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In vivo DNA Methylation of *Escherichia coli* DH5α and Top10F' Strains by Bacterial Cytosine-5 Methyltransferase *M. Msp1**

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Abstract: At the chromatin level, methylated CpG dinucleotides are R.Msp1 resistant compared with nonmethylated counterparts. The DNA of two *E.coli* strains was analyzed following transformation-with bacterial cytosine-5-methyltransferase gene M.Msp1 in mammalian transfection vector pcDNA3. Expression of the M.Msp1 was tested by R.Msp1 digestion. The results suggest that the DNA of both strains was fully methylated at the CCGG sequences, by the active enzyme under the control of T7 and cytomegalovirus (CMV) promoters. Methylated DNA can not be digested and it exhibits higher fragment sizes in 1% agarose gel in contrast to the untransformed cell DNAs in vivo.

Key Words: Bacterial MTase M.Msp1, *in vivo* DNA methylation

Escherichia coli DH5a ve Top10F' Hücre DNA'larının Bakteri Sitozin-5-Metiltransferaz Enzimi, *M. Msp1*, Tarafından *In vivo* Metilasyonu

Özet: Kromatin düzeyinde metillenmiş CpG dinükleotidleri, metil grubu taşımayan nükleotidlerin aksine bir modifiye restriksiyon enzimi olan R. Msp1 enzimine dirençlidirler. Yapısında metillenmiş dinükleotid (mCpG) taşıyan bu serbest DNA aynı restriksiyon enzimince kesilmez. Bu araştırmada, metillenmiş nükleotidlerin bu dirençli olma özelliğinden yararlanarak başka bir bakteri türüne ait (Moroxella spp.), rekombine metiltransferaz enziminin, farklı hücrelerde benzer metilaz aktivite gösterip göstermeyeceği araştırılmak istenmiştir. Bunun için izogenik olmayan iki *E.coli* suşu (DH5α ve Top 10F'), PCR ile 5' ucuna omurgalı nükleer transport sinyali SV40 VP1 ve markır enzim glutatyon-S-transferaz (GST) gen dizileri takılmış bakteriyel sitozin-5-spesifik metiltransferaz (MTaz) M. Msp1 rekombinant gen füzyonu ile transforme edildiler.

Transforme edilmiş her iki hücre DNA' ları R. Msp1 enzimince test edildi, rekombinant genin her iki hücre grubunda da aktif olduğu görüldü. Bir memeli gen transfer vektörü olan $pcDNA_3$ içinde, T7 ve sitomegalovirus (CMV) promotorlarının kontrolünde enzimin tamamen aktif olduğu, in vivo DH5α ve Top10F' hücre DNA'larında CCGG dizilerini metillediği saptandı.

Anahtar Sözcükler: Bakteriyel MTaz M.Msp1, *in vivo* DNA metilasyonu

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Introduction

DNA methyltransferases (MTases) have long been regarded as an attractive model for studying DNA - protein interaction. Over 50 cytosine DNA MTases from organisms ranging from bacteria to mammals have been cloned and sequenced (1-3). DNA methylation is used by a wide range of prokaryotes and eukaryotes for the marking of DNA sequences and regulation of gene expression (including restriction-modification systems), DNA replication and repair. Methylation of cytosine at carbon-5 of pyrimidine ring occurs at postreplication stage of DNA synthesis by the activity of DNA methyltransferase (MTase) (4-6). Cytosine MTases transfer methyl groups from the donor S-adenosyl-L-methionine to the C-5 position of target cytosine in specifically recognized DNA sequences. It is obvious that DNA methylation is the most common form of DNA postmodification in biological systems. Many studies have shown correlations between increased gene expression and decreased DNA methylation (7-8). In mammalian cells, 3-5% of the cytosine residues in DNA are methylated. The haploid mammalian genome contains ($5x10^7$ CpG dinucleotides about 60% of which are methylated at the 5 position. Methylation patterns undergo sweeping reorganization during gametogenesis and early development. In addition to CpG methylation, plant DNA contains methylated bases in the trinucleotide sequence CpNpG, where N can be A ,C,G or T (9). Bacterial MTases are usually found as a part of restriction-modification systems to activate in cell's defense against phage infection in prokaryotes. These enzymes share a common protein architechture. Ten conserved motifs are normally found 10-20 aa in length and a hypervariable sequence called TRD (T: threonine, R: arginine and D: aspartic acid) which is located between motifs VIII and IX (10). Two major classes of DNA MTase exist in nature. First, methylates a pyrimidine ring carbon and forms C-5 methylcytosine (M.Msp1 and M.Hha I). The second class methylates exocyclic nitrogens and forms either N^4- methylcytosine (M.Pvu II) or N^6-methyladenine (M.TaqI) (11-13). HhaI and HpaII DNA MTases bind DNA mismatches which methylate uracil and block DNA repair (14). Bandaru et al. proposed that HpaII MTase was mutagenic in *E. coli*. The presence of the enzyme in *E. coli* causes a substantial increase in C to T mutations at CG sites (15). M.SssI and M.HhaI MTases reveal extensive interactions with the substrate DNA backbone. The two structurally related enzymes display similar specific and nonspecific contacts with DNA while bound to their target sequences (16). Sites for cytosine methylation are known as hot spots for cytosine to thymine mutations (10). It is our claim that because of their biological importance, their sequence specificity and reaction similarity, the C- 5 MTases will be the subject of many studies in the near future.

We aimed to study the gene encoding the above mentioned MTase M.Msp1 whether it is active or inactive with glutathion-S-transferase (GST) and mammalian nuclear localisation transport sequences (NLS) which were fused to the 5' end of gene. For this purpose, the recombinant plasmid, encoding the bacterial M.Msp1 protein and which was used to transform different *E. coli* strains and expression level of the fusion product, was analysed under the control of T7 and CMV promoters. It is reported here, that the protein was found to be fully active and is able to methylate the CCGG sequences of the plasmid encoding the recombinant M.Msp1 in *E.coli* strains used .

Material and Methods

Oligonucleotides and PCR Amplification

Synthesis of all oligonucleotides was performed on a model 381 DNA synthesiser by the biomolecular synthesis service of the Krebs Institue, Sheffield, U.K. About 100 ng bacterial M.Msp1 genomic DNA was served as template and the primers were;

Top strand,

52 mer 5'-CGCG **AAGCTT CATAAG** GCGGGGTATTTTGCGTTTT CCCCTATACTAGGTTAT-3'

HindIII NdeI NLS GST

Bottom strand (AWMSP3 : forward primer to 3' end of M.Msp1 sequence contains EcoRI recognation and stop sites),

39 mer 5'- CGCG **GAATTC TTA** AACGAATTCTAATTCAAAGTTTTCTT -3'

EcoRI Stop M.Msp1

PCR amplification reactions contained 2 μl of each primer (0.5 μM), 1μl template DNA (100 ng), 1μl dNTP's (200 μM for each), 2 μl $MgCl_2$ (2mM), 5 μl of 10X *Taq* DNA polymerase buffer { (500 mM KCL, 100 mM Tris (pH:9.0 at 25 °C), 1 per cent TritonX-100)} and 1μl Taq DNA polymerase (2 Units), (Promega Biotec) were performed in a final volume of 50 μl . PCRs were carried out using the Thermal Cycler Techne Model HL-1. Initial template denaturation was programmed for 1.5 min at 94 °C. The cycle procedure was programmed as follows: 2 min at 50(C (annealing), 3 min at 72 °C (extention) and 1 min at 94 °C (denaturation). Thirty cycles of the profile was run and the final extention step was increased to 10 min.

Bacterial Strains and Chemicals

Escherichia coli strains used for transformations were;

DH5α: [(φ 80 d lac ZΔ M15 , rec A1 gyr A96, thi -1 , hsd R17 (rK-, mK+) sup E44 , rel A1, deoR , Δ (lac zya - argF), U169)]. and

Top10F': F'(lac Tn10 (TerR)] mcr ΔD (mcr- hsd RMS -mcr BC) φ 80 lac ZΔ m15, Δlac X74, deoR , recA1,ara D139 Δ (ara-leu) 7697 gal U , gal K, rps L end A1 nup G.

Both strains were purchased from New England Biolabs (NEB) and Invitrogen respectively. Ultrapure deoxynucleotide solutions were from Pharmacia and ribonuclease A was obtained from Worthington Biochemical Co. DNA weight standards were obtained from NBL Gene Science Ltd. and NEB. Other plasmid DNAs, enzymes and chemicals were obtained from various commerical sources and used according to the manifacturer's instructions.

Construction of Plasmids

The aim of this study was to construct a plasmid that would direct the expression of a recombinant bacterial DNA MTase enzyme in cultered cells. A PCR based strategy was carried out for introduction of three component coding sequences into the mammalian transfection vector pcDNA$_3$ (Invitrogen). First, the gene, encoding the bacterial cytosine-5 specific DNA MTase M.Msp1 was introduced into the general cloning vectors pMTL23 and pNRT2. To this construct the coding sequence for glutathion-S-transferase was added followed by a vertebrate nuclear localisation SV40 VP1 sequence both by PCR based methodology. The complete fusion protein encoding gene was finally introduced into the general purpose transfection vector pcDNA$_3$.

Cell Culture and Transformation

The standard method for preparing competent cells was a modification of the calcium chloride procedure (17). A single colony of *E. coli* strains (DH5α and Top10F'), which was inoculated into 5mls LB medium and incubated overnight at 37 °C. 50 µl of this culture, was then transferred into a further 5 mls of LB and grown until the OD260 of the culture reached 0.6 (~2 hours). 1.5 ml samples were then centrifuged at 12.000 rpm in a microcentrifuge for 2 minutes. The supernatant was removed and the bacterial pellet resuspended in 50 µl of ice-cold $CaCl_2$ (50 mM) solution. After 2 minutes centrifugation, the supernatant was removed and 100 µl of 50 mM $CaCl_2$ solution was added and at the same time between 5-10 µl of a ligation mixture or less than 100 ng (~ 1(l) of a recombinant plasmid DNAs with bacterial M.Msp1 gene fusion were added to the competent cells, which were mixed gently and incubated on ice for 20 minutes. The cells were then heat shocked at 42 °C for 2 minutes and chilled by returning the suspension immediatelly onto ice. 1ml LB medium was added to each sample and subsequently incubated at 37 °C for 1 hour. Each sample was then recentrifuged at 12.000 rpm in a microcentrifuge for 2 minutes, and pellet was resuspended in a volume of 200 µl of LB and plated out onto the appropriate media and antibiotics. DH5α cells were plated out in agar plates containing LB medium (10 gr tryptone+5 gr yeast exract+10 gr NaCl in 1 lt distiled water) with ampicillin (2 µg/ml) and Top10F'cells were plated out in agar plates containing LB medium with ampicillin (2 µg/ml) and neomycin (40 µg/ml).

Preparation of DNA from Transformed Cells

Genomic and plasmid DNAs from transformed DH5α and Top10F' were isolated from 1.5-500 mls of liquid LB culture following alkaline lysis method described in Sambrook *et al*. (18). Recombinant bacterial cultures were grown to late log phase in LB medium with suitable antibiotics and the cells were harvested at 5000 g in a Sorvall centrifuge (GS3 rotor) for 10 minutes at 4 °C. The cells were then washed in solution I (0.184g/ml $CaCl_2$, 0.125 M Hepes, pH: 7.15) and 2ml of freshly prepared solution of lysozyme [100 mg lysozyme and 100 µl Tris (1M) and 10 ml H_2O]. Suspensions were subsequently extracted with phenol-chloroform three times, followed by one-time ice-cold ethanol precipitation. DNA was resuspended in an 40 µl volume of TE buffer (10 mM Tris.Cl, 1mM EDTA, pH : 8.0). Any further purification of plasmid DNA was

carried out by adsorption onto glass beads.

Restriction Activity Assay

Restriction digestion was performed by incubating 1μg DNA from transformed and control group cells in a total volume of 20 μl with 10-20 units of restriction enzyme. Digests were carried out at the suppliers recommended temperatures (usually 37 °C) for 2-24 hours, using specific buffers supplied with the enzymes. The restriction patterns were then analyzed by 1 % agar gel electrophoresis.

Methylation Activity Assay

As a control pUC19, and recombinant plasmids that include NLS +GST + M.Msp1 gene were digested with R.Msp1 and R.HpaII for detection of activity of recombinant gene in *E.coli* strains. Digestion mixture (20 μl) included; 5 μl plasmid DNA + 2 μl R.Msp1 or R.HpaII restriction endonuclease (RE) buffers + 2 μl of RNase (10 μl/ml) +1μl R.Msp1 or R.HpaII RE + 9μl distilled water. The reaction mixture was incubated at 37 °C for 3 hours. Bacteriophage λ DNA digested with HindIII and EcoRI was used as DNA molecular weight standards and unmethylated pUC19 DNA, transformed DH5α and Top10F' plasmid DNAs were spotted onto 1% agar gels and stained with ethidium bromide (EtBr), (4 μg/ml). The amount of DNA present was quantified by comparing the UV induced fluorescence emitted by EtBr molecules intercaleted into the sample DNA with that of a series of markers spotted onto the same gel .

Results

In order to establish the effect of bacterial cytosine-5 M.Msp1 MTase on DH5α and Top10F' cells DNA, we have constructed a fusion of a bacterial cytosine-specific DNA MTase with a vertebrate nuclear targeting signal (NLS-SV40 VP1) and the marker enzyme glutathione-S-transferase (GST). Approximately 100 ng of pNRT2 encoding M.Msp1 gene as template and a 52 mer top strand, a 39 mer bottom strand as primers were used and PCR was set up according to the protocol which was outlined in the expand long template PCR system package (Techne) and explained in materials methods (Figure 1).

These DNA methyltransferase genes were separately cloned into the nonisogenic *E.coli* strains DH5α and Top10F' respectively. Transformed and control group colonies were selected with appropriate antibiotics (Figure 2).

These recombinant plasmids were stably maintained under selective growth conditions. DNA methylation was monitored at different times after induction by determining the susceptibility of the DNA to R.Msp1 digestion. After isolation of small scale (mini- prep) plasmid DNAs, from transformed *E.coli* strains that included NLS+GST+M.Msp1 recombinant gene, were digested with R.Msp1 for detection of methylation activity of fusion protein. This showed that production of bacterial methyltransferase can be expressed by the T7 and CMV promoters. The

fusion protein was found fully active and able to methylate CCGG sequences of the plasmids encoding the M.Msp1 in *E.coli* strains used. The multicopy plasmid DNAs appeared to be completely methylated. We always observed uncut, high-molecular-weight DNA fragments on 1 % agar gels (Figure 3 and 4).

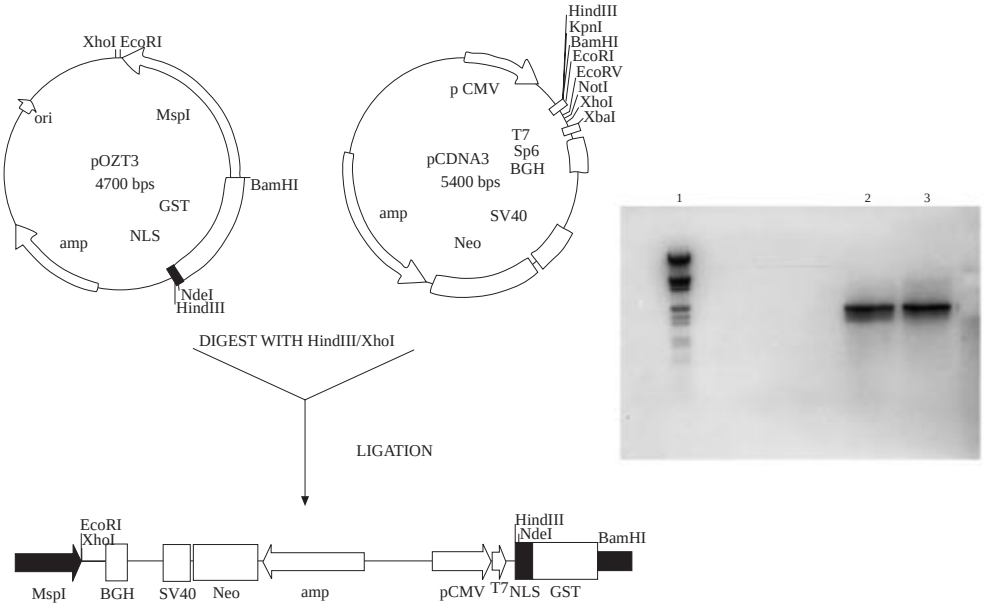

Figure 1. PCR Product Lane 1:HindIII and EcoRI digested (DNA marker Lanes 2-3 : (2000 bps, NLS+ GST+ M.Msp1 gene fusion

Discussion

Approximately 90% of methylated Cs is followed on its 3' side by a G residue. Conveniently, this sequence forms part of the recognition sequence CCGG for two restriction enzymes R.Msp1 and R.HpaII which differ in their ability to digest in this sequence when the C is methylated. Hence, if DNA is digested with either R.Msp1 and R.HpaII, both enzymes will give the same pattern of bands only if all the C residues within the recognition sites are unmethylated (19).

We were able to express monospecific (enzyme modifying a single DNA sequence) cytosine-5 DNA MTase at high levels in two *E.coli* strains as functional fusions with glutathione-S-transferase and vertebrate nuclear targeting signal SV40 VP1. First we constructed the bacterial cytosine specific DNA M.Msp1 MTase gene fusion including a nuclear localisation signal and glutathione-S- transferase and placed it under control of the T7 and CMV promoters (Figure 1).

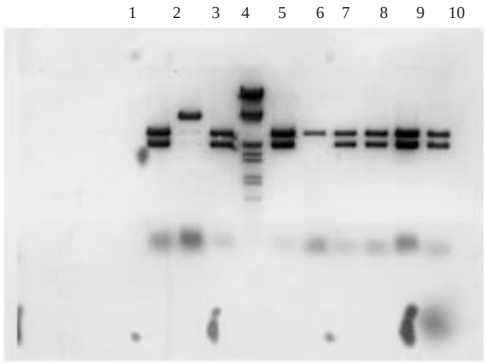

Figure 2. *E.coli* DH5α and Top10F' cells were transformed with PCR product (M.Msp1 gene fusion) and plated out in LB medium and selected with ampicillin for DH5α, ampicillin and neomycin for Top10F'. After mini - prep DNA isolation, DNAs were digested with Hind III and EcoRI restriction endonucleases for selection of fragments carrying the fusion gene (~ 2000 bps). Lanes Lane 1, 2 and 3 : Positive DH5α colonies Lane 4 :λ DNA digested with Hind III and EcoRI Lanes 5, 7, 8, 9 and 10 : Positive Top10F' colonies Lane 6 : Negative Top10F' colony

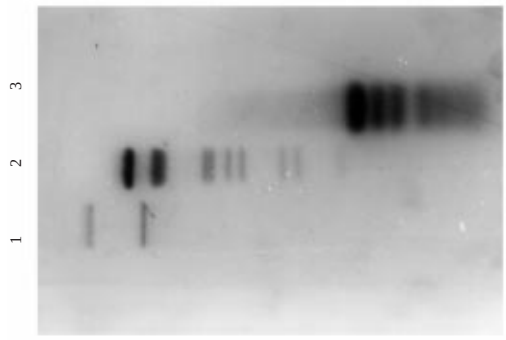

Figure 3. Agar gel electrophoresis (1%) of ethidium bromide stained plasmid DNA from *E.coli* DH5α cells transformed with bacterial cytosine -5- specific DNA MTase M.Msp1 gene fusion .Lanes Lane1: Transformed cells' DNA digested with 10 U of R.Msp1. DNAs were resistant to the enzyme digestion and no fragmentations was found Lane 2 : HindIII and EcoRI digested λ DNA marker Lane 3 : pUC19 plasmid DNA digested with 10 U of R.Msp1.

After transformation of and establishment in two different non-isogenic E.coli cells, with the PCR product of the fusion genes construct, we have found that genes with CpG islands have a significant probability of becoming inactivated by methylation. The results indicate that the primary cause of preferential cutting is specific methylation of the CCGG sites in both strains. Expression of the M.Msp1 gene fusion was tested by the cognate restriction enzyme R.Msp1. Enzyme was active in both strains and methylated DNA could not be cut. Methylated DNA exhibited higher fragment sizes in 1% agar gel (Figure 3 and 4). Resistance to R.Msp1 digestion appeared to require at least 8 hours to achieve sufficient methylation to yield high-molecular-

weight protected fragments.This result suggests that methylation is non-random and certain large regions are inaccessible to bacterial C-5 MTase action within the two *E.coli* DH5α and Top10F' strains. Therefore, foreign MTase genes with regulated expression and vertebrate nuclear targeting signal SV40 VP1 could be useful as an *in vivo* probe to test gene expression and chromatin organization.

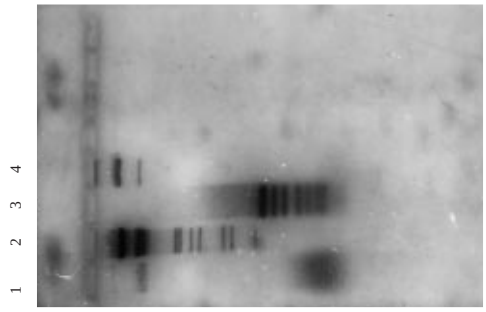

Figure 4. Annealing profiles of transformed *E.coli* Top10F' and untransformed pUC19 plasmids DNA digested with 10 U of R.Msp1. The bacterial cytosine-5 MTase M.Msp1 fusion protein was active in all transformed strains and prevented the DNA fragmentation contrary to the restriction activity of cognate enzyme R.Msp1(which can not cleave if the external cytosine is methylated in its recognition site CCGG). Lanes Lanes 1 and 4 : Transformed *E.coli* Top10F' plasmid DNAs , not fragmentation. Lane 2: HindIII and EcoRI digested λ DNA marker. Lane 3 : pUC19 plasmid DNA digested with 10 U of R.Msp1, high fragmentation.

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