学位論文

Expression and Characterization of Hyperthermophilic DNA methyltransferase M.PabI

（高度耐熱性ＤＮＡメチル化酵素 M.PabI の発現と機能解析）

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ABSTRACT

A novel DNA methyltransferase, M.PabI, of a hyperthermophilic archaeon, *Pyrococcus abyssi*, was purified and characterized. The gene encoding M.PabI was screened through the comparative analysis of the *P. abyssi* genome with that of another Pyrococcal species on the basis of association of restriction-modification genes with genomic rearrangements or polymorphisms. In an earlier work, its constituent restriction enzyme (PabI) was identified as a hyper-thermo-resistant restriction endonuclease, which catalyses cleavage at 5′-GTA/C-3′. In this study, M.PabI, the putative DNA methyltransferase of the PabI restriction-modification system, was over-expressed in *Escherichia coli* and purified by affinity chromatography. The following properties of this modification enzyme were elucidated. First, M.PabI recognized 5′-GTAC-3′ and methylated its adenine residue at the nitrogen of the sixth position. Second, the optimal conditions with respect to pH, temperature and salt concentration were determined, as well as the values of k_{cat} (0.041 sec^{-1}), Km^{DNA} (159 nM) and Km^{AdoMet} (1.28 μM). Third, it was found to be highly thermophilic: it showed methylation activity even at 95°C and retained half of its activity even after 19 min of pre-incubation at 85°C. Fourth, the activation energy of 56.4 kJ mol^{-1} was determined by Arrhenius plot of the initial reaction velocities at 37–85°C. And fifth, of all the examined divalent cations, only Zn^{2+} was found to inhibit M.PabI-catalysed methylation. M.PabI is the most thermophilic of all the characterized DNA methyltransferases. The thermophilicity of this methyltransferase will lead to detailed analyses of mechanisms of DNA methylation and will be widely applicable to DNA engineering.

Abbreviations

AdoHcy, S-adenosylhomocysteine

AdoMet, S-adenosyl-L-methionine

bp, base pair

DTT, dithiothreitol

EDTA, ethylenediamine-N', N', N', N',-tetraacetic acid

E. coli, Escherichia coli

IPTG, isopropyl-β-D(-)-thioglucopyranoside

M, modification

m4C, N4-methylcytosine

m5C, C5-methylcytosine

m6A, N6-methyladenine

[*methyl*-^{14}C]AdoMet, S-adenosyl-L-[*methl*-^{14}C]methionine

ORF, open reading frame

PAGE, polyacrylamide-gel-electrophoresis

PCR, polymerase chain reaction

P. abyssi, Pyrococcus abyssi

R, restriction

RM, restriction and modification

TLC, thin-layer chromatography

TRD, target recognition domain

INTRODUCTION

DNA methylation has a crucial role in diverse biological processes — for example, replication and repair of DNA, expression and silencing of genes, and distinction between self and non-self DNA. DNA methyltransferases require *S*-adenosyl-L-methionine (AdoMet) as the donor of a methyl group (2) (Fig. 1).

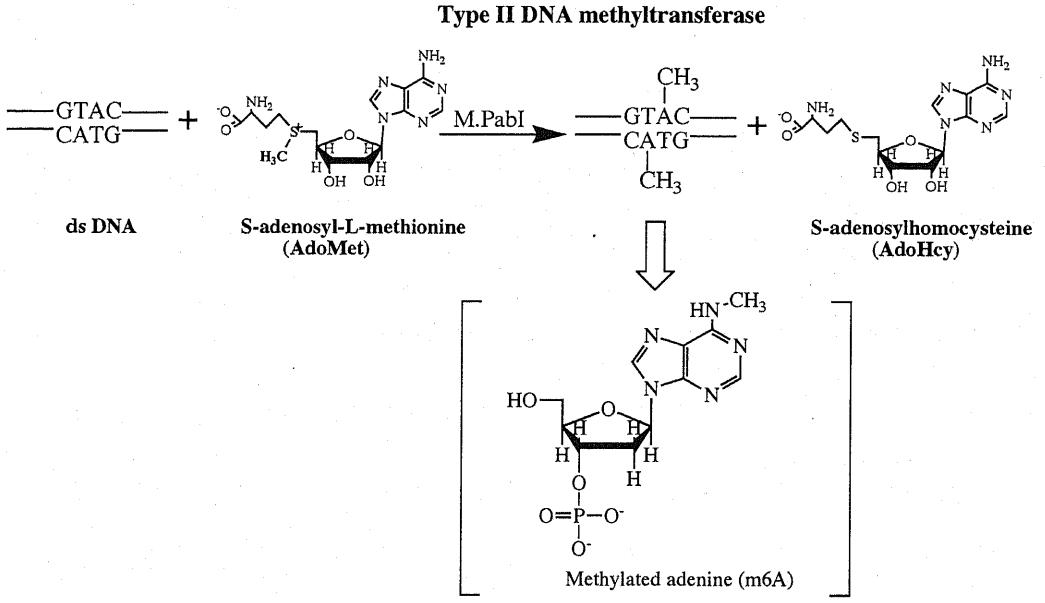


Fig. 1

The common core of AdoMet-dependent methyltransferases is formed by seven-stranded β-sheets (3) and the target base within a double-stranded DNA molecule is installed into its catalytic pocket by a 'base-flipping' mechanism (4). AdoMet-dependent methyltransferases are classified into three groups according to their products: those yielding 5-methylcytosine (m5C), those yielding N4-methylcytosine (m4C) and those yielding N6-methyladenine (m6A). The latter two groups of amino-nitrogen methyltransferases are further subdivided into families of α, β, γ and others according to the order of motifs of the catalytic center and AdoMet-binding region and of target recognition domain (TRD) (5,6). Group α and γ DNA methyltransferase differ in location of the variable segment responsible for DNA sequence recognition. The TRD is at the carboxyl end in the group γ DNA

methyltransferase (and in m5C DNA methyltransferase save for the carboxyl terminal helix associated with motif X), while, in group α DNA methyltransferase, the TRD is found inserted between motifs III and IV. The insertion of substrate-binding domains at a variety of points relative to the core structure has also been noted to occur in other DNA methyltransferase. TRDs are long sequences containing no obviously conserved amino acid motifs, surrounded by well conserved motifs found in all DNA methyltransferase. The extent of amino acid sequence similarity between TRDs is very small, except for some systems that have a common component in the DNA target (7).

Most of the prokaryotic DNA methyltransferases constitute restriction-modification (RM) systems, which can be classified as Type I, II and III (8,9). A typical type I RM system consists of one DNA specificity (S) subunit, two modifications (M) subunits and two restriction (R) endonucleases subunits. Type I restriction enzymes are very large and possess modification, endonuclease and ATPase-driven DNA translocation activities in one enzyme (10). Type III restriction enzymes consist of two mod subunits and two res subunits (11,12). Type III enzymes can function purely as a dimeric DNA methyltransferase in the absence of the res subunit (10).

Most of the known DNA methyltransferases belong to the Type II restriction-modification systems. A classical Type II RM system is composed of two distinct enzymes that share an identical recognition sequence: a restriction (R) endonuclease (restriction enzyme) and a modification (M) enzyme with the DNA methyltransferase activity. The latter methylates one particular base within the recognition sequence and prevents the cleavage of substrate DNA by the former. Type II DNA methyltransferases comprise an AdoMet binding catalytic domain and the TRD. In addition, Dam methyltransferase, although not component of an RM system, has a similar form to the Type II methyltransferase. The phase-encoded methyltransferases, which are used to modify the phage genome in defense against the host RM systems, are also of a similar form to the Type II methyltransferases.

In a bacterial cell carrying an RM system, the modification enzyme protects chromosomal DNA by methylation, whereas the restriction enzyme attacks invading DNA without proper methylation. Therefore, the biological function of RM systems has been construed as being the defense of bacterial cells against invasion by 'non-self' DNA molecules (Fig. 2).

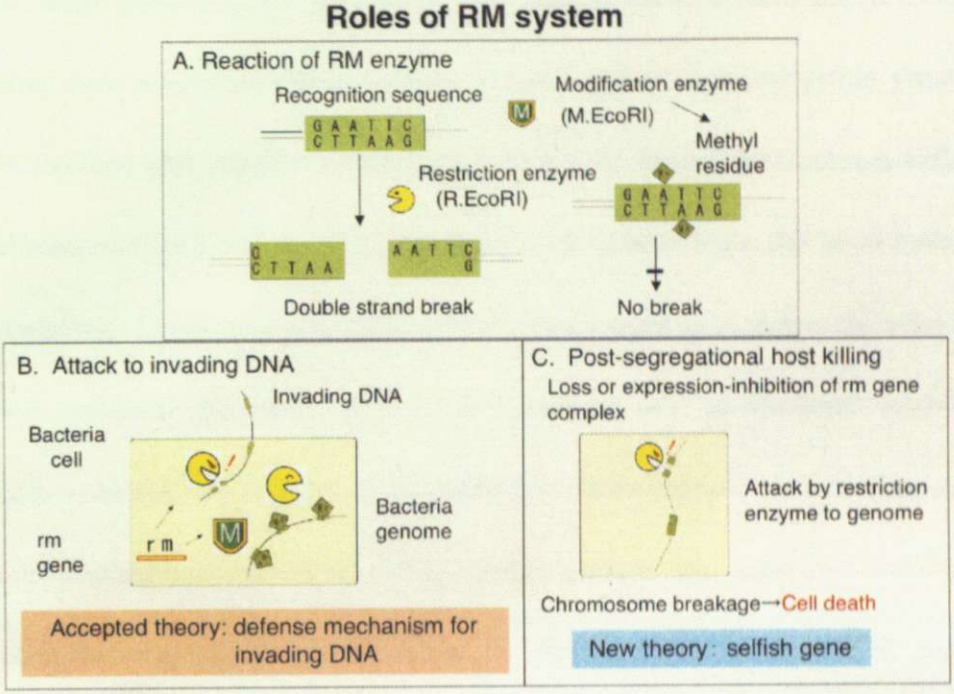


Fig. 2

However, loss of some RM systems was found to lead to cell death, very likely through restriction attack on unmethylated recognition sites in newly replicated chromosomes (13,14).

Several lines of evidence for their mobility have been provided through the decoding of prokaryotic genomes (15). Various types of evolutionary analyses suggest that they have undergone extensive horizontal transfer between distantly-related groups of prokaryotes (16,17). Close examination of the genomic neighborhood of restriction modification gene homologues and its comparison with a closely related genome provide clues as to how restriction-modification gene complexes entered a genome

and caused various genome rearrangements (15,18-20).

Viruses, transposons and other mobile genetic elements all employ unique strategies for their own survival. The strategy used by restriction-modification gene complexes is to destroy non-self DNA that is marked by the absence of proper methylation (Fig. 2). Their attack on invading DNAs represents its simple manifestation. Here there is no conflict between them and their host bacteria. However, when their persistence within the host is threatened by their competitor genetic element, they may kill the host cells together with the competitor (13). When a restriction-modification gene complex is eliminated from a cell, the cell's descendants will contain fewer and fewer molecules of the modification enzyme. Eventually, the modification enzyme's capacity to protect the many recognition sites on newly replicated chromosomes from the remaining pool of restriction enzyme becomes inadequate. Chromosomal DNA will then be cleaved at those exposed sites, leading to cell death (14,21). Such post-segregational host killing would provide the restriction-modification gene complexes competitive advantage {A. Mochizuki, K. Yahara, I. Kobayashi, and Y. Iwasa, Genetic addiction: selfish gene's strategy for symbiosis in the genome. ***Genetics***, *in press*.}

There is increasing lines of evidence for this selfish mobile element hypothesis. Attempts to eliminate restriction-modification gene complexes from a cell led to recovery of variously rearrangemed genomes in the laboratory (22,23). These observations are in accord with their association with genome rearrangements inferrred from genome comparison and genome analyses in *Helicobacter pylori* and other bacteria (18-20,24).

Evidence for the mobility of RM systems has also been obtained by an *in silico* analysis of two *Pyrococcus* genomes (25). The *Pyrococcus* genus represents hyperthermophilic archaea which live in

deep-sea hydrothermal vents with temperatures up to 100°C (26). Its species have been studied with respect to physiology, enzymology and biotechnology and have provided various thermostable proteins, including restriction enzymes (27-29). Comparison of whole-genome sequences of its two species, *Pyrococcus abyssi* (30) (http://www.genoscope.cns.fr/Pab/) and *Pyrococcus horikoshii* (31), has revealed that many cases of large genomic polymorphisms are tightly linked with putative RM genes. A specific region of the *P. abyssi* genome, consisting of a DNA methyltransferase gene homologue (PAB2246, designated as *pabIM* in this work) and its neighbouring open reading frame (ORF) (PAB0105, later designated as *pabIR*) (Fig. 5A), seemed to have been inserted into the *P. abyssi* genome recently according to its lower GC-content and biased codon usage (25) (Fig. 3). A further study demonstrated that PAB0105, indeed, encodes a novel Type-II restriction endonuclease, PabI, which is highly thermophilic and catalyses the cleavage of double-stranded DNA at 5'-GTA/C-3' (32).

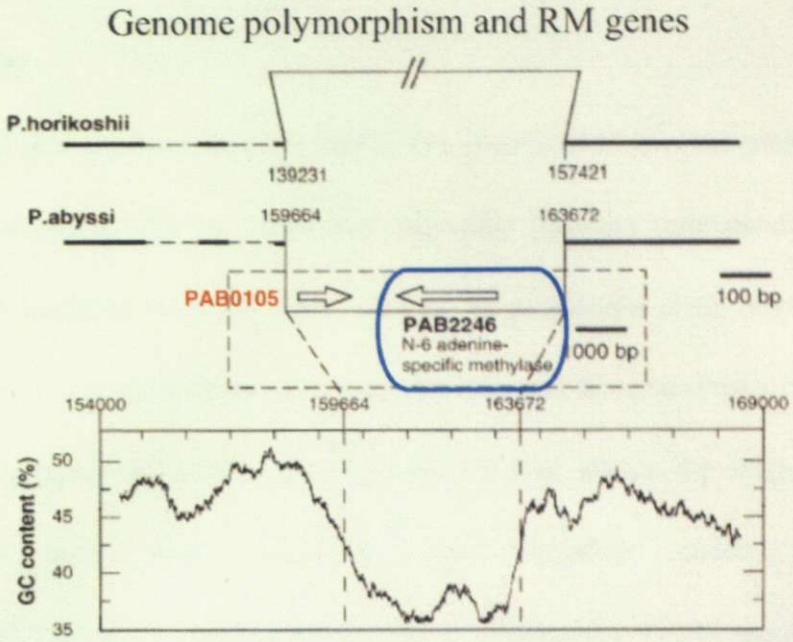


Fig. 3 Chinen *et al. Gene* (2000)

Fig. 3 A DNA segment carrying a predicted DNA methylase and ORF of unknown function (no annotation of function) has apparently become inserted into *P.abyssi* with concomitant target deletion. A thick line and a dotted line indicate regions of more than and less than 70% homology, respectively. An arrow indicates ORF.

Compare with these analysises of restriction endonuclease PabI, DNA methyltransferase gene homologue (M.PabI) was not clear that this DNA methyltransferase was functional or not as proper hyperthermophilic DNA methyltransferase. Therefore, I thought that it was important to provide a basis for functional RM system that exists on large genomic polymorphism in *Pyrococcus abyssi*.

In this study, I demonstrated that *pabIM* indeed encodes a functional DNA methyltransferase recognising the same sequence as PabI, a result in support of the idea of PabI RM system as a mobile genetic element. I also described the expression, purification and detailed enzymatic characterization (optimal conditions, kinetic parameters and thermodynamic parameters) of this enzyme, which turned out to be the most thermophilic of all the reported DNA methyltransferases.

EXPERIMENTAL PROCEDURES

Cloning of pabIM

P. abyssi GE5 genomic DNA was provided by Dr. Yoshizumi Ishino (University of Kyushu, Japan), as described previously (32). A 1,395 base pair (bp) fragment corresponding to *pabIM* ORF (PAB2246) was amplified from this DNA template by polymerase chain reaction (PCR) with the following synthetic oligodeoxyribonucleotide primers (Operon Biotechnologies, K.K., Tokyo, Japan): 5′-TT**GAGCTC**ATGACTTCACAAAAAGAAAAAC-3′ that allows the fragment to introduce a *Sac*I-site (in bold type) next to the initiation codon (underlined) and 5′-CC**AAGCTT**TTAGTCTTTTTGAAATCCTAG-3′ introducing a HindIII site (in bold type) next to the termination codon (underlined). The amplified fragment was then digested with SacI and HindIII, followed by insertion into the corresponding sites of an arabinose-inducible *E. coli* expression vector, pBAD30 (33), which was obtained from The Cloning Vector Collection of National Institute of Genetics, Japan. A pBAD30-derivative clone carrying the expected nucleotide sequence of *pabIM* ORF was designated as pMW1. The insert that was excised from pMW1 was subcloned into another *E. coli* expression vector pET28a(+) (EMD Biosciences, Inc., Novagen Brand, Madison, WI, USA) (34), which was given as a gift by Dr. Masaru Tanokura (University of Tokyo, Japan). This expression vector is inducible with IPTG and had the ability to append 6 × His-tag at the amino terminus of the expressed protein. This pET28a(+) derivative was named pMW2. Both pMW1 and pMW2 plasmids were constructed in *E. coli* strain JM109 (*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 ($r_K^- m_K^+$) *e*14$^-$ (*mcrA*$^-$) *supE*44 *relA*1 Δ(*lac-proAB*)/F' [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*DM15]) (35) (Table 1).

Table 1: Bacterial strains and Plasmids

Strain Names	Strain Genotype	Use	Reference
JM109	(*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 ($r_K^- m_K^+$) *e*14$^-$ (*mcrA*$^-$) *supE*44 *relA*1 Δ(*lac-proAB*)/F' [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*DM15]);	plasmid construction	(35)
MC1061	(*hsdR mcrB araD* Δ(*araABC-leu*)7679 Δ*lacX*74 *galU galK rpsL thi*);	*in vivo* assay	(36)
BL21(DE3)	(F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal* (λ*DE3* (= λ *cI*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7*gene*1));	over-expression	(37)

Plasmid Names	Vectors	Drug resistance	Inserts	Sites	Source of vector	Purpose	Reference
pMW1	pBAD30	Amp	*pabIM*	5'SacI-HindIII	The Cloning Vector Collection (National Institute of Genetics)	*in vivo* expression	This work
pMW2	pET28a(+)	Kan	*pabIM*	5'SacI-HindIII	Masaru Tanokura	over-expression	This work

Expression and activity of M.PabI in E. coli

pMW1 was transformed into *E. coli* MC1061 (*hsdR mcrB araD* Δ(*araABC-leu*)7679 Δ*lacX*74 *galU galK rpsL thi*) (36). The single transformant colonies were cultivated in LB broth (38) supplemented with arabinose at 0.02, 0.04, 0.08 or 0.1%, as well as 50 μg/ml ampicillin with aeration at 37°C. A culture containing 1% glucose instead of arabinose was also prepared as a negative control, repressing the expression of the cloned *pabIM* ORF. After 4 hours of cultivation, plasmid DNA was purified with QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA) from 5 ml of the culture and treated with RsaI (New England BioLabs Inc., Beverly, MA, USA), which recognizes the five 5′-GTAC-3′ sites on pMW1.

Overexpression and purification of M.PabI

The 6 × His-tagged M.PabI protein was expressed in the *E. coli* strain BL21 (DE3) (F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal* (λ*DE3 (*= λ *cI*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7*gene*1)) (37)(Table 1). The plasmid pMW2 was introduced into this strain by electroporation for each protein preparation. A resulting single transformant colony was cultivated in LB medium containing 50 μg/ml kanamycin with shaking at 37°C overnight. Next, 0.5 ml of this pre-culture was diluted into 500 ml of fresh LB medium with kanamycin, to bring the OD_{600} up to 0.6 at 37°C with shaking. 1 mM IPTG was added, and further incubation with mild shaking at 30 °C was continued for another 4 hour. The cells were harvested by centrifugation at 2180 *g* for 30 min and sonicated by an ultrasonic disruptor UD-200 (TOMY Co., Ltd., Tokyo, Japan) in buffer A (50 mM sodium phosphate buffer (pH 7.8), 100 mM NaCl) supplemented with 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation at 6680 *g* for 30 min at 4°C and then by filtration with 0.45 μm cellulose acetate filter (Iwaki Glass Co., Ltd., Chiba-ken, Japan). The resulting supernatant was applied to an Ni-NTA Agarose (Qiagen Inc.) column

of 6 ml volume that had been pre-equilibrated with buffer A containing 20 mM imidazole. After washing with 50 mM imidazole and then 70 mM imidazole in buffer A, the Ni-NTA-bound fraction was eluted out with 150 mM imidazole in buffer A. The fractions containing the tagged M.PabI protein were pooled and concentrated before applying to a Heparin Sepharose CL-6B column (Amersham Biosciences AB, Uppsala, Sweden) that had been pre-equilibrated with buffer B (10 mM Tris-HCl (pH 7.5), 100 mM NaCl). A linear gradient of 0.1–1.0 M NaCl was adopted to elute the heparin-bound fractions. The eluate containing the tagged M.PabI protein was concentrated and dialysed against buffer C (50 mM Tris-HCl (pH 7.5), 100 mM NaCl).

To remove the amino-terminal 6 × His-tag, 50 units of human thrombin protease (EMD Biosciences, Inc., Novagen Brand, Madison, WI, USA) were added to 1 ml of the tagged M.PabI solution. After a treatment period of 9 hours at 16°C, thrombin was removed through a Benzamidine Sepharose 6B column (Amersham Biosciences AB) that had been pre-equilibrated with buffer C. Finally, the purified M.PabI protein was concentrated in buffer C, supplemented with 1 mM DTT, 0.1 mM EDTA and 50% glycerol for storage at –20°C. The purified M.PabI maintained its activity during storage at –20°C for at least 6 months. Every preparation yielded about 2 mg of the final M.PabI fraction from 1 L of the IPTG-induced culture. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay; BioRad Laboratories, Hercules, CA, USA).

In vitro methylation assay using M.PabI

I used synthetic oligonucleotides (Operon Biotechnologies, K.K.,) as the substrates for *in vitro* methylation by the purified M.PabI protein; these oligonucleotides are listed in Table 2. One pair of oligonucleotides (each strand was 1 mM) was first heated at 95°C for 5 min and then cooled down to 25°C for > 30 min for annealing into a duplex form. The purified M.PabI was diluted, if necessary, with the enzyme dilution buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 50 % glycerol). The basal reaction buffer contained 50 mM Tris-HCl (pH 7.5 at 25°C), 100 mM NaCl and 1 mM DTT. To this buffer, appropriate concentrations of a suitable oligo-duplex substrate and the M.PabI enzyme were added. The mixture was pre-incubated in the reaction buffer for 2 min. Then the radioactive methyl donor of [*methyl*-^{14}C]AdoMet (56 mCi/mmol; Amersham Biosciences UK Limited, Amersham Biosciences UK Limited, Buckinghamshire, UK) was added for incubation at 75°C to initiate the methylation, unless stated otherwise. The reaction was terminated by the addition of 0.5% SDS in several experiments, as mentioned below.

Quantification of methylation

For quantitative analysis of methylation, I adopted a filter-binding assay (39,40) that detected the incorporation of ^{14}C-labelled methyl groups into the oligo-duplex substrate. After incubation, the reaction mixture was spotted on a 2.3-cm DE81 filter paper disc (Whatman, Brentford, Middlesex, UK). The filters were air-dried, washed three times with a large excess of 50 mM KH_2PO_4, pure water and 70% ethanol for 10 min each (41). Next, their ^{14}C-label was measured in Clear-sol I scintillation fluid (Wako Pure Chemicals, Tokyo, Japan) in a LS3800 scintillation counter (Beckman Coulter, Fullerton, CA, USA).

Sequence-specific methylation

When I confirmed sequence-specific methylation by M.PabI, 10 μM of oligo-duplex #1 or #2 (Table 2) was added to the *in vitro* methylation assay, as well as 5 μM of the purified M.PabI protein and 12.5 μM [*methyl*-^{14}C]AdoMet. After 1-hour incubation, the oligo-duplex was extracted off the excess AdoMet and M.PabI protein with phenol/chloroform and then precipitated with ethanol and Ethachinmate carrier (Nippongene, Nippongene, Toyama-ken, Japan) for analysis in 20% (w/v) PAGE with 100 mM Tris-HCl, 90 mM borate and 2 mM EDTA. The ^{14}C-methylated substrates in the gel were visualized on a BAS-MS2025 imaging plate (Fuji Photo Film Co., Ltd., Tokyo, Japan) and analysed using a FLA-5100 imaging system (Fuji Photo Film Co., Ltd.).

Thin-layer chromatography analysis of products

A 50-μl reaction mixture in the general reaction buffer, supplemented with 100 μM oligo-duplex #1 (Table 2), 5 μM of the purified M.PabI and 30 μM [*methyl*-^{14}C]AdoMet was incubated at 75°C for 1 hour. The positive control samples of m5C, m4C and m6A were prepared with M.AluI (Takara Bio Inc.,Shiga-ken, Japan), M.BamHI (New England BioLabs Inc.), M.EcoRI (New England BioLabs Inc.) and the oligo-duplexes carrying their recognition sequences (Table 2). To wash out excess AdoMet, the ^{14}C-methylated oligo-duplex was precipitated by adding ethanol and Ethachinmate carrier (Nippongene, Nippongene, Toyama-ken, Japan). Next, the ^{14}C-methylated oligo-duplex was hydrolysed in 10 μl of 60% perchloric acid at 95°C for 1 hour (42). A total of 10 μl of water and 10 μl of 2 M KOH were added to neutralize the reaction, and then the resulting insoluble $KClO_4$ was spun down (43). The ^{14}C-methylated bases in the supernatant were separated on a DC-Platten Cellulose plate (EMD Biosciences Inc. Merck, San Diego, CA, USA) with the liquid phase of isopropanol–water–NH_4OH (60:10:0.1 (v/v/v)) at 25°C for 6 hours. The ^{14}C-methylated bases on the air-dried plate were exposed against an BAS-MS2025 imaging plate and analysed using a FLA-5100

imaging system.

Optimizing reaction conditions

A total of 10 μM of oligo-duplex #1 (Table 2), 0.15 μM of the purified M.PabI and 15 μM [*methyl*-^{14}C]AdoMet were added to the basal reaction buffer (see above). The mixture was incubated at 75°C for 8 min unless otherwise stated, and then the reaction was stopped by addition of 0.5% SDS. Incorporation of ^{14}C-methyl group into the duplex was quantified by the filter-binding assay.

Heat resistance

The purified M.PabI was diluted at 5 μM and pre-heated at 95, 85 or 75°C in the enzyme dilution buffer. Next, the heated aliquot of 0.5 μM M.PabI was added to a reaction mixture to evaluate its remaining activity. These mixtures were supplemented with 10 μM of oligo-duplex and 30 μM of [*methyl*-^{14}C]AdoMet and were incubated at 75°C for 8 min; the reaction was terminated by addition of 0.5% SDS. The methylation yields were quantified by the filter-binding assay, as described above, and used to calculate the percentage of the remaining activity against the methylation activity without preheating.

Temperature-dependence of reaction

A total of 10 μM oligo-duplex #1 (Table 2), 0.15 μM of the purified M.PabI and 15 μM [*methyl*-^{14}C]AdoMet were added to the basal reaction buffer (see above) to analyse the 90-min reaction time course at various temperatures. When the initial velocities for the Arrnhenius plot were determined, 20 μM of the oligo-duplex was supplemented for a 3-min time course. An aliquot was directly spotted onto a filter disc for an immediate measurement (see above). The scintillation counts were evaluated as yields of full-methylated oligo-duplex #1 and expressed as molar concentration of 'Products'. All the series of experiments were performed twice. The thermodynamic parameters for

the rate-determining step of the M.PabI methylation reaction, enthalpy ($\Delta H^{\neq}$), entropy ($\Delta S^{\neq}$) and free energy ($\Delta G^{\neq}$) of activation, were obtained from the Arrhenius plot by:

$\ln(k) = \ln A - Ea/RT$ [1],

according to:

$\Delta H^{\neq} = E_a - RT$ [2],

$\Delta S^{\neq} = R\ln(Ah/ek_BT)$ [3],

$\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$ [4],

where E_a is the activation energy; A is the intercept of Arrhenius equation; R is the gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$); T is the absolute temperature; k_B is the Boltzman constant (6.6256×10^{-34} J·s); and h is the Planck constant (1.3805×10^{23} $J \cdot K^{-1}$) (44,45).

Determination of kinetic parameters

The initial velocity of methylation with 10 nM M.PabI was determined at 75°C with varying concentrations of the substrates. The time course experiment was measured by six samplings during the first 150 sec of the reaction under each condition. An aliquot of each sampling was directly spotted onto a filter disc and immediately quantified (see above). The scintillation counts were evaluated as yields of full-methylated oligo-duplex #1 and expressed as molar concentration of 'Products'.

The K_m^{DNA} for the oligo-duplex substrate #1 was determined in the reaction series, supplemented with 15 μM [*methyl*-^{14}C]AdoMet by varying the concentration of the oligo-duplex from 50 nM to 600 nM. Similarly, the K_m^{AdoMet} was determined within the range of 0.4–6.0 μM [*methyl*-^{14}C]AdoMet with the oligo-duplex concentration fixed at 10 μM. The double reciprocal plots of the resulting initial velocities versus the concentration of respective substrates allowed the determination of K_m and V_{max} values. The turnover number (k_{cat}) was calculated as the ratio of V_{max} to the applied enzyme

concentration. All the obtained data were plotted by regression analysis with the software package of IGOR Pro version 5.03 (Wave Metrics, Inc., Portland, OR, USA).

RESULTS

Sequence comparison

Previous research from our laboratory has predicted that PAB2246 protein encoded by *pabIM* gene is one of the N6-adenine DNA methyltransferases (17,25). A BLASTP 2.2.12 (http://www.ncbi.nlm.nih.gov/BLAST/) search showed that this protein, designated as M.PabI, showed similarities in amino-acid sequences with many methyltransferase homologues, some of which have been well characterized and others being only hypothetical. In the search for homologues with a known three-dimensional structure, I inquired the registration of PAB2246 in GTOP (http://spock.genes.nig.ac.jp/%7Egenome/gtop.html). M.TaqI, from a thermophilic eubacterium *Thermus aquaticus*, was retrieved as having the highest E-value of 1×10^{-9}. M.TaqI is a thermophilic N6-adenine methyltransferase that belongs to the γ-group, and its crystal structures and functions have been analysed in detail (46,47). The γ-group members carry nine amino acid sequence motifs (I through VIII and X) that are specific to amino-methyltransferases as well as a target recognition domain (TRD), a long sequence containing no obviously conserved amino acid motifs and surrounded by well-conserved motifs found in all DNA methyltransferase. They occur in the order of N-terminus-X-I-II-III-IV-V-VI-VII-VIII-TRD-C-terminus (5).

T. Malone *et al.* reported following that: from structural point of view, motif I forms a secondary structure of sheet-loop-helix. The loop (G-loop) binds the methionine moiety of AdoMet. Motif II interacts with the ribose hydroxyls of AdoMet and is followed by a bulky hydrophobic side-chain that makes van der Waals contacts with the adenine moiety of AdoMet. Motif III interacts directly with the exocyclic NH_2 (N6) of AdoMet. The P-loop in motif IV forms the active site. Motif X is suggested to form a helix next β strand (formed by motif I) , with conserved hydrophobic side-chains required at certain positions for packing against the β-strands, and a loop preceding the helix (5).

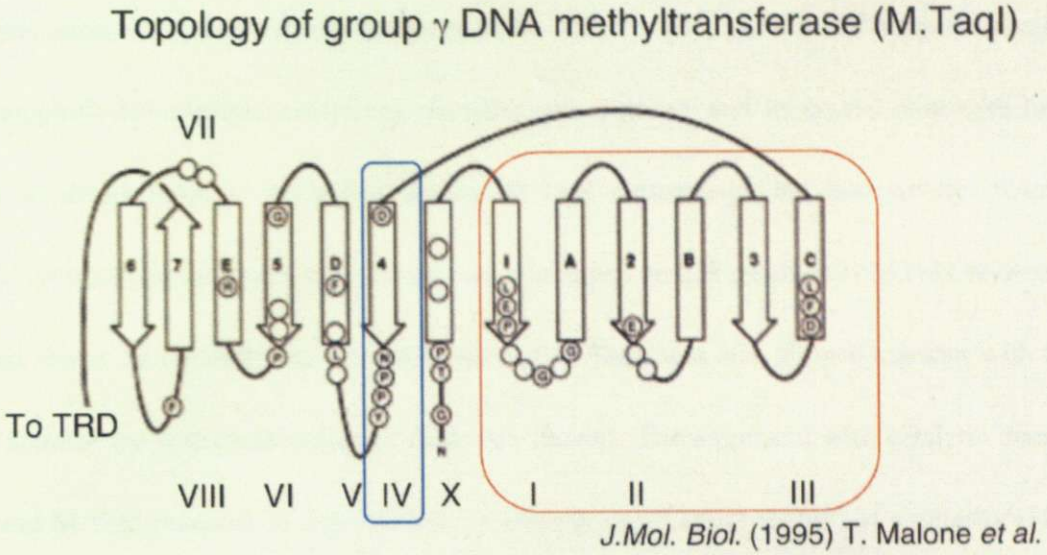


Fig. 4

***In silico* comparative analysis**

When we inquired PAB2246 by NCBI BLASTX 2.2.12 (http://www.ncbi.nlm.nih.gov/BLAST/), the modification (M) subunit of M.AhdI methyltransferase was retrieved with the highest similarity at 8 x 10^{-16} of E-value. M.AhdI requires specificity (S) subunit to exhibit its activity by forming a tetrameric (M_2S_2) complex, resembling a trimeric type-I modification enzyme of M_2S (48). The AhdI-specificity gene (*ahdIS*) was located between the restriction and modification genes. Intriguingly, there was a suggestive 707-bp intermission also between the *pabIR* and *pabIM* genes typically in "tail-to-tail" orientation (49), although no ORF was identified within this interval region and no significant similarity was observed between R.PabI and R.AhdI restriction endonucleases (Fig. 1A). The second best hit of BLASTX search for PAB2246, resulting 3 x 10^{-15} of E-value, was a putative type-I modification subunit (ORF# CUP1744) that had been identified on the whole genome sequence of *Campylobacter upsaliensis* RM3195 (50), see also REBASE Genomes at

http://tools.neb.com/~vincze/genomes/).

As the most related protein structures, on the other hand, chains A, B, and D of M.TaqI methyltransferase were retrieved with E-values of 7×10^{-9}, 7×10^{-9}, and 4×10^{-8}, respectively. M.TaqI is a thermophilic N6-adenine methylases classified into γ-group, and its crystal structures had been analyzed in detail (46,47). According to the M.TaqI structure-guided comparison (5,46), nine conserved motifs of amino-methyltransferases were assigned on 228 residues of M.PabI N-terminus as mentioned above. M.TthHB8I, the closest relative of M.TaqI, was also aligned together with them in order to identify the conserved sequence (date not shown). The alignment with catalytic domains of M.PabI and M.TaqI revealed 26.3% (60/228) of identity or 57.5% (131/228) of similarity (Fig. 5B). The consensus amino acids of group γ (shaded in Fig. 5B) were well conserved within all the nine motifs on M.PabI, and their arrangement of X-I-II-III-IV-V-VI-VII-VIII indicated that M.PabI is a member of γ-group N6-adenine methyltransferases.

The amino-terminal half of M.PabI also showed similarity to several modification subunits of Type I RM systems and related proteins, as well as to several Type II modification enzymes. When I assessed PAB2246 using the NCBI BLASTX 2.2.12, M.AhdI (48) was retrieved as having the highest score. In AhdI RM, an atypical Type II system, two molecules each of modification (M) and specificity (S) subunits combined to form a tetrameric (M_2S_2) modification enzyme, resembling Type-I trimeric (M_2S) modification enzymes. The similarity of parts of M.PabI with some Type I M proteins was considerable (Fig. 5C).

The carboxy-terminal half of M.PabI, on the other hand, was expected to have a TRD as with M.TaqI. A total of 178 amino-acid residues showed similarity to a few putative S subunits of Type-I RM systems in BLASTP 2.2.12 search (Fig. 5D). The most similar ones were found within the

genome of *Nostoc punctiforme* (ZP_00110994), *Marinobacter aquaeolei* (ZP_00816975), or *Clostridium thermocellum* (ZP_00504951). The conserved motifs of the S subunits, pfam01420 and PD314702, were also retrieved. The TRD of M.TaqI was recently reported to show significant structural similarity to the S subunit from a thermophilic archaeon, *Methanococcus jannaschii* (51,52). The presence of TRD at the carboxyl terminus of M.PabI further indicates that it is a member of the γ-group DNA methyltransferases.

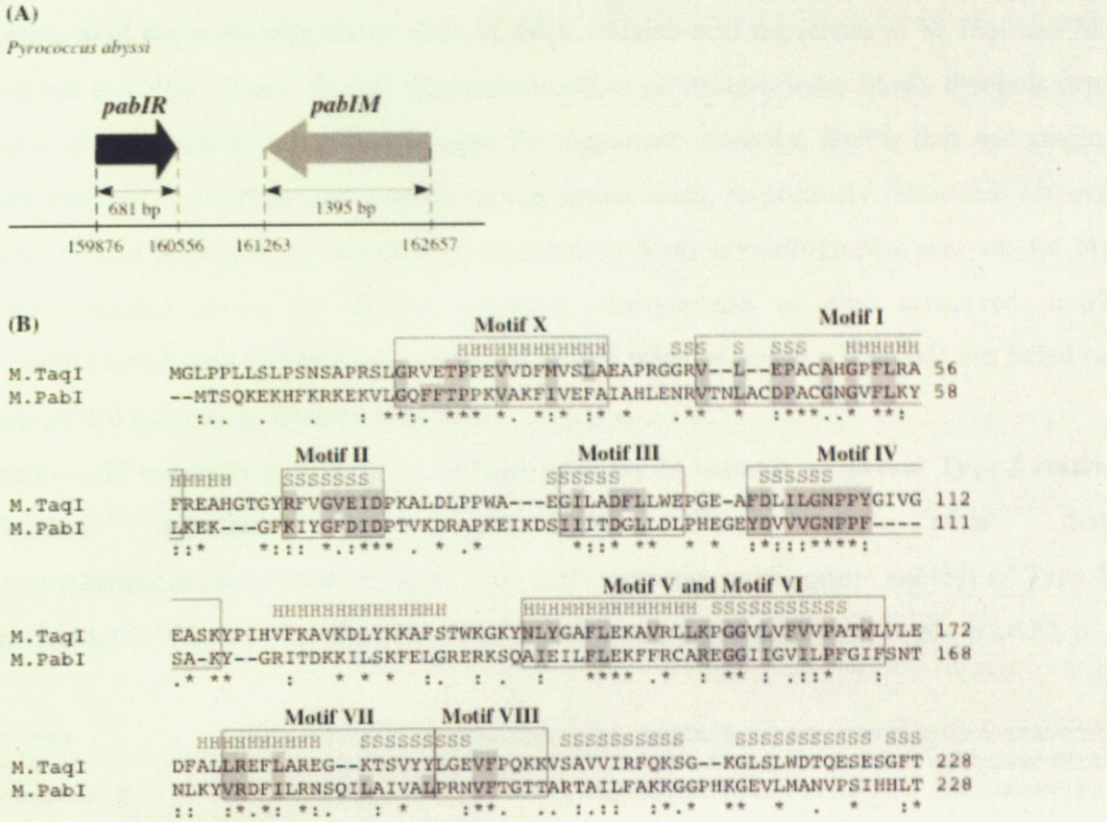


Fig. 5

Fig. 5 Organization of PabI locus and comparative analysis of N-terminal region of M.PabI.

(A) PabI locus. Arrows represent coding regions of *pabIR* (PAB0105; black) and *pabIM* (PAB2246; gray) in the genome sequence of *P. abyssi* (http://www.genoscope.cns.fr/Pab/). The length of each open reading frame is indicated in base pairs (bp). The restriction gene *pabIR* and the putative

modification gene *pabIM* are located in 'tail-to-tail' orientation as is the case in many RM gene pairs (49).

(B) Amino-acid sequence alignment with M.TaqI. Amino-acid sequences of M.TaqI and M.PabI were aligned using the Clustal W 1.82 (http://www.ebi.ac.uk/clustalw/index.html). Symbols denoting the degree of conservation are placed beneath the alignment: asterisks, double dots and single dots represent identical, conserved and semi-conserved amino acids, respectively. Structural elements of α-helices (H) and β-strands (S), which were revealed by X-ray crystallographic analyses for M.TaqI (46), are indicated above the M.TaqI sequence. Assignments of nine conserved motifs as amino-methyltransferases (boxed) and consensus amino acids of group γ (shaded) are based on the structure-guided analysis by Malone et al. (5).

(C) Amino-acid sequence similarity of M.PabI with the M subunit of several Type I restriction modification systems. (i) Motif IV. Based on pfam database (http://www.sanger.ac.uk/Software/Pfam/). Type I, M represent modification subunit of Type I RM system. (ii) Motifs VII and VIII. Based on PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

```
M.PabI_TRD          GIPFISAKVVTPLGIDFTKDKKFIQPNSEMDKKSAHAHVGEIVFVRVGVGTIGRTAVITS 60
Nostoc_S            GTPYIRVGDVKNGQINFESAVKIPITMANVDK-SVGLQIGDIIFTRK--GSFGNSAVVTE 57
Marinobacter_S      GVPFIRGGNLKDMTVTTEG-MLYITAEKNRSMRKGRLQVGDILFVNR--GEIGKLAVIPS 57
                    * *:*    :.   :          .  : .  .   ::*:*:*.. . * :*. **:..

M.PabI_TRD          KEEEGIVDDWSYILTVKSD---KVNPYYLAFYLQAPTIKKQILRYARGVGTITIPQRELK 117
Nostoc_S            LEVNGIISSEIMLVRLTSVSRQEVLPEYVSLFLNSKFGYLQVEHRVHGVAYYSISQPDLA 117
Marinobacter_S      SMNGANLNSQIAYLRVENR---IIDPHYLVHYLASDTIKAEIKAAQEGSVLTQYPISDLA 114
                        . :..    : : .     : * *:  :* :     ::    .*      .  :*

M.PabI_TRD          KIPVLIPPKDFLKKCEEAYKEMVK---------------LRKEGK------IREAKMILN 156
Nostoc_S            NLLIPILPKYQQQKIAEKIKSSFS---------------LKLKSKQLLEIAKTGVERAIE 162
Marinobacter_S      AIHVPVPPKDEQQKISTYLKEQLFSFNVLTSEASNSINLLSERRSALISAAVTGKIDVRN 174
                     : : : **   :*     *. .                 *  . .             :

M.PabI_TRD          SIEKEIEEMIKSKISNLGFQKD- 178
Nostoc_S            TDEATATIWINQQLEALGVKLT- 184
Marinobacter_S      WQPPSDESAFDEEVRQAGMEATA 197
                           :..::   *.:
```

Fig. 5D Amino-acid sequence similarity of the carboxy-terminal half of M.PabI with the S subunit of several Type I restriction modification systems.

DNA methylation in E. coli

As M.PabI was from a hyperthermophile *P. abyssi*, I first examined whether the protein that was expressed at 37°C in *E. coli* exerts DNA modification activity. The *pabIM* ORF was placed under the P_{BAD} promoter, which is induced by arabinose but repressed by glucose, on the expression plasmid pMW1 (EXPERIMENTAL PROCEDURES). I monitored the cleavage at six PabI-sites (5′-GTAC-3′) on pMW1 itself (Fig. 6B). When the *E. coli* MC1061 transformant with pMW1 was cultivated with 1% glucose, the purified pMW1 plasmid was cleaved by RsaI, which also recognizes 5′-GTAC-3′; complete cleavage into five fragments of the expected size was achieved. Once arabinose was added to the culture; expression of M.PabI was arabinose-induced from the pMW1 plasmid (arabinose-inducible *E. coli* expression vector), however, no RsaI-cleavage was detected with the recovered plasmid, even at the concentration of 0.02% (Fig. 6A). These results demonstrated that M.PabI of the *pabIM* ORF product protected pMW1 from RsaI-digestion by at least modifying all of the five RsaI/PabI-recognition sites. Therefore, the M.PabI methyltransferase was expected to recognize the sequence of 5′-GTAC-3′ that correspond to the PabI restriction enzyme (32).

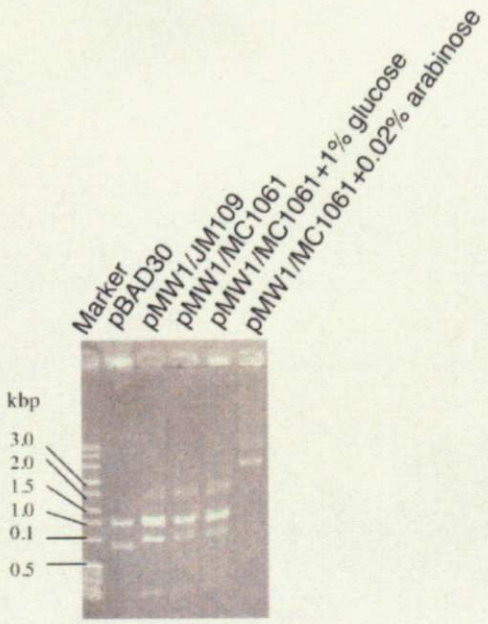


Fig. 6A

Fig. 6. DNA methylation by M.PabI in *E. coli*.
pBAD30 : intact pBAD30 plasmid.
pMW1/JM109 and pMW1/MC1061 were positive controls. The *E. coli* MC1061 transformant with pMW1 was cultivated in 1% glucose or 0.02% arabinose, the purified pMW1 plasmid was cleaved by RsaI; complete cleavage into five fragments of the expected size was achieved. (1% agarose gel and visualized under UV light after ethidium bromide staining.). Each fragment size showed Fig. 6B. (In this experiment, I failed to include uncut pBAD30 or pMW1 as negative control.)

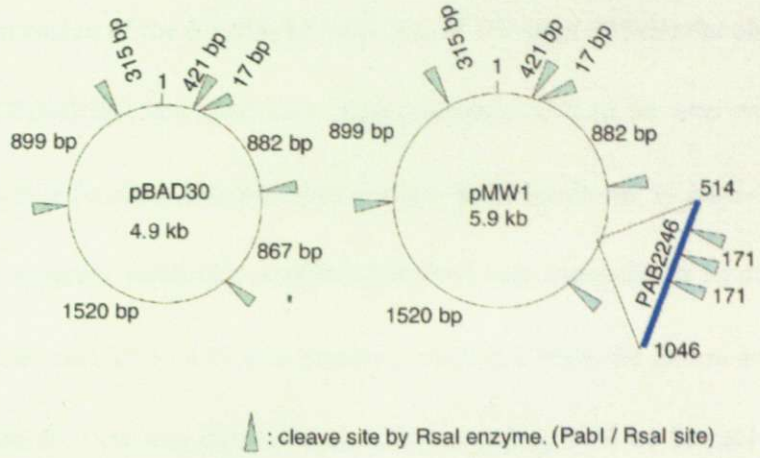


Fig. 6B. Maps of pBAD30 and pMW1. Each number indicates a fragment size (bp; base pair).

Overexpression and purification

Expression of the 6 × His-M.PabI was IPTG-induced from the pMW2 plasmid (EXPERIMENTAL PROCEDURES) in *E. coli* cells. Approximately 60% of the over-expressed M.PabI was recovered in the soluble fraction. The purification steps were monitored by SDS–PAGE analysis. As shown in Fig. 7, the apparent molecular weight of M.PabI was estimated to be 52 kDa. This value was consistent with the mass of 52.455 kDa that was predicted from the amino-acid sequence of *pabIM* ORF. The purified fraction was shown to contain more than 90% of M.PabI protein. About 2 mg of purified M.PabI protein was yielded from 1 L of IPTG-induced culture.

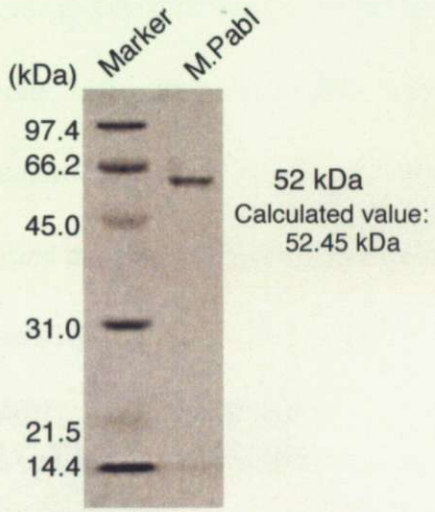


Fig. 7

Fig. 7 The purified M.PabI protein was analyzed using SDS–PAGE with 12% (w/v) acrylamide, together with Molecular Weight Standards, Low Range (Bio-Rad Laboratories), followed by staining with Coomassie Brilliant Blue. The molecular mass of M.PabI (indicated by an arrow) was estimated to be about 52 kDa according to its migration.

In addition, a gel-filtration analysis was performed to observe the subunit constitution of M.PabI. On a HiLoad Superdex 200 column (Amersham Biosciences AB. Sweden), the purified M.PabI was separated as a single peak at the fraction corresponding to 52 kDa (data not shown). This indicated that the enzyme exists as a monomer under a reaction condition.

Methylation resulting in 5'-GTm6AC-3'

I demonstrated specific methylation at the PabI recognition site (5'-GTAC-3') by the purified M.PabI enzyme. The 40-mer oligo-duplex substrates with one or no PabI site (#1, #2, #3, #4 or #5) in Table 2) were applied to an *in vitro* methylation assay. The ^{14}C-labelled methyl group that was derived from [*methyl*-^{14}C]AdoMet was successfully transferred to the substrate that possessed one 5'-GTAC-3' site, whereas no significant radioactive incorporation (non-specific methylation activity) was detected with the substrate (#2, #3, #4 and #5) without a PabI site (Fig. 8). Methylation by M.PabI protected the PabI site in the oligo-duplex from cleavage by RsaI, which can cut 5'-GTAC-3'but not 5'-GTm6AC-3'or 5'-GTAm4C-3'(53)(Fig. 6A). The M.PabI enzyme, on the other hand, did not show significant radioactive methylation activity on the single-stranded oligonucleotides that formed oligo-duplex #1 and #2 in Table 2 (data not shown). Therefore, I concluded that purified M.PabI recognized the 5'-GTAC-3' sequence on double-stranded DNA and modified it specifically by transfer of a methyl group.

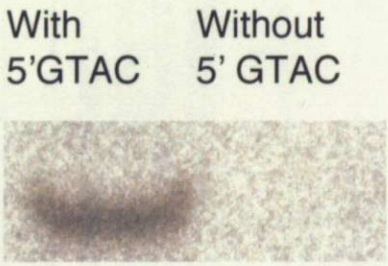


Fig. 8

Fig. 8 *In vitro* methylation of the oligo-duplex substrates (#1 and #2 in Table 2) by the purified M.PabI protein with [*methyl*-^{14}C]AdoMet as the methyl donor, and separation of the products was performed as described in EXPERIMENTAL PROCEDURES.

Table 2: Profiles of oligonucleotides.

Name	Sequence [a]	Length (nt)	Tm (°C) [b]	GC (%)	Use
#1	5' GGACGCTTCACCGGATGTACAGGCATGCGACGACCCCTAG 3' 3' CCTGCGAAGTGGCCTACATGTCCGTACGCTGCTGGGGATC 5'	40	90.2	62.5	Specific methylation by M.PabI
#2	5' GGACGCTTCACCGGATGCTAAGGCATGCGACGACCCCTAG 3' 3' CCTGCGAAGTGGCCTACGATTCCGTACGCTGCTGGGGATC 5'	40	90.2	62.5	Nonspecific methylation by M.PabI
#3	5' GTGAAATGGATCCAAACTG 3' 3' CACTTTACCTAGGTTTGAC 5'	19	63.2	42.1	Specific methylation by M.BamHI
#4	5' GTGAAATAGCTAAACTG 3' 3' CACTTTATCGATTTGAC 5'	17	56.3	35.3	Specific methylation by M.AluI
#5	5' GTGAAATGAATTCAACCTG 3' 3' CACTTTACTTAAGTTGGAC 5'	19	61.1	36.8	Specific methylation by M.EcoRI

[a]: An underline indicates a recognition sequence of each methyltransferase.
[b]: Calculated after Tm = 81.5 + 0.41(%GC) - 675/N(54).

To determine M.PabI modifies the 5′-GTAC-3′ recognition sequence, I separated the ^{14}C-methylated base obtained from the product oligo-duplex by thin-layer chromatography (TLC). The positive control samples of m5C, m4C and m6A were prepared with methyltransferases of M.AluI, M.BamHI and M.EcoRI, respectively. The retention factor (R_f) of the ^{14}C-methylated base produced by M.PabI was indistinguishable from that of m6A but distinct from those of m5C and m4C (Fig. 9). These results indicated that M.PabI transferred methyl group of AdoMet to the sixth nitrogen of adenine residue to generate 5′-GTm6AC-3′. This experimental result was in agreement with the prediction from *in silico* analysis (see Fig. 5B) that M.PabI was one of the γ-group N6-adenine

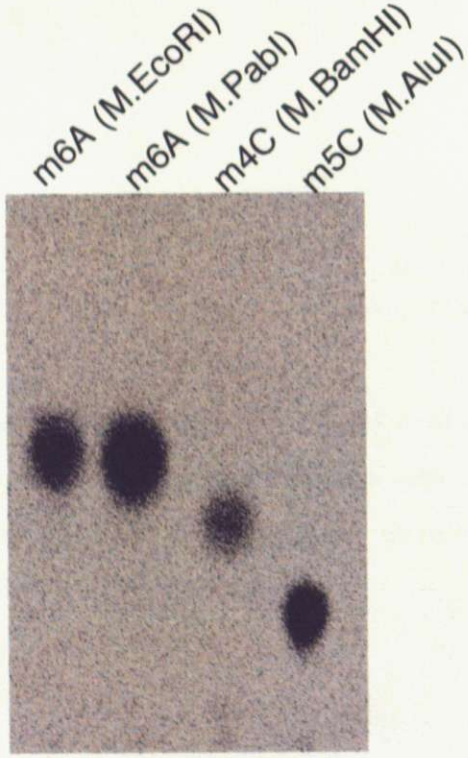


Fig. 9

Fig. 9 Thin-layer chromatography to identify the base modified by M.PabI.
The ^{14}C-methylated bases from the modified oligo-duplexes of four *in vitro* methylation reactions with the indicated methyltransferases were separated on a thin-layer chromatography plate and detected as radioactive spots. Their retention factor (R_f) values are determined and shown beneath the chromatogram. See Table 2 for profiles of the oligo-duplex substrates

Optimal reaction conditions

In consideration of the fact that the hyperthermophile *P. abyssi* GE5 was isolated from a hydrothermal vent of the deep seabed (30), it is reasonably assumed that the M.PabI enzyme is highly thermophilic in Pyrococcal cells. Fig. 10A shows temperature-dependence of the methylation activity of M.PabI. The *in vitro* methylation activity of the purified M.PabI was detectable at a minimum of 35°C and was exerted maximally at 85°C. Impressively, significant activities were detected at 90°C and 95°C (Fig. 10A), even though the 40-mer oligo-duplex and AdoMet are expected to rapidly decompose at these high temperatures (55,56).

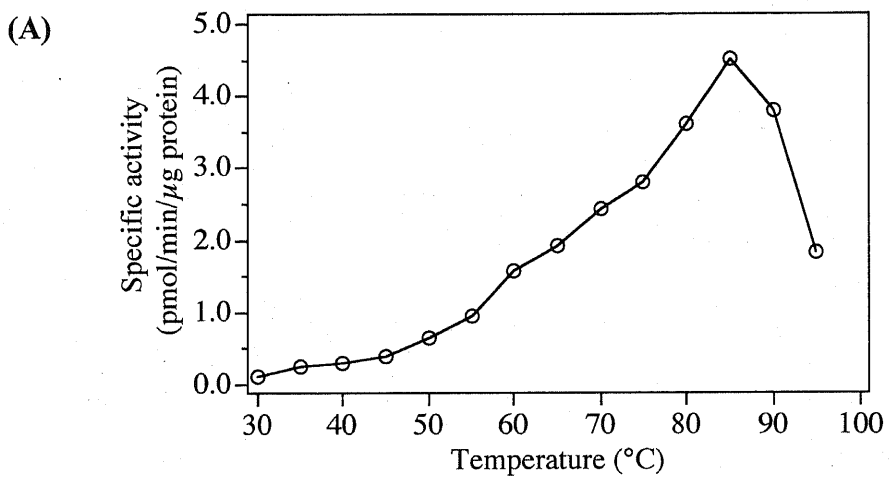


Fig. 10. Effect of reaction conditions on M.PabI activity.

(A) A series of methylation reactions with oligo-duplex #1 (Table 2) were incubated at 30–95 °C, and their methylation activities were quantified by filter-binding assays as described in EXPERIMENTAL PROCEDURES.

I also assessed the activity of M.PabI at pH values ranging from 2.8 to 9.3. As the natural niche of *P. abyssi* was around pH 6.8, I had expected the enzyme to be most active under weak-acidic to neutral conditions. The results shown in Fig. 10B indicated that the optimal pH was 5.8–6.7. In this series of experiments, I tuned each reaction mixture with optimum buffer agents of critic acid, MES, MOPS, Tris-HCl or Na_3BO_3, respectively (see the plot of circles with broken lines). Furthermore, I performed the same measurements with only Tris-HCl to adjust the reaction solution to pH 2.9–8.4 and obtained comparable results: M.PabI showed the highest activity around pH 6.0 (squares with a solid line). In all the following experiments, therefore, the basal reaction buffer (50 mM Tris-HCl pH 7.5) was prepared at 25°C with Tris-HCl and adjusted to pH 7.5, which gives pH 6.0 at 75°C.

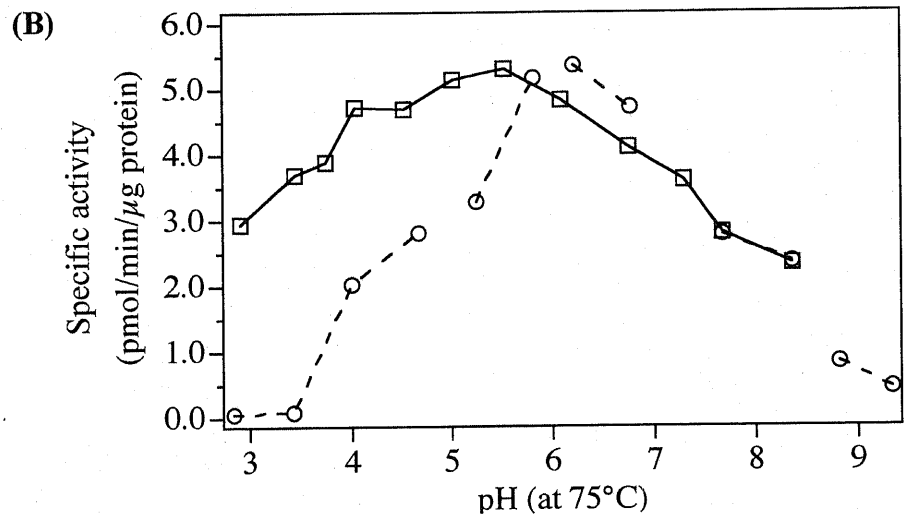


Fig. 10(B) Reaction mixtures containing 100 mM NaCl and 1 mM DTT were adjusted at room temperature with 50 mM of critic acid to pH 3.5–5.5, with MES-HCl to pH 6.0–6.5, with MOPS-NaOH to pH 7.0–7.5, with Tris-HCl to pH 8.0–8.5, or with sodium borate to pH 9.0–9.5 in order to perform M.PabI methylation under pH of 2.8, 3.4, 4.0, 4.7, 5.3, 5.8, 6.2, 6.7, 7.7, 8.4, 8.8 or 9.3 at 75°C of the actual incubation temperature (circle and broken lines). The same assay was performed only with Tris-HCl as the buffer agent to adjust the pH to 2.9–8.4 at 75°C (squares and solid line). Their specific activities were measured by the filter-binding assays as described in EXPERIMENTAL PROCEDURES.

Finally, I investigated the effects of salt concentration to optimize my *in vitro* methylation assay. I found that the optimal NaCl concentration was 200–500 mM (Fig. 10C).

(C)

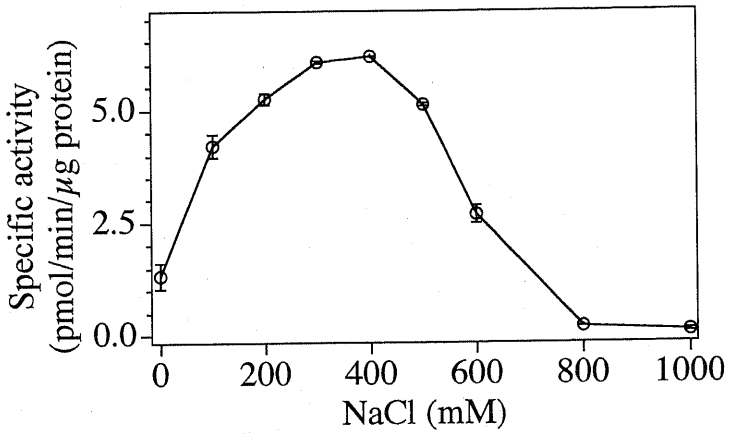


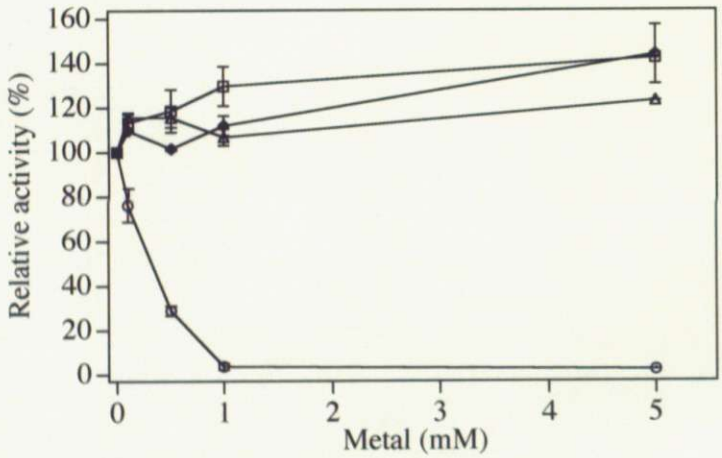
Fig. 10(C) The methylation activities with 0, 100, 200, 300, 400, 500, 600, 800 or 1000 mM NaCl in the reactions were determined. The data from triplicate measurements were plotted with error bars.

Effect of cations

During the investigation of optimal reaction conditions for M.PabI, I found that Zn^{2+} acted as a strong inhibitor (Fig. 11A). Inhibition of the methylation activity to half was seen with 0.3 mM of Zn^{2+}. The other divalent cations examined — Ca^{2+}, Mg^{2+} and Mn^{2+} — did not show any inhibitory effect up to 5 mM (Fig. 11A). To confirm that the above inhibition was caused by Zn^{2+}, I recruited an equal concentration of EDTA as a chelating agent into the M.PabI reaction containing 1 mM Zn^{2+}. As I expected, EDTA restored the reduced methylation activity to 72% of the initial activity (Fig. 11B).

Although NH_4Cl and KCl were also examined at 0.05–5 mM, neither NH_4^+ nor K^+ showed any significant inhibition of M.PabI activity (data not shown).

(A)



(B)

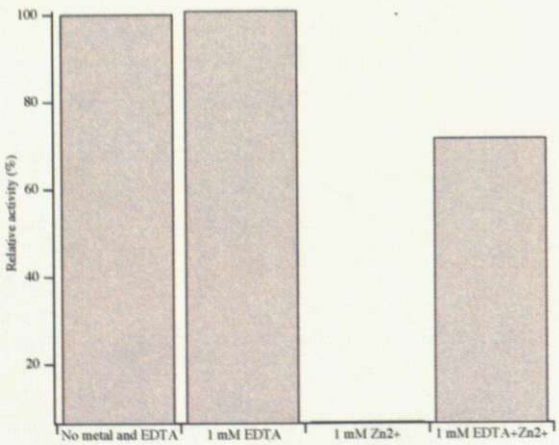


Fig. 11

Fig. 11. Effect of divalent cations on M.PabI activity.

(A) A series of *in vitro* M.PabI reactions was performed either with Zn^{2+} (circle), Ca^{2+} (square), Mn^{2+} (diamond) or Mg^{2+} (triangle), and the methylation activities were quantified using filter-binding assays. The activities relative to the intact methylation in the absence of any divalent metals, which were obtained from triplicate measurements, were plotted with error bars. **(B)** EDTA–mediated reversal of the inhibition by Zn^{2+}. I recruited an equal concentration of EDTA as a chelating agent into the M.PabI reaction containing 1 mM Zn^{2+}.

Hyperthermophilic features

The experiment in Fig. 10A showed that M.PabI is highly thermophilic. To investigate the hyperthermophilic features of M.PabI further, I performed the following series of experiments. First, M.PabI was pre-heated at 75, 85 or 95°C for 5 min to 4 hours before examination of the remaining methylation activity at 75°C for 8 min. As shown in Fig. 12A, the half-lives at 75, 85 and 95°C were approximately 38, 19 and 9 min, respectively.

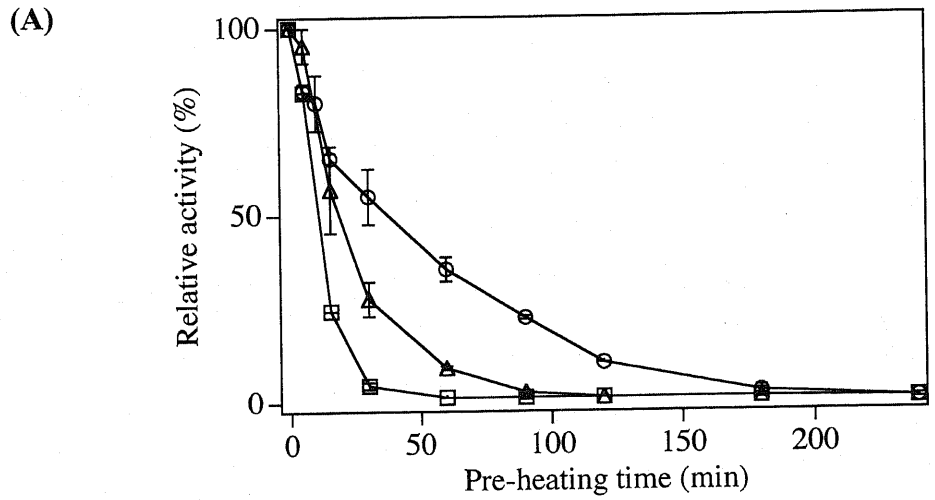


Fig. 12

Fig. 12 Hyperthermophilicity of M.PabI.

All the following series of experiments were carried out at least three times to assess their reproducibility. **(A)** Reduction of M.PabI activity by heat was assessed after pre-heating at 75°C (circles), 85°C (triangles) or 95°C (squares). An aliquot of purified M.PabI was heated in the enzyme dilution buffer at 1.5 μM of protein concentration before the methylation reaction. The remaining activities relative to the intact methylation without pre-heating were plotted with error-bars.

I next performed a series of reaction time-course experiments at various temperatures from 37 to 85°C with excessive (100-fold in molarity of M.PabI protein) oligo-duplex (#1 in Table 2) and [*methyl*-^{14}C]AdoMet to investigate the optimum incubation temperature for M.PabI to exhibit its maximum reaction rate. During this series of experiments and the following kinetic parameter analyses, I evaluated the observed incorporation of ^{14}C-labelled methyl group in terms of molar concentration of full-methylated oligo-duplex #1. Fig. 12B shows time course of M.PabI reactions and the influence of incubation temperature. At 37°C, a 'low' temperature for M.PabI, the methylation time course was fitted into a linear function. Then, at 55–85°C of thermophilic conditions, the M.PabI-catalysed methylation was fitted into a single, exponential function.

(B)

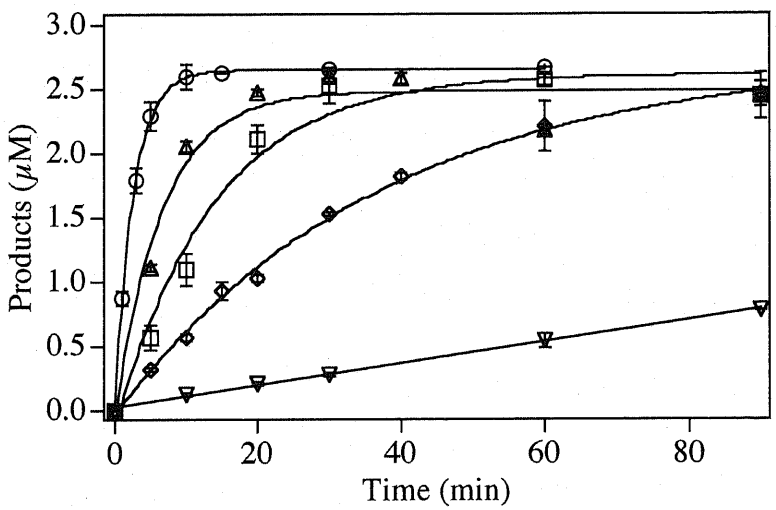


Fig. 12(B) Time courses of M.PabI-catalyzed methylation at 37°C (reverse triangles), 55°C (diamonds), 65°C (squares), 75°C (triangles) or 85°C (circles) were determined.

Fig. 12C also shows the reaction time courses of M.PabI methylation, but over a 180-sec period. All the plots were fitted into linear functions within this period. The obtained initial reaction velocities at 37–85°C resulted in a continuous Arrhenius plot, as seen in Fig. 12D. The thermodynamic parameters for the rate-determining step were calculated from the Arrhenius plot as described in

EXPERIMENTAL PROCEDURES. The activation energy of *in vitro* M.PabI methylation was determined as 56.4 kJ mol^{-1} (Table 3).

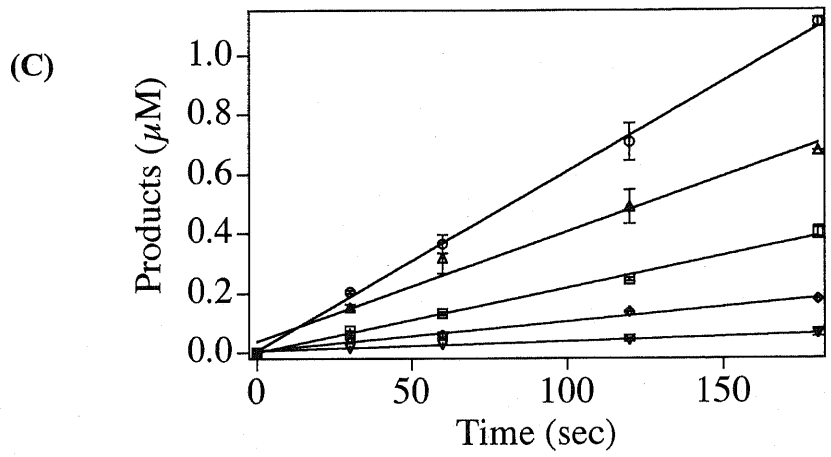


Fig. 12C Effect of incubation temperatures on the initial velocity of M.PabI reaction was observed at 37°C (reverse triangles), 55°C (diamonds), 65°C (squares), 75°C (triangles) or 85°C (circles). The obtained data were plotted with error bars.

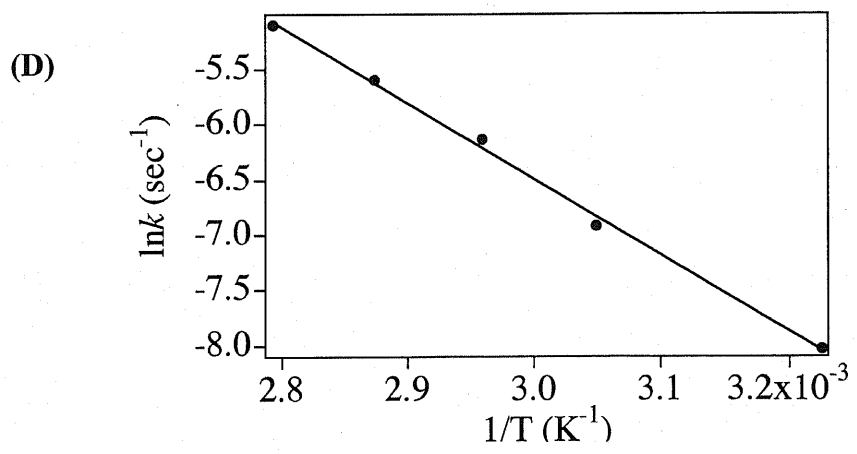


Fig. 12(D) A continuous Arrhenius plot was obtained over a temperature range of 37–85 °C from the initial velocities that were calculated by the chart in panel C.

Kinetic parameters

I performed next experiments to determine the initial reaction velocities. Both the methylation products — methylated DNA and S-adenosylhomocysteine (AdoHcy) — are known to inhibit the methyltransferase activity in some cases (57,58). Therefore, I kept molarity of these products less than 20% of the added oligo-duplex substrate at the start of the reaction. Although the M.PabI enzyme exhibited its maximal reaction rate at 85°C (Fig. 12B), which is considered to be the optimal incubation temperature (Fig. 10A), the M.PabI methylation at this temperature was too rapid to quantitatively analyse its kinetics by manual sampling and the filter-binding assay. Therefore, I determined the kinetic parameters of methylation at 75°C.

According to double reciprocal plots of initial velocities versus substrate concentrations, kinetic parameters (K_m^{DNA}, K_m^{AdoMet} and k_{cat}) were calculated. The catalytic constant of k_{cat} was obtained as the ratio of maximal velocity to enzyme concentration. The specificity constant was determined as k_{cat}/Km^{DNA}. The k_{cat} of M.PabI with the oligo-duplex #1 was calculated as 0.041 sec^{-1}. The double reciprocal plots of $1/v$ *versus* 1/[DNA] (Fig. 13A) and $1/v$ *versus* 1/[AdoMet] (Fig.13B) gave Km^{DNA} and Km^{AdoMet} as 159 nM and 1.28 μM, respectively. Therefore, the k_{cat}/Km^{DNA} of M.PabI was calculated as 2.57×10^5 sec^{-1} M^{-1} (Table 3).

(A)

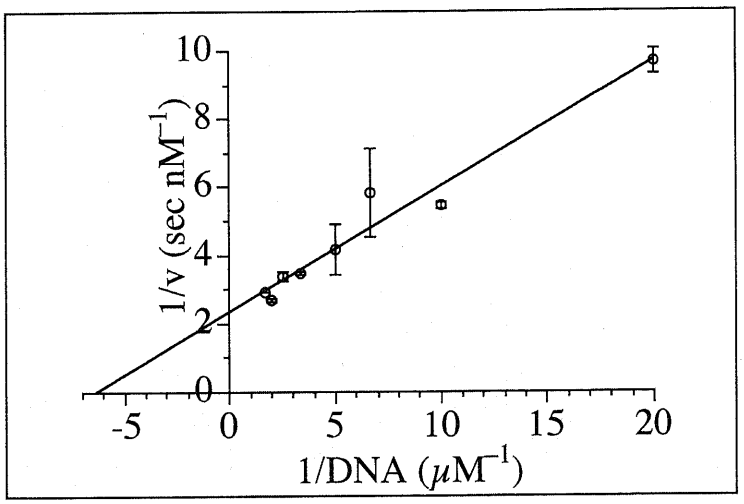


Fig. 13. Steady-state kinetics of M.PabI methylation.

All the following series of experiments were carried out at least three times to assess their reproducibility. (A) Double reciprocal plots of the initial reaction velocity versus the concentration of the oligo-duplex #1. The concentration of [*methyl*-^{14}C]AdoMet was fixed at 15 μM.

(B)

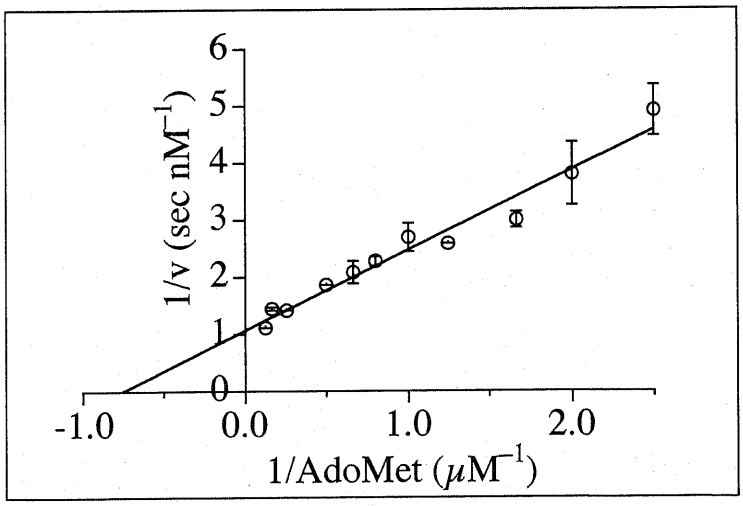


Fig. 13(B) Double reciprocal plots of the initial reaction velocity versus the concentration of AdoMet. The concentration of oligo-duplex #1 was fixed at 10 μM.

Table 3: Thermodynamic and kinetic parameters for methylation by M.PabI.

	at 75 °C	
Thermodynamic parameters [a]	$kJ \cdot mol^{-1}$	$kcal \cdot mol^{-1}$
E_a	56.5	13.5
$\Delta H^{\neq}$	28.1	6.7
$T\Delta S^{\neq}$	-35.2	-8.4
$\Delta G^{\neq}$	63.3	15.1
Kinetic parameters		
k_{cat} (sec^{-1})	0.041	
K_m^{DNA} (nM)	159	
K_m^{AdoMet} (μM)	1.28	
k_{cat}/Km^{DNA} (sec^{-1} M^{-1})	2.57×10^5	

[a]: Changes in activation energy (E_a), enthalpy ($\Delta H^{\neq}$), entropy ($\Delta S^{\neq}$) and free energy ($\Delta G^{\neq}$) at 75°C were calculated from coefficients the Arrhenius equation.

DISCUSSION

M.PabI as an N6-adenine methyltransferase of the PabI RM system

In this study, I provided evidence that M.PabI, the product of *pabIM* (PAB2246), is a methyltransferase that methylates 5′-GTAC-3′ (Fig. 8) on double-stranded DNA to generate 5′-GTm6AC-3′ (Fig. 9). This mode of methylation is as predicted from its similarity with M.TaqI and other DNA methyltransferases.

I analyzed methylation at the PabI recognition site (5′-GTAC-3′) by the purified M.PabI enzyme. The 40-mer oligo-duplex substrates with one or no PabI site (#1, #2, #3, #4 or #5) in Table 2) were used. I confirmed that M.PabI is able to methylate 5′-GTAC-3′ in an 18-mer oligo-duplex (date not shown). These demonstrated that M.PabI can methylate 5′-GTAC-3′.

However, I cannot exclude the possibility that M.PabI can also methylate some other sequences. In this experiment, the sequences present on the negative-control DNA (#2, #3, #4 or #5) GCTA, GACT, AGCT and ATGC were excluded as a recognition sequence. To decide all the possible recognition sequences of the length of 4 bp, I need to examine the following sequences: GTCA, GACT, GCAT, GTAC, TGAC, TAGC, TCAG, TCGA, TAGC, TGCA, ACGT, AGTC, ATCG, ACTG, CTGA, CTAG, CATG, CGTA, CAGT and CGAT.

Moreover, I confirmed that DNA methylated *in vitro* by M.PabI could not be cleaved by R.PabI (date not shown). Similar resistance was obtained *in vivo* methylation by M.PabI and *in vitro* challenge by RsaI, isoschizomer of PabI.

These experiments combined with the genome analysis (25,32) suggest that M.PabI and PabI form a Type II RM system in *P. abyssi*. This conclusion would be made stronger if the genomic DNA of *P. abyssi* is methylated at the PabI sites. This could be examined by its PabI digestion followed by PCR of a PabI site. Homologous regions on *P. horikoshii* genomic DNA would serve as control.

I assigned the nine conserved motifs of amino-methyltransferases within the N-terminal half of M.PabI by the guidance of M.TaqI structural analyses (Fig. 5B), since the catalytic chains of M.TaqI methyltransferase were retrieved as the most related structures for M.PabI protein. Saenger and his collaborators had elucidated the molecular details of binding AdoMet to M.TaqI on their crystallographic and fluorometric analyses (46,47). They had described that adenosine is recognized by the specific hydrogen bonds to Asp89 and Phe90, which are supported by the van der Waals contacts with the side-chains of Ile72, Phe90, and Phe146. In the ribose moiety, the van der Waals contacts to Ala47 and Pro107 assist the single hydrogen bond between carboxylate group and Glu71. The methionine moiety is held in the optimal position by the hydrogen bond with Thr23 and the salt-bridge to Glu45, Ala47, and Cys48. When I applied these AdoMet-interacting amino acids on the putative catalytic domain of M.PabI according to the alignment of Fig. 5B, most of them were conserved as eight identical or two similar (both Glu45 and Glu71 were replaced by Asp) amino acids, except for Phe90 of M.TaqI corresponding to Gly92 of M.PabI. The importance of Tyr108 and Phe196 of M.TaqI had been additionally inspected by enzymological analyses with the substitution mutants (59). These aromatic amino acids, which are significant for stabilizing an extrahelical target adenine in the optimal position for methyl group transfer, were also conserved within the M.PabI catalytic domain as Phe111 and Phe194, respectively (Fig. 5B). Consequently, my motif-assignment for M.PabI is adequate to suggest that the reaction mechanism of M.PabI is probably similar to that of M.TaqI methyltransferase. However, it is necessary to determine the role for these assigned amino acids in the sequence alignment (Fig. 5B) by mutagenesis analysis in order to confirm this point.

On the other hand, together with the restriction enzyme PabI, which is encoded by a neighbouring

gene and cleaves double-stranded DNA at this sequence (32), M.PabI was revealed to constitute a Type II RM system in *P. abyssi*. This finding, combined with genome sequence comparison between *P. abyssi* and *P. horikoshii* (20,49), provides strong evidence for the mobility of the PabI RM system. The similarity of parts of M.PabI with the modification enzymes of diverse eubacteria (Fig. 5B, C) and archaea also suggests its extensive horizontal transfer, as is the case with many RM genes (17,20,49).

Furthermore, the similarity of M.PabI with the S subunit of several Type I RM restriction enzymes in the carboxy-terminal half and with the M subunit of an atypical Type II RM system in the amino-terminal half raise the possibility of some evolutionary relationship with them.

Reaction conditions

To maximally induce the enzymatic activity of M.PabI from the hyperthermophile *P. abyssi*, I investigated the optimal reaction conditions for M.PabI in my *in vitro* assay. The optimal hydrogen exponents of pH 5.5–6.5 and the optimal NaCl concentrations of 200–500 mM (Fig. 10B,C) were common among several Pyrococcal enzymes, such as DNA polymerase (60,61) and the PabI restriction enzyme (32). These conditions may be representative of real-life conditions, as Pyrococcus strains were discovered in deep-sea hydrothermal vents at pH 6.8. The reduction of M.PabI activity under NaCl-free conditions (Fig. 10C) was presumably caused by the instability of oligo-duplex substrates (62).

Zn^{2+} acted as a strong inhibitor of M.PabI-catalysed methylation (Fig. 11A), in contrast to Mg^{2+}, Ca^{2+} and Mn^{2+}, all of which showed no significant inhibition. It has been reported that the methylation activities of M.HindIII (group β), M.LlaCl (group β) and M.EcoVIII (group β) were inhibited by Zn^{2+} (63). Moreover, these enzymes were also inhibited by Mg^{2+}. In case of Mg^{2+}, the modification enzymes might effectively modulate the flow of genes among bacteria by strengthening the restriction

activity of cognate restriction enzymes (63). Although any effects of divalent cations on the activity of the restriction enzyme PabI are yet to be investigated, insight into the biological function of this restriction-modification system might be obtained in the future.

Hyperthermophilic DNA methyltransferase activity

Although the M.PabI protein has been expressed, folded and matured in *E. coli* cells under 'non-thermophilic' conditions, the purified M.PabI exerts extremely hyperthermophilic methylation activity. To my knowledge, this is the most thermophilic of all of the characterized DNA methyltransferases reported so far. M.TaqI was reported to make 5′-TCGm6A-3′ at 60°C (64). M.PspGI (5′-CCWGG-3′), from a Pyrococcus species, was shown to exert its activity *in vivo* at 37°C in *E. coli* cells (28). The expression of any other putative DNA methyltransferases — for example, M.PhoI (31) and M.ApeKI (65) — that have been identified within the genomes of hyperthermophilic archaea has not yet been reported.

M.PabI activity peaked at 85°C in my *in vitro* assay (Fig. 10A). Furthermore, the activity at 95°C was equivalent to that at 65°C, even though the reaction at 95°C must have been restrained both by denaturation of the 40-mer oligo-duplex and decomposition of AdoMet (55,56). M.PabI should lose some activity at the high tempearature (Fig. 12A). Therefore, I cannot exclude the possibility that the optimal temperature for M.PabI *in vitro* itself is higher than 85°C and close to the optimal growth temperature of 96°C for *P. abyssi* (30). In order to increase of M.PabI activity at high temperature (over 85°C), the addition of polyamine to *in vitro* reaction mixture is expected to avoid the composition of 40-mer oligo-duplex and AdoMet. Most organisms, including both prokaryotes and eukaryotes, produce only three standard polyamines. In contrast, extreme thermophiles generally produce two additional types of polyamine: longer linear polyamines and/or branched polyamines (Fig.

14). Theses unusual polyamines in *Thermus thermophilus* stablize the conformation of dsDNA at high temperature(1).

The half-life of the M.PabI activity during pre-heating at 85°C was estimated to be 19 min (Fig. 12A). The restriction enzyme PabI retained half the activity after 1-hour pre-heating at 85°C (32). Both the enzymes of the PabI RM system, therefore, showed comparable thermo-tolerance, even though PabI was synthesized *in vitro* (32) and M.PabI was produced *in vivo*. Additionally, PabI exhibited its appropriate endonuclease activity at approximately pH 6 with 100–200 mM NaCl (32). This optimal reaction condition was also in agreement with that of M.PabI, as I demonstrated in this study.

Moreover, the point that Pyrococcus strains were discovered in deep-sea hydrothermal vents might have to be considered following that: Deep-sea hyperthermophiles thrive in environments with hydrostatic pressures ranging from 200 to 360 atm (hydrothermal vents located as far as 4000 m below sea level. Therefore, strictly, the methylation of M.PabI (with respect to K_m^{DNA}, K_m^{AdoMet} k_{cat} or heat resistant) is might not be able to disregard pressure (66).

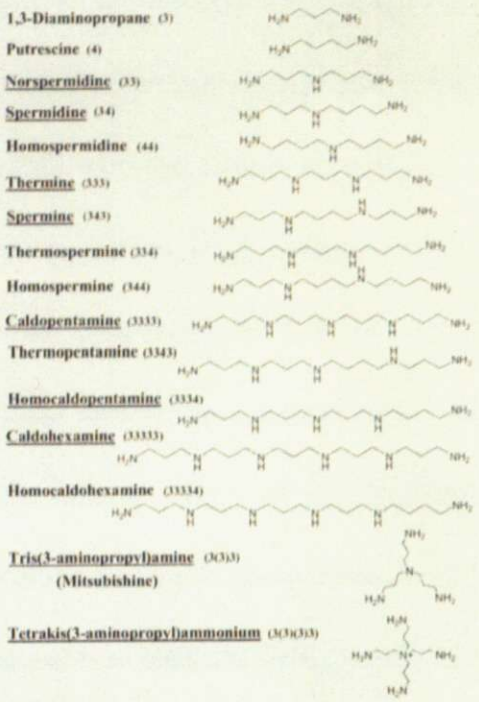


Fig. 14 The 16 polyamines present in the cells of T. thermophilus(1).

Fig. 14

The Arrhenius diagram for M.PabI reactions at 37–85°C showed a linear plot (Fig. 12D), which was typical of thermophilic enzymes (26) and indicated that the reaction velocity of M.PabI-catalysed methylation is dependent on the incubation temperature in a simple manner. This result indicated that the functional conformation of the M.PabI enzyme was essentially invariant throughout the temperature range examined (26,45). Furthermore, I calculated thermodynamic parameters of the methylation, which includes the activation energy of 56.4 kJ mol^{-1}. To my knowledge, this represents the first report of activation energy for DNA methyltransferases. This value was comparable to the known values of activation energy of other thermophilic enzymes, such as RadA recombinase (31.7 $kJ \cdot mol^{-1}$ at 70°C (45)) and D-glyceraldehyde-3-phosphate dehydrogenase (46.8 $kJ \cdot mol^{-1}$ at 52–70°C (67)) (Table 4). The value of the activation energy of M.PabI suggests that the methylation by M.PabI has an advantage over enzymes (such as RNA polymerase with activation energy of 193.2 $kJ \cdot mol^{-1}$ at

28-37°C) derived from *E.coli.* (68) although it is difficult to compare different kinds of enzymes.

Next, I determined the kinetic parameters of the M.PabI reaction, as shown in Fig. 13. The k_{cat} of M.PabI with the 40-mer oligo-duplex #1 was calculated as 0.041 sec^{-1}. The k_{cat} values for other adenine-specific DNA methyltaransferases are reported as follows: 0.124 sec^{-1} of M.EcoRI with 14-mer oligo-duplex (40); 0.015 sec^{-1} of M.T4Dam with 14-mer oligo-duplex (69); 0.733 sec^{-1} of M.TaqI with 36-mer oligonucleotide analogue (59); 0.0022 sec^{-1} of M.KpnI with 38-mer oligo-duplex (70) (Table 4). All the DNA methyltransferases that have been kinetically characterized so far tend to exhibit slow kinetic turnover rates. This low turnover, which is coupled with strong binding to target sequences as indicated by K_m, means that the value of k_{cat}/K_m is high and assures their high specificity for target sequence (70).

The hyper-thermostability of M.PabI is ideal for a DNA methylation reagent, and it will be also a powerful tool for pursuing, in detail, the mechanisms of DNA methylation. As application of M.PabI, the marker such as site specific methylation in high temperature for target DNA including GTAC sequence (ex. genetic engineering or gene diagnosis: SNP *etc.*) might be possible(71). In addition, analyses of structural and functional basis for its hyperthermostability will contribute to protein engineering.

Table 4: Comparison of thermodynamic and kinetic parameters for methylation by M.PabI.

	Thermodynamic parameter		
Thermophilic enzymes	$kJ \cdot mol^{-1}$		Ref.
M.PabI	56.5	at 75°C	this work
RadA recombinase	31.7	at 70°C	(45)
D-glyceraldehyde-3-phosphate dehydrogenase	46.8	at 52–70°C	(67)

	Kinetic parameters				
DNA methyltransferase	K_m^{DNA} (nM)	K_m^{AdoMet} (μM)	k_{cat} (sec^{-1})	k_{cat}/Km^{DNA} (sec^{-1} M^{-1})	Ref.
M.PabI (m6A)	159	1.28	0.041	2.57×10^5 : 75°C	this work
M.TaqI (m6A)	600	3.7	0.733	1.22×10^6 : 60°C	(59)
M.EcoRI (m6A)	2.41	0.125	0.124	0.51×10^8 : 37°C	(40)

FURTHER DISCUSSION

As the further discussion, I realized the necessity of doing some experiments for hyperthernophilic DNA methyltransferase M.PabI in this study.

The *in vitro* methylation activity of the M.PabI was detectable at a minimum of 35°C and was exerted maximally at 85°C. Impressively, significant activity were detected at 90°C and 95°C (Fig. 10A). The decrease at these higher temperatures may have been caused, at least in part, by denaturation of the 40-mer oligo-duplex, decomposition of AdoMet and inactivation of M.PabI itself (Fig. 10A and Fig. 12). Therefore, a shorter incubation time, addition of more oligo-duplex, closure of oligo-duplex ends by a hairpin form, addition of more AdoMet might result in different pattern of temperature-dependence at these temperatures. Furthermore, addition of some polyamines or higher salt concentrations (300~400 mM NaCl) might improve methylation reaction. At the higher salt concentration, the 40-mer oligo-duplex would be stabilized. Some polyamines stabilize the conformation of dsDNA at high temperatur (1). Methylation reaction of M.PabI may also be stabilized by addition of non-specific DNA.

The experiment in Fig. 10A and 12 showed that M.PabI is highly thermophilic. However, this result did not analyze stability of M.PabI in detail. The stability of M.PabI to heat may be estimated from the far-UV CD spectra. The M.PabI might maintain its secondary structure at the high temperature (over 85°C) or be denatured irreversibly above high temperature. The conformational stability of M.PabI might also be estimated from denaturation by treatment with guanidine hydrochloride.

During the investigation of optimal reaction conditions for M.PabI, I found that Zn^{2+} acted as its

strong inhibitor (Fig. 11A). It is not clear how Zn^{2+} acts as an inhibitor of this and other DNA methyltransferases (63). Nobody has examined whether this inhibition might be due to binding of Zn^{2+} to the loop structure of the active site.

On the other hand, the following is suggested for the inhibitory effect of Mg^{2+} in several other modification enzymes. Inhibition of Type II DNA methyltransferase and stimulation of its cognate restriction enzyme by Mg^{2+} cause imbalance between two enzyme activities, which in turn may cause cell death and genome rearrangements, effectively modulating the flow of genes among bacteria (63). The same scheme could apply to Zn^{2+}. Although any effects of divalent cations on the activity of the restriction enzyme PabI are yet to be investigated, insights into the biological function of this restriction-modification system might be obtained in the future. However, we have to remember that Zn^{2+} concentration used in the present study could be much higher than its intracellular concentration.

I determined some optimal conditions of M.PabI *in vitro*. These conditions may be representative of real-life conditions, as Pyrococcus strains were discovered in deep-sea hydrothermal vents at pH 6.8. If PAB2246 (M.PabI) is functional gene in *P. abyssi*, this genomic DNA will methylate and protect by M.PabI. However, I did not attempt to demonstrate whether genomic DNA of Pyrococcus strains was methylated or not by M.PabI enzyme. In order to detect methyaltion by M.PabI in Pyrococcus genome, this genome could be examined by its PabI digestion followed by PCR of a PabI site. Homologous regions on *P. horikoshii* genomic DNA would serve as control.

In this study, together with the restriction enzyme PabI, which is encoded by a neighboring gene (32), M.PabI was revealed to constitute a Type II RM system in *P. abyssi*. This finding, combined with genome sequence comparison between *P. abyssi* and *P. horikoshii* (20,25,49), provides strong evidence for the mobility of the PabI RM system.

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