper.

MATERIALS AND METHODS

DNA isolation. Plasmid DNA of pUC19 [4] and pJW2 [5] and recombinant plasmid DNA from *E. coli* were isolated using Qiagen (USA) kits in accordance with the manufacturer's instructions. Chromosomal DNA of *B. psychrodurans* AC was isolated by the method described previously [6].

Cloning of genes for methyltransferases of the BspACI RM system. For preparation of chromosomal "library", chromosomal DNA fragments obtained during hydrolysis by restrictases *Pst*I and *Zsp*2I (SibEnzyme, Russia) were ligated with the plasmid vector pUC19 linearized by restrictase *Pst*I. The resulting ligase mixture was used for transformation of *E. coli* ER 2267 (F' proA+B+ lacIq Δ(lacZ)M15 zzf::mini-Tn10 (Kanr)/ Δ(argF-lacZ)U169 glnV44 e14- (McrA-) rfbD1? recA1 relA1? endA1 spoT1? Thi-1 Δ(mcrC-mrr) 114::IS10)

competent cells (New England Biolabs, USA). After incubation on selective medium with ampicillin, the total plasmid DNA was isolated from the clones obtained. Isolated DNA was treated with restrictase *BspAC*I and transformed again into *E. coli* ER 2267 cells. All cloning procedures (preparation of competent cells, cell transformation by plasmid DNA) were carried out by standard methods [7]. Electroporation was performed in Easyject Prima (EquiBio, Germany) according to the manufacturer's protocol. The nucleotide sequence of DNA fragments was determined by Sanger's method in an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's protocol.

Expression of the gene for methyltransferase M1.BspACI. PCR was carried out with primers produced by SibEnzyme (recognition sites *FauND*I (CATATG) and *BamH*I (GGATCC) are underlined):

M1ACup 5′-GTGACTAAACATATGAAGAATTTTAA-ATTTATTGATC-3′,

M1AClow 5′-TGTTAAACGGATCCTCAATTCATAT-TATTCAAAC-3′.

The PCR product was treated with restrictases *FauND*I and *BamH*I, purified by elution from agarose gel using Qiagen kits in accordance with the manufacturer's instructions, and incorporated into expression vector pJW2 by the same sites. The clones carrying recombinant plasmid pM1BspACI were selected after electroporation with the ligase mixture of *E. coli* RRI cells (F-Δ(gpt-proA)62 leuB6 glnV44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20 (Strr) xyl-5 mtl-1 RecA+). Exposure of the clones to thermal induction resulted in appearance of a band corresponding to the target protein in 10% SDS-polyacrylamide gel.

Biomass was accumulated in 0.5-liter flasks with 100 ml of the nutrient medium. The culture (5 ml) grown at 30°C overnight was inoculated into a flask with 100 ml of LB nutrient broth containing 100 μg/ml ampicillin. The cells were grown in a thermostatted air shaker at 30°C, 130 rpm, for about 3 h till the mid log growth phase and then exposed to thermal induction at 42°C for 4 h. The cells were harvested by centrifugation at 6000 rpm for 30 min in a J2-21 centrifuge (Beckman, USA). The biomass was stored at −20°C.

Isolation of recombinant M1.BspACI. M1.BspACI was isolated from *E. coli* RRI cells carrying the recombinant plasmid pM1BspACI (derivation of this plasmid is described in "Results and Discussion"). All stages of the process were performed at 4°C. Biomass (4.5 g) was suspended in 15 ml of buffer A (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 5% glycerol) containing 0.3 mg/ml lysozyme and 0.1 mM phenylmethylsulfonyl fluoride for 40 min. The cell suspension was treated with ultrasound in a Soniprep 150 disintegrator (MSE, England) in an ice bath with eight 30-sec pulses and with 30-sec intervals for cooling of the suspension. The supernatant was transferred into a flask, and 15 ml of phosphocellulose P-11 pre-equilibrated with buffer A containing 50 mM NaCl was added. The mixture was stirred for 1 h, applied to a column, and washed with 30 ml of buffer A with 50 mM NaCl. Adsorbed material was eluted by the linear gradient of NaCl concentration (0.05-1.3 M) in buffer A (180 ml). Sixty fractions (3 ml each) were collected.

Fractions with the maximum methylase activity were combined and applied to a column with hydroxyapatite (HAP) (Bio-Rad, USA) (5 ml) pre-equilibrated with buffer P (10 mM K-phosphate, pH 7.2, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 5% glycerol, 50 mM NaCl). The column was washed with two volumes of buffer P. The enzyme was eluted with 200 ml of the same buffer with the K-phosphate concentration linearly increasing to 300 mM. Fifty fractions (4 ml each) were collected. Fractions with the highest methylase activity were combined and diluted three times with buffer A. The preparation was applied to a column with heparin-Sepharose (Bio-Rad) (4 ml) pre-equilibrated with buffer A containing 50 mM NaCl. The column was washed with two volumes of buffer A with 50 mM NaCl. The enzyme was eluted with the linear gradient of NaCl concentration 0.05-1.3 M in buffer P (120 ml). Forty fractions (3 ml each) were collected. Fractions with the maximum methylase activity were combined with addition of BSA to 0.1 mg/ml and dialyzed against 100 ml of buffer containing 50% glycerol, 10 mM K-phosphate, pH 7.3, 0.1 mM EDTA, 7 mM β-mercaptoethanol, and 0.1 M NaCl. The preparation was applied to a column with Sephacryl S-200 (120 ml) pre-equilibrated with buffer A containing 0.6 M NaCl and 0.05% Triton X-100. The enzyme was eluted in 120 ml of buffer A, and 30 fractions (4 ml each) were collected. Fractions with the maximum methylase activity were combined with addition of 5 ml of buffer P and sodium deoxycholate to 0.05% and applied to a column with HAP (4 ml) pre-equilibrated with buffer P. The enzyme was eluted with 200 ml of buffer P with the K-phosphate concentration linearly increasing to 200 mM. Forty fractions (5 ml each) were collected. Fractions with the maximum methylase activity were combined with addition of BSA to a concentration of 0.1 mg/ml and dialyzed against 100 ml of the buffer containing 50% glycerol, 10 mM K-phosphate, pH 7.3, 0.1 mM EDTA, 7 mM β-mercaptoethanol, and 0.1 M NaCl. The absence of foreign proteins in the final preparation was shown by electrophoresis in 10% SDS-polyacrylamide gel.

Detection of DNA methyltransferase activity in eluate. Methylase activity in chromatography eluate was detected by protection of the λ phage DNA from hydrolysis by restrictase *BspAC*I. For this purpose, 2 μl aliquots were taken from the fractions and added to 10 μl of the

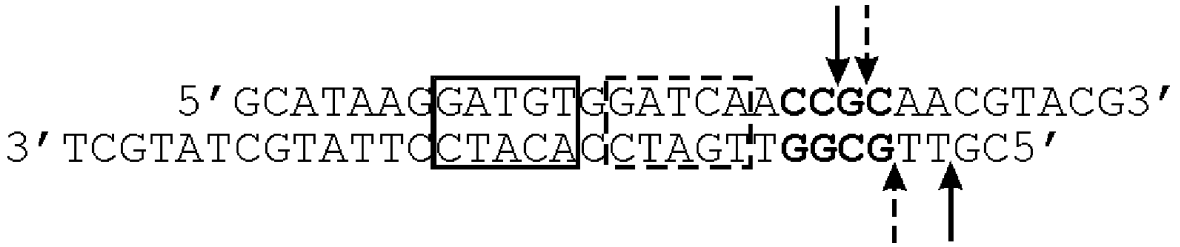

Fig. 1. Structure of synthetic oligonucleotide duplex M1.AC used for detection of methylated nucleotide residue. The M1.BspACI recognition site is in bold; the R.FokI recognition site in a solid frame; the R.AlwI recognition site is in a dashed frame; the sites of DNA cleavage by these enzymes are shown by solid and dashed arrows, respectively.

reaction mixture containing 0.5 μg of the λ phage DNA in SE buffer No. 5 (Y) (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 1 mM dithiothreitol (DTT)) and 160 μM SAM. The mixture was incubated at 30°C for 30 min, followed by addition of 1 μl of the *BspAC*I preparation, and kept at 30°C for 30 min more. The reaction products were separated in 1% agarose gel, stained with ethidium bromide solution, and observed under UV light.

Determination of optimal conditions for the enzyme activity. The optimal temperature, pH, and concentrations of potassium and sodium ions providing the highest enzyme activity were determined by the intensity of labeled SAM incorporation during 60 min of the reaction. The reaction mixture contained 1 μM λ DNA (on the basis of *BspAC*I recognition sites), 49 nM *BspAC*I, 10 μM [^{3}H]SAM, and buffer M (100 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, 0.2 mg/ml BSA, 2% glycerol). The methylation reaction was stopped by applying an aliquot to the DE81 filter (Whatman, England), which then was washed in 0.02 M ammonium bicarbonate solution three times by 20 min and then with water and 70% alcohol. After drying the filter, the quantity of incorporated tritium label was counted in a Beckman Coulter LS 6500.

Detection of methylated base in the M1.BspACI recognition site. The oligonucleotide duplex M1AC (SibEnzyme) was used as a substrate for methylated base detection in the methylase recognition site (Fig. 1).

The duplex was methylated with M1.BspACI using tritium-labeled SAM. For this purpose, an excess of methylase preparation was added for exhaustive methylation to 50 μl of the reaction mixture containing DNA duplex in SE buffer No. 5 (Y) and 10 μM [^{3}H]SAM. The reaction mixture was incubated at 30°C for 3 h. The methylase was inactivated by heating to 70°C for 10 min. Thus labeled oligonucleotide duplex was divided into two equal parts and cleaved by *Fok*I and *Alw*I restrictases in 50 μl of the reaction mixture with the same reaction buffer at 37°C for 3 h. Cleavage products were separated in 40% polyacrylamide gel with 7.5 M urea. For visualization of fragments, the gel was stained with Stains-all dye (Sigma, Germany); then single-chain hydrolysis products were eluted from the gel through passive diffusion and transferred to DE-81 filters. The incorporated label was counted in a Beckman Coulter LS 6500.

Determination of kinetic parameters of DNA methylation. The kinetic parameters of the reaction of λ DNA methylation were determined with 2.15 μM of λ DNA (on the basis of M1.BspACI recognition sites, for determination of methylation rate dependence on SAM), 9.8 nM M1.BspACI, and 10 μM [^{3}H]SAM (for determination of methylation rate dependence on λ DNA) in buffer M, pH 8.0. The reaction was performed for 1 h at the optimum temperature of enzyme activity. The presence of non-canonic methylation was determined under conditions of a single-cycle reaction. The reaction mixture contained 100 nM λ DNA (on the basis of sites), 100 nM M1.BspACI, and 10 μM [^{3}H]SAM in buffer M, pH 8.0.

The catalytic constant and the Michaelis constants were calculated by regression analysis using Origin 5.0.

RESULTS AND DISCUSSION

Cloning of genes for methylases of the BspACI RM system. The obtained chromosomal "library" of the *B. psychrodurans* AC strain contained ~1200 clones. Eight clones were obtained after repeated transformation. All of them carried plasmids resistant to *BspAC*I restrictase and, consequently, bearing the gene for the functionally active methylase of the given RM system. A plasmid isolated from one of the clones was named as pMBspACI and contained an insert of about 4 kb.

Genetic organization of BspACI RM system and cloning of the M1.BspACI methylase gene. Recombinant plasmid pMBspACI was used to determine the nucleotide sequence of the incorporated fragment. Nucleotide sequencing and analysis of the amino acid sequence of the insert revealed two similarly directed open reading frames (ORF), long enough for encoding the genes of the RM system (ORF1, region 3139-4101, and ORF2, region 1771-3138) (Fig. 2). The respective nucleotide and amino acid sequences were deposited in the GenBank database (N).

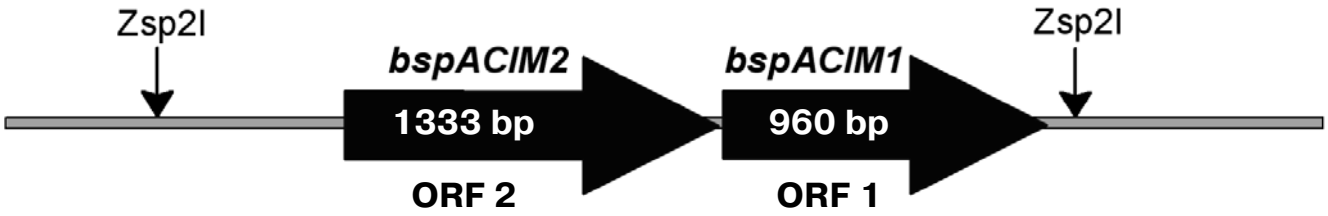

Fig. 2. Scheme of location of the DNA methyltransferase genes from the BspACI restriction–modification system.

The first frame codes for a polypeptide of 320 amino acid residues with the predicted molecular weight of 36.57 kDa. The second ORF of 1367 bp codes for a polypeptide of 455 amino acid residues with the predicted molecular weight of 50.58 kDa. Analysis of amino acid sequences of potential protein products of these two ORFs showed their affiliation with the class of C5 methyltransferases. Plasmid pMBspACI was used for further subcloning. PCR with primers M1ACup and M1AClow was used for amplification of the DNA region of *B. psychrodurans* AC containing ORF1 for further cloning as a part of expression vector pJW2 by the *FauND*I and *BamH*I sites. A recombinant plasmid pM1BspACI carrying the functionally active *bspACIM1* gene was obtained after transformation of ligase mixture into the strain *E. coli* RRI and selection of clones grown at 30°C.

Isolation of BspACI DNA methyltransferase preparation and determination of optimal conditions for the enzyme activity. M1.BspACI was isolated from the cell extract of *E. coli* carrying the recombinant plasmid pM1BspACI with the *bspACIM1* gene by means of column chromatography (Fig. 3a). The solution of homogenous enzyme preparation (4.5 ml) was obtained from wet biomass (4.5 g) at a concentration 0.35 μg/μl (Fig. 3b).

Figure 4 shows the dependence of M1.BspACI activity on temperature, pH, and the concentrations of sodium and potassium ions in the reaction medium. The maximum enzyme activity is observed at 30°C. The maximum rates of DNA hydrolysis by the *BspAC*I restrictase and growth of the *B. psychrodurans* AC strain are also observed at this temperature [3]. All further experiments were carried out at 30°C. The pH value corresponding to the highest enzyme activity was 8.0, being in accordance with the usual pH optimum for DNA methyltransferases. As concerns the enzyme activity dependence on sodium and potassium ion concentrations in the reaction medium, the maximum M1.BspACI activity is observed in their total absence.

Detection of methylated base. Eight single-chain DNA fragments were obtained after the cleavage of H^3-labeled duplex separately by *Fok*I and *AclW*I restrictases. Results of determination of the amount of radioactive label in each fragment are shown in Table 1. One can see that the label is incorporated mainly into fragments of 21 and 20 nucleotides in length. This situation can be

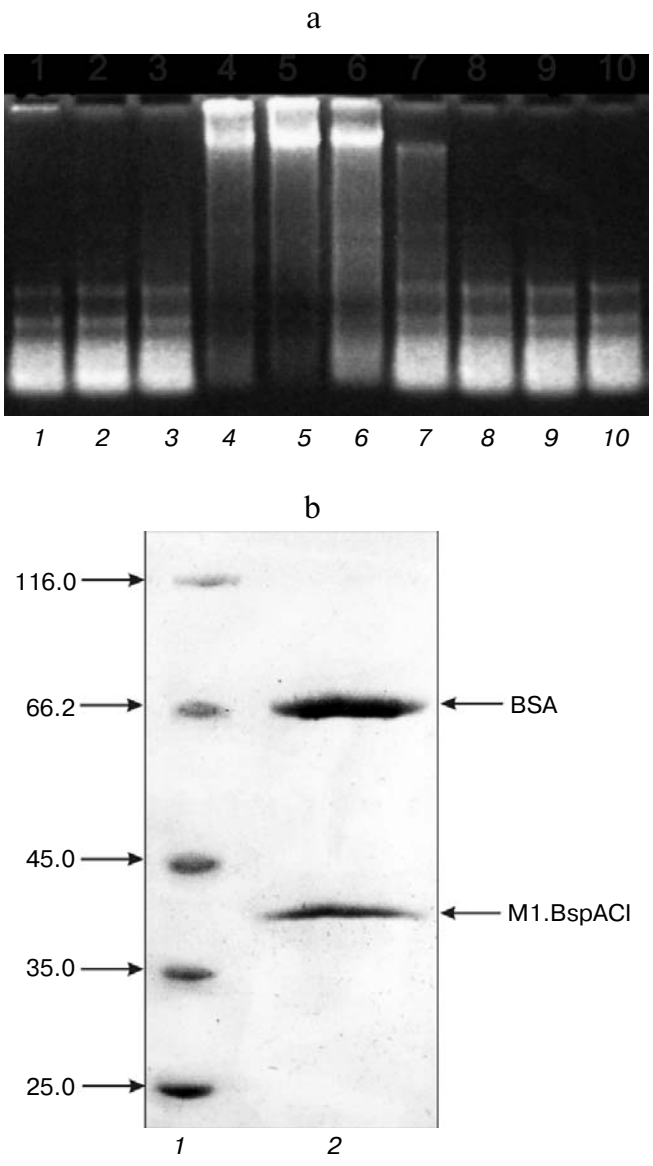

Fig. 3. a) Electrophoregram of the profile of M1.BspACI activity after the last stage of HAP chromatography. The enzyme activity was tested by protection of the λ phage DNA from hydrolysis by R.BspACI. Lanes: *1-10*) each second fraction beginning from the 13th; *4-6*) fractions with considerable DNA methyltransferase activity; b) electrophoregram of high-purity preparation of the enzyme M1.BspACI. Lanes: *1*) molecular weight markers (characters on the left show their molecular weights, kDa); *2*) 20 μl of M1.BspACI preparation.

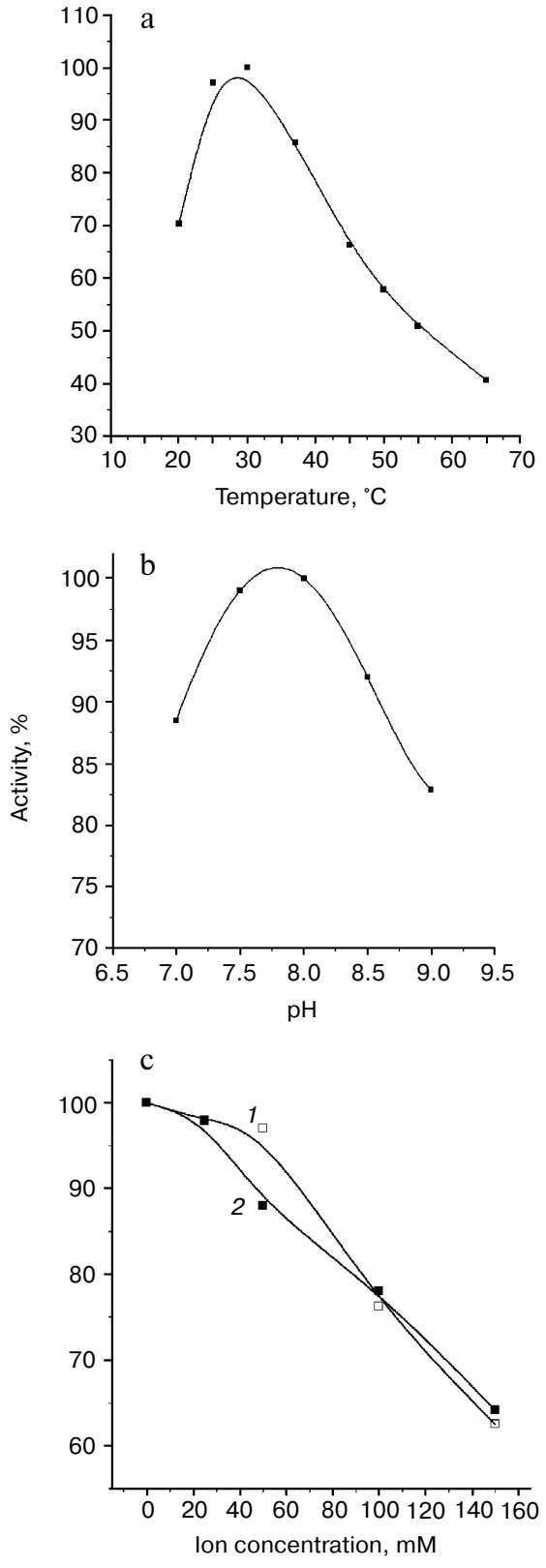

Fig. 4. Dependence of the rate of phage DNA methylation by the enzyme M1.BspACI (concentrations: enzyme, 49 nM; λ phage DNA on the basis of 5′-CCGC-3′ sites, 1 μM; [^{3}H]SAM, 10 μM) on temperature (a), pH (b), and ion concentrations (c) of sodium (*1*) and potassium (*2*).

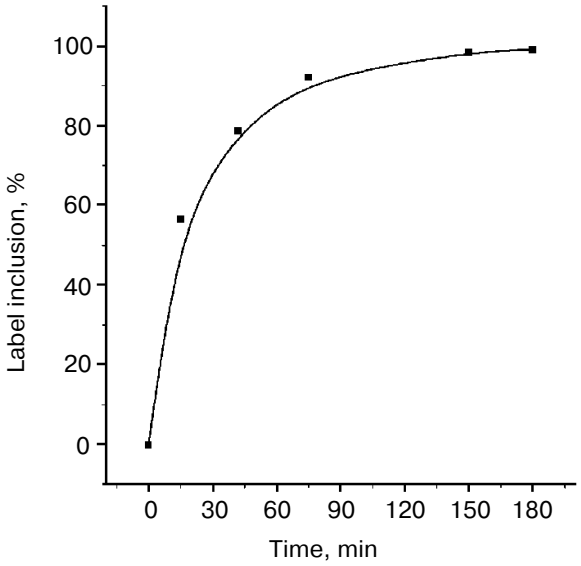

Fig. 5. Time dependence of the concentration of methylated sites of the λ phage DNA. Concentrations: 5′-CCGC-3′ sites of λ phage, 100 nM; M1.BspACI, 100 nM; [^{3}H]SAM, 10 μM.

observed only if M1.BspACI modifies the first cytosine of the 5′-CCGC-3′ sequence.

Detection of non-canonic methylation. Taking into account the frequent occurrence of enzymes with degenerate recognition site among methyltransferases [8, 9], we decided to check the presence of non-canonic methylation in M1.BspACI. Figure 5 presents the results of this experiment. It can be seen that the enzyme completely methylates the λ DNA and there is no tendency to methylation of other sites in addition to 5′-CCGC-3′ in the course of time.

Determination of kinetic parameters of DNA methylation. The dependence of methylation rate on concentration of one of the substrates was measured under saturation with the other substrate. Figure 6a shows the dependence of methylation rate on concentration of λ phage DNA. The Michaelis constant was 0.053 ± 0.007 μM. The catalytic constant was 0.095 ± 0.002 min^{-1}. The dependence of DNA methylation rate on concentration of the second substrate (SAM) was analyzed using the concentration of methylation sites of 2.15 μM. The dependence of methylation rate on SAM concentration is shown in Fig. 6b. Based on the findings, the Michaelis constant is 5.1 ± 0.3 μM.

It has been shown previously in our laboratory that the K_m value of DNA in IIS methyltransferases modifying the substrate with formation of N6-methyladenine or C5-methylcytosine is higher by an order of magnitude than the K_m of DNA in the enzymes recognizing symmetric sequences [2, 9]. The obtained $K_{m\ DNA}$ value for M1.BspACI is in agreement with this regularity: the $K_{m\ DNA}$ of methylase under study is 20-fold higher than

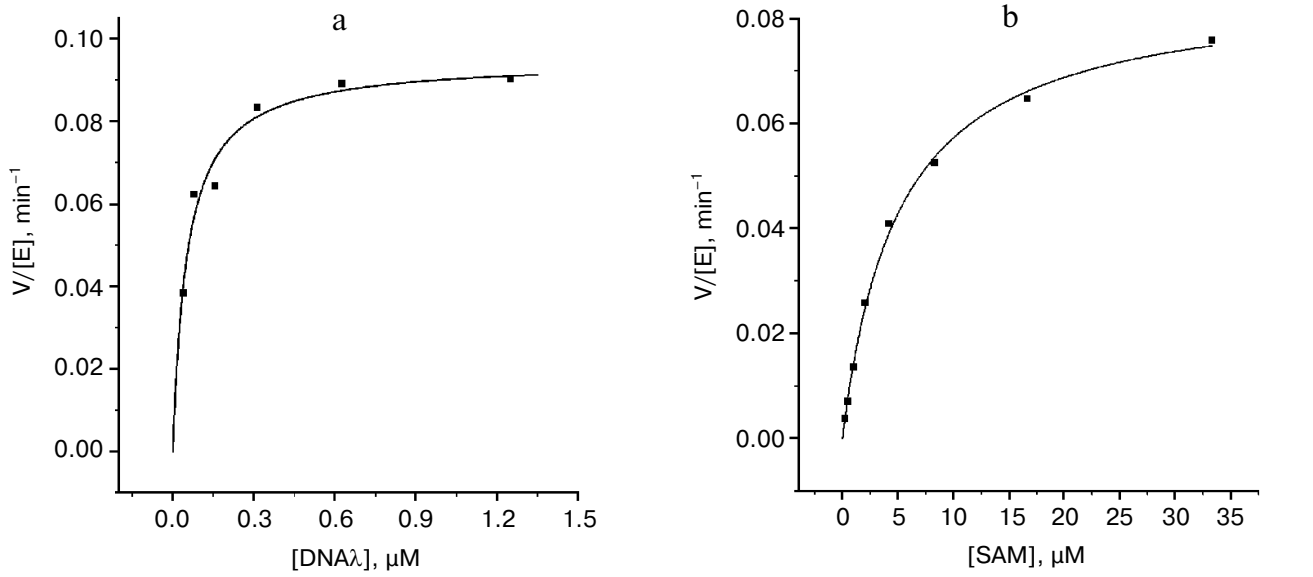

Fig. 6. Dependence of rate of λ phage DNA methylation by M1.BspACI (reaction time, 60 min; enzyme concentration, 9.8 nM) on DNA concentration ([^{3}H]SAM, 10 μM) (a) and SAM concentration (concentration of λ phage DNA on the basis of 5′-CCGC-3′ sites, 2.15 μM) (b).

the maximum value for C5-methylases (M.EcoHK31I) presented in Table 2.

It seems interesting to compare the catalytic constants of DNA methyltransferases recognizing the palindromic and non-palindromic DNA sequences. On the whole, the values of these constants for the enzymes with symmetric recognition sites are higher than for DNA methylases of the IIS type modifying both adenine [10] and cytosine [9]. IIS C5-methyltransferases M1.BspACI and M.FauIA have k_{cat} values of 0.095 and 0.052 min^{-1} [9], respectively, i.e. more than 25 times lower than the minimal value of this parameter for C5-methyltransferases with palindromic recognition sites (2.52 min^{-1} in *Eco*RII) presented in Table 2.

Table 1. Inclusion of tritium label into restriction fragments obtained after cleavage of *Fok*I and *AclW*I oligonucleotide duplex

Restrictase used for hydrolysis	Restriction fragment*	Length, nucleotides	Radioactivity, cpm
*AclW*I	Upper chain		
	5'-GCATAAGGATGTGGATCAA**CC**-3' 5'-**GC**AACGTACG-3'	21 10	1184 75
	Lower chain		
	5'-**CGG**TTGATCCACATCCTTATGCTATCGT-3' 5'-CGTT**G** -3'	28 5	90 56
*Fok*I	Upper chain		
	5'-GCATAAGGATGTGGATCAA**C**-3' 5'-**CGC**AACGTACG-3'	20 11	784 157
	Lower chain		
	5'-T**GCGG**TTGATCCACATCCTTATGCTATCGT-3' 5'-CGT-3'	30 3	223 143

* Bases included in the M1.BspACI recognition site are in bold.

Table 2. Values of catalytic parameters for some DNA methyltransferases of the IIS type

Enzyme, recognition site*	Substrate	k_{cat}, min^{-1}	$K_{m\ DNA}$, nM	$K_{m\ SAM}$, μM	Reference
*EcoR*II C**C**WGG	λ phage DNA	2.52	0.22	–	[11]
*EcoHK*31I YGG**C**CR	plasmid pWM2372	3.0	2	0.58	[12]
*Msp*I **C**CGG	λ phage DNA	10.2	1.8	0.013	[13]
M1.BspACI **C**CGC	λ phage DNA	0.095	53	5.1	this work

* Bases modified by these methylases are in bold.

Thus, in the case of both N6- and C5-methyltransferases, the enzymes recognizing palindromic recognition sites show higher activity and bind to DNA more efficiently than methyltransferases modifying non-palindromic DNA sequences.

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