

M1.Mboll and M2.Mboll type IIS methyltransferases: different specificities, the same target

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Methylation of a base in a specific DNA sequence protects the DNA from nucleolytic cleavage by restriction enzymes recognizing the same sequence. The MbolI restriction–modification (R–M) system of *Moraxella bovis* ATCC 10900 consists of a restriction endonuclease gene and two methyltransferase genes. The enzymes encoded by this system recognize an asymmetrical sequence 5'-GAAGA-3'/3'-CTTCT-5'. M1.MbolI modifies the last adenine in the recognition sequence 5'-GAAGA-3' to N⁶-methyladenine. A second methylase, M2.MbolI, was cloned and purified to electrophoretic homogeneity using a four-step chromatographic procedure. It was demonstrated that M2.MbolI modifies the internal cytosine in the recognition sequence 3'-CTTCT-5', yielding N⁴-methylcytosine, and moreover is able to methylate single-stranded DNA. The protein exists in solution as a monomer of molecular mass 30 000 ± 1000 Da under denaturing conditions. Divalent cations (Ca²⁺, Mg²⁺, Mn²⁺ and Zn²⁺) inhibit M2.MbolI methylation activity. It was found that the isomethylomer M2.NcuI from *Neisseria cuniculi* ATCC 14688 behaves in the same manner. Functional analysis showed that the complete MbolI R–M system, consisting of two methyltransferases genes and the *mbolIR* gene, is the most stable and the least harmful to bacterial cells.

INTRODUCTION

Restriction–modification (R–M) systems are widely distributed among bacteria. The simplest bacterial R–M systems are type II, which comprise distinct DNA restriction and modification enzymes and which require no cofactors other than Mg²⁺ ions (Roberts *et al.*, 2003). Methylation of adenosyl or cytosyl residues by the modifying enzyme results in the protection of cellular DNA against cleavage by restriction endonucleases (Wilson & Murray, 1991; Bickle & Kruger, 1993). Type II R–M systems that recognize nonsymmetrical sites on the target DNA have been designated type IIS. Usually, two different methyltransferase activities are present in type IIS R–M systems, one for each strand of their asymmetrical recognition site.

The MboII restriction endonuclease (R.MboII) of *Moraxella bovis* ATCC 10900 recognizes a pentanucleotide sequence 5'-GAAGA-3'/3'-CTTCT-5' and cleaves DNA 8 and 7 nucleotides downstream of the recognition site in the 5' to 3' direction on the upper strand, leaving a single 3'-protruding nucleotide (Brown *et al.*, 1980). The methyltransferase M1.MboII transfers methyl groups to the 3'-

adenine of the recognition sequence to form N⁶-methyladenine and thereby protects DNA from cleavage by the endonuclease (McClelland *et al.*, 1985). During semi-conservative DNA replication, however, one of the replicated DNA molecules becomes unmethylated and therefore sensitive to restriction, unless another methylase that can methylate the lower strand is available. Since there is no adenine residue in the lower strand of the recognition sequence, such an enzyme must be a C-specific methyltransferase. Recent sequence analysis of the MboII R–M system revealed the presence of a potential open reading frame encoding a second methyltransferase, M2.MboII (Roberts *et al.*, 2003). The position of M2.MboII-mediated methylation has not been determined hitherto. The discovery of a second methyltransferase in the MboII R–M system raised the question of what the biological role might be for the tandem modification enzymes in the proper regulation and functioning of the whole system. To obtain answers to this question it was decided to compare the substrate specificity of the two methyltransferases and their ability to protect host DNA against R.MboII action in more detail.

The presence of two methyltransferases responsible for the recognition and methylation of strand-specific sequences would be advantageous for bacterial cells, because nascent

Abbreviations: AdoMet, S-adenosyl-L-methionine; ME, mercaptoethanol.

strands generated during DNA replication can be simultaneously protected from attack by site-specific endonucleases. In the FokI system, which also recognizes an asymmetrical sequence, the methylase contains two functional domains within a single protein molecule, each of which is responsible for the modification of different strands. Nevertheless, comparison of the two functional domains has shown no sequence similarity except for the tetra-amino acid sequences common to adenine-specific methylases (Kaczorowski *et al.*, 1999). Another type IIS R–M system, HgaI, consists of two separate cytosine methyltransferases which are responsible for methylation of different DNA strands. In this case the sequences of both enzymes are well conserved in their regions overall, suggesting that the two methylases evolved from a common ancestor by gene duplication (Sugisaki *et al.*, 1991).

Our studies have also been motivated by the fact that the MboII R–M system from *M. bovis* (Brown *et al.*, 1980) has the same sequence specificity as another studied in our laboratory, the NcuI R–M system from *Neisseria cuniculi* ATCC 14688 (Furmanek *et al.*, 2007). It was of especial interest to find out what isospecific R–M enzymes have in common at the structural and functional level. The enzymes of these systems recognize the same asymmetrical sequence (McClelland *et al.*, 1985; Bocklage *et al.*, 1991; Furmanek *et al.*, 2001). It has been shown recently that the substrate specificity and the level of activity for two separately existing methyltransferases may be strikingly different, even though they modify either the same or different nucleotides of target DNA (Cerritelli *et al.*, 1989; Merkiene *et al.*, 1998; Kriukiene *et al.*, 2005). The MboII R–M system as well as its isospecific systems NcuI (Furmanek *et al.*, 2007) and HpyAII (Lin *et al.*, 2001), and other homologous R–M systems from *Helicobacter pylori* strains (Ando *et al.*, 2000; Xu *et al.*, 2000), are members of the IIS class, which generally require two methyltransferase activities, one for each strand of their asymmetrical recognition sites. In this case, the nucleotide sequence of the recognition site has profound significance. The two complementary strands are completely different with respect to the type of bases which can be potentially modified. In the top strand of the MboII site only the adenine residues can be the target of methylation, while in the bottom strand it is the cytosine residues.

The data presented in this paper confirm that M1.MboII and M2.MboII differ markedly in their properties.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains DH10B [F^- *mcrA* Δ (*mrr*–*hsdRMS*–*mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *deoR* *recA1* *endA1* *araD139* Δ (*ara*, *leu*)7697 *galU* *galK* λ^- *rpsL* *nupG*], HB101 [F^- Δ (*gpt*–*proA*)62 *leuB6* *glnV* *ara-14* *galK2* *lacY1* Δ (*mcrC*–*mrr*) *rpsL20* (Str^r) *xyl-5* *mtl-1* *recA13* *thi-1*] and ER1992 [F^- λ^- Δ (*argF*–*lac*)U169 *supE44* *e14*⁺ *dinD1::Mu* *dII734* (Kan^R, LacZ⁺) *rfbD1?* *relA1?* *endA1* *spoT1?* *thi-1* Δ (*mcrC*–*mrr*)114::IS10] (New England

Biolabs) were used for routine cloning. Overproduction of M2.MboII and M2.NcuI was performed with *E. coli* Tuner [F^- *ompT* *hsdS_B* (*r_B* *m_B*⁻) *gal* *dcm* *lacY1*] (Novagen). Overproduction of R.MboII was performed with *E. coli* ER2566 [F^- λ^- *fhuA2* *lon* *ompT* *lacZ::T7gene1* *gal* *sulA11* Δ (*mcrC*–*mrr*)114::IS10 R (*mcr-7::miniTn10*–*tetS*)2R (*zgb-210::Tn10*) (Tet^s) *endA1* *dcm*] (New England Biolabs). *E. coli* strains were routinely grown and maintained in LB medium at 37 °C with appropriate antibiotic selection.

Isolation and cloning of the *mboIIM2* and *ncuIM2* genes. The *mboIIM2* gene was amplified from the chromosomal DNA of *M. bovis* ATCC 10900 by PCR (Taq DNA polymerase, Fermentas) using two sets of primers: (1) MboBam 5'-GAGGATCCCTTTTATCTGCCGATG-3' and MboB-T 5'-AGGATCCAATTAATACTTTCTAA-3', containing the underlined BamHI restriction sites in their respective 5' extensions and including a native promoter, or (2) MboNdeI 5'-GTTATAGTCATATGAACACCATATTT-3' and MboB-T, to make translational fusion with T7-phage signals possible. In the first case the PCR product was cloned into the BamHI pre-cut site of pBR322 (Bolivar *et al.*, 1977) in two possible orientations. In the second case, the PCR product was cloned into NdeI/BamHI pre-cut pET24a plasmid (Novagene). Both cloned genes were sequenced to ensure that no errors had occurred during the PCR. The resulting plasmids were named pBRmboIIMB and pET24mboIIMB, respectively. The plasmids were maintained in *E. coli* HB101. The *ncuIM2* gene was amplified from genomic DNA of *N. cuniculi* using primers M2NcuATG and M2NcuTAA. Each primer contained the underlined NdeI and EcoRI restriction site (M2NcuATG: 5'-GATTAGAGACATATGAATACTGTATT-3' and M2NcuTAA: 5'-TGAATTCATTACCAGAGCCTTAAATC-3'). The amplified PCR products were cloned into the pET24a vector. The constructed plasmid was designated pET24aNcuIBM. In all cases PCR amplification was carried out as follows: an initial denaturation at 94 °C for 3 min followed by 30 cycles of amplification (94 °C for 1 min, 48 °C for 1 min and 72 °C for 1.5 min) and an additional extension step at 72 °C for 5 min.

Estimation of R.MboII overproduction level. Two-plasmid systems were employed for the expression of R.MboII. Each system consisted of plasmid pMboR3.0 carrying the *mboIIR* gene (Bocklage *et al.*, 1991) together with (1) pMboM1.1 delivering *mboIIM1* (Bocklage *et al.*, 1991), or (2) pUCMB.3 (BamHI DNA fragment bearing *mboIIM2* cloned into the BamHI site of pUC18; Yanisch-Perron *et al.*, 1985), or (3) pMboMAB carrying both methyltransferase genes (PvuII fragments bearing *P_{lac}::mboIIM1* and *P_{lac}::mboIIM2* from pMboM1.1 and pUCMB.3, respectively, were cloned into pBR322). Expression of all *mboII* genes was dependent on *P_{lac}/LacI* control. Cultures of *E. coli* ER2566 containing the three different plasmid combinations were grown in LB broth (500 ml each) to the early exponential phase following 4 h 1 mM IPTG induction. The concentration of bacterial cells per ml of culture was estimated by the serial dilution method and the wet mass of each culture was weighed. Then, bacterial pellets were resuspended in 5 ml lysis buffer, and the cells were sonicated and subjected to a two-step partial purification, as described elsewhere (Sektas *et al.*, 1992). The cleared cell extracts from each culture were applied, separately, to a phosphocellulose P11 column (Whatman). Proteins were eluted by a gradient of KCl concentration (0–1.0 M) using the same conditions for all extract types, and fractions were examined to assess R.MboII activity. Active fractions were collected and the overall activity was determined, together with densitometric estimation of the R.MboII protein concentration per ml of collected active fractions. One unit of endonuclease activity was defined as the amount of enzyme required to completely digest 1 µg pNH20-Cm.5 plasmid DNA (pNH20 derivative with *cat* gene inserted; Hasan *et al.*, 1989) in 1 h at 37 °C.

Purification of methyltransferases M2.MboII and M2.NcuI. *E. coli* Tuner transformed with pET24aMboIIBM was used to inoculate 1000 ml LB medium containing kanamycin ($25 \mu\text{g ml}^{-1}$). After incubation for 2 h at 37°C , IPTG was added at a final concentration of 1 mM and the mixture was incubated for 4 h at 37°C . Cells were harvested by centrifugation, resuspended in buffer S [10 mM potassium phosphate pH 7.4, 20 mM KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol (ME), 5%, v/v, glycerol, containing 0.1 mM PMSF as a protease inhibitor] and disrupted by sonication at 4°C in 60×10 s bursts. The sonicated cells were centrifuged at 14000 g for 30 min. The resulting supernatant was applied to a phosphocellulose P11 column (2.5×6.5 cm) equilibrated with buffer S without PMSF. Proteins bound to the column were eluted with a 200 ml linear gradient of 0.02–1.0 M KCl in the same buffer. Fractions of 4 ml were collected and assayed for methylation activity. The active fractions were dialysed against buffer HTP (10 mM potassium phosphate pH 7.4, 200 mM KCl, 10 mM ME, 5%, v/v, glycerol), loaded onto a hydroxylapatite column (Bio-Rad; 1.7×2 cm) and eluted with 150 ml of a linear gradient of 0.1–0.5 M potassium phosphate pH 7.4. The active fractions were dialysed against buffer CMS (10 mM potassium phosphate pH 7.4, 25 mM KCl, 1 mM EDTA, 10 mM ME, 5%, v/v, glycerol), loaded onto a CM-Sephadex C-50 column (Pharmacia) (1.7×19 cm), and eluted with 150 ml of a linear gradient of 0.025–1.0 M KCl. The active fractions were dialysed against buffer CMS, applied to a heparin-agarose column (Pharmacia) and eluted with 100 ml of a linear gradient of 0.025–0.8 M KCl. The final preparation of the enzyme was dialysed against a storage buffer containing 10 mM potassium phosphate pH 7.4, 50 mM KCl, 0.1 mM EDTA, 10 mM ME, 60% (v/v) glycerol and stored at -20°C . The enzyme preparation was found to be stable for at least 6 months. Purification of M2.NcuI was carried out by the same set of procedures, except that the heparin-agarose chromatography step was omitted. Protein concentrations were determined by the method of Bradford (1976), with BSA as the standard. Overexpression and purification of M1.MboII and M1.NcuI have been described previously (Furmanek *et al.*, 2007).

Molecular mass determination. SDS-PAGE was carried out in gels containing 10% acrylamide for the separation gel and 4% acrylamide for the stacking gel. After electrophoresis, protein bands were stained with Coomassie brilliant blue R250. The molecular masses of native proteins were estimated by FPLC gel filtration on a Superose 12 HR 10/30 column equilibrated with 10 mM potassium phosphate pH 7.8, 150 mM KCl, 1 mM EDTA, 10 mM ME, 2.5% (v/v) glycerol, using BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and cytochrome *c* (12.4 kDa) as molecular standards.

Enzyme assays. Enzyme activity was determined using a protection assay in which pNH20 plasmid DNA, containing a unique MboII recognition site, was incubated with M2.MboII or M2.NcuI and, following methylation, the DNA was challenged with endonuclease NcuI. A typical reaction assay was carried out in 20 μl reaction mixture containing 10 mM Tris/HCl pH 7, 80 μM *S*-adenosyl-L-methionine (AdoMet), 0.5 μg DNA and 2 μl of column fraction. The reaction mixture was incubated at 37°C for 60 min and then heated at 70°C for 10 min to inactivate the methyltransferase. Following cooling, 2 μl NEB2 buffer and 5 units NcuI were added and incubated for 30 min at 37°C . The samples were analysed by 0.8% (w/v) agarose gel electrophoresis. One unit of modification activity was defined as the minimum amount of methyltransferase that conferred complete resistance to cleavage by the cognate restriction endonuclease. The second assay for M2.MboII/M2.NcuI activity was based on the incorporation of a tritiated methyl group into the substrate DNA. The methylation reactions were carried out in 30 μl reaction mixtures containing 0.5 μg DNA of pNH20 (or synthetic oligonucleotide), 22 nCi [^3H]AdoMet (1 Ci = 3.7×10^{10} Bq), 10 mM Tris/HCl pH 7 and 0.5 unit M2.MboII. The reaction was stopped by adding 30 μl 50%

(v/v) trichloroacetic acid, the sample was centrifuged and the pellet was washed with 1 ml 70% (v/v) ethanol and dried. Scintillation counting was used to estimate incorporated radioactivity.

β -Galactosidase assay. To determine the promoter strength, the regions comprising 111 bp and 281 bp DNA fragments upstream of the coding sequences of *mboIIM1* and *mboIIM2*, respectively, were transcriptionally fused with the reporter gene *lacZ* of pTL61 vector (Linn & St Pierre, 1990). β -Galactosidase was assayed as described by Miller (1972).

Characterization of M2.MboII and M2.NcuI activity optima. The effects of divalent cations at different concentrations on the activity of M2.MboII and M2.NcuI was investigated in 10 mM Tris/HCl buffer pH 7. Methylation reactions were carried out at 37°C in 20 μl reaction mixtures for 1 h using the same amount of enzyme and 0.5 μg pNH20 plasmid DNA.

Determination of the methylated base in DNA. To determine the base methylated by M2.MboII a method employing type IIS restriction endonucleases was used (Posfai & Szybalski, 1988). In order to obtain an appropriate template, two synthetic DNA fragments, MboAde (5'-AATTCCGGATGGCTAGCAAGA-3'/3'-GCCTACCGATCGTTCTCTTCGA-5') and MboCyt1 (5'-AATTCCGGATGTTGCTAGCGGAAGA-3'/3'-GCCTACAACGATCGCCCTCTTCGA-5'), carrying the underlined M2.MboII recognition site overlapped by the endonuclease FokI cut site were cloned into plasmid pGEM3Zf(+) double digested with EcoRI and HindIII, resulting in plasmids pMboAde and pMboCyt1, respectively. Plasmid pMboCyt2 without the MboII site was used as a control. In order to identify the base that is methylated, the DNA fragments (357 bp and 359 bp) were amplified using primers complementary to pGEM3Zf(+) vector (ADE1 5'-TTACGCCAGCTGGCGAAAG-3' and ADE2 5'-CATTAATGCAGCTGGCAC-3') and methylated by M2.MboII. The methylation reaction was performed in a 20 μl mixture containing 200 ng of the aforementioned DNA fragments, 10 mM Tris/HCl pH 7, 22 nCi [^3H]AdoMet and 0.5 unit M2.MboII (1 h, 37°C). Cleavage by FokI endonuclease of the [^3H]methyl-labelled 357 bp and 359 bp DNA fragments separates the adenines in the upper strand from the cytosines in the lower strand and the two neighbouring cytosines in the lower strand respectively, within the M2.MboII recognition site. After electrophoresis, DNA fragments were transferred to DEAE-cellulose membrane. Scintillation counting was used to estimate incorporated radioactivity.

Analysis of the N-terminal amino acid sequence of M2.MboII. N-terminal protein sequence analysis was performed at BioCentrum Ltd (Krakow, Poland). The sequentially detached phenylthiohydantoin derivatives of amino acids were identified using the Procise 491 automatic sequence analysis system (Applied Biosystems), according to the standard protocol of the manufacturer.

RESULTS

Protein sequence analysis and specificity of MboII methyltransferases

Type IIS R–M systems often contain three proteins: one restriction enzyme and two methylases, with each methylase recognizing and modifying bases on the opposite strand of the recognition site. According to the available *M. bovis* genomic fragment sequence database (Roberts *et al.*, 2003), and based on our sequence analysis, the MboII R–M system revealed the presence of two ORFs coding for

homologous isomethylomer of *N. cuniculi* (data not shown).

Isospecific systems MboII and NcuI have a similar genetic organization (Furmanek *et al.*, 2007). However, in contrast to the rather compact gene structure of the NcuI R–M system, the two MboII methyltransferases are separated by an intergenic region of 684 bp (Fig. 1b). In this DNA fragment we identify two ORFs, 654 bp and 375 bp long, which are in the same frame, with no sequence similarity to known expressed genes. We surmise that these ORFs flanked by the two MboII methyltransferase genes might encode a regulatory protein. Work is in progress to elucidate the possible significance of this region.

Overproduction of M2.MboII in *E. coli*

Analysis of the expression of the *mboIIM2* gene in *E. coli* with plasmid pBRmboIIMB.4 revealed that the natural $P_{mboIIM2}$ promoter is too weak to protect recognition sites against R.MboII digestion (Fig. 2, lane 5). The gene can give a sufficient level of protein to protect all MboII sites only after its fusion with an additional constitutive promoter, P_{tetA} (Fig. 2, lane 7). In contrast, the expression of the *mboIIM1* gene from its natural promoter is fully sufficient to give protection to all cellular MboII sites (data not shown). Study of the promoter strength of both methyltransferases in the heterologous background of *E. coli* showed that they are relatively weak, but $P_{mboIIM1}$ is fourfold stronger than $P_{mboIIM2}$ (reporter β -galactosidase activity 2500 Miller units, versus 630 Miller units for both

M1.MboII and M2.MboII). This raises the question of whether the M2.MboII methylase is dispensable in protection of the host DNA against the cognate restriction endonuclease. For that reason, we were interested in further study of the functional interplay between the M1.MboII/M2.MboII and R.MboII enzymes, reflected either in their expression control level or in differences in biochemical properties.

In order to isolate the *mboIIM2* gene originated from the *M. bovis* genomic DNA in *E. coli* cells, two pairs of primers were designed to clone the gene with its own promoter or to obtain transcriptional/translational fusion with T7 expression signals for M2.MboII overproduction. We found that DNA methylation by M2.MboII, but not M1.MboII, triggered the methylation-related autorestriction process, lethal for the vast majority of cells of the $Mcr^+ Mrr^+$ *E. coli* hosts (data not shown). Thus, we could only obtain clones carrying the *mboIIM2* gene in *E. coli* deficient for McrBC and Mrr (Raleigh *et al.*, 1988). To purify the M2.MboII and M2.NcuI enzymes the T7 phage expression system was employed. Results of the purification are summarized in Fig. 3 and Table 1. The standard procedure consisted of ion-exchange chromatographic steps. After the last step of purification the specific activity of M2.MboII and M2.NcuI was approximately 3000-fold higher than that of the crude lysate. The final enzyme preparation did not show the presence of non-specific nucleases. From 5 g of bacteria 15 mg homogeneous M2.MboII preparation was obtained with an overall yield of 60%. N-terminal amino acid sequencing of M2.MboII was carried out by automated Edman degradation. The sequence of the first 10 amino acid residues was Met₁-Asn₂-Tyr₃-Ile₄-Phe₅-Phe₆-Lys₇-Asp₈-Ser₉-Arg₁₀ and was found to be identical to the amino acid sequence of

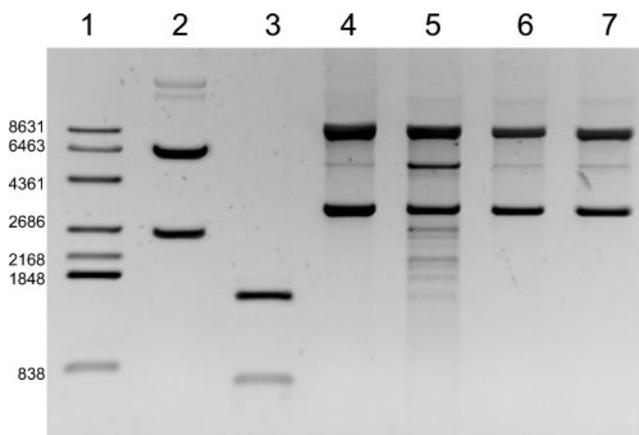


Fig. 2. Protection of the MboII site against R.MboII digestion *in vitro*, dependent on the level of constitutive expression of the *mboIIM2* gene. Plasmid preparations were isolated from *E. coli* ER1992, then each sample was electrophoresed on 0.8% agarose gel. Lanes: 1, DNA size markers (bp); 2, intact pBR322 (11 MboII sites); 3, pBR322 after R.MboII digestion; 4, intact pBRmboIIMB.4 carrying *mboIIM2* gene with natural promoter (12 MboII sites); 5, pBRmboIIMB.4 after R.MboII digestion; 6, intact pBRmboIIMB.20, carrying *mboIIM2* with additional P_{tetA} promoter (12 MboII sites); 7, pBRmboIIMB.20 after R.MboII digestion.

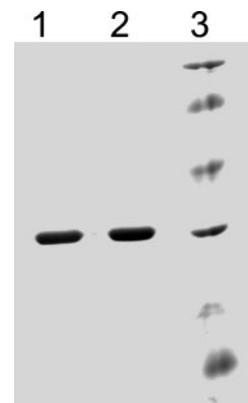


Fig. 3. Molecular mass determination of methyltransferases M2.NcuI and M2.MboII: Coomassie-blue-stained SDS-PAGE gel of the purified proteins. Lanes: 1, sample of M2.NcuI (200 ng); 2, sample of M2.MboII (200 ng); 3, molecular mass markers (phosphorylase *b*, 97 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α -lactalbumin, 14 kDa).

Table 1. Purification of methyltransferases M2.MboII and M2.NcuI

M2.MboII/M2.NcuI purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (fold)
Cell-free extract	80 000/52 500	90/68	889/772	100/100	1/1
Phosphocellulose	80 000/33 000	75/52	10 669/634	100/63	1.2/0.8
Hydroxylapatite	75 000/14 250	60/39	12 501/305	94/27	1.4/0.47
CM-Sephadex	55 000/16 000	40/6	1375/2667	69/30	2.25/3.4
Heparin-agarose	40 000	15	3200	60	3.6

M2.MboII, confirming the identity of the protein. M2.NcuI was purified to homogeneity, yielding approximately 6 mg protein from 2.5 g cell paste. The molecular mass of M2.MboII and M2.NcuI and the purity of the preparations were determined by SDS-PAGE. The enzymes showed a single band on the gel corresponding to a molecular mass of $30\,000 \pm 1000$ Da (Fig. 3), which is close to the values of 32 111 and 32 676 Da deduced from the nucleotide sequence of the *mboIIM2* and *ncuIM2* genes, respectively. The same value was estimated by gel filtration on Superose 12 (data not shown), suggesting that M2.MboII exists in solution as a monomer.

Comparative study of R.MboII production in the presence of MboII methyltransferases

Most type II R–M systems consist of two enzymic activities: a restriction endonuclease that recognizes a specific DNA sequence for cleavage and a cognate methyltransferase that modifies adenine or cytosine at recognition site. These two activities must be carefully balanced to ensure protection of the host chromosome (Nagornykh *et al.*, 2008). To gain some preliminary data on the interplay between MboII methyltransferases which participate in the regulation of the MboII R–M system's function we compared the viability of the host cells and levels of R.MboII production dependent on the presence of particular kinds of MboII methyltransferase, separately or together. We co-transformed *E. coli* strain ER2566 with three plasmid pairs, where all the investigated genes were dependent on the P_{lac} promoter control. Each plasmid pair included pMboR3.0, which delivers an *mboIIR* gene, with a combination of one of three alternative plasmids: pMboM1.1 (M1.MboII⁺), pUCMB.3 (M2.MboII⁺) or pMboMAB (bearing both methyltransferase genes). We found that during IPTG induction the most stable and fast-growing cultures were those with the pMboMAB helper plasmid. This system gave maximal production of R.MboII protein in *E. coli* cells (8.5 ± 1.2 µg per ml of collected active fraction after preliminary purification, versus 2.5 ± 0.6 µg ml⁻¹ and 5.1 ± 1.8 µg ml⁻¹ for M2.MboII⁺ and M1.MboII⁺, respectively). This result was also reflected in the highest wet bacterial paste mass (1.46 g versus 1.29 and 1.02, for M1.MboII⁺ and M2.MboII⁺ respectively), which was in agreement with the doubling time and maximum cell viability assessed for this culture. The slowest growth was observed in the case of M2.MboII⁺ cells, while the

M1.MboII⁺ cells showed an intermediate growth rate (Fig. 4).

Determination of the methylation position

In order to determine the base methylated by M2.MboII a method employing type IIS restriction endonucleases was used. Two kinds of DNA fragments with the MboII site next to the recognition site of the FokI enzyme were used. An amplified DNA fragment (357 bp) carrying the M2.MboII recognition site (see Methods), after methylation with M2.MboII in the presence of [³H]AdoMet, was cleaved by endonuclease FokI into three portions: 29 bp, 103 bp and 225 bp (Fig. 5a, d). Cleavage by FokI endonuclease of the [³H]methyl-labelled DNA fragment separates adenines in the upper strand from cytosines in the lower strand within the M2.MboII recognition site. Most of the radioactivity was observed in the 103 bp fragment (B), which suggests that one of the cytosine residues in the sequence 5'-TCTTC-3' is modified by M2.MboII. In the next step, the amplified DNA fragment (359 bp), after methylation with M2.MboII, was cleaved by

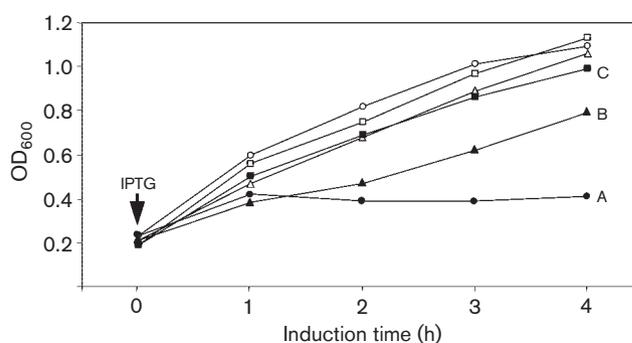


Fig. 4. Effect of MboII R–M two-plasmid expression systems on growth and cell viability of *E. coli* ER2566 harbouring pMboR3.0 (bearing *mboIIR*) plus one of pUCMB.3 (○, ●), or pMboM1.1 (△, ▲) or pMboMAB (□, ■) (bearing *mboIIM2*, *mboIIM1* or both methyltransferase genes, respectively). All *mboII* genes were under P_{lac} promoter control; therefore IPTG induction for their expression was applied (4 h at 1 mM). Growth curves for uninduced (open symbols) and induced (filled symbols) cultures are shown. The final c.f.u. ml⁻¹ for cultures A, B and C were 1.1×10^7 , 7.7×10^7 and 2.25×10^8 , respectively.

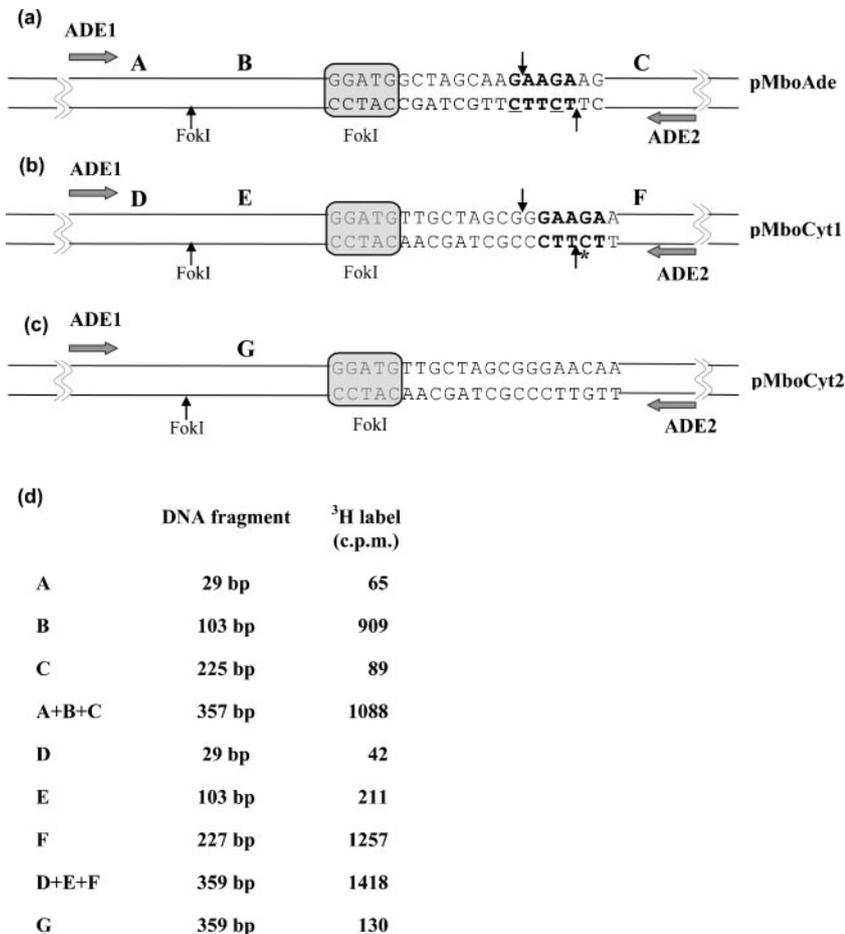


Fig. 5. Analysis of the methylation pattern of M2.MboII. DNA fragments (357 bp in panel a; 359 bp in panels b and c) were amplified using primers ADE1 and ADE2. As templates, plasmids pMboAde (a), pMboCyt1 (b) and pMboCyt2 (c) were used. PCR products containing a unique MboII site (shown in bold) were methylated by M2.MboII using [³H]AdoMet. After cleavage with FokI, the DNA was electrophoresed in an agarose gel (1.5%) and particular restriction fragments (designated by upper-case letters) were isolated. The labelled methyl group contents of the fragments are shown in c.p.m. in (d). The putative methylation sites of M2.MboII are underlined; an asterisk indicates the base modified by M2.MboII. The FokI cleavage sites are marked by arrows.

FokI into three portions: 29 bp, 103 bp and 227 bp (Fig. 5b, d). This time, cleavage of the [³H]methyl-labelled DNA fragment ensured separation of the two cytosines within the MboII recognition site. Most of the radioactivity was observed in the 227 bp fragment (F). These results showed that M2.MboII methylates the internal cytosine residues of the strands carrying 3'-CTTCT-5'. In the control experiment a 359 bp DNA fragment lacking the MboII site failed to be a substrate for M2.MboII modification activity (Fig. 5c, d). Similar results were recorded for M2.NcuI (data not shown).

Enzyme characterization

Methyltransferase M2.MboII is active over a relatively wide pH range, with maximum activity at pH 7–8, and seems to be more stable at alkaline than acid pH values (data not shown). The activity of M2.MboII is influenced by divalent cations. In general, methylation was much less effective in the presence of Ca²⁺, Mg²⁺, Mn²⁺ or Zn²⁺. We found that Mn²⁺ and Zn²⁺ acted as strong inhibitors of the enzyme (IC₅₀ 0.4 mM), while Ca²⁺ and Mg²⁺ inhibited the enzymes less efficiently (IC₅₀ 4 mM and 5 mM, respectively). We also found that M2.NcuI behaves in the same way. Comparison of the sensitivity of M1.MboII and

M2.MboII to Mg²⁺ showed that the latter is more tolerant to this cation.

Methylation of single-stranded and double-stranded DNA

The substrate specificity of the methyltransferases was analysed by comparing their enzymic efficiencies *in vitro* with two types of DNA substrates. In the first series, DNA molecules of biological origin were probed as substrates. The methylation reactions contained identical amounts of activity units of each methyltransferase, [³H]AdoMet and DNAs that were supplied in equivalent amounts of 5'-GAAGA-3' and 3'-CTTCT-5' sites. As shown in Fig. 6(a) both MboII methyltransferases were able to methylate efficiently the control DNA, pUC18. Most interestingly, M2.MboII proved to be equally efficient on the single-stranded or double-stranded form of the phage PhiX174 DNA. In contrast, no detectable activity on the single-stranded substrate was observed with M1.MboII. Moreover, double-stranded DNA was less efficiently methylated than pUC18.

The specificity studies were extended to include oligonucleotide substrates which allowed the determination of

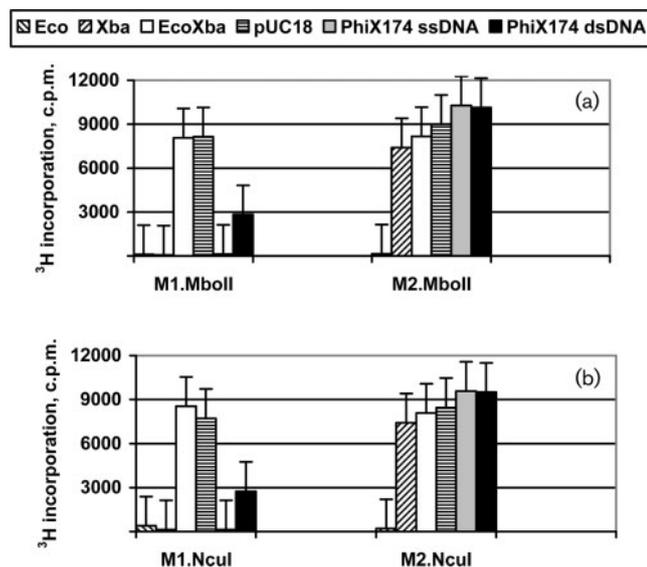


Fig. 6. Enzymic incorporation of [^3H]methyl groups into single-stranded (ss) and double-stranded (ds) DNA by isomethylomers M1.MboII and M2.MboII (a), and M1.NcuI and M2.NcuI (b). Methylation assays were performed as described in Methods. Eco and Xba refer to complementary oligonucleotide sequences as follows: Eco, 5'-AATTCGAAGATGTGAAGATGTGAAGATGTGAAGATGT-3'; Xba, 5'-CTAGACATCTTCACATCTTCACATCTTCACATCTTC-3' (the underlined regions represent the MboII/NcuI recognition sequences). pUC18 and PhiX174 dsDNA contain seven and eleven MboII sites, respectively; PhiX174 ssDNA contains four 5'-GAAGA-3' and seven 5'-TCTTC-3' sequences.

methylase specificity with respect to the individual strands. Consistent with our earlier results, M2.MboII was able to modify a single-stranded oligonucleotide at the same level as that observed for the specific duplex. As expected, M1.MboII showed high activity on the duplex oligonucleotide containing the MboII sites, but no detectable methylation on the constituent strands. Both methyltransferases of the NcuI R-M system showed similar properties (Fig. 6b). Studies in our laboratory indicated that PhiX174 single-stranded DNA could be methylated but not restricted by R.MboII (data not shown).

DISCUSSION

The enzymic transfer of a methyl group to specific nucleotides in DNA is the most common form of biological DNA modification. In prokaryotes all three natural types of DNA methylation are observed: N^6 -methyladenine, N^4 -methylcytosine and C^5 -methylcytosine. DNA methylation is introduced enzymically by DNA methyltransferases after DNA replication (Jeltsch, 2002). The R-M systems of bacteria constitute the most diverse group of methylases, and although only accounting for a small percentage of the total methylated DNA, they play an

important role in bacterial survival. R-M systems are found in a vast range of bacteria, with over 3000 examples recognized to date (Roberts *et al.*, 2007). Methylation results in a modification pattern in the bacterial DNA specific to the individual enzymes encoded by the host. This allows the host bacterium to distinguish between its own specifically methylated DNA and the unmethylated (or differently methylated) DNA entering the bacterial cell. The distinction between self and nonself has traditionally been associated with defence against bacteriophages but has been more recently implicated in speciation and evolution in general (Jeltsch, 2002).

Sequence analysis of the MboII R-M system

The type IIS R-M system MboII from *M. bovis* ATCC 10900 as well as the isomeric NcuI system is composed of two (N^6 -methyladenine and N^4 -methylcytosine) methyltransferases and a single restriction enzyme. The requirement for two methylases can be rationalized, first by the asymmetry of the recognition site, and second by the fact that each chain of such a site contains different types of bases that are to be methylated. Thus for methylation in both strands, cytosine-specific and adenine-specific methyltransferases are required. Sequence analysis of the MboII R-M system revealed the presence of an open reading frame encoding a second cytosine N^4 -methyltransferase, M2.MboII, in the vicinity of the genes specifying the previously characterized adenine N^6 -methyltransferase M1.MboII and restriction endonuclease R.MboII. M2.MboII contains all the conserved motifs common to N^4 -methylcytosine methyltransferases, including a previously overlooked, weakly conserved sequence patch present in most of the N^4 mC methylase sequences. This patch, labelled in Fig. 1(a) as motif IX-N4 (N/Q/D-V/I-W-N/E/D-I/V), can be found after motif VIII, between the variable region and motif X (Bujnicki & Radlinska, 1999). We also noted a close relationship between M2.MboII and M2.NcuI in the nucleotide sequence coding regions of these proteins, showing 83% identity. The amino acid similarity between the two methyltransferases is even higher (88% identity), indicating that these proteins have emerged from a common ancestor.

Enzymic properties of M2.MboII

The biochemical characteristics (molecular mass, activity dependency on divalent cations) determined for homogeneous preparations of M2.MboII and M2.NcuI were very similar. In both cases we found that Mn^{2+} and Zn^{2+} , even at low concentrations, inhibited the enzymes very efficiently. In the presence of 5 mM Mg^{2+} M2.MboII and M2.NcuI retained over 50% of their activity; it was also observed that a significant amount of activity was lost (70%) in the presence of 5 mM Ca^{2+} . On the other hand, it has been demonstrated that Mg^{2+} ions are necessary for the restriction activity of R.MboII and R.NcuI but in the

case of M1.MboII and M1.NcuI, Mg^{2+} inhibited methylation activity (Furmanek *et al.*, 2001, 2007). Assuming that the physiological concentration of Mg^{2+} in *M. bovis* and *N. cuniculi* cells is similar to that of *E. coli* (1–4 mM; Lusk *et al.*, 1968), these conditions ensure high restriction activity while the activity of both methyltransferases of the investigated R–M systems are inhibited. This finding underlines the defensive character of R–M systems and also explains the recombinogenic role of restriction endonucleases (McKane & Milkman, 1995). Most restriction endonucleases require Mg^{2+} to promote enzyme activity; however, magnesium can function as a strong inhibitor of modification. Thus, changes in the Mg^{2+} concentration might act as a regulator for the proper functioning of R–M systems to ensure a balance between restriction and modification activities and to prevent genomic stability. In an earlier report we described the significance of this issue in more detail (Furmanek *et al.*, 2007).

Methyltransferases M1.MboII and M2.MboII were purified from the *E. coli* expressing the *mboIIM1* and *mboIIM2* genes separately, and their enzymic efficiencies *in vitro* were compared with a variety of DNA substrates. Both enzymes act on 5'-GAAGA-3'/3'-CTTCT-5' sites in double-stranded DNA; however, M2.MboII can also, with comparable efficiency, modify the specific targets in single-stranded DNA. The specific activity of M2.MboII for double- and single-stranded DNA is almost the same. The primary biological role of M2.MboII may be similar to that suggested for DpnA, namely facilitation of the natural transfer of plasmid DNA between strains by the transformation pathway, in which incoming plasmid DNA is in a single-stranded form (Cerritelli *et al.*, 1989). This is in accordance with experiments performed with naturally competent members of the genera *Moraxella* and *Neisseria*, which also demonstrated that single-stranded DNA can transform these bacteria (Johnsborg *et al.*, 2007). It remains to be determined whether the single-stranded-DNA-specific methylases facilitate horizontal gene transfer, and together with gene loss and duplication may shape the bacterial genomes.

Most of the characterized methyltransferases bind to DNA as monomers (Bheemanaik *et al.*, 2006). This is in agreement with the observation that a typical methylase DNA substrate is hemimethylated; therefore, it is asymmetrical and needs only a single methyl transfer to return to a fully methylated state. On the other hand, in the absence of DNA, methyltransferases exist predominantly as dimers, especially at high protein concentrations. It has been reported that M.RsrI shows partial dimerization, and two methyltransferases from *Streptococcus pneumoniae* appear to exist as dimers (Cerritelli *et al.*, 1989; Thomas & Gumpert, 2006). In the case of M.MspI the enzyme dimerizes at higher protein concentrations, which may reflect a tendency to aggregate (Dubey *et al.*, 1992). This is in agreement with our earlier finding that methyltransferases M1.MboII and M1.NcuI purified from overexpressing clones exist in both monomeric and dimeric forms (Furmanek *et al.*, 2007).

Expression of methylation and restriction genes in *E. coli*

A complete MboII R–M system consisting of two methyltransferase genes and the *mboIIR* gene is the most stable functionally and genetically. The first report on *mboIIR* gene expression cloned in *E. coli* in the presence of M1.MboII only indicated a 20-fold lower level of R.MboII activity in the *E. coli* host than in *M. bovis* cells (Bocklage *et al.*, 1991). The transfer of a PCR-amplified tandem of genes (*mboIIM1::mboIIR*) under T7 promoter control always gave rise to mutations on the *mboIIR* encoding sequence, which decreased R.MboII activity (our unpublished observations). A similar effect was obtained when the *E. coli* host was used, which allows for the premodification of the MboII sites by expression of the *mboIIM1* methylase gene. It was shown that the natural $P_{mboIIM2}$ promoter is too weak to protect restriction sites against R.MboII digestion in *E. coli* hosts. In contrast, the expression of the *mboIIM1* gene from a natural promoter is fully sufficient to give protection to all cellular MboII sites. Study of the promoter strength of both methyltransferases showed that $P_{mboIIM1}$ is four times stronger than $P_{mboIIM2}$. We compared the viability of the host cells and levels of R.MboII production depending on the presence of particular kinds of MboII methyltransferase, separately or together. All the investigated genes were uniformly dependent on P_{lac} promoter control, to make expression independent of their natural promoters, which all differ in strength. Each plasmid pair included pMboR3.0, which delivers an *mboIIR* gene, with a combination of one out of three alternative plasmids, supplying M1.MboII, M2.MboII or both methyltransferases. We found that during IPTG induction the most stable and fast-growing were cultures where the plasmid delivered two methyltransferase activities. This system gave maximal production of the R.MboII protein in *E. coli* cells as well bacterial mass in comparison to any of the systems containing a single M.MboII. It was also associated with the shortest doubling time and high cell viability. The worst rate of growth was obtained in the case of bacterial cells bearing the M2.MboII⁺ plasmid, and an intermediate value in the presence of M1.MboII⁺. These results show that functional imbalance between restriction and modification, observed in the presence of any single methylase, is not necessarily a matter of the promoter's strength in relation to particular methyltransferase genes, but is something which underlies their specific biochemical properties and the asymmetrical structure of the target sequence.

Gene arrangements within MboII and NcuI R–M systems

The enzymes of the MboII R–M system are functionally identical with those of the NcuI R–M system. Since the genes are also very similar, the MboII and NcuI R–M systems, encoded by two closely related species, apparently have a common evolutionary origin. However, while the

two MboII methyltransferase genes are separated by 684 nucleotides which encode two small ORFs, the genes encoding the NcuI R–M system are closely linked and arranged in tandem. To further investigate the putative role of this intergenic region we performed a PCR procedure, using primers corresponding to the 5′- and 3′-terminal parts of the DNA sequence located between the *mboIIM2* and *mboIIM1* genes, with chromosomal DNA of five different *M. bovis* strains as a template (Kakuda *et al.*, 2006). In all cases the sequential analysis of the amplification products showed the presence of a 684 bp DNA fragment containing two ORFs identical to those of *M. bovis* ATCC 10900. Comparison of the location of *mboIIM2* in the genetic structure of the MboII R–M system with the locations of their isomethylomers suggests independent evolutionary routes at least for this element among MboII-like R–M systems. The difference in the gene arrangement suggests that the two methyltransferases of the MboII and NcuI R–M systems may have become joined in the two organisms in independent, stepwise events. Alternatively, different gene arrangements may have evolved following joint transfer of the ancestral genes into the different hosts. A long evolutionary history for the present MboII and NcuI R–M systems is suggested by the fact that the nucleotide sequences immediately flanking the R–M genes are different in *M. bovis* and *N. cuniculi*.

Concluding remarks

This study shows the unusual ability of methyltransferases M2.MboII and M2.NcuI, belonging to the class IIS R–M system, to methylate single-stranded DNA. Each enzyme of the MboII or NcuI system is responsible for the modification of different strands. Similar results were obtained with the BcnI system; however, both BcnI methyltransferases are isospecific on double-stranded DNA, modifying the same cytosine residue in the recognition sequence (Vilkaitis *et al.*, 2002). Moreover, subcloning experiments indicated that M1.MboII alone is sufficient to support the growth of *E. coli* cells carrying the R.MboII gene; this could not be demonstrated for M2.MboII. Taken together with their dissimilar primary structures, this disparity indirectly suggests that the two MboII methyltransferases may have distinct functional roles. We have demonstrated the substrate specificity of M2.MboII, which, together with M1.MboII, methylates both strands of their recognition sequences and shows different strand-specificity: an adenine residue is methylated on the top strand and a cytosine residue on the bottom strand. The biological meaning of the coexistence of a restriction endonuclease and two individual methylases specific for forms of DNA remains to be elucidated.

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