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M.*Bst*F5I-2 and M.*Bst*F5I-4 DNA Methyltransferases from *Bst*F5I Restriction–Modification System of *Bacillus stearothermophilus* F5

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Abstract—The *Bst*F5I restriction–modification system from *Bacillus stearothermophilus* F5 includes four site-specific DNA methyltransferases, thus differing from all known restriction–modification systems. Here we demonstrated for the first time that one bacterial cell can possess two pairs of methylases with identical substrate specificities (methylases *Bst*F5I-1 and *Bst*F5I-3 recognize GGATG, whereas methylases *Bst*F5I-2 and *Bst*F5I-4 recognize CATCC) that modify adenine residues on both DNA strands. Different chromatographic methods provide homogenous preparations of methylases *Bst*F5I-2 and *Bst*F5I-4. We estimated the principal kinetic parameters of the reaction of transfer of methyl group from the donor S-adenosyl-L-methionine to the recognition site 5′-CATCC-3′ catalyzed by *Bst*F5I-2 and *Bst*F5I-4 DNA [N6-adenine]-methyltransferases from the *Bst*F5I restriction–modification system.

Key words: DNA methyltransferase, restriction–modification system, *Bacillus stearothermophilus*, kinetics of an enzymatic reaction

DNA methyltransferases (DMTs) play a crucial role in life cycles of both prokaryotes and eucaryotes ensuring genome integrity during transfer of genetic information, control of transcription, and regulation of gene expression. A broader involvement of DMTs in cellular metabolism has been discussed [1]. DMTs catalyze the transfer of a methyl group from S-adenosyl-L-methionine to the N6-position of adenine or to the C5- or N4-positions of cytosine residues located in a specific site [2]. DMTs are interesting models for the study of the site-specific DNA–protein interactions due to high specificity and hypothetical simple structural organization. In addition, the elucidation of the mechanisms of action of these enzymes remains an important objective in the field of biological methylation of DNA.

The region of recognition for the enzymes of type II R–M systems is mainly symmetrical; one DMT that modifies the site on both strands is sufficient for protection of the host DNA. In type IIs R–M systems the recognized sequences are non-palindromic and two different DMTs are needed for DNA protection; each DMT methylates one DNA strand in the common recognition site. The exceptions are homologous R–M systems *Fok*I [3] and *Sts*I [4] that include only one DMT. However, it was demonstrated that these enzymes consist of two domains possessing DNA methyltransferase activity and modifying different strands in the recognition site [5]. Cloning and expression resulted in two catalytically active domains—DMT *Fok*IN and DMT *Fok*IC. Each of these methylates only one DNA strand in the recognized sequence [6].

We isolated earlier the strain *Bacillus stearothermophilus* F5 that produces the restriction endonuclease *Bst*F5I. This enzyme recognizes the same specific sequence 5′-GGATG-3′/5′-CATCC-3′ as the restriction endonucleases *Fok*I and *Sts*I, but cleaves DNA in position 2/0 [7], whereas the restriction enzymes *Fok*I and *Sts*I cleave DNA differently (9/13 and 10/14, respectively). While studying the *Bst*F5I R–M system, we cloned

Abbreviations: DMT) DNA methyltransferase; R–M system) restriction–modification system; SAM) S-adenosyl-L-methionine; PMSF) phenylmethylsulfonyl fluoride.

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and determined the nucleotide sequences of four DMTs from this strain—*Bst*F5I-1 [8], *Bst*F5I-2 [9], *Bst*F5I-3 [10], and *Bst*F5I-4 [11]. It was shown that DMTs *Bst*F5I-1 and *Bst*F5I-3 methylate adenine on the upper strand in the recognition site. Comparative analysis of the primary structures of the DMTs genes revealed that DMT *Bst*F5I-2 and DMT *Bst*F5I-3 are homologous to the C- and N-terminal domains of the enzymes—isoschizomers *Fok*I and *Sts*I (approximately 80% homology) [9, 10]. We studied earlier the kinetic properties of DMTs *Bst*F5I-1 and *Bst*F5I-3 [12].

Here we describe the purification and isolation of homogenous preparations of the enzymes DMTs *Bst*F5I-2 and *Bst*F5I-4, estimation of their substrate specificity, and comparative study of the kinetic properties of these DMTs that are responsible for methylation of the same DNA strand in the recognition site 5′-CATCC-3′.

MATERIALS AND METHODS

Strains and reagents. In this study we used the bacterial strains *Bacillus stearothermophilus* F5 from the collection of JSC SibEnzyme (Novosibirsk, Russia) and *Escherichia coli* RRI (New England Biolabs, USA). We also used the following reagents and the components of nutrient media: Tris (Promega, USA); acrylamide, KH_2PO_4, ampicillin (Helicon, Russia); bis-acrylamide, EDTA (Fluka AG, Switzerland); agarose (Hybaid-AGS, Germany); glycerol, 2-mercaptoethanol, Triton X-100 (ICN, USA); lysozyme (Serva, Germany); S-adenosyl-L-methionine (SAM) (New England Biolabs); dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA); tryptone, yeast extract (Organotechnie, France); hydroxyapatite, heparin-Sepharose (BioRad, USA); phosphocellulose P-11 (Whatman, England); Sephacryl S-200, [^{3}H-CH_3]SAM (Amersham, England); and enzymes and DNA preparations (SibEnzyme, Russia). All other reagents were from Russian suppliers.

Production of DMT *Bst*F5I-2 and DMT *Bst*F5I-4 biomass. *Bst*F5I-2 and *Bst*F5I-4 genes were amplified and cloned in pJW2 vector [13] under thermoinducible promoter of λ phage using the restriction sites *Bam*HI and *Fau*NDI. The strain *E. coli* RRI was used for transformation of the recombinant plasmids. The cells transformed with the plasmid pJW-*bst*F5IM-2 or pJW-*bst*F5IM-4 were inoculated into LB medium containing tryptone (10 g/liter), yeast extract (5 g/liter), NaCl (5 g/liter), pH 7.0-7.3, with addition of ampicillin to concentration 100 μg/ml and grown on a thermostatic shaker at 30°C and 180 rpm to A_{550} 0.8-0.9. Then the cells were thermoinduced at 42°C for 4 h. The cells were collected by centrifugation at 3000*g* (4°C) and stored at −20°C.

Isolation of DMT *Bst*F5I-2 enzyme. All procedures were carried out at 4°C. Eight grams of biomass were suspended in 30 ml of buffer A (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 5% glycerol) with addition of lysozyme (0.3 mg/ml). After 30 min the cells were broken with a Soniprep 150 ultrasound disintegrator (MSE, England) 6 times for 30 sec. After a 1 min-long treatment with Triton X-100 (concentration 0.1%), cellular debris was eliminated by centrifugation at 5000*g* for 30 min using a J2-21 centrifuge (Beckman, USA). The supernatant was transferred into a vessel, then proteinase inhibitor PMSF, NaCl to concentration 200 mM, and 16 ml of phosphocellulose P-11 equilibrated with buffer A were added. The suspension was incubated for 1 h at 4°C and then applied on a column (16 mm × 20 cm) and washed with two volumes of buffer A with 200 mM NaCl. The sample was eluted with 160 ml of linear NaCl gradient from 0.2 to 1.5 M in buffer A. Fractions that contained the enzyme were collected, diluted 2-fold with buffer A, and applied on a column (10 mm × 20 cm) with 5 ml of hydroxyapatite, washed with two volumes of buffer P (10 mM KH_2PO_4, pH 7.2, 0.1 mM EDTA, 200 mM NaCl, 10 mM 2-mercaptoethanol, 5% glycerol). The sample was eluted with 120 ml of linear gradient of potassium phosphate from 50 to 400 mM in buffer P. Enzymatically active fractions were collected, diluted 3-fold with buffer A, and applied on a column (10 mm × 10 cm) with 4 ml of heparin-Sepharose equilibrated with buffer A. The column was washed with two volumes of buffer A with 200 mM NaCl, and the enzyme was eluted with 100 ml of linear NaCl gradient from 200 mM to 1.5 M in buffer A. Active fractions without admixture proteins (according to SDS-PAGE) were collected together and concentrated against buffer C (10 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 50% glycerol). The resulting methylase preparation was stored at −20°C.

Isolation of DMT *Bst*F5I-4 enzyme. Chromatographic purification of methylase *Bst*F5I-4 was carried out using the above indicated buffers and reagents at 4°C. Seven grams of biomass of *E. coli* cells containing DMT M.*Bst*F5IM-4 were suspended in 20 ml of buffer A with addition of lysozyme (0.3 mg/ml) for 30 min. The suspension was subjected to ultrasound treatment 5 times for 1 min each. Cellular debris was eliminated by centrifugation at 5000*g* for 30 min. The supernatant was transferred into a vessel, then proteinase inhibitor PMSF was added together with NaCl to concentration 100 mM and 7 ml of heparin-Sepharose equilibrated with buffer A. The suspension was incubated for 1 h at 4°C and applied on a column (10 mm × 20 cm) that was washed with two volumes of buffer A with 100 mM NaCl. The sample was eluted with 150 ml of linear NaCl gradient from 100 to 800 mM in buffer A. Fractions that contained the target protein were pooled, diluted 2-fold with buffer A, and applied on a column with 3 ml of hydroxyapatite (10 mm × 10 cm). The column was washed with two volumes of buffer P containing 70 mM potassium phosphate. The sample was eluted with 100 ml of linear gradient of potassium phos-

phate from 70 to 700 mM in buffer P. The fractions with target activity were pooled, and the protein was pelleted by addition of ammonium sulfate (75% saturation) at 4°C overnight. Afterwards the suspension was centrifuged at 5000*g* for 30 min. The pellet was suspended in 1 ml of buffer A and applied on a column (16 mm × 70 cm) with Sephacryl S-200 equilibrated with buffer A with 0.3 M NaCl. After elution, the fractions containing methylase *Bst*F5I-4 were pooled and rechromatographed on 3 ml of hydroxyapatite. The fractions that were shown to contain the targeted homogenous protein were pooled. The enzyme was concentrated against buffer C and stored at −20°C.

Testing of methylase activity in a profile. Methylase activity in chromatographic profiles was tested by protection of λ phage DNA from hydrolysis by restriction endonuclease *Bst*F5I. λ phage DNA was incubated with aliquots from fractions as follows: 2-μl aliquots from fractions were diluted 5-fold with SE-buffer Y (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 1 mM DTT) and added to 20 μl of the reaction mixture that contained 1 μg of λ phage DNA in SE buffer Y and 80 μM SAM. The mixture was incubated for 15 min at 65°C, then 1 μl of restriction endonuclease *Bst*F5I was added followed by incubation for 15 min at 65°C. The reaction products were separated in 1% agarose gel, stained with a solution of ethidium bromide, and visualized under UV light. The rate of purification of the enzyme was monitored by electrophoresis (25-μl aliquots were subjected to electrophoresis in denaturing 10% SDS-polyacrylamide gels).

Measurement of the specific activity of homogenous methylase preparations. A unit of specific methylase activity was defined as the minimal quantity of the enzyme sufficient for protection of 1 μg of λ phage DNA from hydrolysis by restriction endonuclease *Bst*F5I (during 1 h at 55°C in 10-μl reaction mixture). For measurement of the activity of DMT preparations we used reaction buffer Met containing 50 mM Tris-HCl (pH 7.9 for DMT *Bst*F5I-2 and pH 8.7 for DMT *Bst*F5I-4), 1 mM EDTA, 1 mM DTT, and 80 μM SAM. Enzyme preparations were diluted with Met buffer 2-, 4-, 8-fold, etc., and then added to 10 μl of reaction mixture and incubated for 30 min at 55°C. Afterwards 2 μl of 10× SE buffer Y, 8 μl of H_2O, and 1 μl (5 U) of restriction endonuclease *Bst*F5I were added and the mixture was incubated at 65°C for 30 min. The reaction products were separated in 1% agarose gel, and the rate of λ phage DNA protection from hydrolysis by restriction endonuclease *Bst*F5I was assessed.

Measurement of methylase activity at different salt concentrations by the quantity of incorporated ^{3}H label. We assessed the effect of different concentrations of K^+ and Na^+ on the activities of methylases *Bst*F5I-2 and *Bst*F5I-4 in reactions of methylation of λ phage DNA by the quantity of incorporated ^3H-CH_3-groups. Three NaCl and KCl concentrations were tested: 50, 100, and 150 mM; the standard reaction buffer was taken for the control. Concentrations of SAM, DMTs *Bst*F5I-2 and *Bst*F5I-4 were 5 μM, 8 nM, and 57 nM, respectively. The reaction was carried out for 20 min. Radioactivity was counted as described below.

Determination of the strand modified by methylases *Bst*F5I-2 and *Bst*F5I-4. To detect which strand was modified by methylases, we used SAM with ^{3}H-labeled methyl group and a combination of duplexes that contain modified adenine residue in the recognition site of restrictase *Bst*F5I (recognition site is underlined, M = N6-methyladenine):

I 5-CGGCCAGCGGATGCGGGAGCG-3,

Im 5-CGGCCAGCGGMTGCGGGAGCG-3,

II 5-GCCGCTCCCGCATCCGCTGGCCGGC-3,

IIm 5-GCCGCTCCCGCMTCCGCTGGCCGGC-3.

The methylation reaction was carried out at 37°C for 2 h. Concentrations of DMTs *Bst*F5I-2, *Bst*F5I-4, duplex, and [^3H-CH_3]SAM were 0.32, 0.35, 1.0, and 20 μM, respectively. Aliquots from reaction mixtures were applied on DE81 filters (1 × 1 cm, Whatman). Filters were washed three times with 0.02 M NH_4HCO_3, twice with water, and once with 75% ethanol. Then they were dried and ^{3}H-radioactivity was measured in toluene scintillator in a Mark III radioactivity counter.

Measurement of kinetic parameters. Reaction mixture for methylation contained 100 mM Tris-HCl (pH 8.5 for DMT *Bst*F5I-2 and pH 9.0 for DMT *Bst*F5I-4), 1 mM EDTA, 1 mM DTT, BSA (0.2 mg/ml), and 5% glycerol. Concentrations of the substrates and the enzyme varied depending on the experimental conditions. Tritium-labeled SAM was used in the reactions; the time course of the reaction was chosen to ensure measurement of the initial velocities of the reactions. After appointed intervals, aliquots were taken from the reaction mixtures and applied on DE81 filters. Radioactivity was measured as described above. All experiments were performed at least two times.

RESULTS AND DISCUSSION

Isolation of DMT *Bst*F5I-2 enzyme. The presence of the active methylase in the fractions after each chromatography was detected by the two above described methods. After the first chromatography, the enzyme was eluted at NaCl concentrations from 700 mM to 1.12 M (Fig. 1). After the second and third chromatographies, the enzyme was eluted at potassium phosphate concentrations 140-330 mM and NaCl concentrations 0.5-

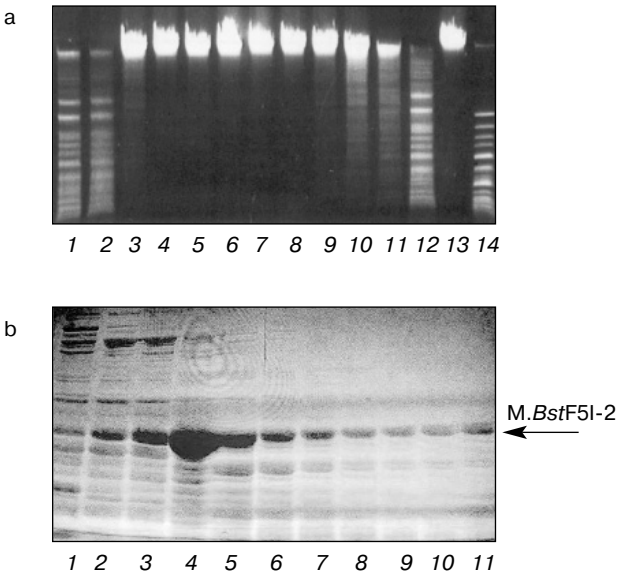

Fig. 1. Electrophoregrams of the profile of activity (a) and protein (b) for DMT *Bst*F5I-2 after the first step of chromatography on phosphocellulose. a) Testing of the enzymatic activity by protection of λ phage DNA from hydrolysis by restriction endonuclease *Bst*F5I (1% agarose). Lanes: *3-10*) peak of methylase activity; *13*) initial reaction mixture; *14*) λ phage DNA + restrictase *Bst*F5I. b) Testing by the protein (10% SDS-PAGE gel is stained with Coomassie brilliant blue dye). Lanes: *3-11*) peak of the target protein.

1.3 M, respectively (data not shown). Finally, we obtained 15 ml of homogenous DMT *Bst*F5I-2 preparation with protein concentration 0.45 mg/ml (Fig. 2). The specific activity of the enzyme was 60 U/μl. The molecular weight was 34.7 kD.

Isolation of DMT *Bst*F5I-4 enzyme. The results of testing of the enzyme activity after chromatography on heparin-Sepharose (the enzyme was eluted at NaCl concentrations 350-660 mM) are presented in Fig. 3. During the following chromatographic steps, the enzyme was eluted at potassium phosphate concentrations from 180 to 570 mM and from 300 to 500 mM. The purification resulted in 4.8 ml of DMT *Bst*F5I-4 preparation with protein concentration 0.1 mg/ml (Fig. 2) and specific activity 7 U/μl. The molecular weight of the enzyme was 43.6 kD.

Measurement of methylase activities at different salt concentrations. The influence of different concentrations of K^+ and Na^+ on the activity of DMTs *Bst*F5I-2 and *Bst*F5I-4 is presented in Fig. 4. DMT *Bst*F5I-2 is maximally active in the presence of 50 mM NaCl or KCl, as, for example, DMTs *Bam*HI, *Eco*RI, and *Hae*III [14]. The increase of K^+ concentration leads to a more rapid inhibition of the enzyme activity in comparison to Na^+. Salt concentration influences the activity of DMT *Bst*F5I-4 differently. This enzyme is maximally active in the absence of both K^+ and Na^+, and the increase in salt concentrations is accompanied by large decrease of the enzy-

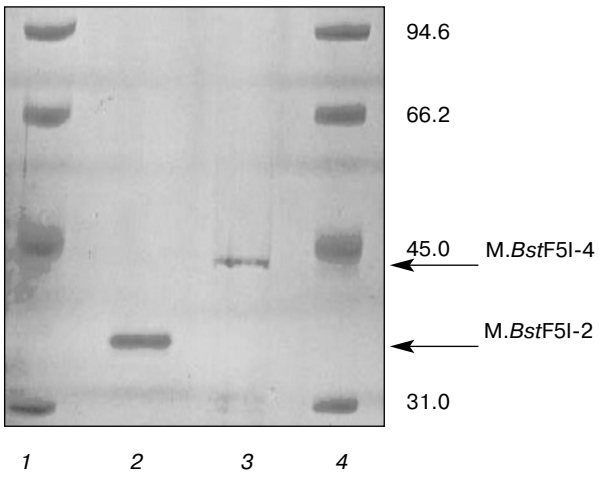

Fig. 2. Preparations of the enzymes DMTs *Bst*F5I-2 and *Bst*F5I-4. Electrophoresis in 10% SDS-polyacrylamide gels. Lanes: *1*, *4*) protein ladder; *2*) preparation of DMT *Bst*F5I-2; *3*) preparation of DMT *Bst*F5I-4. The molecular weights of proteins in kD are indicated on the right.

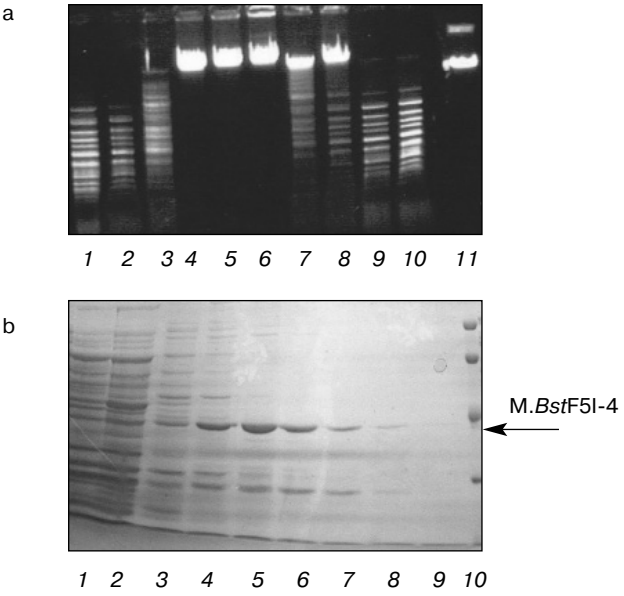

Fig. 3. Electrophoregrams of profiles of activity (a) and protein (b) of DMT *Bst*F5I-4 after the first step of chromatography on heparin-Sepharose. a) Testing by activity (1% agarose). Lanes: *4-7*) peak of methylase activity; *11*) initial reaction mixture. b) Testing by protein (10% SDS-PAGE). Lanes: *4-7*) peak of the target protein; *10*) protein ladder.

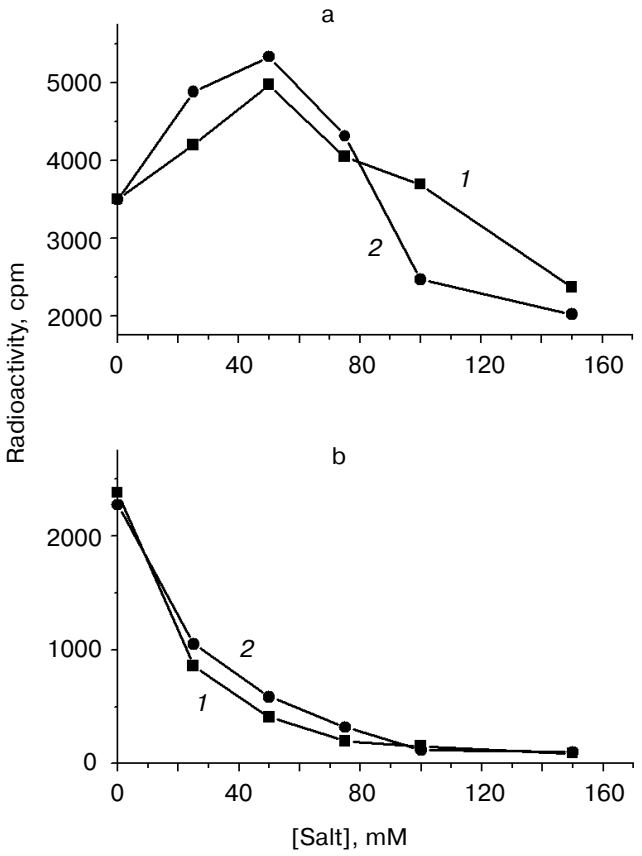

Fig. 4. Dependence of the velocity of methylation by DMTs *Bst*F5I-2 (a) and *Bst*F5I-4 (b) on concentration of NaCl (*1*) and KCl (*2*) measured by the quantity of incorporated ^{3}H-label.

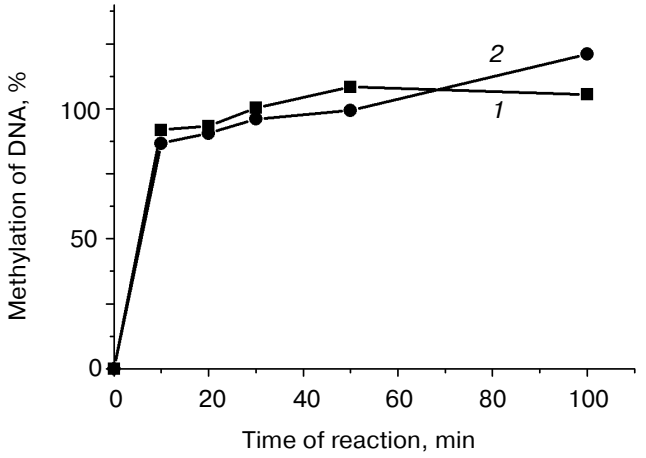

Fig. 5. Maximal methylation of λ phage DNA by DMTs *Bst*F5I-2 (*1*) and *Bst*F5I-4 (*2*). Reaction mixture contained 100 nM of the enzyme, 100 nM of sites of λ phage DNA, and 5 μM SAM. The reaction was carried out at 55°C and terminated after 50 min, when DNA was precipitated by 96% ethanol and the reaction was repeated.

matic activity. It is known that the enzymes DMT *Alu*I, Dam *E. coli*, and DMTs *Hha*I and *Hpa*II are also maximally active in the absence of monovalent ions [14].

Determination of the strand methylated by DMTs *Bst*F5I-2 and *Bst*F5I-4. To detect which DNA strand was methylated by the methylases, we used combinations of oligonucleotides containing modified adenine residue in the recognition site of the restriction endonuclease *Bst*F5I. It was suggested earlier that DMT *Bst*F5I-2 methylates adenine residue in the lower strand of the recognized sequence like DMTs *Fok*IC and *Sts*IC. This suggestion was based on the high homology between amino acid sequences of DMT *Bst*F5I-2 and C-terminal domains of DMTs *Fok*I and *Sts*I. Data presented in Table 1 indicate that the label is incorporated only in the lower DNA strand. Thus, DMTs *Bst*F5I-2 and *Bst*F5I-4 modify the DNA strand that contains the sequence 5′-CATCC-3′.

Comparison of the substrate specificities of DMTs *Bst*F5I-2 and *Bst*F5I-4. Both DMTs modify adenine residue in the non-palindromic sequence 5′-CATCC-3′. The reaction of maximal methylation of λ phage DNA was used to check the substrate specificity of the methylases (Fig. 5). The plateaus of methylation curves for the two enzymes coincide and are close to 100% modification of the region 5′-CATCC-3′ indicating high specificity of these enzymes. A slight exceeding over the 100%-level of specific methylation could be explained by lower (by one or several orders) unspecific DNA methylation. Thus, both DMTs have the same specificity and modify the DNA sequence 5′-CATCC-3′.

Stable coincidence of two DMTs with identical specificities in *Bst*F5I cells could be related to some peculiarities of functioning of these enzymes in the cells of the microorganism *Bacillus stearothermophilus* F5.

Dependence of the enzymatic activities on the temperature and pH. The temperature optimum for the DMTs is close to 55°C (Fig. 6), thus correlating with the optimal

Table 1. Combination of oligonucleotides and incorporation of the ^{3}H-CH_3-group in DNA substrate in methylation reactions catalyzed by DMTs

Combination of oligonucleotides	Incorporated ^{3}H-label, radioactivity, cpm	
	*Bst*F5I-2	*Bst*F5I-4
I + II	44 826	91 706
I + IIm	1824	1646
Im + II	39 913	95 985
Im + IIm	1313	1734
Background	1204	1551

temperature for growth of *B. stearothermophilus* (55°C) [7]. A model in which only two ionized protein groups are taken into account can be used for approximate description of the pH dependence of the enzymatic activity. According to this model, an enzyme is considered as a dibasic acid HEH (E is enzyme, H are hydrogen atoms of the ionized groups of the enzyme) with two different acid groups. In this case, experimental curves could be well enough described by the Michaelis function [15]:

$$V = V_m/(1 + K_1/[H^+] + [H^+]/K_2), \quad (1)$$

where $[H^+]$ is the concentration of hydrogen ions, $K_1 = [E^{2-}]\cdot[H^+]/[HE^-]$ and $K_2 = [HE^-]\cdot[H^+]/[HEH]$. Constants K_1 and K_2 are assumed to be molecular constants, since generally they cannot be attributed to ionization of individual groups. The total curve characterizing the influence of pH on the enzymatic activity reflects a complicated effect of pH on a number of factors that influence the velocity of an enzymatic reaction. The values of pK_1 obtained for both DMTs (10.3 for DMT *Bst*F5I-2 and 9.6 for DMT *Bst*F5I-4) are close to pK_a of the sulfhydryl group of cysteine, while the pK_2 values (5.5 and 7.7, respectively) are close to the pK_a of the imidazole group of histidine [16]. Their state of ionization may determine the portion of the active enzymatic form.

Figure 7 demonstrates that the two dependences differ significantly from each other. The results of adjustment of Eq. (1) to the experimental curves show that the graph of dependence of the activity for DMT *Bst*F5I-2 is characterized by a relatively broad pH range (7.0-9.0), but the optimal pH value is between pH 7.5 and 8.5 that is typical for the majority of DMTs. DMT *Bst*F5I-4 is maximally active in more basic conditions and is characterized by a sharp peak of activity at pH 8.7.

Determination of the standard kinetic parameters of the reactions catalyzed by DMTs *Bst*F5I-2 and *Bst*F5I-4. The reactions catalyzed by DMTs *Bst*F5I-2 and *Bst*F5I-4

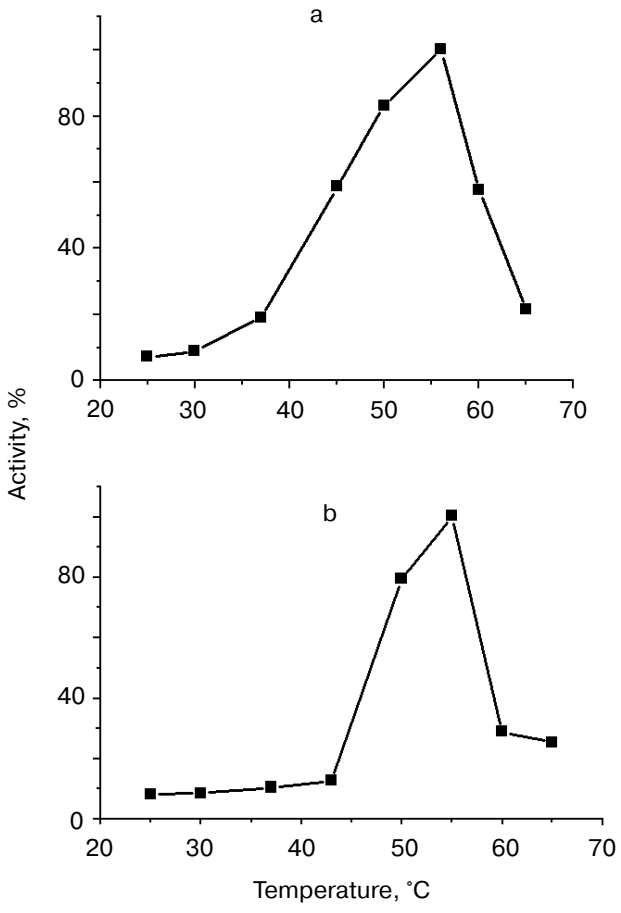

Fig. 6. Temperature dependence of the activity of DMT *Bst*F5I-2 (a) and DMT *Bst*F5I-4 (b). Both reactions were carried out at 1.3 μM of restriction sites of λ phage DNA and 5 μM SAM. Concentration of DMT: *Bst*F5I-2, 5 nM; *Bst*F5I-4, 30 nM. The reaction was carried out for 20 min.

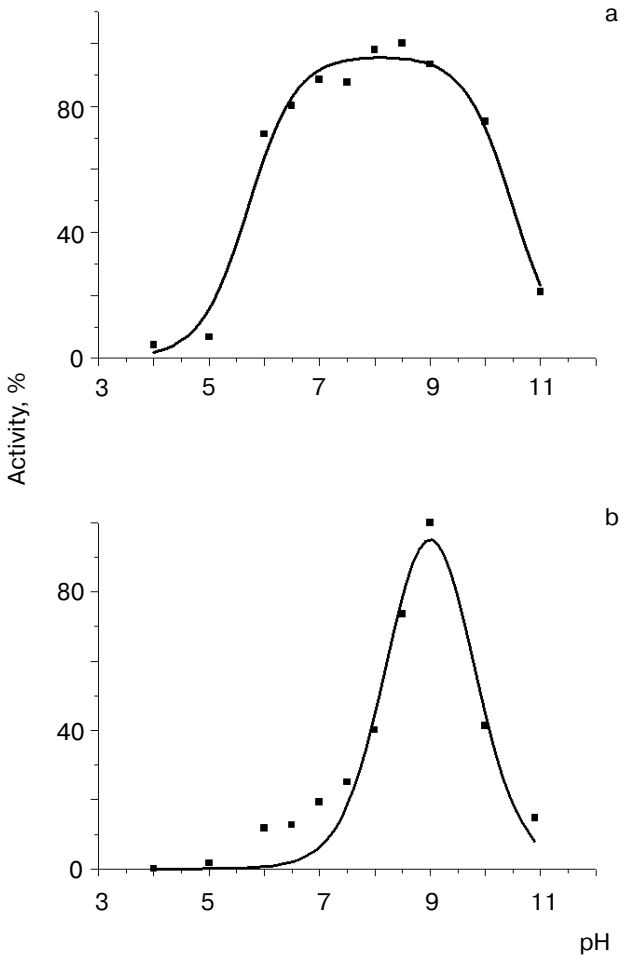

Fig. 7. Dependence of the activity of DMT *Bst*F5I-2 (a) and DMT *Bst*F5I-4 (b) on pH. Both reactions were carried out at 1.19 μM of restriction sites of λ phage DNA and 5 μM SAM. Concentration of DMT: *Bst*F5I-2, 5 nM; *Bst*F5I-4, 30 nM. The reaction was carried out for 20 min.

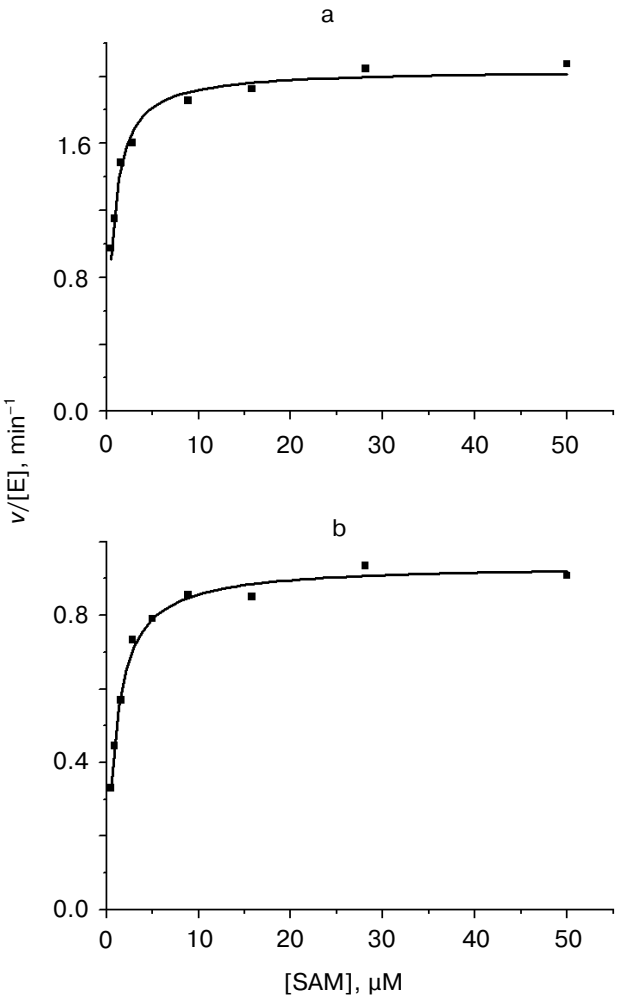

Fig. 8. Dependence of velocity of methylation of λ phage DNA by DMTs *Bst*F5I-2 (a) and *Bst*F5I-4 (b) on SAM concentration. Reaction mixture for DMT *Bst*F5I-2 contained 5.2 nM of the enzyme, 8 μM of methylation sites of λ phage DNA; for DMT *Bst*F5I-4, 15 nM of the enzyme, 9 μM of methylation sites of λ phage DNA. The reaction was carried out for 20 min.

are two-substrate reactions; therefore, the kinetic curves may be complicated due to two- or multi-stage type of reactions. However, in the studied interval of substrate concentrations the kinetic curves are close to the classic curve of Michaelis. For determination of the kinetic parameters, we measured the dependences of the reaction velocity on concentrations of the substrate λ phage DNA and SAM (donor of methyl group). When the concentration of λ phage DNA was calculated, we used concentrations of methylation sites 5′-CATCC-3′ obtained by multiplication of molar concentration of λ phage DNA by the number of potential sites of modification in the DNA molecule (150 recognition sites). The catalytic and Michaelis constants were calculated by adjustment of the experimental data to the ordinary Michaelis curve. Concentration dependences of the velocities of the reactions catalyzed by DMTs *Bst*F5I-2 and *Bst*F5I-4 can be easily described by the Michaelis equation. The dependence of the reaction velocity on SAM (Fig. 8, a and b) and DNA (Fig. 9, a and b) concentrations for both enzymes coincides with a hyperboloid curve using which $K_{m\ SAM}$ and $K_{m\ DNA}$ were calculated (Table 2). In the case of modification of λ phage DNA, the affinity to this substrate of certain other DMTs described in the literature is slightly higher: 0.17 μM for *Eca*I [17], 0.22 nM for *Eco*RII [18], and 1.8 nM for *Msp*I [19].

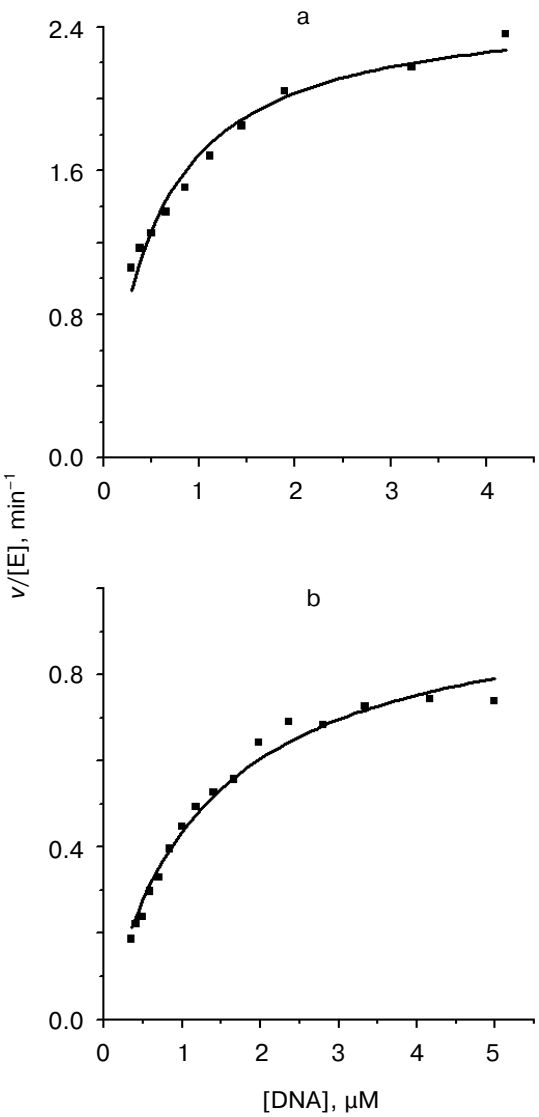

Fig. 9. Dependence of methylation velocity of λ phage DNA by DMTs *Bst*F5I-2 (a) and *Bst*F5I-4 (b) on concentration of methylation sites of λ phage DNA. The reaction mixture for DMT *Bst*F5I-2 contained 5.2 nM of the enzyme and 30 μM SAM; for DMT *Bst*F5I-4, 15 nM of the enzyme and 10 μM SAM. The reaction was carried out for 20 min.

Table 2. Kinetic parameters of reactions catalyzed by DMTs *Bst*F5I-2 and *Bst*F5I-4

Kinetic parameter	M.*Bst*F5I-2	M.*Bst*F5I-4
k_{cat}, min^{-1}	2.56 ± 0.39	0.99 ± 0.04
$K_{m\ DNA}$, μM	0.72 ± 0.24	1.29 ± 0.11
$K_{m\ SAM}$, μM	0.67 ± 0.26	0.94 ± 0.063
$k_{cat}/K_{m\ DNA}$, $\mu M^{-1}\cdot min^{-1}$	3.56	0.77
$k_{cat}/K_{m\ SAM}$, $\mu M^{-1}\cdot min^{-1}$	3.82	1.05

The affinity of both DMTs to the universal donor of methyl groups is also comparable with K_m for SAM for known DMTs; for example $K_{m\ SAM}$ for *Eca*I is 1.75 μM [17], for *Eco*RI 0.21 μM [20], and for T4Dam 0.49 μM [21]. The catalytic constants (k_{cat}) of the studied enzymes are comparable to that of such site-specific DMTs, as *Eco*RI, 8.4 min^{-1} (on plasmid DNA) [20]; *Eco*RII, 2.52 min^{-1} (on λ phage DNA) [18]; *Eca*I, 1.14 min^{-1} (on λ phage DNA) [17].

The comparison of the properties of DMT *Bst*F5I-2 and DMT *Fok*I that consists of two independent domains united in one protein is of particular interest. M. *Fok*I recognizes the same DNA sequence, but is able to modify both adenine residues in the asymmetric sequence 5′-GGATG/CATCC-3′. Methylation of the sequence 5′-CATCC-3′ is the function of the C-terminal domain that is highly homologous to DMT *Bst*F5I-2. We determined the kinetic parameters of the reactions catalyzed by DMT *Bst*F5I-2 using as the substrates the combinations of oligonucleotides for production of unmodified (I/II) and semi-methylated duplexes (Im/II).

These results allowed for the first time to compare the catalytic constants for DMT *Bst*F5I-2 with data obtained for both the native DMT *Fok*I protein and homologous C-domain [22] (Table 3). Although the affinity of DMT *Bst*F5I-2 to DNA is one order lower, the k_{cat} value for DMT *Bst*F5I-2 is one order higher in comparison to the native enzyme and C-domain. The comparison of the specificity coefficients leads to the conclusion that all enzymes prefer unmodified substrate to the semi-methylated one.

Thus, the determined kinetic parameters indicate that both enzymes *Bst*F5I-2 and *Bst*F5I-4 are involved in DNA modification in bacterial cells. However, one may suggest that in conditions of incomplete saturation with substrates, DMT *Bst*F5I-2 will have several-fold higher enzymatic activity than DMT *Bst*F5I-4 due to higher affinity of DMT *Bst*F5I-2 to the substrate DNA and the donor of methyl groups SAM and to high specificity coefficient (k_{cat}/K_m).

Since DMT *Bst*F5I-2 has a broader pH optimum, greater catalytic constant, and affinity to DNA in comparison to DMT *Bst*F5I-4, it appears that the second methylase would be sufficient for methylation of the host unmethylated DNA in a cell. Therefore it is reasonable to suggest that the fourth methylase of the studied R–M system is necessary first of all for methylation of another substrate type, for example of semi-methylated double-stranded DNA and/or of single-stranded substrate. Data on methylation of these types of substrates will be analyzed in the following studies.

Table 3. Kinetic parameters of reactions catalyzed by DMTs *Bst*F5I-2, *Fok*I, and *Fok*IC on oligonucleotide duplexes

Enzyme	Substrate*	$K_{m\ DNA}$, μM	k_{cat}, min^{-1}	$k_{cat}/K_{m\ DNA}$ (K_{sp}), $\mu M^{-1}\cdot min^{-1}$
M.*Fok*I	non-methylated	0.16	0.027	0.17
	semi-methylated	0.40	0.035	0.088
M.*Fok*IC	non-methylated	0.23	0.024	0.11
	semi-methylated	0.41	0.0065	0.016
M.*Bst*F5I-2	non-methylated	1.228	0.581	0.47
	semi-methylated	3.589	0.590	0.16

* Semi-methylated substrate contained N6-methyladenine instead of A in the sequence GGATG (GG^{m}ATG/CATCC).

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