

# MmeI: a minimal Type II restriction-modification system that only modifies one DNA strand for host protection

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## ABSTRACT

**MmeI is an unusual Type II restriction enzyme that is useful for generating long sequence tags. We have cloned the MmeI restriction-modification (R-M) system and found it to consist of a single protein having both endonuclease and DNA methyltransferase activities. The protein comprises an amino-terminal endonuclease domain, a central DNA methyltransferase domain and C-terminal DNA recognition domain. The endonuclease cuts the two DNA strands at one site simultaneously, with enzyme bound at two sites interacting to accomplish scission. Cleavage occurs more rapidly than methyl transfer on unmodified DNA. MmeI modifies only the adenine in the top strand, 5'-TCCRAC-3'. MmeI endonuclease activity is blocked by this top strand adenine methylation and is unaffected by methylation of the adenine in the complementary strand, 5'-GTYGGA-3'. There is no additional DNA modification associated with the MmeI R-M system, as is required for previously characterized Type IIG R-M systems. The MmeI R-M system thus uses modification on only one of the two DNA strands for host protection. The MmeI architecture represents a minimal approach to assembling a restriction-modification system wherein a single DNA recognition domain targets both the endonuclease and DNA methyltransferase activities.**

## INTRODUCTION

MmeI is a restriction endonuclease from *Methylophilus methylotrophus* that cuts DNA two turns of the helix away from its asymmetric recognition sequence, 5'-TCCRACN<sub>20</sub>/N<sub>18</sub>-3' (1). The 20-bp reach between the recognition site and cutting position is the longest of the Type II enzymes and makes MmeI useful for applications that capture the sequence information of the 20

unspecified bases such as LongSAGE (2) or paired end sequence reads (3).

Restriction-modification (R-M) systems are classified into types based on their architecture and cofactor requirements (4). As more restriction systems are characterized, not only their diversity but also the evolutionary interrelatedness of the various types of R-M systems is becoming more apparent. In one example crossing the Type I and Type II boundaries, systems have been characterized that have a typical Type II endonuclease paired with a Type I-like methyltransferase composed of separate M and S proteins (5). The availability of genomic sequences has provided new opportunities to observe the wealth of systems that modify and cut DNA, especially systems that have been underrepresented in traditional screens for restriction activity. Thus our understanding of the diversity of R-M systems continues to grow as more and more variations on the theme of restriction and modification are characterized. The MmeI system we describe represents one such new variation on the restriction-modification theme.

There are three main groups of R-M systems. The Type I restriction systems are the most complex, composed of three separate proteins for restriction, modification and specificity that function as a single protein complex requiring *S*-adenosyl-L-methionine (AdoMet) and the energy from ATP hydrolysis in addition to magnesium to support cleavage. Somewhat less complex are the Type III systems composed of two proteins: the Mod protein, which combines DNA recognition and methyltransferase functions, and the Res protein that pairs with the Mod protein to accomplish DNA cleavage. Like the Type I systems these enzymes require ATP, AdoMet and magnesium for endonuclease activity. Unlike the Type I enzymes, DNA cleavage occurs at a discrete position to one side of the recognition sequence. Type III enzymes utilize both DNA looping and translocation to bring enzyme bound at two sites together to accomplish DNA scission, which occurs next to one of the two interacting sites (6–8). The single DNA methyltransferase of Type III systems modifies only one DNA strand (9). Type III endonucleases

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normally require two recognition sites oriented in a head-to-head direction for DNA cleavage to occur (10). This requirement effectively places DNA modification on both strands, ensuring that newly replicated host DNA will be protected from the action of the endonuclease.

The Type II systems familiar in molecular biology are less complex than the Type I and Type III systems, though there is considerable variation among these systems as reflected in their various subtype classifications (4). The Type II enzymes do not require energy input from ATP for cleavage. Most require only magnesium ions as cofactor, though some, including MmeI, additionally require the methyl-donor cofactor AdoMet (11). Normally these systems have separate DNA methyltransferase and endonuclease proteins that recognize and act independently upon the same DNA target sequence. Together, the two separate enzymes partner to accomplish host protection and foreign DNA restriction. The Type IIS subgroup enzymes such as FokI are distinct in that they recognize asymmetric sequences and cleave outside the recognition sequence. Because the linear sequence of bases on each DNA strand differs, these systems require two DNA methyltransferases, one to modify each strand (12). The Type IIG enzymes are enzymes wherein DNA methyltransferase and endonuclease activity reside in a single polypeptide. These proteins have a common DNA target recognition domain that directs the competing enzymatic functions. The methyltransferase function modifies one DNA strand within the recognition sequence, while the endonuclease function cleaves unmodified DNA at some distance away from the recognition sequence. The Type IIG enzymes are formally designated RM.(enzyme name), i.e. RM.MmeI, to indicate they have both functionalities in the same polypeptide. However, for simplicity we refer to RM.MmeI as MmeI in this report. The modification activity in the Type IIG restriction enzymes modifies only one strand of their recognition sequence. The Type IIG enzymes characterized to date therefore partner with a second, separate DNA methyltransferase to accomplish the modification on the complement strand of the recognition sequence necessary to protect newly replicated host DNA. For example, the well-characterized Eco57I restriction enzyme could not be cloned and expressed in *Escherichia coli* in the absence of the second methyltransferase (13–15).

MmeI was previously reported to be a bi-functional Type IIG restriction enzyme (16). MmeI requires both magnesium and AdoMet for endonuclease activity, with a more recent study indicating AdoMet enhances binding to the specific recognition site (17). It was reported that MmeI modified the adenine in the top strand of the recognition sequence, 5'-TCCRAC-3', but that this modification did not block MmeI endonuclease activity. A second methyltransferase was isolated and reported to block MmeI activity through modification of the adenine in the bottom strand of the MmeI recognition sequence, 5'-GTYGGGA-3' (16).

Here we report the cloning, sequence and organization of the MmeI R-M system. Biochemical characterization of MmeI reveals significant differences from those previously reported. The methylation produced by this system and

the sensitivity to methylation we observe for the MmeI endonuclease are key to understanding this unusual restriction system. Our findings indicate MmeI differs from previously characterized R-M systems and represents a new sub-group within the Type II restriction enzymes.

## MATERIALS AND METHODS

MmeI, AdoMet, all restriction endonucleases, T4 DNA Ligase, Phusion DNA polymerase, DNA size standards and competent cells were obtained from New England Biolabs (Ipswich, MA). All synthetic DNA oligonucleotides were from New England Biolabs, Organic Synthesis Division (Ipswich, MA). The synthetic oligonucleotides are listed by number in Supplementary Table S1. [methyl-3H]-AdoMet was obtained from GE Healthcare (Pittsburgh, PA). Gamma-<sup>33</sup>P-ATP was obtained from Perkin Elmer (Boston, MA). Plasmid miniprep and DNA purification spin columns were from Qiagen (Valencia, CA) or Zymo Research (Orange, CA). Pre-cast Novex TBE acrylamide gels were obtained from Invitrogen (Carlsbad, CA).

### MmeI endonuclease and methyltransferase assays

MmeI endonuclease activity requires magnesium ions and AdoMet, while methyltransferase activity requires only AdoMet. Endonuclease activity was assayed by incubating various amounts of MmeI at 37°C in MmeI reaction buffer (NEBuffer 4: 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, supplemented with 100 µg/ml BSA and 80 µM AdoMet) containing 1 µg substrate DNA per 50 µl. Reactions were terminated by addition of stop solution (50 mM EDTA, pH 8.0, 50% glycerol, 0.02% bromophenol blue), and reaction products were analyzed by electrophoresis in 1% LE agarose or 3% NuSieve GTG agarose gels alongside DNA size standards lambda-HindIII with PhiX174-HaeIII, or lambda-BstEII with pBR322-MspI. Methyl transfer activity was assayed in the same buffer that contained no magnesium ions with 80 µM AdoMet or 1 µM [methyl-3H]-AdoMet.

### Cloning and expression of the MmeI RM system

The gene encoding MmeI in *M. methylotrophus* was identified in the following way and then cloned into *E. coli* for efficient expression of the MmeI endonuclease. MmeI endonuclease protein was purified to near homogeneity from 750 g *M. methylotrophus* cells by chromatography over six columns: Heparin Hyper D (500 ml), Source 15 Q (100 ml), Heparin TSK (56 ml), Source 15 S (75 ml), Superdex 75 (21) and Ceramic HTP (16 ml). Two milligrams of highly pure MmeI protein was obtained. Amino acid sequence was determined from the amino terminus of the protein and from internal cyanogen bromide cleavage products. Degenerate DNA primers corresponding to several short stretches of these sequences were synthesized and used to PCR amplify a portion of the MmeI gene. A 650-bp portion of the MmeI gene from the 7.5-kDa CNBr fragment (forward) to the 14-kDa

CNBr fragment (reverse) was amplified using primers 1 and 2 (Supplementary Table T1). A 1-kb portion of the MmeI gene from the 7.5-kDa CNBr fragment to the 25-kDa CNBr fragment was amplified with primers 1 and 3. The DNA sequences of these portions of the MmeI gene were determined. The DNA sequence of the entire MmeI endonuclease gene and flanking DNA sequences were obtained through inverse PCR. Finally, the complete MmeI gene was amplified from *M. methylotrophus* genomic DNA using primers 4 and 5, cut with PstI and BamHI, ligated into an expression vector, pRRS (18) and transformed into *E. coli* ER2683. One construct, pTBMmeI.1, was found to express 20 000 U of MmeI endonuclease activity per gram of cells.

### MmeI methyltransferase product

The methylated base produced by the methyltransferase of MmeI was identified using antibodies specific for N6-methyl adenine or N4-methyl cytosine. An unmodified DNA substrate was formed by PCR amplification of pMMH1 using primers NEB1224 and primer 29 to yield a 685-bp DNA having two MmeI sites, one EcoRI and one BamHI site. This DNA was *in vitro* modified by MmeI, M.EcoRI (positive control for N6-adenine methylation) and M.BamHI (positive control for N4-cytosine methylation). All three enzymes produce two modified bases per DNA molecule. The modified DNAs were spotted onto nitrocellulose filters alongside positive control DNAs for N6-adenine methylation (*dam* methylase modified pUC19) and N4-cytosine methylation (M.EsaBC3I modified pUC19 derivative). Antibodies specific for N6-methyl adenine or N4-methyl cytosine were incubated with the DNAs and detected as previously described (19).

### MmeI methyltransferase base target

The incorporation of labeled methyl groups by MmeI was measured on synthetic DNA substrates in which the adenines in the MmeI recognition site were either unmethylated or methylated. The DNAs were incubated with MmeI and 1  $\mu$ M [methyl-3H]-AdoMet, the unincorporated label was removed using a DNA spin column and the incorporated radioactivity counted in a scintillation counter. Synthetic oligonucleotides were synthesized and annealed to form dsDNA containing two unmodified adenines, N6-methyl adenine in the top strand only (5'-TCCG(m6A)C-3'), in the bottom strand only (5'-GTCCG(m6A)-3') or in both strands. The synthetic DNAs had restriction sites overlapping the MmeI site for Hpy188I (TCNGA), HinfI (GANTC), MboI (GATC) and BfuCI (GATC). Hpy188I, HinfI and MboI are all blocked by methylation of the adenine in their recognition sequences, both by hemi-methylation on one strand only or by full modification on both strands (20). BfuCI is not affected by adenine methylation and served as a positive control. These endonucleases were used to verify the methylation status of the synthetic DNAs. Fully unmodified DNA, 'UU', was formed from oligonucleotides 8 and 10. Top strand modified DNA, 'MU', was formed from oligonucleotides 9 and 10. Bottom strand

modified DNA, 'UM', was formed from oligonucleotides 8 and 11. DNA modified in both strands, 'MM', was formed from oligonucleotides 9 and 11. MmeI was incubated with 40 pmol of the differentially modified dsDNAs in 100- $\mu$ l methyltransferase buffer containing 1  $\mu$ M [methyl-3H]-AdoMet. Modified DNA was then purified from unincorporated [methyl-3H]-AdoMet using a Qia-QUICK Nucleotide Removal spin column (Qiagen) and eluted in 50  $\mu$ l TE. Fifteen microliters of the eluate was electrophoresed on a 3% NuSieve GTG agarose gel while 25  $\mu$ l was mixed with 1.5 ml scintillation fluid and counted in a scintillation counter. As a positive control the same reactions were performed using *dam* methyltransferase.

### MmeI endonuclease sensitivity to m6-adenine methylation

The sensitivity of the MmeI endonuclease to methylation at the two adenine bases in its recognition sequence was tested by digesting the differentially modified synthetic dsDNAs 'UU', 'MU', 'UM' and 'MM' (10 pmol in 50  $\mu$ l) with MmeI (4 U). The DNAs were also digested with Hpy188I, HinfI, MboI and BfuCI to verify their methylation status.

### MmeI methylation effect on MmeI endonuclease activity

The effect of the *in vivo* methylation produced by the cloned MmeI gene on MmeI endonuclease activity was tested by digesting the MmeI expressing plasmid, pTBMmeI.1, with MmeI. As a positive control the reaction mixture was split and 1  $\mu$ g PhiX174 RFI DNA was added to one aliquot. A point mutation plasmid construct derived from pTBMmeI.1 carrying an inactive MmeI gene, pRMMmeN773D, was digested with MmeI to confirm the presence of the sites in the pTBMmeI.1 construct. The presence of the three MmeI recognition sites in the pTBMmeI.1 plasmid DNA was also confirmed by DNA sequencing.

The effect of *in vitro* MmeI methylation was tested using a plasmid substrate, pMMH1, designed to introduce an MmeI site in the pUC19 polylinker region that overlaps a 5' MboI site (GATC) and a 3' HinfI site (GANTC): 5'-... cccgggGATCCGACTCcggtcgac...-3'. pMMH1 was formed from pUC19 using the Phusion Site-Directed Mutagenesis Kit (NEB) with primers 6 and 7. The HinfI site in pUC19 at position 427 was removed by the mutagenesis. pMMH1 was grown in a *dam* methylase-proficient host, ER2683, or in a *dam* methylase-deficient host, ER2925 (to avoid 5'-GATC-3' adenine methylation), purified and sequenced to confirm the mutagenic alteration. pMMH1 DNA from the *dam* minus host was modified *in vitro* by MmeI, purified over a spin column and digested with MmeI, MboI and HinfI. The MmeI reaction was split and 1  $\mu$ g unmodified pMMH1 DNA was added to one aliquot as a positive control. Unmodified pMMH1 was similarly digested with MmeI, HinfI and MboI. The fragments produced from the MmeI modified and unmodified DNAs were resolved on a 1% agarose gel. The HinfI and MboI digests were compared to identify any differences due to the MmeI modification. pMMH1 from the *dam*-proficient host was also digested with MmeI, MboI and DpnI.

Additionally, the pMMH1 polylinker region containing the single MmeI site overlapping the MboI and HinfI sites was PCR amplified using primers NEB1224 and NEB1233. The amplified DNA was *in vitro* methylated by MmeI, purified and digested with MmeI, HinfI and MboI.

#### Relative rates of MmeI cleavage and methyltransferase activities

The relative rates of the endonuclease and methyltransferase activities of the MmeI enzyme were examined by incubating a DNA substrate with MmeI and [methyl-<sup>3</sup>H]-AdoMet in endonuclease buffer, removing aliquots of the reaction at various time-points and purifying over a spin column, then measuring the extent of endonuclease digestion on an agarose gel and the extent of methyltransferase activity by counting the incorporation of tritiated methyl groups into the DNA. The same time course was performed in methyltransferase buffer; however, the purified samples were digested subsequently with MmeI to measure the extent of modification. In a separate experiment, lambda DNA was digested with MmeI (2 U/μg). Aliquots were removed at various time-points, purified and ligated overnight into concatamers. An aliquot of the ligated DNA was then re-cut with MmeI.

#### Construction of plasmid DNA containing a single MmeI recognition site, or MmeI recognition sites in head-to-head orientation

MmeI has two recognition sites in pUC19, at positions 996 and 1180, oriented in the same direction. The site at 1180 was removed by site-directed mutagenesis using primers 12 and 13 to form a single site substrate, p996SS1. Similarly the orientation of the site at 996 was reversed by mutagenesis using primers 14 and 15 to form a substrate having two head-to-head sites, pUCHH1. An MmeI site was added to pUC19 at position 50 in either orientation. The head-to-tail orientation relative to the 996 and 1180 sites, pUC50HT1, was formed using primers 16 and 17, while the head-to-head orientation relative to the 996 and 1180 sites, pUC50HH1, was formed using primers 18 and 17.

#### Effect of site number and orientation on MmeI endonuclease activity

The single site substrate p996SS1 closed circular DNA was digested with 0.125–16 U of MmeI. In a separate test p996SS1 was linearized with PstI prior to MmeI digestion. pUC19, a two site, head-to-tail DNA, was digested with MmeI as a supercoiled circular DNA and as a linear DNA (PstI cut). The head-to-head configuration substrate, pUC19HH1, was digested both as closed circular DNA and linear DNA. Once linearized by PstI the two pUC19HH1 sites are oriented tail to tail on the linear molecule. pUC50HT1 and pUC50HH1 were similarly digested.

#### Single recognition site activation *in trans*

Short dsDNAs having a recognition site for MmeI were supplied *in trans* to reactions containing MmeI and the single site substrate p996SS1. Synthetic oligonucleotides

were synthesized in pairs and annealed to form dsDNA that extended either beyond the point of DNA cleavage: M30 (oligonucleotides 19 and 20), to the point of DNA cleavage: M20 (oligonucleotides 21 and 22), or short of the point of DNA cleavage: M12 (oligonucleotides 23 and 24). A DNA lacking an MmeI site, NmeA30 (oligonucleotides 25 and 26) was also tested, as was a DNA of the same sequence as M12 that contained m6-adenine in the MmeI site (oligonucleotides 30 and 24). One microgram (0.57 pmol) p996SS1 was digested with 0.125–16 U MmeI as supercoiled or linear (PstI cut) DNA in the presence of 10 pmol or 1 pmol *trans* DNA in a 50-μl reaction and the extent of cleavage measured on a 1% agarose gel.

#### Bioinformatic analysis

Multiple sequence alignment of the MmeI protein and homologous proteins was performed on the PROMALS web server (21). Structure prediction for the MmeI protein was performed on the PHYRE web server (22).

#### Genomic *M. methylotrophus* DNA methylation status

*Methylophilus methylotrophus* genomic DNA was tested to determine if there was any modification present in the bottom strand, 5'-GTYGGGA-3', of the MmeI recognition sequence in the host DNA to protect against MmeI endonuclease cleavage. Twenty-microgram genomic *M. methylotrophus* DNA was digested with HincII and BsaAI to produce a discrete 527-bp fragment that contained two MmeI recognition sites oriented in the same direction and purified over a spin column. For synthesis of unmodified top strand MmeI sites, 1 pmol of synthetic primer #27 was 5'-end labeled with <sup>33</sup>P-γ-ATP (NEG302H) using T4 polynucleotide kinase. Ten micrograms of the HincII–BsaAI cut genomic DNA was placed in 500 μl 1X Phusion HF reaction buffer containing 0.25-μM dNTPS and 5 U Phusion HotStart DNA polymerase. The reaction mix was denatured at 98°C for 1 min, then held at 94°C while the labeled primer was added, then the primer was extended for 5 min at 72°C (one extension cycle). The DNA was purified over a spin column. The same procedure was performed for synthesis of unmodified bottom strand MmeI sites using a complement strand primer, #28. The labeled hybrid DNA was digested with MmeI, or with BspHI as a control. As a positive control for MmeI activity, top strand extension product DNA was added to one reaction of MmeI digestion of the bottom strand synthesis DNA. The products were resolved on a 6% acrylamide TBE gel, the gel was dried onto a Hybond N+ nylon membrane and the products detected by autoradiography.

## RESULTS

#### MmeI has been cloned and is a bi-functional protein that has both endonuclease and DNA methyltransferase activities

The gene encoding MmeI was cloned and expressed in *E. coli*. The amino acid sequences obtained from Edman degradation protein sequencing of the native MmeI peptide were: (M)ALSWNEIRRKAIEF from the amino terminus, (M)KISDEFGNYFARIPLKSTXXIXEXNALQ

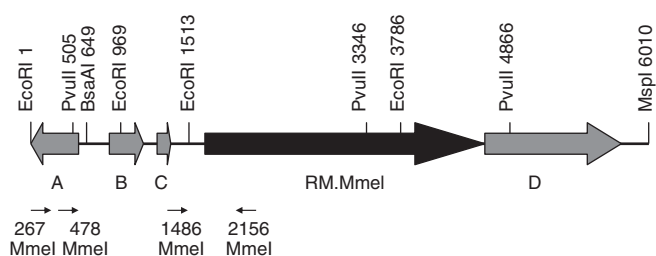
from a 25-kDa CNBr fragment, (M)DAKKRRNLGAHYTSEANILKLI KPLLLDELWVVFXXKVN from a 14-kDa CNBr fragment and (M)KSRGKDLKAYDQALDYFSGIAER from a 7.5-kDa CNBr fragment. Degenerate oligonucleotide primers corresponding to portions of these amino acid sequences were synthesized and used to obtain the full sequence of MmeI from genomic DNA. The DNA sequence of the *MmeI* gene and 1.6 kb of flanking DNA on each side was determined (GenBank accession # EU616582). MmeI is a single polypeptide of 919 amino acids that possesses site-specific DNA methyltransferase activity and site-specific DNA endonuclease activity (GenBank accession # ACC85607). The calculated molecular weight for MmeI, 105 130 Da, is very close to the previous estimate of 105 kDa (23).

Alignments of the amino acid sequence (Supplementary Figure S1) and *in silico* structure predictions (22) indicate MmeI is composed of three functional domains. The amino terminal domain contains the conserved catalytic motif of the metal-dependent P(D)-(D/E)XK endonucleases: VD<sub>70</sub>(X9)E<sub>80</sub>MK<sub>82</sub>. The central portion of the protein contains conserved DNA methyltransferase motifs of the amino DNA methyltransferases (24,25). Sequences for motif X: G<sub>312</sub>AHYTS, motif I, the AdoMet binding site: F<sub>359</sub>FDPACGCGNFL, and motif IV, encoding the conserved catalytic residues, G<sub>480</sub>NPPF, are readily identifiable. These motif sequences are most similar to those found in N6-methyl adenine producing methyltransferases and occur in the order of the gamma class of N6-adenine methyltransferases, which include M.TaqI and the Type I methyltransferases. Structure homology modeling indicates the methyltransferase domain of MmeI is significantly similar to that of M.TaqI (PDB: 1G38). The C-terminal portion of MmeI from position 625 to 820 models onto the DNA recognition domain of the M.TaqI methyltransferase, suggesting this part of MmeI may be responsible for recognition of the 5'-TCCRAC-3' DNA target.

The expression construct produces 20 000 U of MmeI endonuclease per gram of cells in a crude extract, where 1 U is the amount of enzyme required to completely cut 1 µg PhiX174 DNA in a 50-µl reaction volume in 1 h at 37°C. No differences in function were observed between the native and recombinant enzymes. Purified MmeI has a specific activity of ~30 000 U per milligram of protein, which is comparable to that of type IIS restriction endonucleases such as FokI (26).

### Gene organization flanking the MmeI locus

DNA sequence for a 6-kb contig of the *M. methylotrophus* genome containing the 2.8-kb MmeI gene and flanking sequences was determined (Figure 1). Significantly, no additional methyltransferase is observed on either flank of the MmeI gene. Orf A and Orf B, upstream of mmeIRM, are each similar to a widely distributed protein family of unknown function: Orf A to pfam09694 and Orf B to pfam04380 and COG2960. These homologs are not associated with known or candidate R-M systems by proximity in their genome contexts. Likewise, Orf C, which appears to be a C-terminal truncation of an



**Figure 1.** DNA sequence containing MmeI and predicted flanking genes from *M. methylotrophus* genomic locus. (A–D) are uncharacterized putative ORFs. RE sites are numbered from the 5'-EcoRI site (in bp). The four MmeI sites are listed with the arrow indicating the orientation of the site: 5'-TCCRACN20/N18-3'.

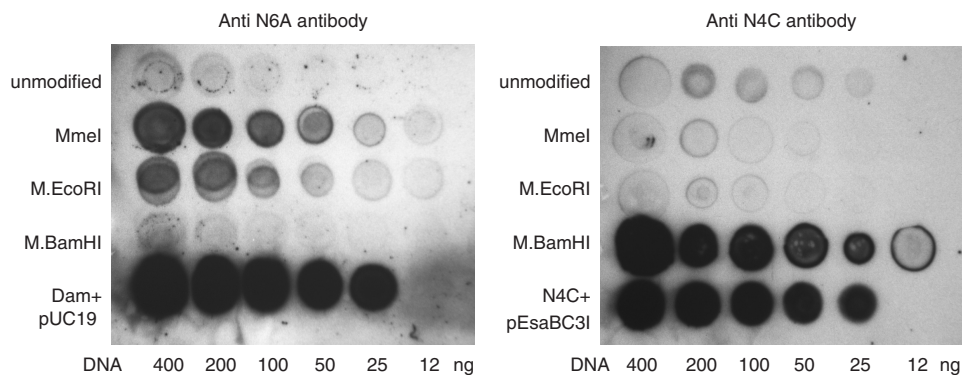
Mg<sup>2+</sup> chelatase related protein similar to COG0606 and pfam01078, is also not observed to be associated with R-M systems. It is possible ORF C may have been interrupted by a DNA mobility event that brought the MmeI gene into the *M. methylotrophus* genome. ORF D lies 3' to the *MmeI* gene and codes for a putative protein of 426 amino acids. This protein currently has only one significant BLAST hit in GenBank, to a much smaller hypothetical protein that is not located near a restriction system.

### MmeI DNA methyltransferase activity produces N6-adenine methylation

The conserved DNA methyltransferase motifs in MmeI are most similar to the gamma class of N6-methyl adenine DNA methyltransferases. MmeI was confirmed to produce N6-methyl adenine by the use of antibodies that specifically recognize N6-methyl adenine (Figure 2). MmeI did not produce any detectable m4C methylation. The antibody results confirm the previous report that MmeI produces N6-methyl adenine (16).

### MmeI methyltransferase modifies the adenine in the 5'-TCCRAC-3' (top) strand

The MmeI recognition sequence has two adenine bases, one in each strand, that are potential targets for modification. We measured MmeI incorporation of labeled methyl groups using DNAs that were unmethylated or pre-methylated at these adenines. Labeled <sup>3</sup>H-methyl groups were incorporated into the DNA when the adenine in the top strand, 5'-TCCRAC-3' was unmodified, independent of the methylation status of the bottom strand adenine, 5'-GTYGGA-3'. Labeled methyl groups were not incorporated into the DNA when the top strand adenine was methylated, even when the bottom strand adenine was unmodified (Table 1). In a separate approach *in vitro* MmeI modification was shown to block HinFI cleavage when the MmeI recognition sequence overlaps the HinFI recognition sequence (GANTC) at the top strand adenine, 5'-TCCGACTC-3', indicating MmeI modifies the top strand adenine. However, MmeI modification did not block MboI cleavage at the site where the MboI recognition sequence (GATC) overlaps the bottom strand adenine of the MmeI recognition sequence, 5'-GTYG GATC-3', indicating this bottom strand adenine is not modified by MmeI (Figures 3 and 4B).

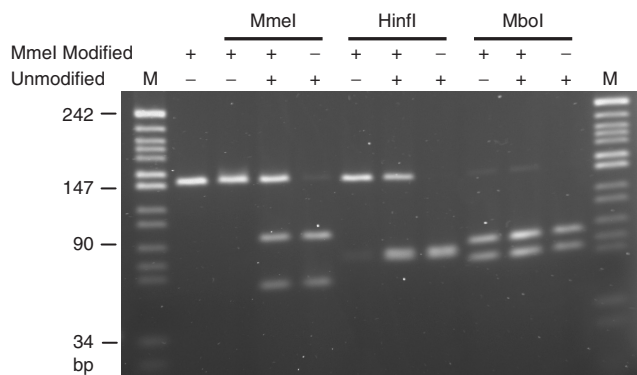


**Figure 2.** Detection of MmeI modification using antibodies specific for N6-methyladenine (m6A) and N4-methylcytosine (m4C). (Top row) unmethylated DNA; (second row) MmeI *in vitro* modified DNA; (third row) M.EcoRI *in vitro* modified DNA; (fourth row) M.BamHI *in vitro* modified DNA. Positive controls: m6A antibody panel; (fifth row) dam (m6A) *in vivo* methylated pUC19 DNA (400–25 ng dilution), m4C antibody panel; (fifth row) M.EsaBC3I (m4C) *in vivo* methylated plasmid DNA (400–25 ng dilution).

**Table 1.**  $^3\text{H}$  counts per minute incorporated from [methyl- $^3\text{H}$ ]-AdoMet into the variously modified DNAs by MmeI or dam methyltransferase

$^3\text{H}$ counts per minute	MmeI	Dam	No enzyme
UU	18 440	38 969	549
MU	433	31 201	ND
UM	4969	26 534	ND
MM	436	19 295	ND

ND indicates no data.



**Figure 3.** Endonuclease digestion of MmeI *in vitro* modified or unmodified DNA (150-bp PCR product across pMMHI polylinker region) containing a HinfI site overlapping the top strand of the MmeI site: 5'-TCCGACTC-3' and an MboI site overlapping the bottom strand of the MmeI site: 5'-GTCGGATC-3'. The restriction endonucleases were mixed with buffer, aliquoted into three reactions to which were added MmeI modified DNA, unmodified DNA or both DNAs. Size standard: pBR322-MspI.

### MmeI methylation prevents cleavage by MmeI

The single strand modification produced by MmeI prevents MmeI endonuclease cleavage. Expression of MmeI *in vivo* renders the MmeI expressing plasmid, pTBMmeI.1, completely resistant to MmeI endonuclease digestion *in vitro* (Figure 4A). The complete cleavage of (unmodified) PhiX174 DNA in the same reaction verifies MmeI activity was present in the reaction but did not cut due to the *in vivo* modification. However, a point mutation in the

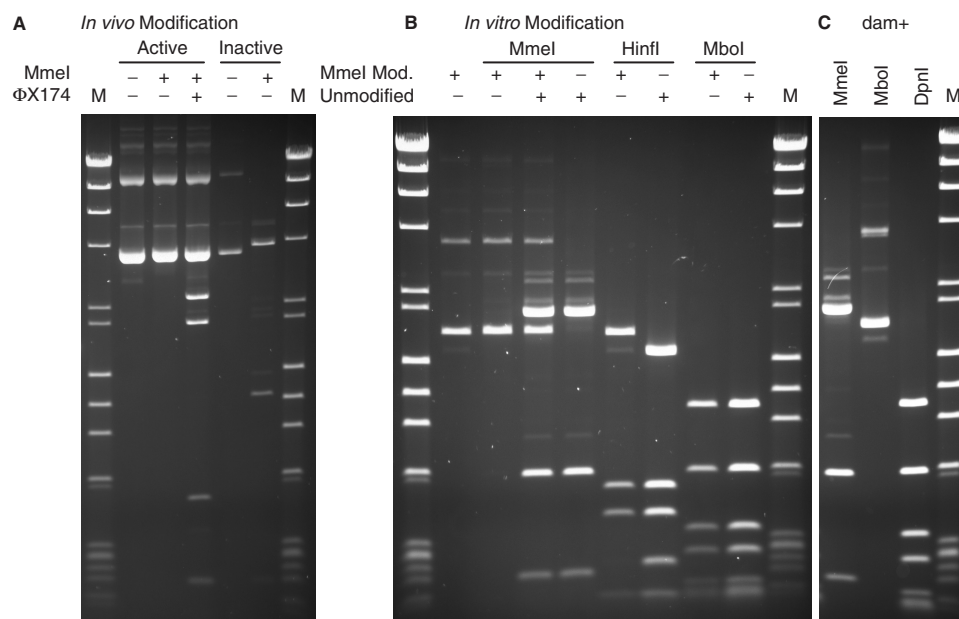
MmeI gene that inactivates MmeI (N<sub>773</sub>D) makes the resulting plasmid construct cleavable by MmeI. MmeI modification performed *in vitro* in the absence of magnesium ions blocks subsequent MmeI endonuclease digestion (Figures 3 and 4B). The *in vitro* MmeI modification also blocked HinfI cleavage at the overlapping site but not MboI. MmeI cut its site even when it was modified at the bottom strand adenine by the *in vivo* action of the *E. coli* dam methyltransferase (Figure 4C). The action of the MmeI methyltransferase is thus sufficient to protect DNA from the action of the endonuclease.

### MmeI endonuclease is sensitive only to top strand methylation

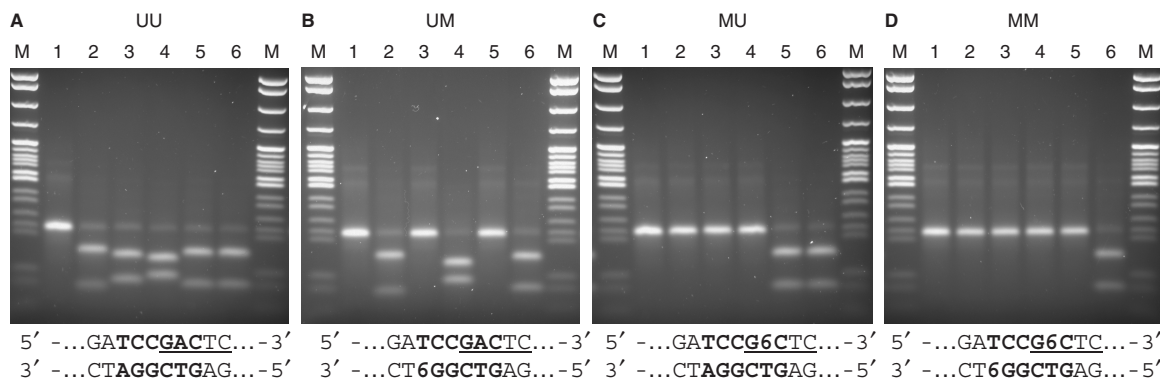
MmeI endonuclease sensitivity to m6A methylation of each strand was explicitly tested using a set of DNA substrates containing N6-methyl adenine in the top strand only, the bottom strand only, both strands, or neither strand (Figure 5). MmeI cut the DNAs in which the top strand was unmethylated (UU and UM), but was blocked when the top strand was methylated (MU and MM). That MmeI cut the top strand unmodified, bottom strand modified substrate, UM, indicates MmeI is not sensitive to bottom strand N6-methyl adenine modification. The results for the control enzymes Hpy188I, HinfI, MboI and BfuCI verify the modification status of the DNA substrates. Additionally, MmeI cut pMMHI plasmid DNA prepared from a dam methylase expressing host even though dam methylase modifies the bottom strand adenine at the MmeI site introduced into the plasmid (Figure 4C).

### MmeI endonuclease specific activity and turnover

The protein concentration of a highly purified preparation of MmeI was measured at 2.17 mg/ml (27). Calculating from its molecular mass of 105 130 g/mol, the concentration of MmeI in this preparation was 20.6 nmol/ml. This preparation of MmeI was measured to have 64 000 U of endonuclease activity per ml, where 1 U is the amount of enzyme required to cut 1  $\mu\text{g}$  PhiX174 DNA to completion in 50  $\mu\text{l}$  in 1 h. One unit of MmeI thus corresponds to 0.32 pmol of protein. One microgram PhiX174 DNA is 0.28 pmol and has five MmeI sites. The number of substrate



**Figure 4.** (A) *In vivo* MmeI Modification. MmeI endonuclease digestion of purified plasmid DNA from the construct expressing active or inactive MmeI. 'Active' is the MmeI expression plasmid, pTBMmeI.1. 'Inactive' is a plasmid DNA derived from pTBMmeI.1 that carries a single point mutation (N<sub>773</sub>D) that renders MmeI inactive. Addition of PhiX174 DNA verifies that the added MmeI endonuclease is active in the reaction wherein pTBMmeI.1 DNA is not cut. (B) *In vitro* MmeI modification. pMMH1 DNA from a dam-deficient host, either modified *in vitro* by MmeI or not modified, digested with MmeI, HinI or MboI. (C) pMMH1 DNA from a dam-proficient host digested with MmeI, MboI and DpnI. M: Size standards: Lambda-HindIII, PhiX174-HaeIII.



**Figure 5.** Digestion of synthetic DNAs containing modified or unmodified adenines in the MmeI recognition sequence. (A) UU, fully unmethylated DNA. (B) UM, containing N6-methyl adenine in the bottom strand, 5'-GTYGG(m6A)-3', of the MmeI recognition sequence. (C) MU containing N6-methyl adenine in the top strand, 5'-TCCR(m6A)C-3', of the MmeI recognition sequence. (D) MM containing N6-methyl adenine in the both strands of the MmeI recognition sequence. Lanes: 1 = no enzyme; 2 = MmeI (TCCRAC); 3 = Hpy188I (TCNGA); 4 = HinI (GANTC); 5 = MboI (GATC); 6 = BfuCI (GATC). M: pBR322-MspI size standard. DNA recognition sites: 6 = m6-adenine, MmeI site 5'-TCCGAC-3' in 'bold', HinI site 5'-GANTC-3' top strand underlined, MboI and BfuCI site 5'-GATC-3' bottom strand underlined.

sites for MmeI in the reaction is thus 1.4 pmol, while the number of molecules of MmeI is 0.32 pmol. This indicates one molecule of MmeI on average cuts ~4.4 sites in the standard unit definition reaction conditions. While the standard unit definition allows 1 h for the digest, MmeI endonuclease activity occurs rapidly and is largely complete within 5 min. MmeI remains active in the standard reaction conditions used, retaining >50% of its endonuclease activity toward unmodified DNA substrates after 1 h incubation at 37°C, with or without DNA present in the initial reaction mixture. Adding <1 U and allowing more time does not result in increased DNA cleavage.

#### MmeI methylates its recognition site following DNA cleavage

Since DNA cleavage by MmeI does not disrupt the enzyme's DNA recognition sequence, MmeI can bind and modify its recognition sequence even after endonuclease cleavage. DNA cleaved in the presence of AdoMet and purified after 1 or 5 min incubation, i.e. shortly after completion of cleavage, could subsequently be cut by MmeI after ligation of the DNA into concatamers, indicating that most MmeI sites had not been modified. However, DNA incubated for 1 h under endonuclease digestion conditions was refractory to subsequent

MmeI digestion, indicating that after cutting the DNA MmeI modified the still intact recognition sites, rendering them resistant to MmeI endonuclease activity when the DNA was reassembled (Figure 6).

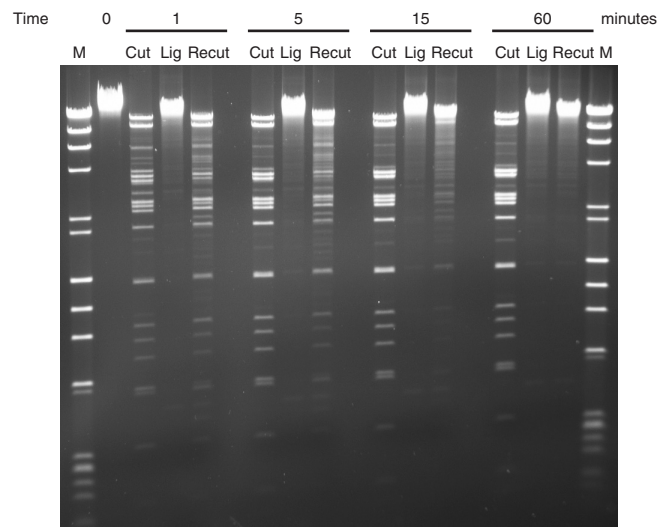
### Relative rates of MmeI cleavage and methyltransferase activities

The MmeI protein functions as both methyltransferase and endonuclease, but the relative rates of the competing reactions differ in standard endonuclease digestion conditions. When presented with DNA substrates having multiple unmodified recognition sites, the activity of the MmeI endonuclease is rapid, achieving substantial cutting in seconds, ~90% cutting in one minute and complete cutting in 5–15 min. In contrast, at 1 min only 5% of the MmeI sites have been modified. The rate of modification was linear through ~15 min, at which point ~70% modification had been achieved. Complete modification was achieved in 1 h (Figure 7). The rate of methylation in buffer without magnesium is comparable to that in endonuclease buffer (data not shown), indicating that  $Mg^{2+}$  is not required for methylation. Cleavage in the absence of  $Mg^{2+}$  is negligible, however, indicating that  $Mg^{2+}$  is essential for endonuclease activity.

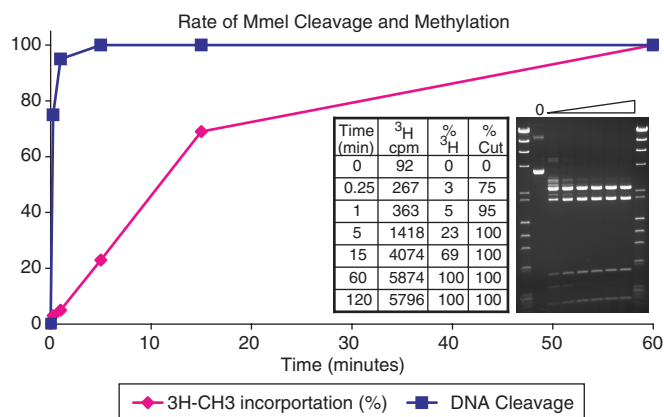
### MmeI requires two sites for efficient DNA cleavage

MmeI endonuclease cleavage efficiency on DNA substrates containing single or multiple recognition sites was compared. Some Type II endonucleases bind individual recognition sites and cleave their sites independently. Others require two or more sites for efficient cleavage, with the multiple sites either acting cooperatively to effect cleavage, or with one site binding to an effector position in the endonuclease to effect a conformational change required for DNA cleavage competence (4,28–30).

MmeI cleaves a single site plasmid DNA incompletely, whether supercoiled (data not shown) or linear, achieving ~70% cleavage with 1–16 U of enzyme (Figure 8A). In contrast, the same single site plasmid DNA is nearly completely cleaved when a second recognition site is provided *in trans* by adding a synthetic DNA containing the MmeI recognition site (Figure 8B and C). The DNA bearing the recognition site need not be capable of being cleaved itself, as a DNA having only 12 bases 3' to the recognition site facilitates cleavage of the single site plasmid as well as a DNA extending to or beyond the position of cleavage. Cleavage stimulation is dependent upon the MmeI recognition sequence, as addition of a similar DNA lacking an MmeI site did not increase cleavage (Figure 8D). Likewise, a DNA in which the MmeI site is methylated at the top strand adenine does not stimulate cleavage (Figure 8E). The concentration of sites supplied *in trans* needed to stimulate cutting of the single site DNA was approximately equimolar (0.01–0.02  $\mu$ M) with the number of recognition sites (0.011  $\mu$ M) in the single site DNA (Figure 8F), in a reaction that contained 0.026  $\mu$ M (4 U/50  $\mu$ l) MmeI.



**Figure 6.** MmeI modification following endonuclease cleavage. 'Cut' indicates lambda DNA digested with MmeI (2 U/ $\mu$ g DNA) for 1, 5, 15 and 60 min; 'Lig' indicates the cut lambda DNA purified from MmeI and ligated into concatamers; 'Recut' indicates subsequent MmeI digestion of the ligated DNA concatamers.

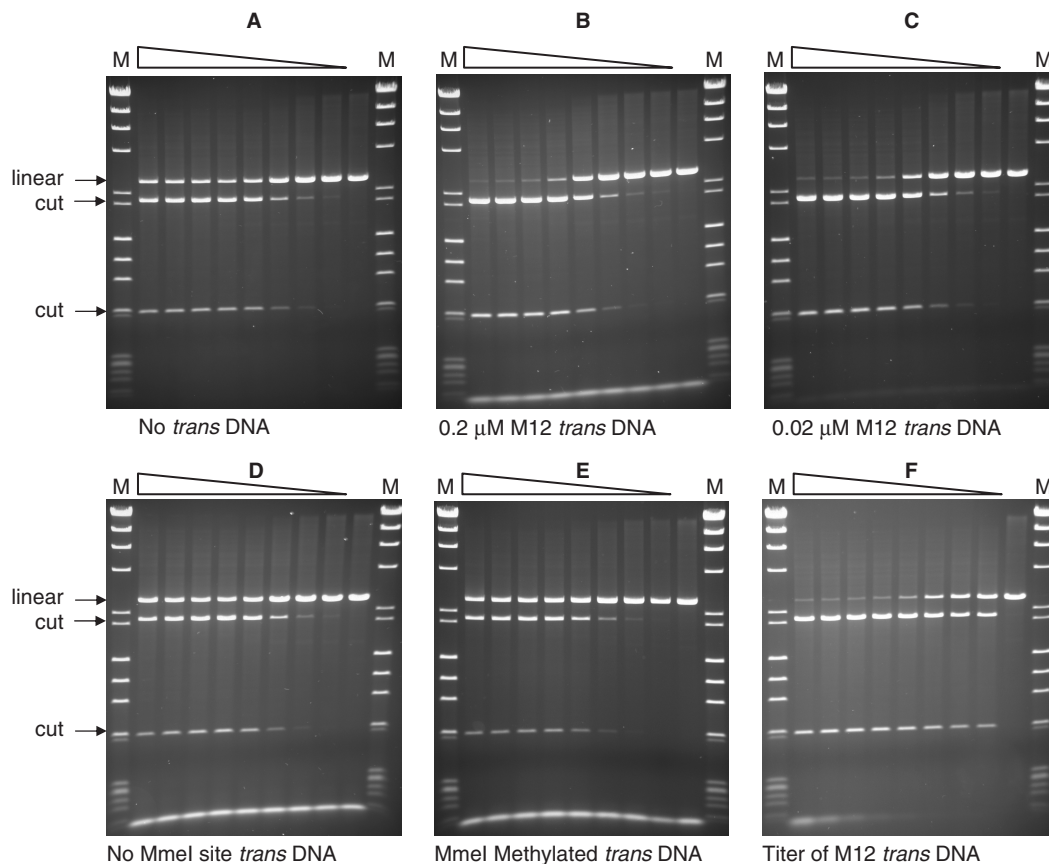


**Figure 7.** Relative rates of MmeI endonuclease cleavage and DNA methyltransferase activities. MmeI (2 U/ $\mu$ g) digestion of Phix174 DNA with 1  $\mu$ M [methyl- $^3H$ ]-AdoMet over a time course of 0, 0.25, 1, 5, 15, 60 and 120 min.  $^3H$  counts are reported for an equal amount of DNA to that on the gel. The extent of endonuclease cleavage was estimated from the MmeI fragment pattern. The extent of methylation was calculated as a percent of the maximal counts reached at 60 min, subtracting the background counts from each count.

### MmeI cuts recognition sites in any orientation

MmeI cuts DNA regardless of the relative orientations of the recognition sites, unlike the Type III enzymes such as EcoP15I that require two sites in a head-to-head orientation (10). The two MmeI sites that occur in pUC19 are oriented in the same direction, i.e. head to tail, as are the four sites that occur in pBR322, yet these DNAs are cut by MmeI (Supplementary Figure 2A). The efficiency of cleavage on pUC19 and pBR322 is similar whether cutting supercoiled or linear DNA. On a plasmid having head-to-head sites, such as pUCHH1 or pUC50HH1, MmeI produces the same final digestion products as the head-to-tail plasmids, pUC19 or pUC50HT1.





**Figure 8.** MmeI cleavage of a single site substrate is incomplete but can be stimulated by *in trans* DNA containing an MmeI site. (A–E) MmeI digestion in a 2-fold serial dilution from 16 U/μg DNA to 0.125 U/μg DNA on p996SS1 DNA previously linearized by digestion with PstI. (A) no *trans* DNA; (B) 0.2 μM M12 *trans* DNA; (C) 0.02 μM M12 *trans* DNA; (D) 0.2 μM NmeA30 *trans* DNA that does not contain an MmeI site; (E) 0.2 μM N6-adenine methylated (5'-TCCG(m6A)C-3') M12 *trans* DNA; (F) 2-fold serial dilution of the M12 *trans* DNA from 0.16 μM to 0.001 μM in a digestion reaction containing 4 U (0.026 μM) MmeI/μg substrate DNA. Arrows indicate 'linear' DNA (uncut by MmeI) and 'cut' DNA that is the MmeI cleavage products.

Circular pUCHH1 DNA is not only head to head around the 2.5 kb between the two MmeI recognition sites, but also represents a tail-to-tail configuration across the 184 bp between the sites. When cut within the 2.5 kb between the head-to-head sites, the resulting linear molecule has the two sites in a tail-to-tail configuration. The linear tail-to-tail and the circular head-to-head substrates were cleaved to the same extent (Supplementary Figure S3). These results suggest the enzyme has little preference regarding site orientation.

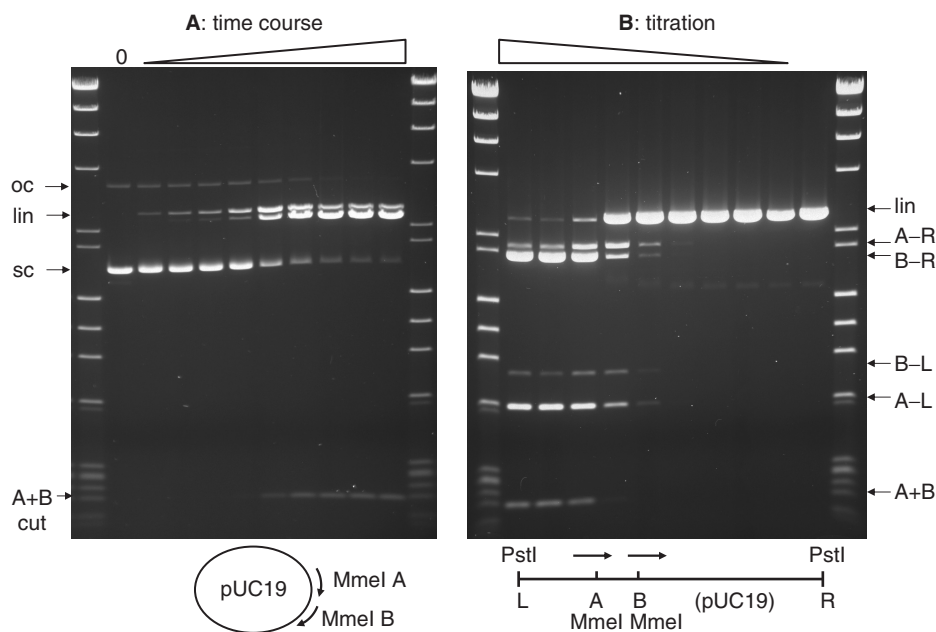
#### MmeI cleaves the two DNA strands coordinately one site at a time

MmeI endonuclease activity cleaves DNA at one site at a time in a reaction wherein both DNA strands are cut coordinately. A time course of MmeI digestion on the two-site substrate pUC19 reveals that the supercoiled DNA is converted directly to linear DNA. No increase in nicked open circular DNA is observed, indicating both strands of the duplex DNA are cleaved simultaneously, rather than the enzyme introducing a nick in one DNA strand followed by cleavage of the second strand (Figure 9A). MmeI endonuclease activity acts at

one site at a time. The two-site circular substrate pUC19 is cut first at one of the two sites to yield full-length linear DNA, followed by cutting at the second site to produce the two fragments of complete digestion. The linear form of the pUC19 substrate is cut first at one site, with no marked site preference between the two sites, before cutting within the same molecule at the second site (Figure 9B). This indicates enzyme bound at two cognate sites interacts to cut next to only one of the recognition sites.

#### Modification of the MmeI site in the *M. methylotrophus* host occurs on only one DNA strand of the duplex recognition sequence

DNA substrates that consisted of a hybrid of one *M. methylotrophus* genomic DNA strand and one newly synthesized, and therefore unmethylated, DNA strand were produced from a single round of primer extension on *M. methylotrophus* genomic DNA. MmeI cut the DNA in which the bottom strand of the recognition sequence, 5'-GTRGGA-3', was derived from the host *M. methylotrophus* genomic DNA and the top strand was newly synthesized, indicating the host DNA has no modification



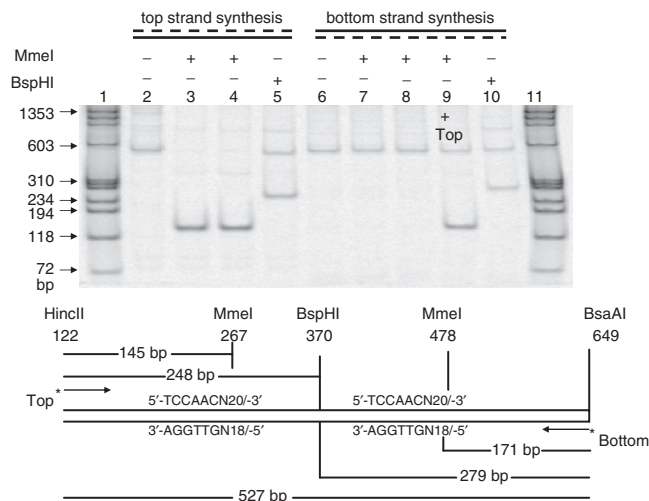
**Figure 9.** MmeI cleaves the two DNA strands at one site simultaneously. (A) Time course of MmeI digestion of supercoiled pUC19 DNA (2 U/μg) for 10s, 20s, 30s, 1, 3 10, 20, 30 and 60 min. Supercoiled plasmid (sc) is converted directly to linear (lin) DNA, with no accumulation of open circular DNA (oc). ‘A + B cut’ indicates the 184-bp product of MmeI cleavage at both pUC19 sites. (B) MmeI digestion of linear pUC19 DNA (previously cut with PstI), in a 2-fold serial dilution from 8 to 0.03 U/μg. MmeI cuts at a one site, forming products from either site A or site B, before forming product from cleavage at both sites (A + B). ‘lin’ indicates linear pUC19, ‘A-R’ and ‘A-L’ indicate the cleavage products from MmeI cutting at the 996 site, ‘B-R’ and ‘B-L’ indicate the cleavage products from MmeI cutting at the 1180 site, while ‘A + B’ indicates the cleavage product from MmeI cutting at both sites.

on the bottom strand to block cleavage by MmeI. DNA in which the top strand of the recognition sequence, 5'-TCCRAC-3', was located in the genomic *M. methylotrophus* strand and the bottom strand was newly synthesized was not cut by MmeI (Figure 10). These results indicate the top strand modification produced by MmeI is present in the host *M. methylotrophus* DNA and able to block MmeI cleavage. However, there is no modification present in the *M. methylotrophus* DNA on the bottom strand of the MmeI recognition sequence to block MmeI endonuclease activity. These results confirm that the entire modification used by the MmeI restriction system is the single-strand methylation of the adenine in the top strand produced by the MmeI enzyme.

**DISCUSSION**

MmeI expresses well in *E. coli*, where it represents ~1% of the cellular protein in the construct described. The MmeI protein architecture and the significant similarity to *M. TaqI* imply MmeI likely arose from a fusion of an endonuclease module onto the amino terminal end of a gamma class N6-adenine DNA methyltransferase.

The endonuclease activity of MmeI exhibits characteristics of the Type IIS endonucleases, such as FokI (30,31), BsgI and BpmI (32). MmeI has one PD-ExK endonuclease motif per molecule, suggesting that it has only one catalytic site for cleavage and must therefore dimerize to achieve double-strand cleavage. Single site DNAs are cut incompletely even with a large excess of enzyme,



**Figure 10.** MmeI cleaves DNA containing a newly synthesized (unmodified) top strand and a genomic *M. methylotrophus* bottom strand. MmeI digestion of DNAs from one round of primer extension. Lanes 2-5 are the newly synthesized top strand, while lanes 6-10 are the newly synthesized bottom strand. Lanes 2 = uncut; 3 = 20 U MmeI; 4 = 5 U MmeI; 5 = 10 U BspHI; 6 = uncut; 7 = 20 U MmeI; 8 = 5 U MmeI; 9 = 20 U MmeI digestion of both newly synthesized bottom strand and top strand DNA (as a positive control for MmeI activity); 10 = 10 U BspHI. Lanes 1 and 11: PhiX174-HaeIII size standard. MmeI cuts the DNA containing a genomic *M. methylotrophus* bottom strand and an unmodified top strand (lanes 3, 4 and 9), but not the DNA containing a genomic *M. methylotrophus* top strand and an unmodified bottom strand (lanes 7, 8 and 9). The native host DNA from *M. methylotrophus* is thus modified to prevent MmeI cleavage only in the top DNA strand of the MmeI recognition sequence.

however cleavage can be enhanced by supplying MmeI recognition sites *in trans*, as has been observed for a number of restriction endonucleases (33). The *trans* DNA enhancement requires specific recognition at an MmeI site. DNA catalysis is not required, since *trans* DNA that does not extend to the position of cleavage is competent to enhance cutting of the single site plasmid DNA. This indicates that only molecules bound to cognate recognition sequences are competent to dimerize and then cleave. Because the point of DNA cleavage is far from the recognition sequence, MmeI can remain bound to the intact recognition sequence after DNA cleavage has occurred, but prior to methyltransfer, and may interact with MmeI bound at an uncut site to accomplish cleavage. MmeI cuts sites one at a time in a sequential process. Coordination of one endonuclease motif from each of two MmeI molecules would provide the correct geometry to accomplish double strand cleavage at one site. The absence of nicking on supercoiled substrates indicates MmeI cuts both DNA strands in one catalytic event, implying that even upon specific binding the endonuclease domain is not catalytically active until it interacts with a second MmeI molecule. This requirement likely represents a safeguard to prevent random DNA cutting. MmeI is thus a further example of a Type II endonuclease that has to interact with two sites before cleaving the DNA (28). The need for two sites not only can enhance discrimination of cognate from noncognate sites, but may also be important to the unusual biology of using only single-strand modification for host genome protection.

In characterizing MmeI we found significant differences from previous reports regarding the interplay of its methylation and endonuclease activities (16). As reported, MmeI methylates the top strand adenine in its recognition sequence. However, MmeI endonuclease is blocked by methylation of this top strand adenine, indicating the action of the MmeI methyltransferase is sufficient to protect DNA from the action of the endonuclease. It was reported previously that methylation of the adenine in the bottom strand blocked MmeI cleavage (16). However, we found MmeI to be insensitive to m6A methylation in the bottom strand. A second methyltransferase was reported to modify this bottom strand adenine (16), but we find no evidence for such a methyltransferase in MmeI host cells or as part of the MmeI system. It is possible that the reported methyltransferase was in fact the methyltransferase of the MmeII system, which recognizes GATC (1,23) and would have the potential to modify the bottom strand adenine in the MmeI recognition sequence of the synthetic DNA substrate used in the earlier study (16).

The surprising finding in cloning MmeI was the absence of a second-strand methyltransferase. No methyltransferase was observed flanking the MmeI gene. Although a second methyltransferase might be located at a distance from the *MmeI* gene in the *M. methylotrophus* genome, we have demonstrated that MmeI is not blocked by methylation at the adenine in the complement strand. Because MmeI is not sensitive to this modification there is no selective advantage driving MmeI to partner with an additional N6-adenine DNA methyltransferase, since such

a methyltransferase would not provide the host DNA any additional protection. Methylation in Type II R-M systems can be of three types: the N6 position of adenine and the N4 and C5 positions on cytosine can be methylated (34,35). In addition to the one adenine, the bases present in the MmeI bottom strand, 5'-GTYGGA-3', do not include any invariant cytosine or additional adenine bases. Guanine and thymine are not normal targets for modification in Type II restriction systems, while the degenerate pyrimidine position, if modified at cytosine, would at best protect only half the MmeI sites, making this position an unlikely target for modification. Further, there is at least one homolog of MmeI, BsbI (5'-CAACACN20/N18-3') (20), that has neither adenine nor cytosine base in the complement strand. There are, thus, no additional targets for normal Type II base modification in the complement strand of MmeI, nor any targets at all in some homologs. An examination of the gene context for MmeI homologs in sequenced bacterial genomes reveals no additional DNA methyltransferase associated with any of these homologs. The absence of a cytosine or adenine base target for modification in the complement strand demonstrates MmeI cannot employ a normal Type II R-M system methylation strategy to protect the bottom strand.

Because it is difficult to envision how single-strand modification can provide effective host protection, we tested whether MmeI might use some alternative form of modification in the bottom strand of its recognition sequence to achieve host protection. MmeI cut hybrid DNA containing the *M. methylotrophus* derived bottom strand and a newly synthesized, and thus unmodified, top strand, demonstrating there is no modification present on the MmeI site bottom strand in the host DNA to prevent MmeI cleavage. MmeI did not cut the hybrid DNA containing the host derived top strand, confirming that the hemi-methylation present in the top strand of the MmeI recognition site is sufficient to protect against MmeI endonuclease activity. The MmeI R-M system thus relies on only one-strand modification for host protection, namely the hemi-methylation produced by the MmeI enzyme.

Single-strand methylation presents a biological challenge in that one of the two daughter DNA molecules will be unmodified following replication, and thus would be expected to be subject to restriction. However, MmeI can be successfully cloned and expressed at high levels in *E. coli* even though it produces only single-strand modification, in contrast to previous results for Type IIG enzymes such as Eco57I, for example, which cannot be expressed without its companion second-strand methyltransferase (15). MmeI seems to have evolved to balance the competing activities of DNA scission and modification. The endonucleolytic activity of MmeI is relatively fast compared to the methyltransferase activity, but is active in a relatively narrow range of enzyme and substrate site concentration. Were the methyltransferase activity more active than the endonuclease, very few sites would ever be cut. Were the endonuclease free to cut whenever an unmodified site was encountered, the result would be little or no DNA protection

following replication. The requirement for cooperation between two molecules bound at unmodified sites may have the effect of increasing the likelihood a DNA that presents multiple unmodified sites at the same time will be cut, as would occur when a foreign DNA invades the host, while decreasing the likelihood unmodified sites presented sequentially as host DNA is replicated will be cut before being modified. Just how this system is able to avoid deleterious cutting of newly replicated host DNA is an area for further investigation.

MmeI represents perhaps the simplest possible form of an R-M system, comprising a single protein composed of one endonuclease module, one methyltransferase module and one DNA specificity module. Typical Type II restriction systems have separate endonuclease and methyltransferase proteins, each of which separately recognizes the same DNA sequence. MmeI effectively eliminates the need for two separate DNA recognition modules. Since the target recognition domain of MmeI directs both host protection and endonuclease activity, any mutations that alter DNA recognition will simultaneously alter the target site for both host protection and foreign DNA endonuclease digestion. The absence of the requirement for a second methyltransferase frees MmeI and similar enzymes from the constraints imposed by relying on a separate protective methyltransferase. This simplicity implies these systems are uniquely adapted for the rapid evolution of DNA specificity, which may explain their wide distribution in spite of the challenge presented by single-strand modification. Systems such as Eco57I are constrained to have a methylation target adenine in the bottom strand corresponding to that in the top strand, thus effectively fixing a second position in their recognition sequences as a thymine. By eliminating second-strand modification MmeI and homologs have greater freedom regarding the sequences they can recognize, since only the one top strand adenine is required for protective methylation. This observation predicts that the putative proteins observed in genome sequences that are strikingly similar to MmeI and to each other may yet recognize diverse DNA sequences, and recent work (Morgan, R.D. manuscript in preparation) shows this to be the case. This simple organization provides a facile natural system to rapidly evolve endonucleases having different DNA specificity, which may provide a selective advantage to bacteria in the continuing arms race between phage and microbial hosts, since restriction systems are most effective if they are variable and fluid within a bacterial population (36).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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