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New DNA methyltransferase M.AgsI produces TTSA(m6A)

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ABSTRACT

Agrococcus species 25 DNA was cloned in pUC19 plasmid of *Escherichia coli*. Cloned DNA fragment contained two Opened Reading Frames with 8 amino acid motives which belonged to amino DNA methyltransferases. Thus M.AgsI can be the first of subunit adenine-(N6)-DNA methyltransferase. The enzyme was purified from the recombinant strain by chromatography on P-11 Phosphocellulose, Heparin-Sepharose and Hydroxyapatite. M.AgsI specificity was determined by a study of protection of lambda DNA methylated with M.AgsI against cleavage with some restriction endonucleases. A sensitivity of restriction endonucleases to M.AgsI-methylation was studied.

KEYWORDS

Gene cloning;
Enzyme isolation;
Subunit DNA methyltransferase;
Enzyme specificity;
Restriction endonuclease;
Methylation sensitivity.

INTRODUCTION

DNA methyltransferases (MTases) transfer the methyl group from S-adenosyl-L-methionine (SAM) to cytosine or adenine in double-stranded DNA. The modified bases are 5-methylcytosine (5mC or m5C), N4-methylcytosine (N4mC or m4C) or N6-methyladenine (N6mA or m6A)[1]. The MTases differ in their recognition sequence and the base to be modified within this site. DNA methylation of eukaryotes is involved in processes of an expression of genes, embryonal development, differentiation of cells, protection against viruses, carcinogenesis and aging. DNA methylation of bacteria plays role in DNA replication, reparation, recombination, cellular differentiation, pathogenicity, and also in Restriction-Modification (RM) systems of protection against foreign DNA.

The subject of this work is DNA MTase from *Agrococcus* species 25, which produces the unique ENase AgsI splitting DNA in the site TTS^AA[2]. Therefore MTase M.AgsI must also have unique specificity for protection of cognate DNA like a work of RM systems[3,4]. The purpose of this work is M.AgsI cloning and study of the recombinant enzyme properties and substrate specificity. We have performed a comparative study of sensitivity of different ENases to DNA methylation by M.AgsI.

EXPERIMENTAL

Enzymes, DNA and reagents

Restriction endonucleases, alkaline phosphatase (calf intestinal) and T4 DNA ligase as well as pUC19,

phage λ (dam-, dcm-) and T7 DNAs, ATP, reaction buffers were obtained from Sibenzyme Ltd, Russia. Lysozyme was from Helicon, Russia. Bovine serum albumin (fraction V) (BSA) was from Americanbio.com, USA. S-adenosyl-L-methionine (SAM) – from New England Biolabs Inc, USA.

Strains and culture medium

Escherichia coli K-12 strain RR1 ("mcrC-mrr recA+) was from New England Biolabs Inc, USA. *Agrococcus species* 25 with RM-system AgsI was from Sibenzyme Ltd, Russia. L-broth contained 10 g/L Tryptone (Organotechnie, France), 5 g/L Yeast extract (same firm), 5 g/L NaCl, 0.5 g/L $MgCl_2$, 1 mg/L thiamin. For plates L-broth was supplemented with 15 g/L agar (C.T. Roeper GmbH, Germany). Ampicillin was added up to 100 μg/mL, streptomycin – up to 25 μg/mL.

Transformation and colony screening procedures

A. species 25 cells were grown in the flasks containing 300 mL L-broth at 25°C with 130 rpm for 40 h. 10 μg *A. species* 25 DNA, isolated as described [5], was digested in the reaction mixtures of 200 μL with 20 units of PstI (CTGCA^G) for 1 h at 37°C. Digested DNA was cleared by phenol and precipitated with ethanol. Hybrid plasmids of pUC19 were constructed by ligating 10 μL of PstI-digested *A. species* 25 DNA with 3.0 μg phosphatase-treated pUC19/PstI by using 2,000 units of T4 DNA ligase. The reaction was allowed to proceed for 16 h at 4°C in 20 μL of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP. The ligated DNA was precipitated with ethanol and dissolved in 12 μL of water.

2.5 μg DNA of the ligation mixture was added to approximately 3.0×10^9 RR1 cells in 50 μL and a mixture was treated for the electroporation in "Easyject Prima" according to the manufacturer's instructions (EquiBio, UK). After electroporation the mixture was added to 1.0 mL of L-broth and incubated for 1 h at 37°C. For calculation of transformants 10 μL of the culture were plated on L-agar with ampicillin (50 clones were grown after 16 h at 37°C). The genomic library (1.0 mL) was grown in 100 mL of L-broth with ampicillin for 16 h at 37°C with shaking.

Total DNA of hybrid plasmids was isolated from 100 mL culture of 5000 transformants with use QIAGEN Plasmid Maxi Kit (Germany) according to the manufacturer's instructions. 10 μg DNA was digested with 10 units of AgsI for 4 h at 37°C, precipitated with ethanol and dissolved in 15 μL of water. According to the protocols[5] the digested DNA was incubated with 3.0×10^7 RR1 cells that had been treated with $CaCl_2$. Cells were plated on L-agar containing ampicillin and 120 clones were obtained after 16 h at 37°C. The each clone was grown in L-broth with ampicillin and plasmid DNA was isolated with QIAGEN Plasmid Miniprep Kit (Germany). DNAs from clones were cut with AgsI and analyzed by electrophoresis in 10 g/L agarose gel. AgsI doesn't cleave DNA of plasmid pM.AgsI-106 and E.coli RR1 was transformed with this plasmid. The obtained clone was named *E. coli* N106 (pM.AgsI).

DNA sequencing

The sequencing of DNA was carried out with ABI 3130xI Genetic Analyzer device (Applied Biosystems, USA).

Purification of AgsI MTase from *Escherichia coli* N106 (pM.AgsI)

E. coli N106 (pM.AgsI) was grown in flasks containing 300 mL L-broth with ampicillin at 30°C with stirring for 20 h. Cells were harvested at 8000 x g and stored at -20°C.

The enzyme purification was carried out at 4ÚC with Buffer A (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 7 mM β-mercaptoethanol) or Buffer B (10 mM K-phosphate pH 7.2, 0.1 mM EDTA, 7 mM β-mercaptoethanol). 12 g of harvested cells, suspended in 60 mL of Buffer A with 0.2 M NaCl, 5 g/L Triton® X-100 (non-ionic detergent, Sigma) and 0.1 mM PMSF (proteases inhibitor), were disrupted by ultrasound on Soniprep 150 (MSE, UK). Cell debris was removed by centrifugation at 20,000 x g for 1 h. The supernatant was loaded onto a Phosphocellulose P11 (Whatman, UK) of 40 mL bed volume and eluted with 500 mL of a 0.2 to 0.6 M NaCl gradient in Buffer A. Fractions were assayed for M.AgsI activity as described below. The activity-containing fractions were pooled, and, after a dialysis against 500 mL of Buffer A with 0.05 M NaCl were loaded onto a Heparin-Sepharose (Bio-Rad, USA) of 5 mL bed volume column. Protein was eluted with 100 mL of 0.05-0.6 M NaCl gradient in Buffer A. Fractions containing activity

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were loaded onto a Hydroxyapatite (Bio-Rad, USA) of 10 mL of bed volume column and eluted with 200 mL of a 0.01-0.3 M K-phosphate gradient, pH 7.2 in Buffer B. Fractions containing activity were pooled, dialysis against 100 mL of Buffer A with 500 g/L glycerol, 0.05 M of NaCl and stored at -20°C.

DNA methyltransferase activity assay

MTase assay was based on a protection methylated DNA from cognate ENase. M.AgsI sample was incubated in 50 µL with 50 µg/mL λ DNA (dam–, dcm-), 1 mM SAM, 33 mM Tris-AcOH, pH 7.9, 1 mM EDTA, 66 mM KAc 1 mM DTT, 0.1 mg/mL BSA in a well of 96 Well Microplate (Medpolymer, Russia) at 25°C for 1 h. Then, Microplate was warmed up on a bath at 65°C for 10 min to inactivate the enzyme. To cleave not modified DNA the mixture was supplemented with 5 µL of 1000 u/mL AgsI restriction endonuclease diluted with 10 mM Tris-HCl pH 7.6, 50 mM KCl, 0.1 mM EDTA, 200 µg/mL BSA, 1 mM DTT and 100 mM $MgAc_2$. After incubation at 37°C 1 h the mixture was analyzed by electrophoresis in 8 g/L agarose gel as described[5]. One unit of M.AgsI activity methylated 1 µg of λ DNA in 1 h blocking the activity of AgsI restriction endonuclease.

DNA methylation with M.AgsI

Reaction mixture containing 0.4 mg/mL λ or T7 DNA, 10 mM SAM, 33 mM Tris-AcOH pH 7.9, 1 mM EDTA, 66 mM KAc, 1 mM DTT, 0.1 mg/mL BSA and 100 u/mL M.AgsI enzyme in a total volume of 1.0 mL, was incubated at 25°C for 20 h. Then, 0.1 mL of 100 g/L SDS (Sigma) and 0.2 mL of 3 M KCl were added. The mixture was warmed at 65°C for 10 min to dissolve SDS-precipitate, cooled at 0°C for 15 min for precipitate formation. The supernatant, pooled after centrifugation at 10,000 x g for 3 min, was mixed with 0.6 volume of i-PrOH. DNA pellet was rinsed with 800 g/L EtOH, dried on air and dissolved in 0.7 mL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) to the concentration 0.5 mg/mL.

DNA cleavage with restriction endonucleases and electrophoresis in agarose gel

Native or M.AgsI-methylated λ or T7 DNA were cleaved in 50 µl reaction mixture containing 50 µg/mL DNA, 0.1 mg/mL BSA, appropriate SE buffer, 125 u/mL restriction endonuclease in a cell of 96 Well Microplate (Medpolymer, Russia) for 2 h at the temperature recommended by the manufacturer (Sibenzyme Ltd). Reaction was stopped by addition 10 µL of 0.25 M Na-EDTA, pH 8.5, 500 g/L sucrose and 5 g/L bromphenol blue. The Microplate was warmed up on a bath at 65°C for 10 min and cooled on ice.

The electrophoresis was carried out in 8 g/L of LE agarose (Segenetic) in TAE buffer with 0.5 µg/mL ethidium bromide (Sigma) at 5 V/cm for 2.5 h as described[5]. The fluorescence of DNA was revealed on the Herolab GmbH device.

Determination of DNA methyltransferase specificity

M.AgsI recognition sequence and a methylated nucleotide were determined based on the methylation sensitivity of restriction endonucleases[2] and DNA cleavage pattern simulation as described earler[6]. Native and M.AgsI-methylated λ DNA (dam-, dcm-), were cut by ENases AgsI (^TTSAA), AluI (AG^CT), HindIII (A^AGCTT) and EcoRI (G^AATTC). Then DNA fragments were analyzed by the electrophoresis in 8 g/L agarose gel. Protection of DNA cleavage with restriction endonucleases due to AgsI-methylation was simulated with PC to reveal an overlapping of methylation and cleavage sites. Methylation of TTSA(mA) in λ DNA was simulated by replacement of TTSAA by NTSAN and designated (T=>N)TSA(A=>N). Then, the simulated cleavage patterns of native DNA and DNA with the replaced nucleotides by restriction endonucleases were constructed with Vector NTI program. In this program a restriction endonuclease didn't cut recognition site if any of bases was replaced by "N". M.AgsI specificity was determined by an analysis of experimental data and simulated patterns of DNA cleavage.

RESULTS AND DISCUSION

Selection of clones carrying the M.AgsI gene and the sequence analysis

MTase clones were selected by the resistance of recombinant DNA to the cognate restriction endonuclease hydrolysis according to[7]. A genomic

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library of *A. species* 25 DNA was obtained in pUC19/ PstI vector DNA as 5000 *E. coli* RR1 transformants. A total DNA of hybrid plasmids was digested with AgsI and used for additional RR1 transformation. Plasmid DNAs from 120 clones were cut with AgsI and analyzed by electrophoresis in 10 g/L agarose gel. A DNA of pM.AgsI-106 plasmid resistant to AgsI cleavage was used for *E.coli* RR1 retransformation. The obtained clone was named *E. coli* N106 (pM.AgsI). The insertion of pM.AgsI-106 was sequenced. A restriction map of this recombinant plasmid with ORF of M.AgsI gene is shown in Figure 1.

The cloned *A. species* 25 DNA fragment contained two Opened Reading Frames (ORF) named *ags*IMA and *ags*IMB. Their nucleotide and amino aside sequences were deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov/nuccore/LN869920.1). The deduced amino acid sequences corresponding to *ags*IMA and *ags*IMB contained 9 conservative motives which are specific for adenine (N6) – and cytosine (N4) amino MTases[8]. The main motives I (with A-G-G) and IV (NPPF) are located in M.AgsIB and M.AgsIA, respectively. Amino acids analysis with Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed a homology with amino acid sequence of M.TaqI (http://www.ncbi.nlm.nih.gov/protein/AAA27506.1) [8], which modifies adenine in a recognition site TCGA (Figure 2).

Surprisingly, M.AgsIB contained motives I, X and presumable motives II and III. These four motives

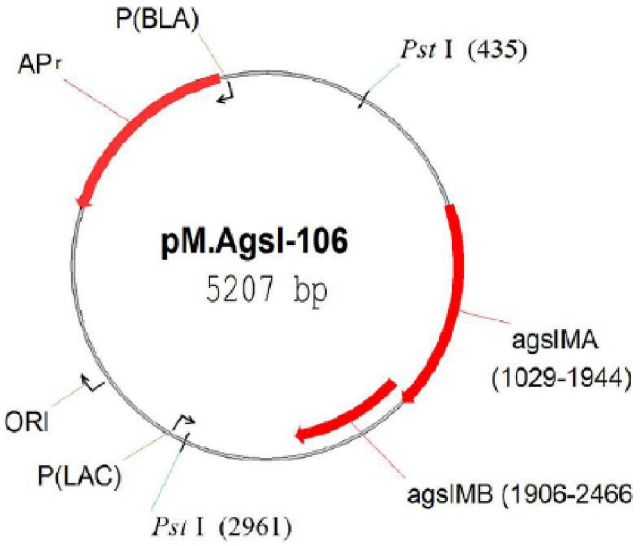

Figure 1 : Restriction map of pM.AgsI-106 plasmid DNA: PstI, ligation, sites (bp); 435 – 2961 bp, *A.* species 25 DNA; 2961 – 435 bp, pUC19 DNA; ORI, replication start; P(BLA), β-lactamase promoter; Ap^r, ampicillin resistance; P(LAC), β-galactosidase promoter; agsIMA and agsIMB, ORFs of DNA methyltransferase M.AgsI subunits. The map was simulated with Vector INT program

corresponded to SAM-binding domain[8]. Another subunit M.AgsIA contained other motives IV-VIII. The sequence NPPF in catalytic motive IV is typical for N6-Mtases[8]. Thus we believe that M.AgsI enzyme consists of two subunits.

Now there are only three known DNA MTases M.AquI, M.EaeI and M.EcoHK31I which consist of two subunits. All of them belong to the class of C5 MTases[2]. M.AgsI is a first N6-MTase, which consists

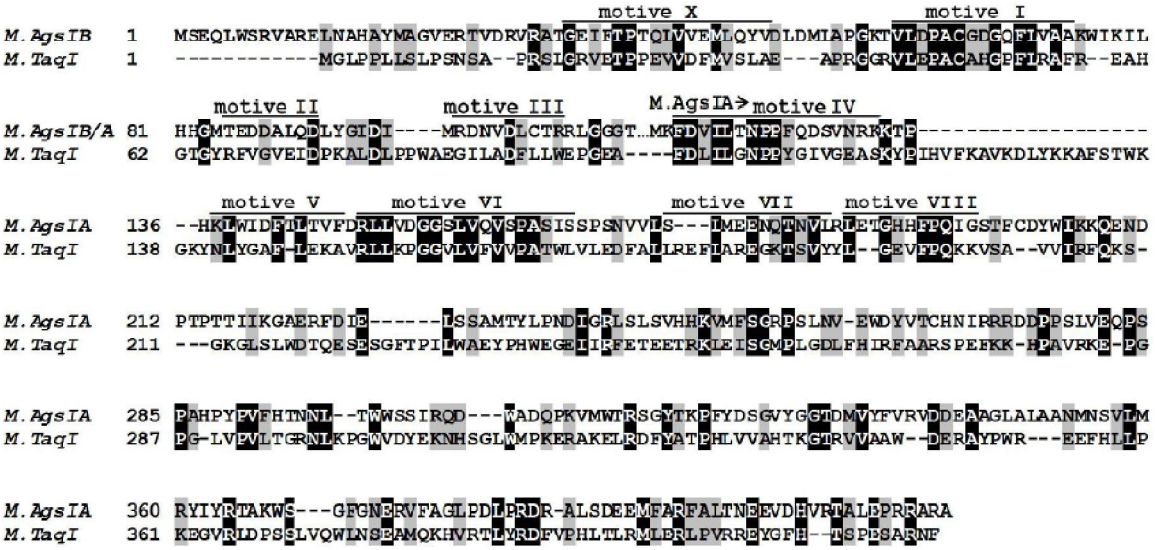

Figure 2 : Amino-acid alignment of the subunits B and A of M.AgsI with M.TaqI MTase (TCGm6A): A coincidence of identical amino acids is marked by black, of similar – by gray

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of two subunits. C-terminal part of M.AgsIB protein has no homology to any of known proteins and its role remains unclear. We believe that binding of two subunits of the enzyme may be one of possible role of this part of MTase.

M.AgsI purification and characterisation.

E.coli N106 (pM.AgsI) was grown and the enzyme was purified from cellular extract by subsequent chromatographic steps on Phosphocellulose P11, Heparin-Sepharose, and Hydroxyapatite as described in "Materials and Methods". M.AgsI was obtained with activity 10 u/μL.

The specificity of M.AgsI was determined based on the methylation sensitivity of restriction endonucleases and DNA cleavage pattern of native and M.AgsI-methylated λ DNA simulation[6] as described in "Materials and Methods". Figure 3 (A and B) shows that the methylation of λ DNA with M.AgsI (Lanes: 2) completely blocks DNA hydrolysis with cognate ENase AgsI (^TTSAA). Simulation showed that M.AgsI recognition site is overlapped with a part of recognition

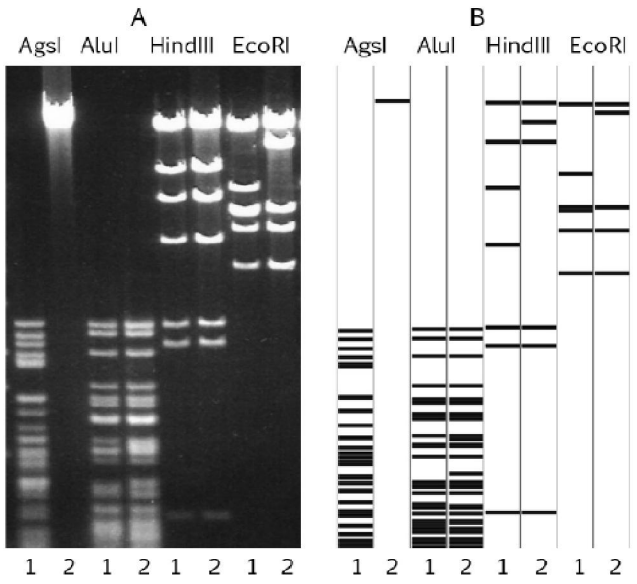

Figure 3 : Determination of M.AgsI-methylation specificity by blocking of restriction endonucleases (A) and by modeling (B): Lanes: 1, unmethylated λ DNA; 2, λ DNA methylated with M.AgsI (A) or edited (T=>N)TSA(A=>N) (B). DNA was digested with AgsI (^TTSAA), AluI (AG^CT), HindIII (A^AGCTT) and EcoRI (G^AATTC): Electrophoresis was performed in 8 g/L agarose, simulation with Vector NTI program

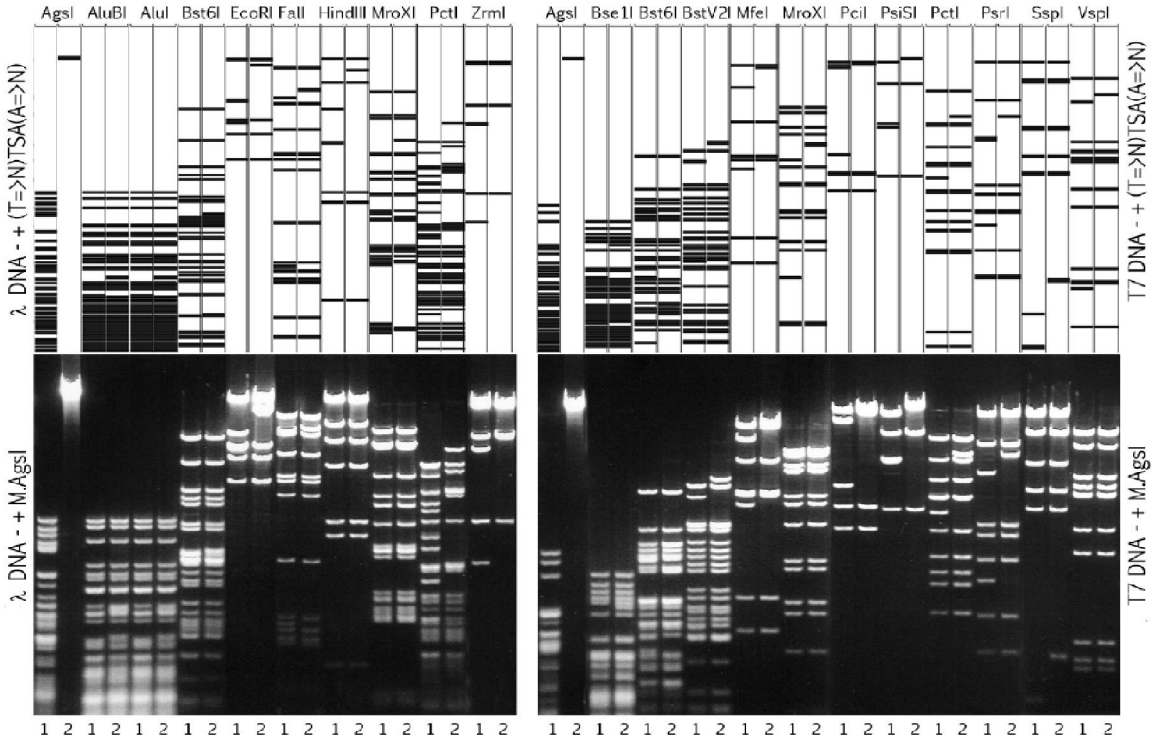

Figure 4 : Simulated and experimental cleavage of native and M.AgsI-methylated λ and T7 DNA with restriction endonucleases: Lanes: 1, native nucleotide sequence and DNA; 2, sites of the sequence were edited as (T=>N)TSA(A=>N), or DNA methylated with M.AgsI. Simulation was performed with Vector NTI program. Cleavage was carried out with each endonuclease activity sufficient for 5-fold excess of DNA. DNA bands in 8 g/L agarose were visualized by fluorescence with ethidium bromide

TABLE 1 : Methylation sensitivity of some restriction endonucleases.

ENase	Methylated site	Cleavage (%)*	DNA
AgsI	5'-TTSA(m6A)-3' 3'-(m6A)ASTT-5'	0*	λ, T7
AluBI	5'-(m6A)GCT-3' 3'-TCGA-5'	0	λ
AluI	5'-(m6A)GCT-3' 3'-TCGA-5'	0	λ
Bse1I	5'-(m6A)CTGG-3' 3'-TGACC-5'	0*	T7
Bst6I	5'-CTCTTC-3' 3'-GAG(m6A)AG-5'	0*	λ, T7
BstV2I	5'-GA(m6A)GAC-3' 3'-CTTCTG-5'	0*	T7
EcoRI	5'-GA(m6A)TTC-3' 3'-CTTAAG-5'	0	λ
FalI	5'-A(m6A)GNNNNNCTT-3' 3'-TTCNNNNNGAA-5'	0*	λ
HindIII	5'-A(m6A)GCTT-3' 3'-TTCGAA-5'	100	λ
MfeI	5'-CA(m6A)TTG-3' 3'-GTTAAC-5'	0*	T7
MroXI	5'-GA(m6A)NNNNTTC-3' 3'-CTTNNNNAAG-5'	100*	λ, T7
PciI	5'-(m6A)CATGT-3' 3'-TGTACA-5'	0*	T7
PciSI	5'-GCTCTTC-3' 3'-CGAG(m6A)AG-5'	0*	T7
PctI	5'-GA(m6A)TGC-3' 3'-CTTACG-5'	0*	λ, T7
PsrI	5'-GA(m6A)CNNNNNNTAC-3' 3'-CTGNNNNNNATG-5'	0*	T7
SspI	5'-A(m6A)TATT-3' 3'-TTATAA-5'	0*	T7
VspI	5'-(m6A)TTAAT-3' 3'-TAATTA-5'	100*	T7
ZrmI	5'-(m6A)GTACT-3' 3'-TCATGA-5'	0*	λ

The sensitivity of ENases was tested by cleavage λ and T7 DNA methylated by M.AgsI TTSA(m6A). Single letter code: R = A or G, Y = T or C, M = A or C, K= G or T, W = A or T, S = G or C, D = A or G or T (not C), H = A or C or T (not G), B = C or G or T (not A), V = A or C or G (not T), N = A or C or G or T. * Data are obtained for the first time

sites of AluI, HindIII and EcoRI restriction endonucleases. HindIII doesn't cut a sequence (m6A)AGCTT[9,10], however, HindIII cleaves DNA sequence TTSAAGCTT overlapping with M.AgsI. Thus, M.AgsI didn't produce TTS(m6A)A. At the same time, methylation with M.AgsI blocks the cleavage of sites TTSAAGCT and TTGAATTC with AluI (AG^CT) and EcoRI (G^AATTC), respectively. It demonstrates that M.AgsI produces TTSA(m6A), because both ENases are sensitive to methylation even on one DNA strand: AluI doesn't cut (m6A)GCT[11], EcoRI doesn't cut GA(m6A)TTC[12] but cuts G(m6A)ATTC[11].

Thus the results of gene analysis and the probing of the methylated site by restriction endonucleases show that M.AgsI forms 5 '-TTSA(m6A)-3' on DNA and belongs to adenine-(N6)-DNA methyltransferases (EC 2.1.1.72)[1]. M.AgsI possesses a new specificity among known analogs[2] and it can quite be of interest to molecular and genetic works.

Methylation sensitivity of restriction endonucleases.

An essential characteristic of restriction endonucleases is methylation sensitivity of these enzymes. A number of ENases were tested for methylation sensitivity with λ and T7 DNAs methylated by M.AgsI on TTSA(m6A). The model and experimental results are shown in Figure 4 and are interpreted in TABLE 1. If some cases the methylation of particular sequences was simulated as described[6,13].

Some results shown in TABLE 1 confirm known data about methylation sensitivity of restriction endonucleases[2]. Other results were obtained for the first time (* noted cleavage %).

CONCLUSIONS

The gene of a new DNA methyltransferase AgsI, from bacterial strain *Agrococcus* species 25 was cloned in *Escherichia coli*. Recombinant M.AgsI enzyme was isolated and it was shown that this MTase modified the external adenine residue in the recognition sequence TTSAA producing 5'-TTSA(m6A)-3'. M.AgsI differs from all known MTases by the recognition site [2]. The new data on sensitivity of different restriction endonucleases to M.AgsI-methylation have been obtained.

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DECLARATION

The manuscript is original and is not published or communicated for publication elsewhere either in part or full Vladimir Sergeevich Dedkov, Ph.D. 20 October 2015

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