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Relaxed specificity of prokaryotic DNA methyltransferases results in DNA site-specific modification of RNA/DNA heteroduplexes

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Abstract RNA/DNA hybrid duplexes regularly occur in nature, for example in transcriptional R loops. Their susceptibility to modification by DNA-specific or RNA-specific enzymes is, thus, a biologically relevant question, which, in addition, has possible biotechnological implications. In this study, we investigated the activity of four isospecific DNA methyltransferases (M.EcoVIII, M.LlaCI, M.HindIII, M.BstZ1II) toward an RNA/DNA duplex carrying one 5′-AAGCUU-3′/3′-TTCGAA-5′ target sequence. The analyzed enzymes belong to the β-group of adenine N6-methyltransferases and recognize the palindromic DNA sequence 5′-AAGCTT-3′/3′-TTCGAA-5′. Under standard conditions, none of these isospecific enzymes could detectibly methylate the RNA/DNA duplex. However, the addition of agents that generally relax specificity, such as dimethyl sulfoxide (DMSO) and glycerol, resulted in substantial methylation of the RNA/DNA duplex by M.EcoVIII and M.LlaCI. Only the DNA strand of the RNA/DNA duplex was methylated. The same was not observed for M.HindIII or M.BstZ1II. This is, to our knowledge, the first report that demonstrates such activity by prokaryotic DNA methyltransferases. Possible applications of these findings in a laboratory practice are also discussed.

Keywords Prokaryotic DNA methyltransferase · Relaxed specificity · RNA/DNA hybrid

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Introduction

RNA/DNA hybrid duplexes occur naturally, not only following reverse transcription, but also with R-loop formation during normal transcription, where they can affect genome stability and human disease (Groh and Gromak 2014; Hamperl and Cimprich 2014). These hybrid duplexes are structurally distinct from DNA duplexes (Shaw and Arya 2008), and it is not yet easy to predict which DNA-specific enzymes also act on RNA/DNA hybrids.

Restriction-modification (RM) systems may be regarded as sophisticated molecular machines that can protect bacteria against the invasion of virulent phages (Vasu and Nagaraja 2013; Mruk and Kobayashi 2014), and may play additional roles as well (Vasu and Nagaraja 2013). Typically, they contain two enzymatic activities embodied in a restriction endonuclease (ENase) and a DNA methyltransferase (MTase). Both activities generally recognize the same specific nucleotide sequence—the restriction ENase cuts it unless the sequence is modified by the cognate MTase. Thus, the methylation of specific sequences enables the cell to distinguish between self and non-self DNA. Structural and functional criteria divide the RM systems into four classes (Roberts et al. 2003). Enzymes belonging to the type II RM systems are the most abundant, and appear to be extremely accurate in recognizing their canonical sequences within the DNA (Pingoud et al. 2014). Studies on target recognition have shown that restriction/modification of specific sequences which differ by one base pair from the original target site is significantly lower, though in many cases still detectable both in vitro and in vivo (Woodbury et al. 1980; Reich et al. 1992; Smith et al. 1992; Ramsahoye et al. 2000; Aoki et al. 2001; Gowher and Jeltsch 2001). In general, DNA MTases are believed to be somewhat less specific than their cognate restriction ENases (Pingoud and Jeltsch 1997, 2001). As it was proposed, this feature may serve as a starting point to

achieve/develop enzymes with new specificities, since any changes in ENase alone would be lethal (Cohen et al. 2002).

For many ENases [e.g., R.EcoRI (Robinson and Sligar 1998), R.HindIII (Nasri and Thomas 1986), R.BamHI and R.EcoRV (Robinson and Sligar 1995)] as well as MTases [e.g., M.EcoRI (Woodbury et al. 1980) and M.HaeIII (Cohen et al. 2002)], relaxed specificity (star activity) can be easily detected under certain reaction conditions. Neutral solvents like dimethyl sulfoxide (DMSO), glycerol, or ethanol, which generate changes in osmotic pressure, are especially efficient in evoking star activity (Robinson and Sligar 1993). An important role of water molecules in mediating specific interactions between protein and DNA has been confirmed in case of many proteins, e.g., Trp repressor (Carey et al. 1991), or ENases, such as R.EcoRI (Robinson and Sligar 1993, 1998), R.BamHI or R.PvuII (Robinson and Sligar 1995). It was shown for R.EcoRI, for example, that the binding of a nucleotide sequence that differs by one base pair from the canonical site is associated with the release of more water molecules than when the enzyme binds the original target sequence (Robinson and Sligar 1998).

Here, we test whether relaxed-specificity conditions allow DNA-specific MTases to modify RNA/DNA duplexes. As far as we know, there are no other reports on prokaryotic MTases modifying RNA/DNA hybrids. However, RNA-directed DNA methylation was observed in plants (Wassenegger et al. 1994). A number of type II restriction ENases have the ability to act on RNA/DNA heteroduplexes. Some of them specifically digested the DNA strand in RNA/DNA hybrids [e.g., R.EcoRI, R.SalI, R.HindIII, R.AluI, R.HhaI, R.TaqI, R.MspI, and R.HaeIII (Molloy and Symons 1980)]. Cleavage of the RNA strand was undetectable with the techniques used. In another report, 223 type II ENases were analyzed for RNA/DNA cleavage (Murray et al. 2010) and four ENases, R.AvaII, R.AvrII, R.BanI, and R.TaqI, were found to digest both strands of the RNA/DNA heteroduplex; surprisingly, R.HinfI preferentially digested the RNA strand and R.HaeIII only acted on the RNA strand.

In our study, we analyzed four prokaryotic DNA MTases that are isospecific: M.EcoVIII from *Escherichia coli* E1585–68 (Mruk and Kaczorowski 2003), M.LlaCI from *Lactococcus lactis* subsp. *cremoris* W15 (Mruk et al. 2003), M.HindIII from *Haemophilus influenzae* Rd (Roy and Smith 1973), and M.BstZ1II from *Bacillus stearothermophilus* 14P (Mruk 2004; Mruk and Kaczorowski 2007). Although they come from different bacterial orders, all of them recognize the same specific palindromic sequence 5′-AAGCTT-3′ and catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the first adenine in the sequence (Roy and Smith 1973; Mruk et al. 2003; Mruk and Kaczorowski 2003, 2007; Mruk 2004). We, nevertheless, found them to be substantially different in their activities on RNA/DNA duplexes under conditions of relaxed specificity.

Materials and methods

Protein purification

The DNA MTases used in this study, M.EcoVIII, M.HindIII, and M.LlaCI, were purified to an apparent electrophoretic homogeneity using simple procedures based on ion exchange, molecular sieve, and affinity chromatography, described in detail previously (Mruk et al. 2001, 2003; Mruk and Kaczorowski 2003). All MTases used in this study were not tagged. The final preparations were free of non-specific endonucleases. The M.BstZ1II MTase was prepared from *E. coli* MO 20–1 transformed with pT7MBstZ1II. This plasmid was constructed by cloning into a pT7-6 vector (Tabor and Richardson 1985) linearized with BamHI and EcoRI, a 1.7-kb DNA fragment carrying the M.BstZ1II gene that was obtained by polymerase chain reaction (PCR) followed by double digestion with BamHI and EcoRI. The forward and reverse primers were 5′-CATTTGGATCCCCGACACC-3′ and 5′-CAAAGAATTCAGAGAATG-3′, respectively (the restriction sites are underlined). Plasmid pGP10 (BstZ1II R^-M^+; Mruk and Kaczorowski 2007) was used as a template in the amplification reaction (PCR). In recombinant plasmid (pT7MBstZ1II, 3.9 kb), the start codon of *bstZ1IIM* is located 428 nt downstream from the ϕ10 promoter of phage T7. Bacteria carrying the overproducing plasmid were cultivated at 30 °C in 1 l of TY broth (Sambrook et al. 1989) supplemented with ampicillin (100 μg ml^{-1}) and tetracycline (12.5 μg ml^{-1}). Overproduction of the enzyme was induced at OD_{600}=0.3 by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, followed by incubation at 37 °C for 3 h. The cells were then harvested by centrifugation and stored at −70 °C. All purification steps were carried out at 4 °C. Upon protein loading, each chromatographic column was extensively washed with adequate buffer. The progress of protein purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For enzyme purification, frozen cells (2.5 g) were resuspended in 30 ml of PB buffer (10 mM K/PO_4 pH 7.6; 0.16 M KCl; 1 mM EDTA; 10 mM β-mercaptoethanol; 5 % glycerol *v*/*v*) supplemented with phenylmethylsulfonyl fluoride (PMSF) (25 μg ml^{-1}) and disrupted by sonication (4 °C, 60 bursts of 10 s at an amplitude of 12 μm; MISONIX Sonicator XL2020, USA). The lysate obtained was clarified by centrifugation (4 °C, 14,000×*g*, 40 min) and applied to a phosphocellulose P11 column (2.5×4 cm, Whatman) equilibrated with the PB buffer. Proteins bound to the column were eluted with a 200-ml linear gradient of KCl (0.16–1.2 M) in the PB buffer. Active fractions, after dialysis against the BA buffer (10 mM K/PO_4 pH 7.6; 0.17 M KCl; 1 mM EDTA, 10 mM β-mercaptoethanol, 5 % glycerol *v*/*v*), were loaded onto a blue agarose column (1×5 cm, Pharmacia), and adsorbed proteins were eluted with a 150-ml linear gradient of KCl (0.17–1.2 M)

in the same buffer. Active fractions were dialyzed against the CM buffer (10 mM K/PO_4 pH 7.6; 0.12 M KCl; 1 mM EDTA, 10 mM β-mercaptoethanol, 5 % glycerol *v*/*v*) and applied to a CM-Sephadex C-50 column (1.8×10 cm, Pharmacia). Bound proteins were eluted with a 150-ml linear gradient of KCl (0.12–1.2 M) in the CM buffer. Fractions with the highest M.BstZ1II activity were collected and concentrated by overnight dialysis against storage buffer (10 mM K/PO_4 pH 7.5; 0.05 M KCl; 0.5 mM EDTA; 10 mM β-mercaptoethanol, 50 % glycerol *v*/*v*) and stored at −20 °C.

Oligonucleotides

Oligonucleotides DNA1 (5′-TGCAGTCGCGAAGCTTGGTC ACCTTGAGG-3′) and DNA2 (5′-TGCCTCAAGGTGAC CAAGCTTCGCGACTG-3', the HindIII site is underlined) were synthesized by Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Poland) while RNA1 (5'-UGCA GUCGCGAAGCUUGGUCACCUU-GAGG-3') was synthesized by DNA Integrated Technology (Germany). Oligonucleotides and oligoribonucleotide were dissolved in RNase-free TE buffer (Sambrook et al. 1989) at a concentration of 100 μM. To prepare 29-bp double stranded homoduplex DNA1/DNA2 or heteroduplex RNA1/DNA2, an equimolar mixture of both oligos was heated to 95 °C and slowly cooled to room temperature to ensure complete annealing.

In vitro methylation assay

The assay was based on the enzyme-catalyzed transfer of [^{3}H]methyl groups from [methyl-^{3}H]SAM to DNA1/DNA2 homoduplex or RNA1/DNA2 heteroduplex. Methylation was performed in a 20-μl reaction mixture containing DNA1/DNA2 homoduplex or RNA1/DNA2 heteroduplex (0.15 μM), 10 mM MOPS pH 7.0, 0.5 μM [methyl-^{3}H]SAM (69.5 Ci/mmol), and enzyme (0.3 μM). The reaction was carried out for 1 h at 37°C followed by enzyme inactivation (65°C, 15 min). Finally, the reaction was stopped by adding two volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 4.8, Sigma). The samples were centrifuged (10 000 g, 40 min, 4 °C) and the pellet was washed with 700 μl of 70% ethanol, centrifuged and dried. Reactions were performed in triplicate. Scintillation counting was used to estimate the incorporated radioactivity.Relaxed specificity of isospecific DNA MTases was investigated using pMet (0.15 μg) as a substrate and DMSO, glycerol or ethanol at elevated

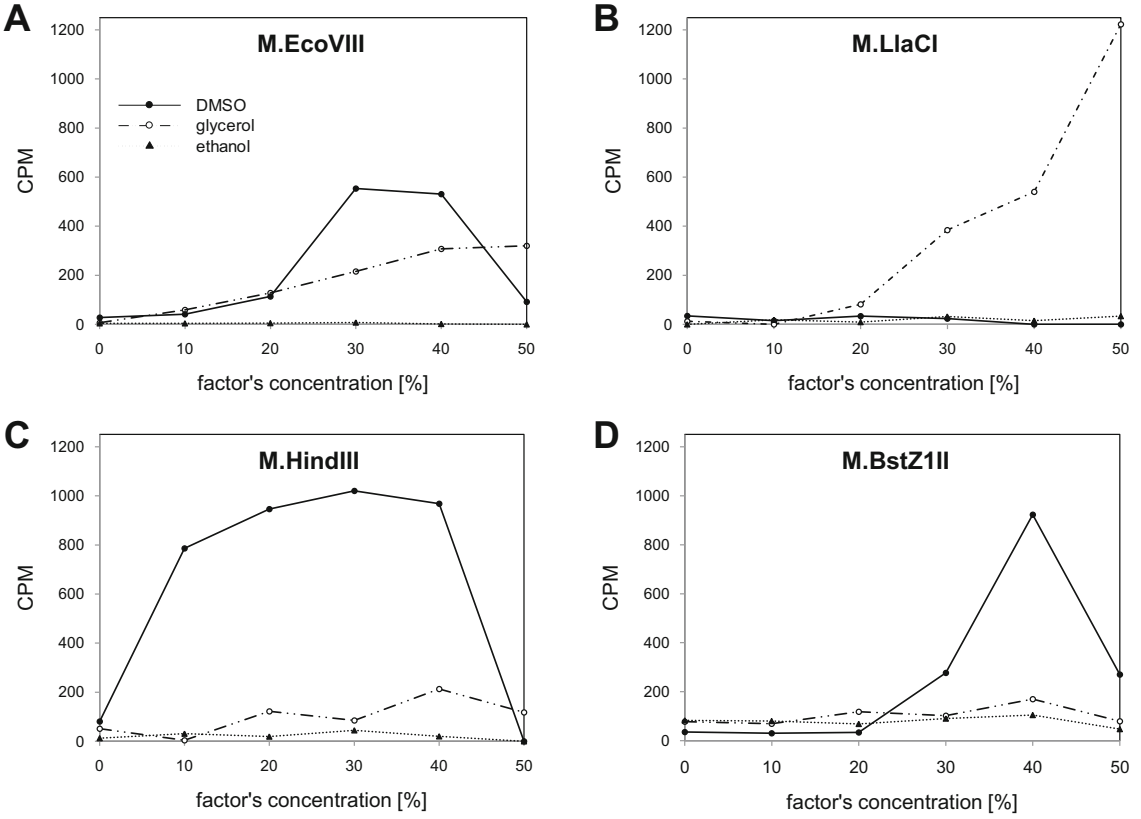

Fig. 1 Effect of factors that induce star activity of isospecific DNA MTases: M.EcoVIII (**a**), M.LlaCI (**b**), M.HindIII (**c**), and M.BstZ1II (**d**). Plasmid pMet (pBR322-derivative, 4,365 bp) deprived of its 5′-AAGCTT-3′ target sequence was used as a substrate in methylation reactions with [*methyl*-^{3}H]-*S*-adenosyl-L-methionine. Methylation activity in the presence of DMSO (*filled circles*), glycerol (*open circles*), and ethanol (*triangles*) was assayed

concentrations (up to 50 %) as star activity-inducing agents. pMet is a derivative of pBR322 (Bolivar et al. 1977) deprived of a singular HindIII site. It was constructed by the digestion of pBR322 with HindIII, followed by filling in 5′-protruding sticky ends with Klenow Fragment (Fermentas). Then, the plasmid was ligated and introduced into *E. coli* MM294 (Sambrook et al. 1989). The resultant clones were selected for resistance by plating on Luria–Bertani (LB) agar plates with ampicillin (100 μg ml^{-1}). Recombinant clones were verified by digestion with HindIII enzyme. The clone (pMet) that was resistant to HindIII digestion was used in further experiments. In the standard methylation assay, pMet plasmid was not modified by DNA MTases used in this study. Plasmid pMet was deposited in the Collection of Plasmids and Microorganisms, University of Gdansk, Gdansk, Poland.

Results

Isospecific DNA MTases can modify DNA at secondary sites

In order to induce star activity of four isospecific DNA MTases (M.EcoVIII, M.LlaCI, M.HindIII, M.BstZ1II), three solvents were used at 0–50 % concentrations: DMSO, glycerol, and ethanol. In all experiments, a pMet plasmid deprived of its 5′-AAGCTT-3′ cognate target sequence was used as a substrate. Computational analysis of the pMet nucleotide sequence revealed 19 potential secondary sites differing by one nucleotide from the target. It was shown previously that reduced specificity of restriction ENases and DNA MTases is strongly correlated with osmotic pressure (Woodbury et al. 1980; Robinson and Sligar 1995). Water molecules play a crucial role in creating specific interactions between a protein and the target sequence. Neutral organic solvents, like DMSO, glycerol, or ethanol, change the osmotic pressure by altering the access of free water molecules, which may lead to improper interactions with the DNA, resulting in the recognition of secondary specific sites (Robinson and Sligar 1995).

We have found that relaxed specificity of M.EcoVIII, M.HindIII, and M.BstZ1II was most effectively induced by DMSO at a concentration of 30 % for M.EcoVIII and M.HindIII, and 40 % in the case of M.BstZ1II (Fig. 1a, c, d). To a lesser extent, star activity was observed when glycerol was used at concentrations of 40–50 % for M.EcoVIII, M.LlaCI, and M.HindIII (Fig.1a, b, c). We found that no enhanced star activity was seen for either enzyme in the presence of ethanol (Fig. 1). In control experiments, when star activity-inducing

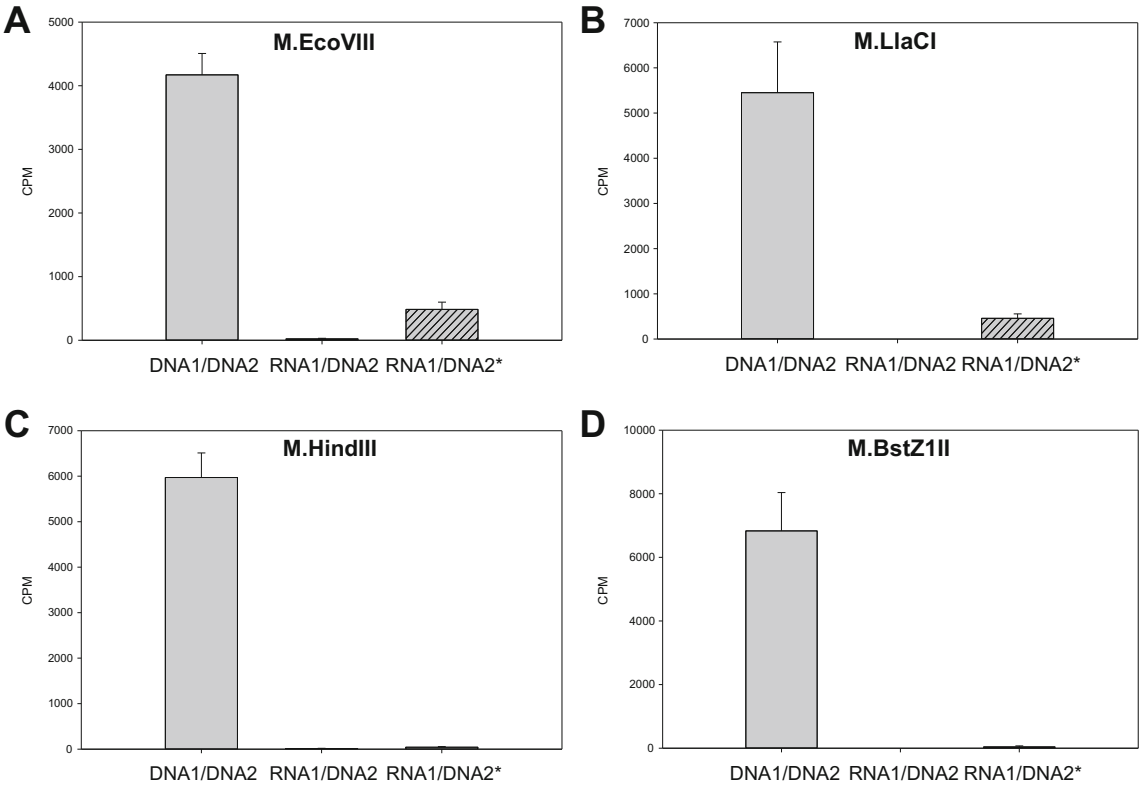

Fig. 2 Methylation of the RNA/DNA heteroduplex by isospecific DNA MTases under standard (RNA1/DNA2) and star (RNA1/DNA2*) conditions. DMSO was used to induce star activity of M.EcoVIII (30 % DMSO), M.HindIII (30 % DMSO), and M.BstZ1II (40 % DMSO). In case of M.LlaCI, glycerol at concentration of 50 % was used. In a control reaction, the DNA1/DNA2 homoduplex was used as a substrate. The *error bars* represent the standard deviations of the means of three assays

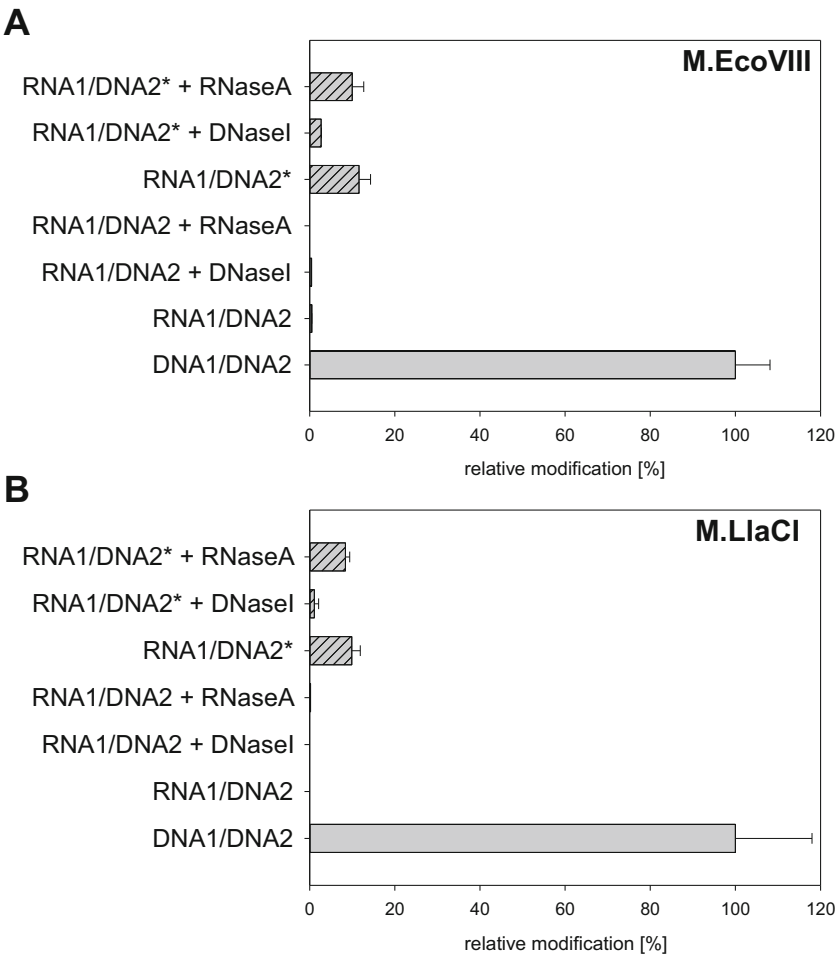

Fig. 3 Methylated strand determination after RNA1/DNA2 heteroduplex modification by M.EcoVIII (**a**) and M.LlaCI (**b**). Reaction was followed by treatment with DNase I or RNase A. *RNA1/DNA2* stands for heteroduplex methylated under standard conditions, *RNA1/DNA2** stands for heteroduplex methylated under star conditions (30 % DMSO for M.EcoVIII, 50 % glycerol for M.LlaCI). In a control reaction, the DNA1/DNA2 homoduplex was used as a substrate. The *error bars* represent the standard deviations of the means of three assays

factors were not present, we did not observe any methylation of the specific site-ablated pMet DNA (data not shown).

DNA MTases can modify the RNA/DNA heteroduplex

Next, we tested the possibility of modification of the RNA/DNA heteroduplex by the four isospecific DNA MTases, using a cognate site but with one strand RNA and the other DNA (5′-AAGCUU-3′/3′-TTCGAA-5′). Methylation reactions were performed in both standard and star activity-inducing conditions. Under standard conditions, none of the investigated MTases catalyzed detectable methylation of the RNA/DNA hybrid (Fig. 2). However, the addition of DMSO (M.EcoVIII) or glycerol (M.LlaCI) to the standard reaction mixture resulted in methylation of the RNA1/DNA2 heteroduplex (Fig. 2a, b). This activity was not observed for M.HindIII or M.BstZ1II (Fig. 2c, d). In each case, the obtained modification level was lower by an order of magnitude when compared to the control (homoduplex DNA1/DNA2).

In order to determine which strand of the RNA1/DNA2 heteroduplex was methylated, products of the modification reaction were treated with RNase A (free of DNase) or with DNase I (free of RNase, Thermo Scientific, Germany). We found that DNase I treatment eliminated the recoverable radioactivity, while digestion of the RNA strand did not change the level of recovered radioactivity (Fig. 3a, b). This indicates that only the DNA strand of the RNA1/DNA2 heteroduplex is methylated by M.EcoVIII and M.LlaCI. In a control experiment, we found that DMSO and glycerol at the concentrations used did not influence the activity of either DNase I or RNase A (data not shown).

We have also tested whether observed methylation of the RNA/DNA heteroduplex might be a result of the modification of single-stranded DNA, since some MTases have that ability (Sistla et al. 2004). In this experiment, the DNA2 oligonucleotide was used as a substrate. We found that ssDNA, indeed, is a substrate for M.EcoVIII (Fig. 4a), but not for M.LlaCI (Fig. 4b). In the case of M.EcoVIII, however, the methylation level of ssDNA is ten times or twice (standard and star reaction conditions, respectively) lower than when dsDNA was used as the substrate. Oligonucleotide DNA2 could self-hybridize at the 6 nt substrate site itself, but even without glycerol, the melting temperature (T_m) would be ~20 °C, and the reactions were carried out at 37 °C.

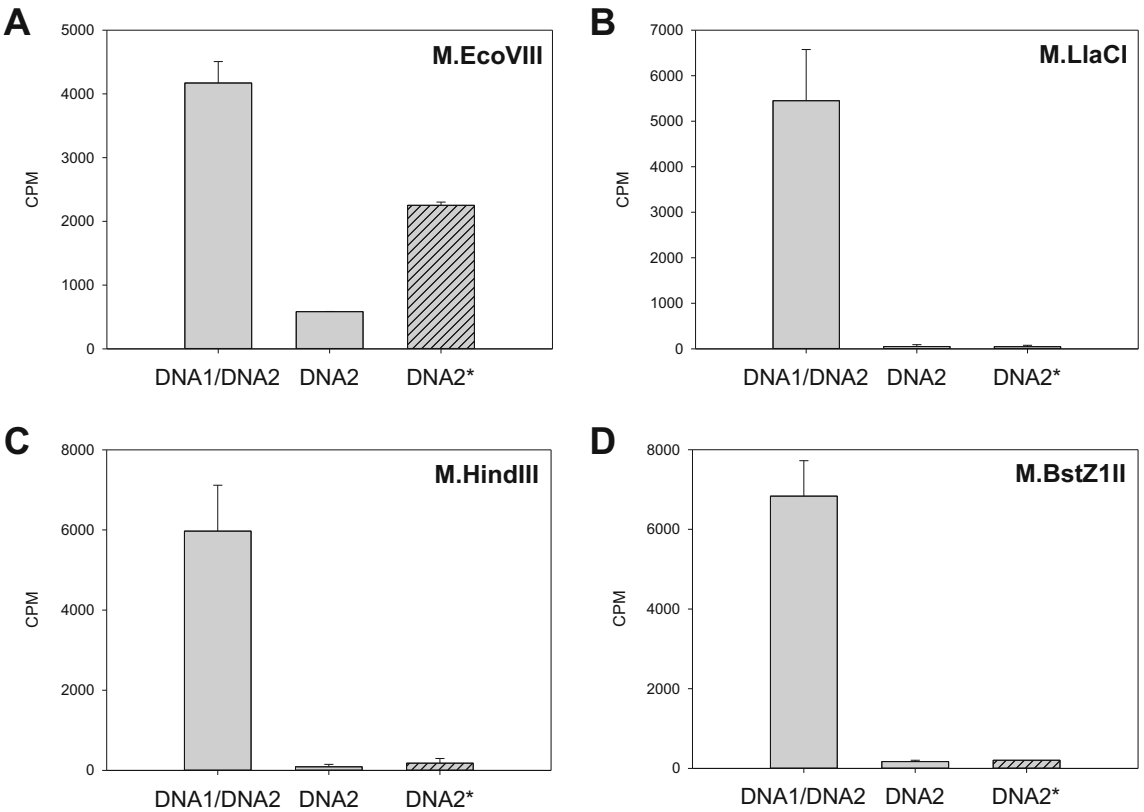

Fig. 4 Methylation of single-stranded DNA catalyzed by M.EcoVIII (**a**) and M.LlaCI (**b**). As a substrate, the DNA2 single-stranded oligonucleotide was used. Methylation reactions were carried out under standard (*DNA2*) or star conditions (*DNA2**). For star activity induction, DMSO (30 %) was used for M.EcoVIII, while glycerol (50 %) was used for M.LlaCI. In a control reaction, the DNA1/DNA2 homoduplex was used as a substrate. The *error bars* represent the standard deviations of the means of three assays

Discussion

In the present study, we investigated the activity of isospecific DNA MTases toward the RNA/DNA heteroduplex carrying the 5′-AAGCUU-3′/3′-TTCGAA-5′ target sequence. All of the studied enzymes belong to the β-group of N6-MTases and, thus, have the same structural permutation (Gong et al. 1997). Putative target recognition domains (TRDs) of the analyzed proteins, located between conserved motifs VIII and X, are markedly similar at the amino acid sequence level (Mruk and Kaczorowski 2007). There are 18 identical amino acids residues in each TRD. However, antiserum against M.EcoVIII cross-reacts only with M.LlaCI, and not with M.HindIII or M.BstZ1II, suggesting that M.EcoVIII and M.LlaCI have at least some identical or very similar epitopes (Mruk and Kaczorowski 2003).

Enzymes acting on DNA use two mechanisms to recognize their specific nucleotide sequence. The first is based on direct readout of the specific sequence. The recognition of bases takes place in major and minor grooves of the DNA helix, and it is reached by forming hydrogen bonds between the enzyme recognition domain and nucleotides of the specific sequence. The most important contacts are formed in the major groove, since each base pair generates a unique pattern of hydrogen bonds with the protein interface (Seeman et al. 1976). For this, it is noteworthy that the RNA strand contains two uracil bases in place of the normally recognized thymines, which removes consecutive methyl groups from the major groove.

The second mechanism of target recognition relies on indirect readout. It is based on the local deformation of a double helix which arises from interaction with the nucleotide sequence. This depends on base pairs which are not directly contacted by the protein (Rohs et al. 2010). The structure of RNA/DNA heteroduplexes is unique; its conformation is neither the B-type helix characteristic for dsDNA nor the A-type adopted by dsRNA (Noy et al. 2005). The sugar puckering of both components is different; ribose is in a C2′-endo conformation (North), while deoxyribose is in a C3′-endo (South) or an O4′-endo (East) (Egli et al. 1993; Salazar et al. 1993). Moreover, because of the ribose hydroxyl group, the whole structure is more rigid and, therefore, less influenced by local deformation resulting from the nucleotide sequence (Noy et al. 2005). Additionally, the hydroxyl group itself may cause a steric barrier. The topology of the grooves is also changed; in a B-type helix, the sugar-phosphate backbone creates a wide major groove and a narrow minor groove, while in the A-type helix, the major groove is narrower and deep, whereas the

minor groove is shallow and wider. The recognition of specific DNA helix is mainly achieved by contacts between enzymes' functional groups in the major groove, but in the case of RNA, the indirect mechanism must be involved, as the edges of the bases are hardly accessible in the narrowed and deepened major groove of the A-type helix (Egli et al. 1993). Thus, compared to double-stranded DNA, RNA/DNA hybrids have many features which may affect both direct and indirect readout.

The unique conformation of RNA/DNA heteroduplex may affect the target recognition of enzymes acting on DNA. This kind of hybrid is a rather difficult substrate for enzymes normally acting on dsDNA. Moreover, DNA MTases rotate their target base from the double helix. Although there are still open questions concerning this "base flipping" pathway, there is evidence that it can proceed through the DNA major groove (Horton et al. 2004; Shieh et al. 2006; Bianchi and Zangi 2013). Thus, the spatial architecture of the RNA/DNA heteroduplex may affect the ability of DNA MTases in the recognition and methylation of target sequences. Despite that possibility, surprisingly, two of the analyzed isospecific MTases (M.EcoVIII and M.LlaCI) were able to modify the DNA strand in the RNA/DNA heteroduplex.

In a study on the activity of restriction ENases toward RNA/DNA heteroduplexes, it was shown that, out of 223 enzymes tested, seven were able to cut the target site in both DNA and RNA strands (Murray et al. 2010). These enzymes belong to type II restriction ENases recognizing palindromic or interrupted palindromic target sequences (type IIP). According to the authors, the ability to cleave heteroduplex is an exception rather than the rule for type II restriction ENases. RNA-specific cleavage was also reported for a fusion of ribonuclease H with a zinc finger (Sulej et al. 2012).

On the other hand, the analysis of crystal structures of DNA in complex with restriction ENases revealed that their conformation resembles an A-type helix in the case of R.PvuII and R.EcoRV (Lu et al. 2000). As it was found for R.PvuII, the majority of sugar puckering is A-type, and what is more, this enzyme extensively contacts the recognition sequence from the minor groove side, and the global helical twist is reduced at the two central bases. Since A-type helices were found in crystal structures with many enzymes that act on phosphodiester linkage, restriction ENases may be more prone to recognize RNA/DNA hybrids than DNA MTases, whose function requires access to the modifiable base. Our observation may be applicable for RNA/DNA duplex labeling by site-specific methylation in cases when DNA ends are blocked or difficult to modify. In addition, such MTase-mediated labeling may also be used to introduce other chemically modified groups (methyl group analogues), which should be helpful for a wide variety of reporter group techniques and their substrate detection, e.g., in the SMILing DNA technique (sequence-specific methyltransferase-induced labeling of DNA; Schmidt et al. 2008; Hanz et al. 2014). The potential for in vivo application may also be linked with genome editing technology based on bacterial CRISPR-Cas nuclease systems (Chen et al. 2014). In light of the growing interest in RNA-directed DNA methylation and its impact on epigenetics, our findings, though limited to in vitro observation, may provide some useful insights on as yet unexplored properties of some prokaryotic MTases.

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Conflict of interest The authors declare that they have no conflict of interest.

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