Mutagenesis and biochemical characterization of signature motifs in Type III RM enzyme

A thesis submitted in partial fulfillment of the requirements of BS-MS Dual Degree Program

Indian Institute of Science Education and Research, Pune

By

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Abstract

Restriction-modification (RM) system in bacteria has evolved to protect bacterial cell from foreign DNA that enters the cell. Type III RM system is one of the classes of RM system involved in such a defense mechanism. It is comprised of a Mod and a Res subunit that has methylation and restriction function respectively. Though the Type III system is extensively studied through EcoP15I and EcoP1I enzymes, many structural and mechanistic details are still to be known. In this study, mutations and biochemical characterization have been carried out in hope to understand the important motifs for methylation activity. A highly conserved stretch GDN in EcoP15I was characterized by mutation and found to be involved in the binding of the cofactor for methylation. Structural analysis of different classes of methyltransferases has shown that a presence of a negative charge in the cofactor binding pocket is a characteristic feature of all methyltransferases. We propose that this particular GDN motif in EcoP15I is the actual Motif III in Type III RM enzyme, which is assigned wrongly at present and is important in the SAM (S-adenosyl methionine, the cofactor for methylation) binding.

Characterization of the MboIII protein was done to find the functional relevance of a loop in the methyltransferase domain. In contrast to the hypothesis proposed, the residues in the loop were found to be important in the restriction activity and not methylation activity. Based on the data obtained, we suggest that the loop T128 to S156 is an allosteric regulator for the function of Res subunit. The function of this loop could be replaced by the binding of the ligand in the cofactor pocket of methyltransferase. Together with the bound ligand and an ordered loop that interacts to the ligand, arrays of interactions are initiated from the Mod subunit all the way up to the Res subunit resulting in the cleavage of DNA. Detailed experiments have to be carried out to characterize this long-range interaction.

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Aathira Gopinath P

Certificate

This is to certify that this dissertation entitled "Mutagenesis and biochemical characterization of signature motifs in Type III RM enzyme" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Aathira Gopinath P at IISER Pune under the supervision of Dr Saikrishnan Kayarat, Associate Professor, Department of Biology during the academic year 2017-18

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Declaration

I hereby declare that the matter embodied in the report entitled "Mutagenesis and biochemical characterization of signature motifs in Type III RM enzyme" are the results of the work carried out by me at the Department of biology, IISER Pune under the supervision of Dr Saikrishnan Kayarat and the same has not been submitted elsewhere for any other degree.

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Introduction

Bacteria are constantly facing attack from bacteriophages and other foreign particles. To counter such threats, these single-celled organisms have cleverly come up with various systems as the defense against foreign DNA. Some of the strategies employed by bacteria include the Restriction-Modification (RM) system which works similar to the innate immune system, CRISPR-Cas system which works similar to the adaptive immune system and the toxin-antitoxin. (Makarova., 2013). The restriction modification system has been extensively studied and has applications in molecular recombinant technology. RM system works by discriminating self DNA from non-self DNA like the immune system of higher eukaryotes. When a virus attacks a bacterial cell, it releases its genome into the cell, which interferes with the host genome and starts viral replication using host machinery thereby destroying the host cell. To avoid this, bacteria produce restriction enzymes that bind to specific DNA sequences of the viral genome and cleave the DNA via its endonuclease activity. To protect its own genome from self-cleavage the same enzyme methylate the adenine/cytosine base of the target sequence present in bacterial genome after DNA replication.

There are four kinds of Restriction-Modification (RM) system known to date: Type I, Type II, Type III and Type IV. The classification is done based on sequence recognition, molecular structure, cleavage position and cofactor requirements (Rao et al., 2013). Type II is NTP independent and is widely used in recombinant DNA technology. They bind to specific palindromic sequences and cleave the target site giving rise to staggered or blunt end cuts. The Type I and III use ATP to cleave foreign DNA and use S-adenosyl methionine (SAM) to modify DNA. They bind to asymmetric DNA sequences and cleave DNA away from the target site. Type IV system, in contrast to the others, cleave only modified DNA and do not have any methylation activity. Phages over time have evolved to invade bacteria with RM system by having methylation at the target site. Type IV system has arisen to invade such phages by having restriction activity on methylated DNA. (Loenen et al., 2013)

1.1 Type III RM systems

Type III RM enzymes are composed of two subunits: Mod and Res both encoded by a single operon. The Mod subunit methylates genomic DNA after replication to be recognized as self. Res subunit restricts any unmethylated DNA. Thereby, foreign DNA that has entered into the cell will be recognized as non-self as it doesn't have any methylation and will eventually be cleaved. EcoP15I and EcoP1I are the extensively studied enzymes of Type III class.

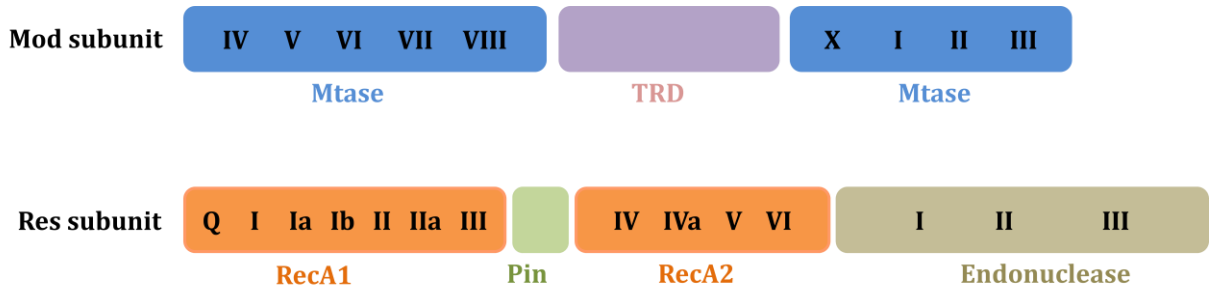

Fig 1.1 Domain architecture of the Mod and Res subunits of Type III RM enzymes. The motifs of each domain are marked by roman numerals.

The mod subunit is composed of a methyltransferase domain (Mtase) and a target recognition domain (TRD). The Mtase belongs to β class of N6-adenine methyltransferase. Enzymes of this class methylate exocyclic nitrogen atom at the N6 position of the adenine ring giving rise to N6-methyladenine (Scavetta et al., 2000). β class of Mtase is composed of nine signature motifs: sequentially arranged as IV-VIII (catalytic motifs), Target recognition domain (TRD), X, I-III (motifs for SAM binding) as shown in Fig 1.1. The active site for methylation is a loop formed by Motif IV, VI and VIII (Malone et al., 1995). Type III RM enzymes use two Mod subunits for the methylation activity. One of the Mod recognizes the target sequence, which is usually 5-6 bp long, via its TRD. The adenine in the sequence is then flipped out of the DNA helix into the catalytic site of the second Mod. A CH_3 group bound to the sulfur atom of the cofactor gets transferred to the adenine base giving rise to N6-methyl adenine. (Gupta et al., 2015)

The Res subunit is composed of an ATPase domain and an endonuclease domain. The ATPase of Type III belongs to Superfamily2 (SF-2) characterized by eleven signature motifs (Tuteja and Tuteja, 2004) as in Fig 1.1. The endonuclease domain belongs to Archeal Holliday Junction Resolvase family (AHJR) which has three main motifs that together form the metal ion binding pocket (Nishino et al., 2001). For restriction to happen two Mod and a Res subunit has to come together to form a heterotrimeric complex. A Mod_2Res complex can cleave an unmethylated DNA with target sites in head to head orientation (HtH). The TRD in Mod will recognize the sites and bind to it. Upon the binding of ATP, the ATPase domain gets activated. It uses ATP hydrolysis to switch to another conformation of the enzyme that will allow it to diffuse along the DNA. Once the enzyme is in a diffusive state no further ATP is required. Hence the ATP required by Type III RM system is very minimal in contrast to 1 ATP/bp for Type I systems. Whenever a diffusing enzyme collides with a site bound enzyme, the endonuclease domain gets activated and results in cleavage (Gupta et al., 2015). Cleavage occurs 25-27 bp downstream to one of the target sites (Rao et al., 2013). Apart from the HtH orientation of site, Type III is also observed to cleave single site DNA substrate.

In this study, two homologous enzymes have been used as model system to characterize the methylation activity, EcoP15I and MboIII. The EcoP15I protein recognizes the target sequence, 5'-CAGCAG-3' and methylate the second adenine in the sequence (Humbelin et al., 1988). MboIII is another Type III RM enzyme identified and characterized in our lab. It contains a Mod and a Res subunit with the typical domain architecture shown in Fig 1.1. MboIII recognizes the target sequence 5'-YAATC-3' (Y=T/C) and methylates the second adenine in the target sequence (Ahmad et al., unpublished data).

1.2 Conserved motif GDN

Previous comparative studies of Type III RM enzymes in the laboratory had led to the identification of conserved non-canonical motifs (Manasi., 2016). The same study had

identified 6 new motifs in the MTase domain by sequence alignment of many Type III enzymes. Among them a GDN motif was found to be conserved in all the enzymes checked. GDN was found to be located N-terminal to the motif IV (Fig 1.2 a). When this motif was mapped into the structure of EcoP15I, a type III restriction enzyme, it was found to be in the AdoMet binding region (Fig 1.2b). Since EcoP15I structure does not have SAM as a ligand, SAM was docked into the EcoP15I structure using another homologous enzyme of the same class, Rsr1. The Asp residue of the GDN motif was found to interact with the N6-adenine of SAM. The high conservation of this motif among type III led us to think that it could be an important residue needed for methylation activity of the protein. In this study, I have mutated the Asp of GDN motif in EcoP15I to find the functional relevance of this conserved motif.

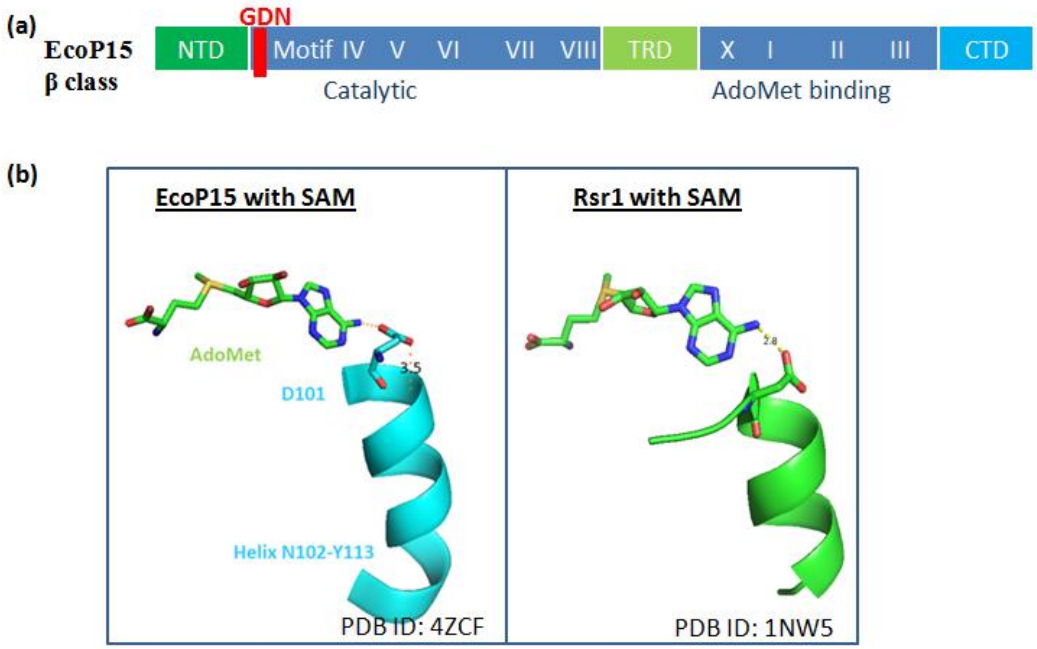

Fig 1.2 GDN motif in EcoP15I. (a) The position of GDN in MTase of EcoP15I enzyme (b) The interaction of Asp to N6-adenine in EcoP15I (left) and Rsr1 (right)

1.3. Interaction of Mod to Res via a Loop

The crystal structure of a dimer of the Mod subunit of MboIII, which is a β-class of N-6 adenine methyltransferase, with sinefungin (SNF) determined in our laboratory is shown in Fig 1.3a. The crystal structure of MboIII has two monomers in the asymmetric unit corresponding to the dimer of Mod subunit. Based on the structure of EcoP15I (Gupta et al., 2015), it is expected that in an active enzyme complex of MboIII, one of the Mod (ModA) will be bound to the target sequence in the DNA and the second subunit (ModB) will catalyze the methylation reaction. A close look at the SAM binding pocket led us to find certain differences between the two subunits. A region from T128 to S156 was found to be completely disordered in the ModB subunit whereas it had electron densities in the ModA subunit. In the ModA, it forms a short alpha helix followed by a loop. The D134 residue in this region forms a hydrogen bond with the 3'-OH of ribose ring of SNF. This additional interaction present in ModA might help it to firmly hold the SAM in place. The absence of same interaction in the latter subunit led us to think that the ModA is positioned in such a way as to facilitate methylation and ModB for target recognition. Although the division of labor between Mod dimer for the two functions has been proposed earlier, the mechanism by which this happens is not known (Gupta et al., 2015). Binding of SAM to the catalytic site of ModA might make the loop more ordered from its flexible state. As a result, the catalytic site becomes a closed pocket and SAM is now firmly bound to the pocket. Presence of a flipped adenine residue will now initiate the reaction of methylation.

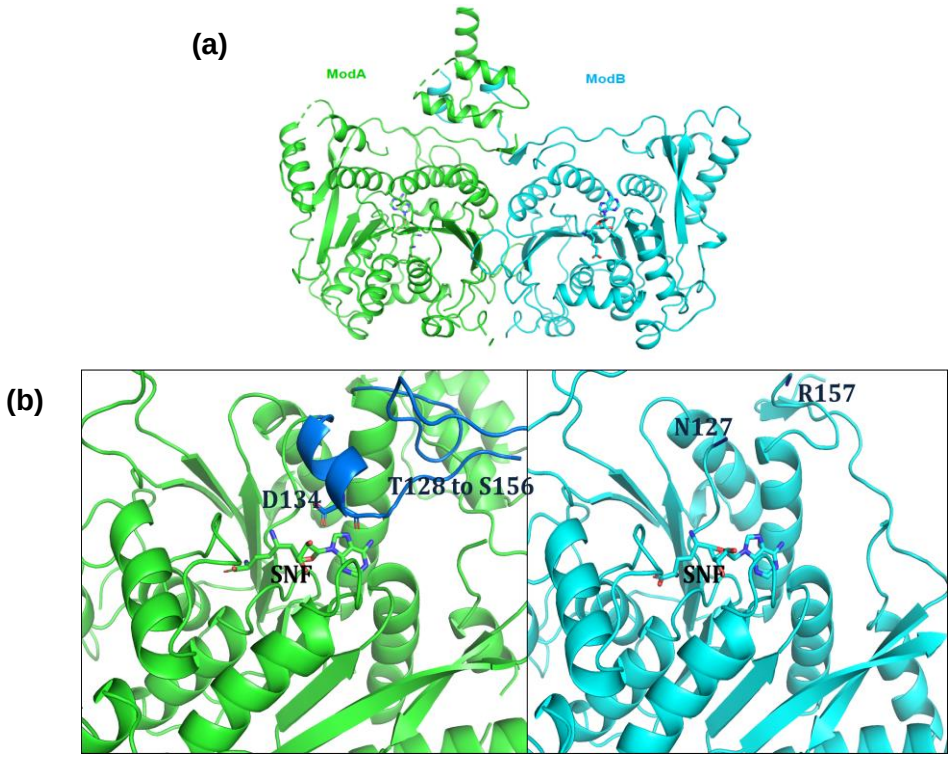

Fig 1.3. Crystal structure of MboIII and position of the loop (a) Crystal structure of MboIII (b) The position of loop T128 to S156 in the structure in ModA subunit is shown in dark blue color in the left. The D134 forms H bonding with SNF. Absence of Loop in ModB subunit in the right panel.

The interaction of 3'-OH of ribose ring in SNF with D134 should be a conserved one among the similar class of enzymes if the hypothesis is true. To check this, ModA subunit was superimposed onto the structure of M.MboII (PDB ID:1G60) and Rsr1 (PDB ID:1NW5) (Fig 1.4). In M.MboII, the Asp residue was replaced by a tryptophan (W40), which can create a similar interaction through pi-stacking. An earlier study in M.MboII has proposed that the loop region including the W40 is a flexible arm that closes the SAM binding pocket (Osipiuk., 2003). In contrast, the superimposed structure of MboIII to Rsr1 did not show any interaction at the position. Rsr1 being monomeric, methylation and target recognition function is carried out by the same subunit. Hence, there is no need for the distinction between the functionality of Mod subunits. This could be the reason for the absence of interaction in Rsr1.

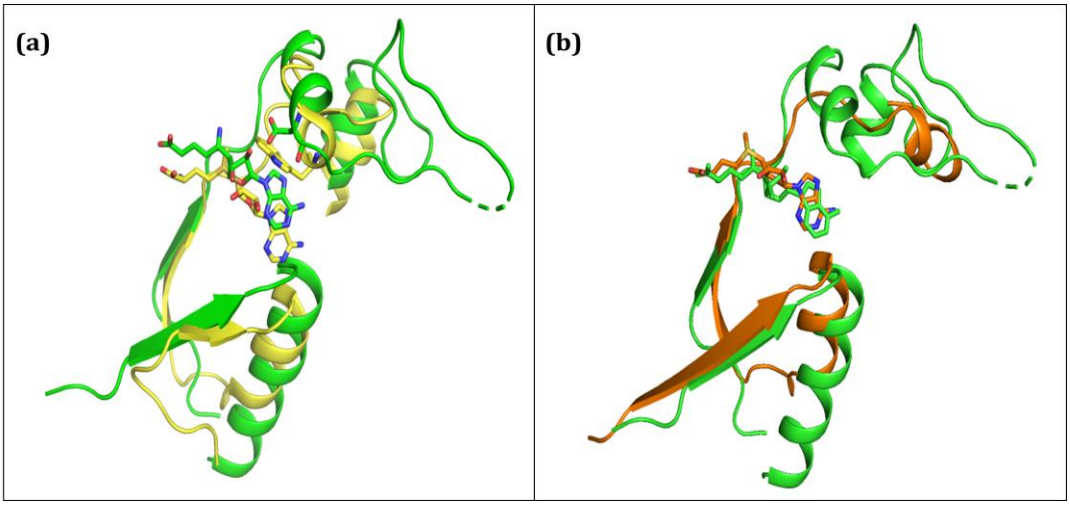

Fig 1.4 M.MboIII superimposed to M.MboII and Rsr1. (a) M.MboIII superimposed to M.MboII (b) M.MboIII superimposed to Rsr1. M.MboIII structure is in green, M.MboII in yellow and Rsr1 in orange. The residues interacting with SAM in MboIII and MboII are shown as sticks

To gain insights into the function of the loop, I mutated D134, which interacts with the ribose ring of SAM, to alanine. Also, the entire loop from T128 to S156 was deleted and replaced by four glycine residues. This construct, I imagined, will be equivalent to the loop being disordered in both the subunits of Mod.

Materials and methods

Table 2.1 Primers used in PCR reactions

Sl No	Primer Name	Sequence (5' -> 3')
1	SK D101A	CAATACTTCGAGATTAGCCCCTTTGATTAACAGG
2	SK D101N	CAATACTTCGAGATTATTCCCTTTGATTAACAGG
3	EP15MPHISF	GTTTAACTTTAAGAAGGAGATATACATATGAAAAAAGAAA CGATTTTTTCCG
4	EP15RPHISR1	CTTTTAATGATGATGATGATGATGGGATCCTGGTAATGC GCTCTTGATTAAC
5	MxMreB-f	GTTTAACTTTAAGAAGGAGATATACAT
6	MxMreB H6r	GCTTTTAATGATGATGATGATGATGGG
5	MboIII D134A Rev	CACTTAGATTATTGCCAGCAGAAAGTGAGC
6	Mbo Gly loop	CATATTTAGCCAACCAGTACGGCCGCCGCCGCCATTATA AGGAGGATCG
7	MboprsfF	GATATACCATGGCACATCACCACCACCATCACATGAATA CTATAAAACAG
8	ModRevMCO	CGCAGCAGCGGTTTCTTTACCCTCGAGTTACTTAATTGC ATCAGT
9	235 For	GGTGATGTACGAAGAGGAGTTCACCATAATCAACGCCGT TTG
10	235 Rev	GTGGAACTGGTTGGGGAACTCGTTCTCCCTAATCAGCTT GGC

2.2 Cloning

2.2.1 Site directed mutagenesis by Polymerase chain reaction

Mutations were introduced into the gene by two steps of polymerase chain reaction (PCR). PCR was carried out using purified Pfu polymerase. In the first PCR, the primer with respective mutation (Table 2.1. mutations made are marked in red) and a gene primer is used to amplify a short fragment/megaprimer. The megaprimer is purified from the reaction mix using Qiagen PCR purification kit and then used in the next PCR to amplify the whole gene. The amplified gene with the desired mutation is then purified from the reaction mix. The standard PCR reaction cycle and conditions are given below.

Standard protocol for PCR amplification

10X Pfu Buffer	: 5 µl (1 X)
dNTPs (2.5 mM each)	: 4 µl (200 µM)
For primer	: 0.6 µM
Rev primer	: 0.6 µM
Template	: 100 ng
Pfu polymerase	: 0.8 µl

MilliQ to final reaction volume of 50 µl

PCR Conditions:

Initial Denaturation	*: 95°C for 2'*	
Denaturation	*: 95°C for 30''*	} *x 30 cycles*
Annealing	*: 55-60°C for 30''*	
Extension	*: 72°C at 1kb/min rate*	
Final extension	*:72°C for 10'*	

Stored at 4°c

2.2.2 Double digestion of vector and gene

The PCR purified gene with the desired mutation was double digested by restriction enzymes to obtain sticky ends for cloning into a vector system. The 5 kb EcoP15I gene mutants and 2.6 kb pHis vector was double digested with BamHI-HF and NdeI in CutSmart buffer at 37°C for 3 hours. The MboIII gene mutants and 3.7 kb pRSF vector

were double digested with XhoI and NcoI-HF in a similar manner. The reaction mix was then gel extracted on a 1% agarose gel and eluted using Qiagen Gel extraction kit.

2.2.3 Ligation and transformation

The double digested gene and the vector were used in the ratio of 1:3 (0.02 pmol vector and 0.06 pmol gene) for the ligation reaction. Ligation was carried out in NEB T4 DNA ligation buffer using NEB T4 DNA ligase at 25°C for 2 hours. The vector was treated with TSAP phosphate beforehand to remove 5'-OH in order to prevent self-ligation of the vector. The ligation mix was then used to transform NEB Turbo electrocompetent cells by the method of electroporation and plated onto LB agar plates with ampicillin antibiotic for EcoP15I and kanamycin antibiotic for MboIII. The colonies got after 12 hours of incubation at 37°C was then cultured and the plasmid was purified using the conventional method of plasmid extraction. The plasmid was digested with the respective enzyme using a quick double digestion method and checked on a 1% agarose gel. Positive plasmids were sent for sequencing to Sigma-Aldrich with required primers for sequencing the entire gene.

2.3 Culturing and expression of protein

The EcoP15I gene in the pHis17 vector was expressed in BL21(AI) cells. BL21(AI) cells are tightly regulated by araBAD operon and need L-arabinose for induction. The Mod MboIII gene in pRSF-1b and Res MboIII in pHis were expressed together in BL21(DE3) cells which were regulated by lac operon. Lac operons were induced with IPTG. The plasmids to be expressed were transformed by heat shock method to cells and plated onto LB agar plates with the respective antibiotic. The plates were incubated at 37°C for 9 hours. Colonies are then scraped to a 100ml primary LB culture and was grown till OD 0.6 was reached. This culture was used for inoculation of the secondary culture, which was grown at 37°C with 180 rpm shaking till OD reached 0.6-0.7. The culture was induced with 2 g/L of L-arabinose or 0.5 mM IPTG based on the expression strain and grown at 18°C for 12 hours.

An expression test was done to check the solubility of the protein. The 1 ml cell pellet from the culture was resuspended in both 100 µl lysis buffer and 5X Tris-Glycine-SDS (TGS) buffer. The cells were then sonicated for 1 minute in a 5 s on 5 s off cycle with 60% amplitude. The cells resuspended in lysis buffer were spun at 15000 rpm for 10 min at 4°C. From this fraction, the supernatant and pellet were separated and the pellet was resuspended in 5X TGS buffer. Protein in the supernatant indicated the soluble fraction of the pellet. To visualize the protein on an SDS PAGE, equal volume of 2X SDS gel loading dye was added to the fractions. The samples were loaded onto a 10% SDS PAGE (Polyacrylamide gel electrophoresis) gel and run at 230V. Protein bands on the PAGE were visualized using coomassie blue staining method.

2.4 Protein purification

2.4.1 Resuspension and Ultracentrifugation

The 4-6 L cell pellet was resuspended in 200-300 ml lysis buffer (50 mM Tris HCl, 500 mM NaCl, 5 mM $MgCl_2$, 10% Glycerol, 10 mM Imidazole). The solution was then sonicated with 1s on and 3s off cycle for 3 minutes at 60% amplitude. The cycle was repeated 3 times with 8 minutes gap. The solution is kept on ice to avoid heating. The sonicated sample was then ultracentrifuged in Optima TM XE ultracentrifuge at 37,000 rpm using Ti45 rotar for 50 minutes at 4°C. The supernatant from the spin was used for the first step of purification.

2.4.2 Affinity chromatography by Ni-NTA column

Ni-NTA column was used to purify proteins with a Histidine tag. EcoP15I and MboIII proteins used have an N-terminal hexahistidine tag. The 5 mL HisTrap HP Ni-NTA column (GE Healthcare) was equilibrated with buffer A (50 mM Tris HCl, 500 mM NaCl, 12 mM Imidazole). The supernatant after the spin was loaded onto the column. The His-tag in the protein was expected to bind to the Ni^{2+} in the column resin during this step. The protein was then eluted with a step gradient of increasing buffer B (50 mM Tris HCl, 500 mM NaCl, 500 mM imidazole). Ni^{2+} have a higher affinity for imidazole and

hence imidazole can replace the histidine. Fractions of 10 ml were collected from 2% buffer B to 100% buffer B. 20µl of the fractions were then collected to check the purity of fractions on a 10% SDS PAGE gel. Fractions of protein were pooled and dialyzed against 2 L buffer B15 (50 mM Tris HCl, 15 mM NaCl, 1 mM EDTA, 1 mM DTT) overnight at 4°C.

2.4.3 Anion exchange chromatography

Protein after dialysis was spun at 18,000 rpm for 20 minutes at 4°C using Avanti J-26XP to remove aggregates of protein if present. The supernatant was then loaded onto an 8 mL Mono Q™ 10/100 GL column (GE Healthcare). The column was equilibrated with buffer B50 (50 mM Tris HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) before loading. The protein was loaded to the column through a 50 ml super loop. The column works by the principle of anion exchange. At pH 8, the protein (pI of protein is 5) is negatively charged and stays as an anion and hence will bind to the positively charged resin. Elution was done with increasing gradient of buffer B (50 mM Tris HCl, 1000 mM NaCl, 1 mM EDTA, 1 mM DTT). As the buffer B increases, the anions bound to the column would be replaced by chloride ions of NaCl. Protein elution was viewed by the UV absorption at 280 nm wavelength. Fractions with protein of interest were confirmed by loading on a 10% SDS-PAGE. Protein was pooled and concentrated to 500-700 µl in Vivaspin®2 (GE Healthcare) protein concentrator (MWCO 30 kDa).

2.4.4 Size exclusion chromatography

Superose 6 Increase (GE Healthcare), a 23 ml column was used for size exclusion chromatography as the last step of purification. The column was equilibrated with buffer R (10 mM Tris HCl pH 8, 100 mM KCl, 1 mM DTT). The concentrated protein from the last step was manually injected to a 1 ml loop into the column. Proteins were eluted based on the size of the protein. Smaller protein traverses a longer path through the small pores in the beads and takes a longer time to elute. Larger proteins unable to pass through the pore will traverse a shorter path and elute earlier. Fractions of the

eluted protein were checked by SDS PAGE and then concentrated using Vivaspin®2 (GE Healthcare) protein concentrator (MWCO 30 kDa). After concentration, protein concentration was analyzed by Nanodrop and aliquoted in 2.5 µl and flash frozen using liquid N_2 and stored at -80°C until further use.

2.5 Substrates for assays

1035 bp DNA :

ATGTTTGACTGGCTTCACACCCTCTTCTCGCGTGACCTCGCCATCGACCTCGGCAC
GGCGAACACGCTCATCTACATCCGCGGCCAGGGCATCGTCTCCAACGAGCCCTC
GGTGGTGGCCGTCCAGCAGGACGCGCGCGGGGGCAAGAAGGTCCTCGCGGTGG
GCAAGGAAGCCAAGGAAATGCTCGGGCGCACCCCGGGTAACATCGTGGCCATCC
GCCCGATGAAGGACGGCGTCATCGCGGACTTCGAAATCACGGCCGCGATGCTGC
GCTACTTCATCCAGAGCGCGCACAACCGCAAGACGCTCGTCAATCCGCGCATCAT
CATCGGCATCCCCTCCGGCATCACCGAGGTGGAGCGCCGCGCGGTGCGCGAGG
CGGCGGCCAACGCGGGCGCCCGCGAGGTCTACCTCATCGAGCAGCCCATGGCG
GCGGCCATTGGCGCGGGCCTTCCGGTGACGGAGCCCAGCGGCAACATGATAGTG
GACATCGGCGGTGGCACGTCCGACGTCGCGGTCATCAGCCTCGCCGGCATCGTG
TTCGCCAAGAGCGTGCGCATCGGCGGCGACAAGCTGGACGAGGCCATCATCCAG
TACGTCAAGCGCAAGTACAACCTGCTCATCGGTGAGCGCACGGCGGAGCTCATCA
AGATGGGCATCGGCACCGCGTACCCGACGGACGAGGTCATGACCATGGAGATCA
AGGGTCGCGACCTGGTGGCCGGCGTGCCGCGCACGCTCACGGTGTCCAGCGAC
GAAGTGCGTGACGCGCTGGCGGAGCCCGTCAACGGCATCGTCGAGGCGGTGAAG
CTGACGCTGGAGCGCACTCCCCCCGAGCTCGCCGGCGACATCGCCGACCGCGG
CATCGTCCTGGCCGGTGGCGGCGCGCTCCTGAAGAACCTGGACACGCTGCTGCG
CGAGGAGACGGGCCTGCCCGTGTTCCTCGCCGAGGACCCGCTCTCCGCCGTCGT
GATTGGCGCGGGCAAGGCGCTGGAGTCGCTCGACATCCTCCGGCAGGTCTGCCA
GCCGGGCTGA

235 bp DNA:

GGTGATGTACGAAGAGGAGTTCACCATAATCAACGCCGTTTGCGACCGGCTTACC
AAGGACGCGAACGCGAAGGTGGTCTTCCTCGTCGACAAGAACGGGCAGCTCATCT
CCTCCGCGGGTCAGACGCAGAACATCGACACCACGTCACTGGCCTCGCTGACGG
CCGGTAACGTGGCCGCGATGGGTGGCCTGGCCAAGCTGATTAGGGAGAACGAGT
TCCCCAACCAGTTCCAC

The DNA was amplified by PCR using the primers listed in Table 2.1. The recognition sites are marked in red in the DNA sequence above. The purity of DNA was checked on a 1% agarose gel. The reaction mix was then purified using Qiagen PCR purification kit and concentration was checked by Nanodrop measurement.

2.6 Cleavage assay of EcoP15I wt, EcoP15I D101A, EcoP15 D101N

Cleavage assay was done with a 1035 bp DNA having two recognition sites in head to head orientation. 300 nM protein and 30 nM DNA were pre-incubated at 25°C in the presence of buffer D+ (50 mM Tris HCl pH 8, 50 mM KCl, 10 mM MgCl2, 1 mM DTT, 100 µg/ml BSA) for 40 minutes. 1 mM ATP was added to initiate cleavage and kept at 25°c for 30 min. 0.5 volumes of Stop buffer (10 mM Tris pH 8, 100 mM EDTA, 40% w/v sucrose, 0.025% SDS, 0.03% BPB) was added to stop the reaction. EDTA in the stop buffer will chelate the Mg^{2+} ion and prevent any further use of ATP and nuclease activity. Samples was analyzed by loading onto a 1% agarose gel and electrophoresed at 100V. The gel was imaged using GeneSnap scanner from Syngene.

2.7 Methylation assay of EcoP15I wt, EcoP15 D101A, EcoP15 D101N

The 30 nM DNA and 300 nM protein were incubated in buffer D^+ in the presence of S-adenosyl methionine (SAM) for 1 hour at 25°C. Different concentration of SAM, ranging from 100 nM to 100 µM was used. The reaction mix was then purified using PCR purification column to separate the DNA. Concentrations of DNA were checked using Nanodrop. This DNA was then used to check its cleavage efficiency with EcoP15I wt

protein. The cleavage will indirectly tell us the amount of methylation that has happened on the DNA. If the DNA is methylated, it will be refractory to cleavage. Cleavage assay was done as mentioned above.

2.8 Cleavage assay of MboIII, MboIII D134A and MboIII loop mutant

A 235 bp DNA with recognition sites for MboIII in inverted orientation was used for the assay. 50 nM DNA and 300 nM protein was pre-incubated for 10 minutes at 37°C in buffer M (10 mM Tris-acetate pH 8, 10 mM KCl acetate, 10 mM MgCl2, 1 mM DTT). 4 mM ATP is then added to the reaction and kept further for 45 minutes at the same temperature. Native stop buffer (10 mM Tris pH 8, 60 mM EDTA, 60% glycerol, 0.025% SDS, 0.03% BPB) was added to stop the reaction at the end of 45 minutes and heated at 65°C for 20 minutes to heat inactivate the enzyme. Samples were loaded onto 12% native PAGE gel (12% Acrylamide: Bis-Acrylamide (29:1), 1X TBE, APS, TEMED). The gel was pre-run for 20 minutes and wells are washed thoroughly before loading of samples. Electrophoresis was carried out at 150V and gel was post-stained in an ethidium bromide solution for 5 minutes. Gel image was taken using Typhoon TRIO+ variable mode imager (GE Healthcare).

2.9 Methylation assay of MboIII, MboIII D134A and MboIII loop mutant

Methylation assay was carried out with 50 nM DNA and 300 nM protein in presence of different concentrations of SAM for 1 hour at 37°C in buffer M. The DNA was then purified by Qiagen PCR purification column and cleavage assay was carried out with the DNA.

Results & Discussion

RESULTS

3.1 Cloning of EcoP15I mutants

Mutations were introduced to the gene by two-step of PCR reaction. In the first PCR, a fragment size of 318bp was amplified (Fig 3.1.1a & 3.1.2a). The overlap extension PCR using 318bp as forward primer gave an amplified product of 5 kb corresponding to the gene (Fig 3.1.1b & 3.1.2b). 7 clones were checked by double digestion from the recombinant plasmids of EcoP15ID101A. Upon digestion 5 of the cloned plasmids cleaved into a 2.7 kb and a 5 kb fragment (Fig 3.1.1c). 4 clones were checked from the EcoP15I D101N mutant. Upon digestion of this plasmid, all of them cleaved into a 2.7 kb and a 5 kb fragment (Fig 3.1.2c).

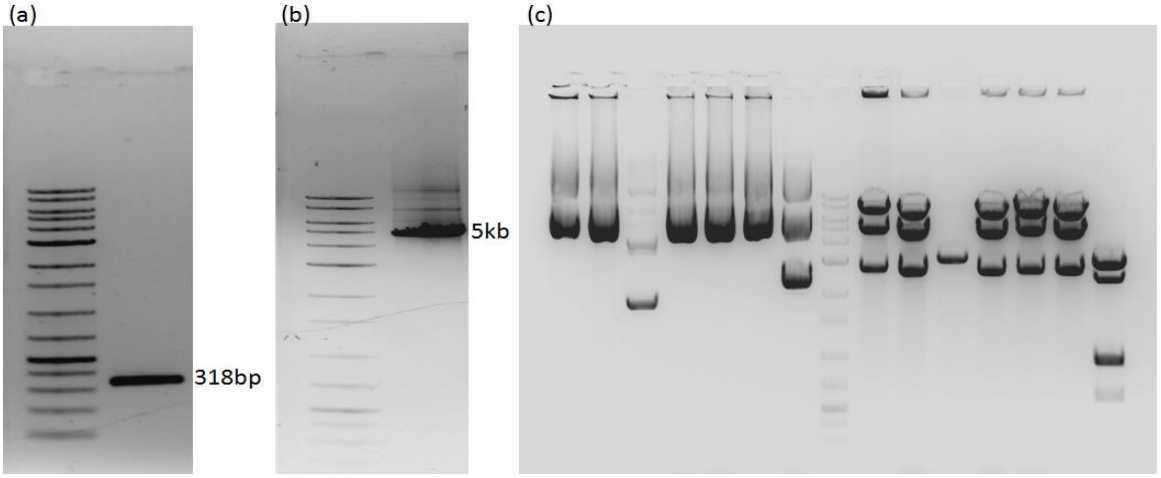

Fig 3.1.1 Cloning of EcoP15I D101A. (a) First PCR to incorporate the mutation. (b) Complete amplification of 5kb EcoP15I gene (c) Clones double digested with BamHI and NdeI. Lanes 1 to 7 are the plasmid isolated after cloning. Lanes 9-15 are corresponding double digested plasmids

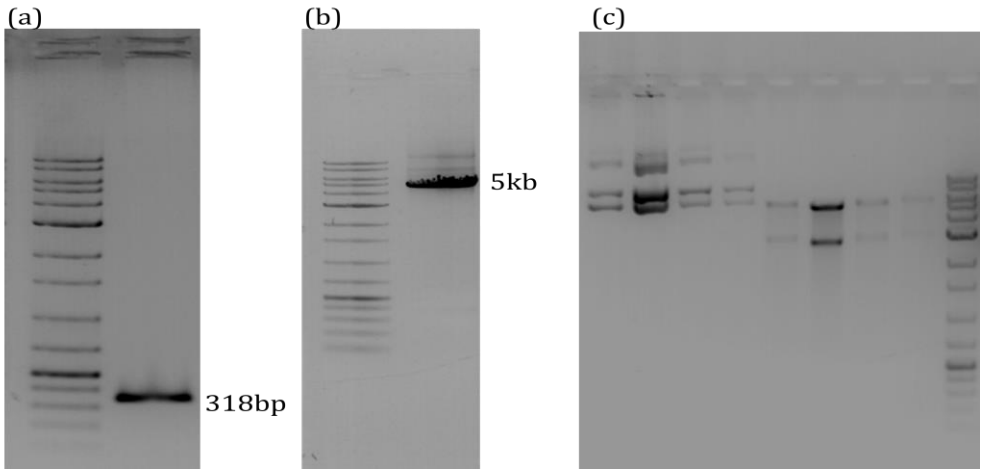

Fig 3.1.2 Cloning of EcoP15I D101N. (a) First PCR to incorporate the mutation. (b) Complete amplification of 5kb EcoP15I gene (c) Clones double digested with BamHI and NdeI. Lanes 1 to 4 are the plasmid isolated after cloning. Lanes 5-8 are corresponding double digested plasmids

3.2 Cloning of MboIII mutants

MboIII D134A and MboIII Loop deletion constructs were also cloned in the same way. In the MboIII D134A construct, a fragment of 304 bp was amplified after the first PCR (Fig 3.2.1a). The second PCR reaction amplified the entire MboIII gene of 1.6 kb (Fig 3.2.1b). The recombinant plasmids from three colonies were checked by double digestion. After the double digestion, plasmid got cut into a 1.6 kb and 3.7 kb fragments (Fig 3.2.1c). In the loop deletion construct, first PCR amplified a fragment of 414 bp (Fig 3.2.2a) and second PCR amplified the 1.6 kb gene (Fig 3.2.2b). Double digestion of recombinant plasmids from 3 colonies cleaved into required band sized fragments of 1.6 kb and 3.7 kb (Fig 3.2.2c).

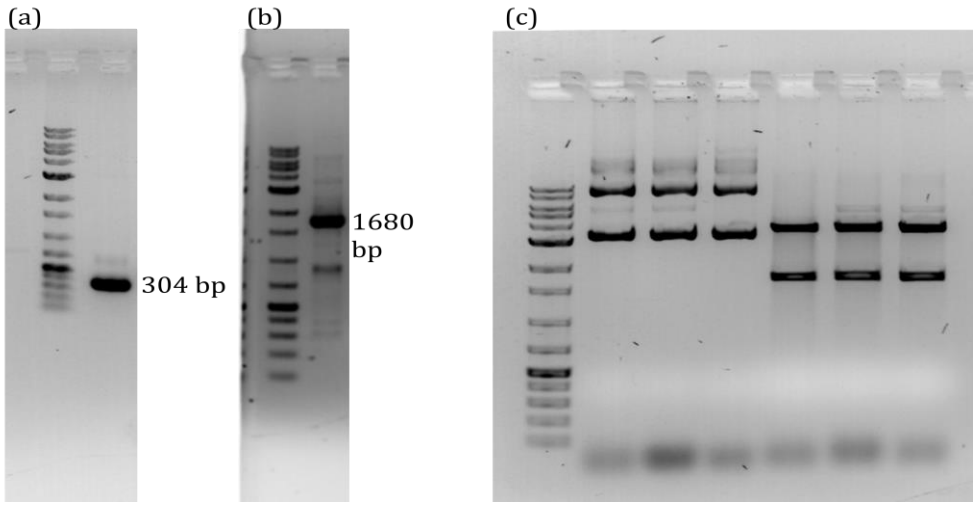

Fig 3.2.1 Cloning of MboIII D134A (a) First pcr to incorporate the mutation. (b) Complete amplification of 1.6kb MboIII gene using the first PCR product (c) Clones double digested with XhoI and NcoI. Lanes 2 to 4 are the plasmid isolated after cloning. Lanes 5-7 are corresponding double digested plasmids

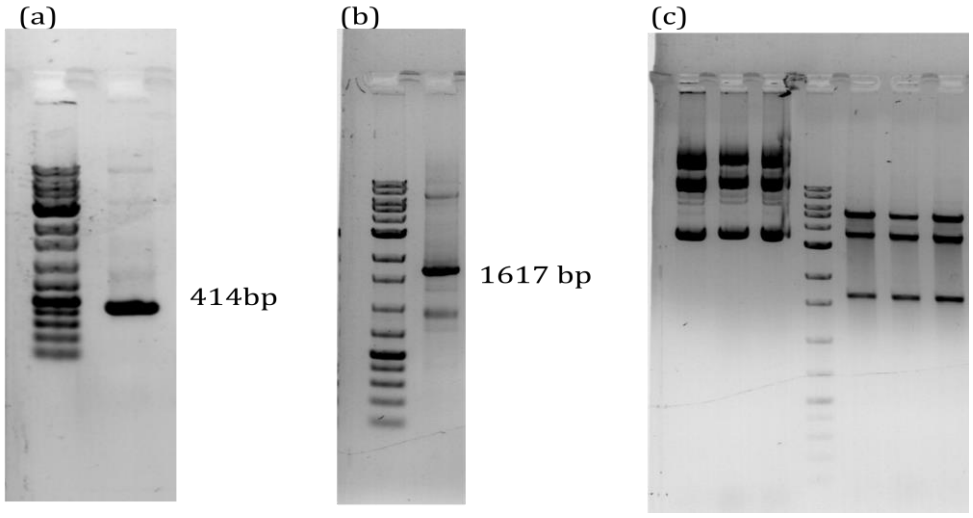

Fig 3.2.2 Cloning of MboIII Loop deletion (a) First pcr to incorporate the mutation. (b) Complete amplification of 1.6kb MboIII gene using the first PCR product (c) Clones double digested with XhoI and NcoI. Lanes 1 to 3 are the plasmid isolated after cloning. Lanes 5-7 are corresponding double digested plasmids

3.3 Sequencing result of the mutants

The sequencing of the gene of all mutants showed that the mutations are incorporated as desired (Fig 3.3). In the Loop deletion construct of MboIII, 63 basepairs were deleted and 4 stretches of GCC (Gly amino acid) was added (Fig 3.3d).

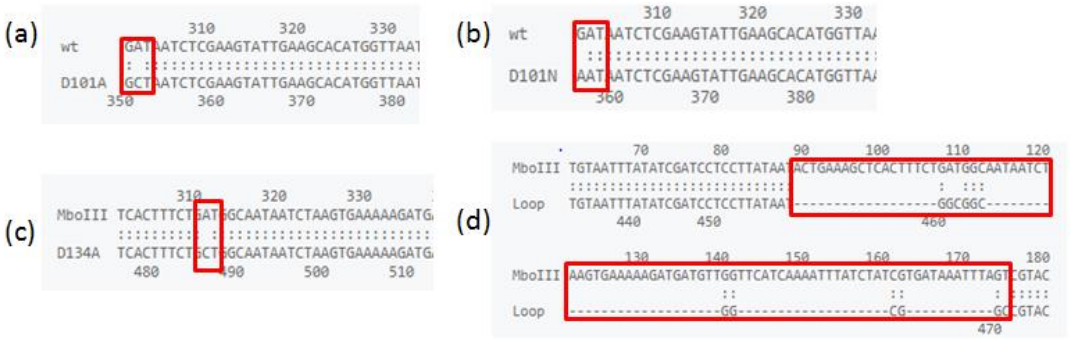

Fig 3.3 Sequencing results of all the mutations made. (a) EcoP15I D101A (b) EcoP15I D101N (c) MboIII D134A (d) MboIII Loop deletion. Red boxes indicate the codon in the wild type protein changed for the desired mutation.

3.4 Protein expression of EcoP15I mutants

Mutants of EcoP15I, EcoP15 D101A & EcoP15 D101N, had the protein overexpressed in BL21AI cell lines (Fig 3.4). An analysis on SDS-PAGE had a band of 75 kDa and 110 kDa as marked by red arrows corresponding to Mod and Res subunit respectively.

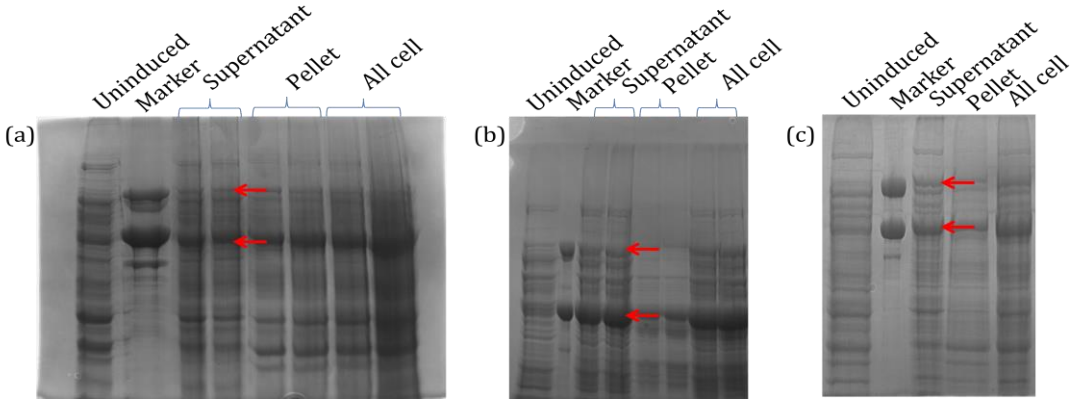

Fig 3.4 Protein expression check of EcoP15I mutants (a) EcoP15I wt (b) EcoP15I D101A (c) EcoP15I D101N. The red arrow indicates 110 kDa band of Res subunit and 75 kDa band of Mod subunit.

3.5 Protein expression of MboIII mutants

MboIII D134A and MboIII Loop deletion construct expressed the protein in BL21(DE3) cells. A band of 66 kDa of Mod and 94 kDa Res was observed in the SDS-PAGE gel (Fig 3.5). Most of the protein was present in the pellet fraction which could be due to an improper sonication.

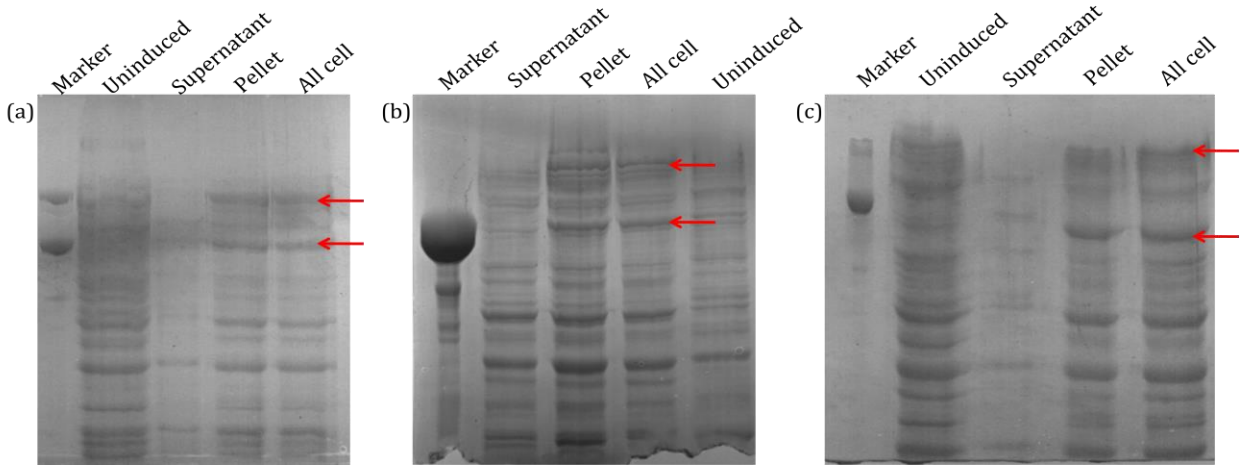

Fig 3.5 Protein expression check of MboIII mutants (a) MboIII wt (b) MboIII D134A (c) MboIII loop deletion. The red arrow indicates 94 kDa band of Res subunit and 66 kDa band of Mod subunit.

3.6 Protein purification

Purification was done in three steps. After each column, SDS-PAGE was used to analyze protein fraction (Fig 3.6.1 to Fig 3.6.6). The purest fractions were then selected and used further. All the mutants were purified and concentrated. The concentration of each protein batch is as given below.

EcoP15I wt	: 200 µl of 19 mg/ml
EcoP15I D101A	: 200 µl of 19 mg/ml
EcoP15I D101N	: 100 µl of 1 mg/ml
MboIII wt	: 60 µl of 1.6 mg/ml

MboIII D134A : 200 µl of 11 mg/ml

MboIII Loop deletion : 200 µl of 8.6 mg/ml

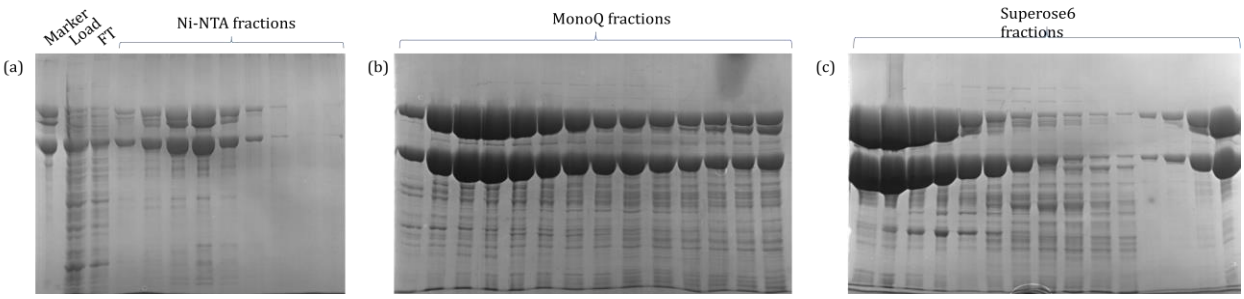

Fig 3.6.1 Purification of EcoP15 wt

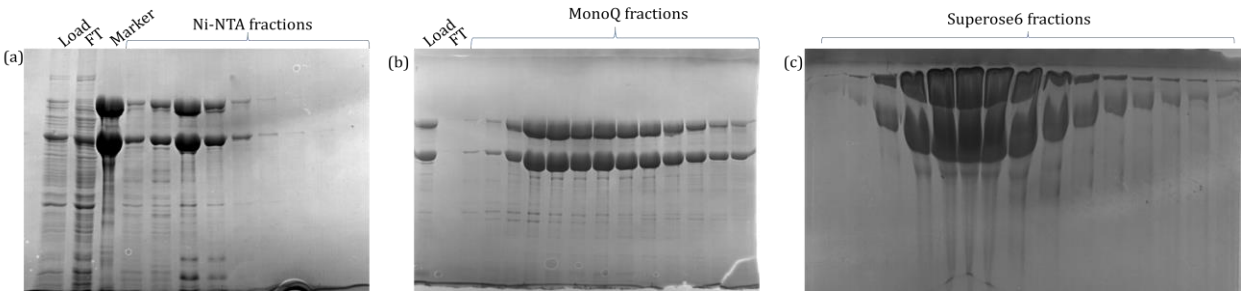

Fig 3.6.2 Purification of EcoP15I D101A

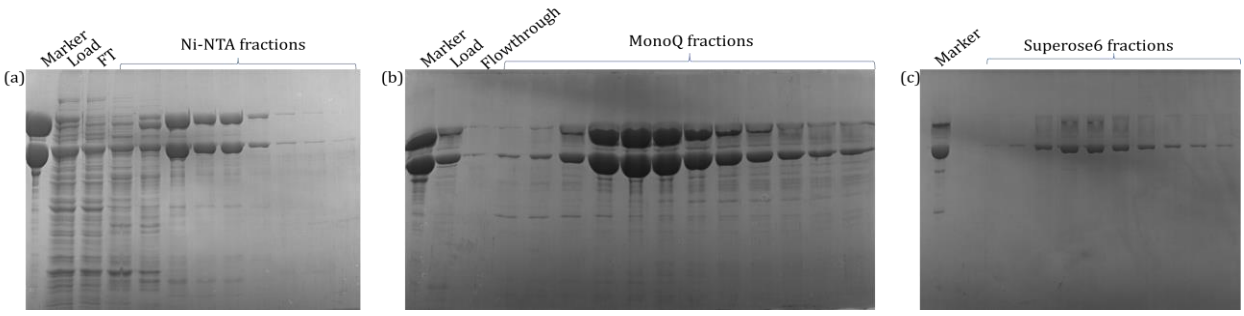

Fig 3.6.3 Purification of EcoP15I D101N

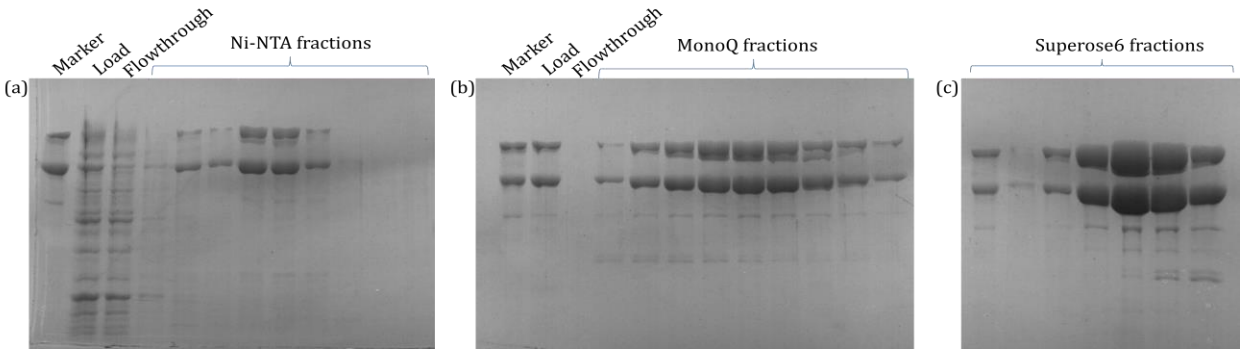

Fig 3.6.4 Purification of MboIII wt

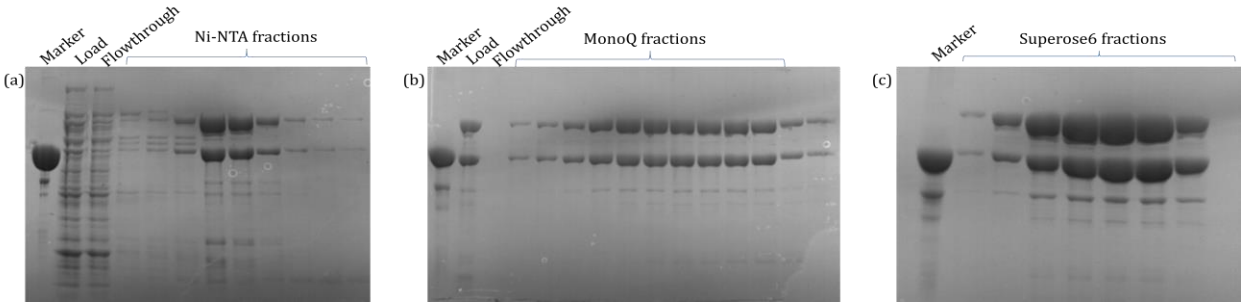

Fig 3.6.5 Purification of MboIII D134A

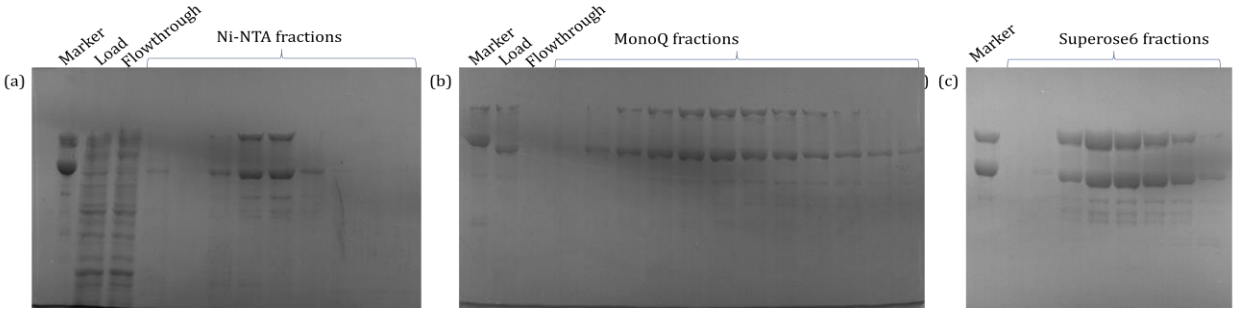

Fig 3.6.6 Purification of MboIII Loop mutant

3.7 Cleavage assay of EcoP15I wt at different protein concentrations

Cleavage assay of EcoP15 wt was done with 1035 bp DNA at different ratios of protein and DNA (Fig 3.7). From 1:2 concentrations of DNA and protein, cleavage was observed. Complete cleavage requires a 1:4 concentration of DNA and protein as seen

is Lane 6 of Fig 3.7. Further increase in protein doesn't have any evident change. Hence the concentration of DNA and protein was fixed to be 30nM and 120nM for further assays.

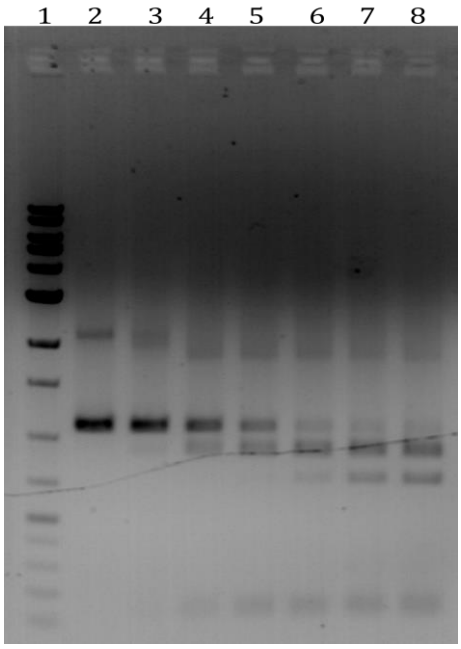

Fig 3.7 Cleavage of 1035 bp DNA with different concentrations of protein. Lane 1: DNA Ladder, Lane 2: 30 nM DNA, Lane 3: 30 nM DNA + 30 nM EcoP15I wt, Lane 4: 30 nM DNA + 60 nM EcoP15I wt, Lane 5: 30 nM DNA + 90 nM EcoP15I wt, Lane 6: 30 nM DNA + 120 nM EcoP15I wt, Lane 7: 30 nM DNA + 240 nM EcoP15I wt, Lane 8: 30 nM DNA + 300 nM EcoP15I wt

3.8 Cleavage assay of EcoP15I wt, EcoP15I D101A, EcoP15I D101N

Cleavage assay of the EcoP15I wild-type, EcoP15I D101A, EcoP15I D101N was done to check the nucleolytic activity of the mutants. The mutants of Aspartate (D101A & D101N) were nuclease active as it cleaved the DNA substrate as efficiently as the wild type enzyme (Fig 3.8). This was expected as the mutation in SAM binding pocket should not have any effect on restriction activity. Cleavage was also done in presence of Sinefungin (SNF), an analogue of SAM to check if it has any effect in cleavage. Presence of SNF did not affect the nucleolytic activity of the proteins.

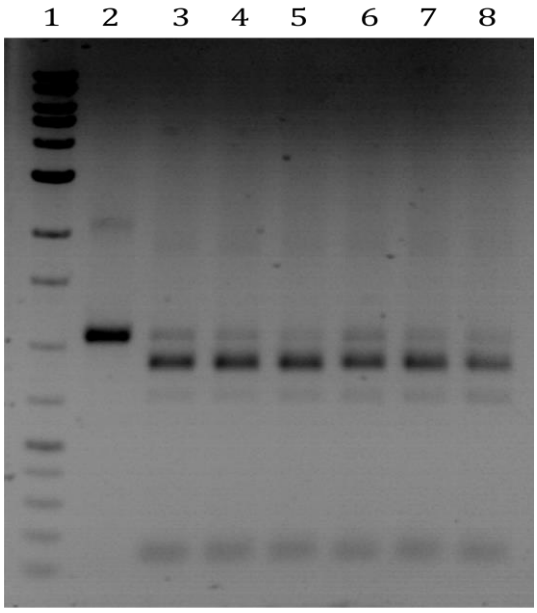

Fig 3.8 Cleavage of 1035 bp DNA with EcoP15I wt, EcoP15I D101A & EcoP15I D101N.
Lane 1: DNA Ladder, Lane 2: 30 nM DNA, Lane 3: 30 nM DNA + 120 nM EcoP15I wt, Lane 4: 30 nM DNA + 120 nM EcoP15I D101A, Lane 5: 30 nM DNA + 120 nM EcoP15I D101N, Lane 6: 30 nM DNA + 120 nM EcoP15I wt + 20 μM SNF, Lane 7: 30 nM DNA + 120 nM EcoP15I D101A + 20 μM SNF, Lane 8: 30 nM DNA + 120 nM EcoP15I D101N + 20 μM SNF

3.9 Methylation assay of EcoP15I D101A

Methylation activity of mutants was analyzed by an indirect method of methylation assay followed by cleavage check of the methylated DNA. As a control, EcoP15I wt was used. DNA methylated by EcoP15I D101A was refractory to cleavage by EcoP15I wt protein (Fig 3.9.1). There was a slight difference in methylation of EcoP15I wt and EcoP15I D101A. At 100 μM of SAM concentration, EcoP15Iwt and EcoP15ID101A could methylate the DNA as the DNA methylated by them could not be cleaved by wild-type protein. But, the EcoP15I D101A had a slight band at 880bp corresponding to cleaved product (Fig 3.9.1). Hence, a methylation assay at different concentrations of SAM was carried out. At different concentrations of SAM, the effect of mutation was clearer. While EcoP15I wt methylated DNA completely at 1 μM, EcoP15I D101A needed 100 μM of SAM to attain the same methylation (Fig 3.9.2). This indicates that the aspartic acid at 101 position helps in binding of SAM. Since there are redundant interactions for SAM to

bind to the pocket, mutation at this position is not effective at higher SAM concentrations.

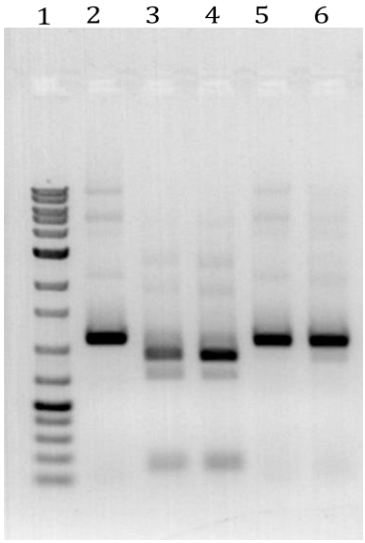

Fig 3.9.1 Methylation assay in the presence of 100 µM SAM. Lane 1 : DNA Ladder, Lane 2 : DNA, Lane 3 : DNA + EcoP15I wt + ATP, Lane 4 : DNA + EcoP15I D101A + ATP, Lane 5 : DNA methylated by EcoP15I wt + EcoP15I wt + ATP, Lane 6 : DNA methylated by EcoP15I D101A + EcoP15I wt + ATP

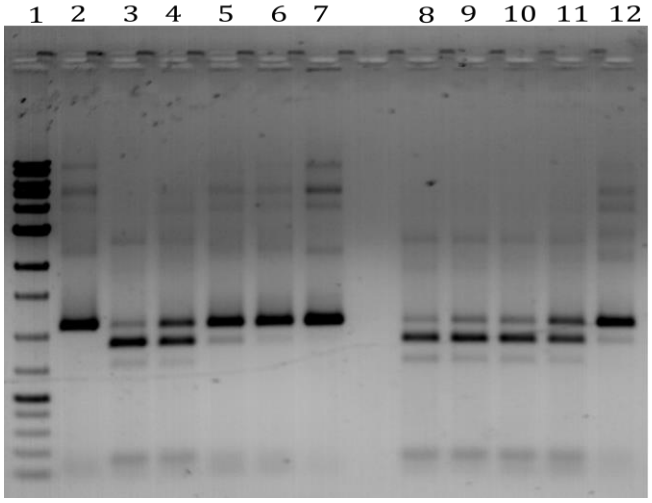

Fig 3.9.2 Methylation assay in different concentrations of SAM. Lane 1 : DNA Ladder, Lane 2 : DNA Control, Lane 3 : DNA + EcoP15I wt, Lane 4 : DNA + EcoP15I wt + 100 nM SAM, Lane 5 : DNA + EcoP15I wt + 1 µM SAM, Lane 6 : DNA + EcoP15I wt + 10 µM SAM, Lane 7 : DNA + EcoP15I wt + 100 µM SAM, Lane 8 : DNA + EcoP15I D101A , Lane 9 : DNA + EcoP15I D101A + 100 nM SAM, Lane 10 : DNA + EcoP15I D101A + 1 µM SAM, Lane 11 : DNA + EcoP15I D101A + 10 µM SAM, Lane 12: DNA + EcoP15I D101A + 100 µM SAM

3.10 Methylation assay of D101N

Methylation was carried out at different SAM concentrations for EcoP15I D101N and EcoP15I wt. The methylation of EcoP15I D101N was closer to the methylation pattern of EcoP15I wt. The mutant methylated completely at 10 μM of SAM. A mutation from Asp101 to Asn did not seem to affect the methylation activity of EcoP15I as much as a mutation from Asp101 to Ala. This indicates that the aspargine, through a negative charge present at its side chain, is able to interact with SAM similar to aspartic acid.

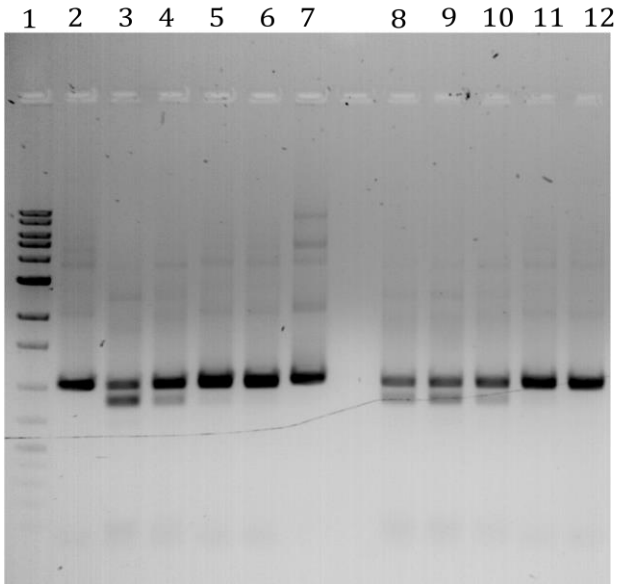

Fig 3.10 Methylation assay of EcoP15I D101N mutant in different SAM concentrations.
Lane 1 : DNA Ladder, Lane 2 : DNA Control, Lane 3 : DNA + EcoP15I wt, Lane 4 : DNA + EcoP15I wt + 100nM SAM, Lane 5 : DNA + EcoP15I wt + 1 μM SAM, Lane 6 : DNA + EcoP15I wt + 10 μM SAM, Lane 7 : DNA + EcoP15I wt + 100 μM SAM, Lane 8 : DNA + EcoP15I D101N, Lane 9 : DNA + EcoP15I D101N + 100 nM SAM, Lane 10 : DNA + EcoP15I D101N + 1 μM SAM, Lane 11 : DNA + EcoP15I D101N + 10 μM SAM, Lane 12: DNA + EcoP15I D101N + 100 μM SAM

3.11 Comparison of different methyltransferase

Methylation is used by both prokaryotic and eukaryotic cells for various purposes like gene silencing, transcriptional control, self protection from phages, etc. There are different kinds of methyltransferase proteins that methylate DNA, RNA, protein and

small molecules. DNA MTase and protein MTase have certain common features in terms of the cofactor binding pocket and the catalytic mechanism. Hence different classes of MTase were analyzed to find out the level of conservation of the non-canonical motif GDN.

The DNA Mtases are of mainly three kinds based on the nucleotide methylated in the DNA: N6-adenine methyltransferase, N4-cytosine Mtase and C5-cytosine MTase (Scavetta et al., 2000). Protein structures from each class of AdoMet Dependent MTase shown above were analyzed for the presence of a GDN like motif in the binding pocket of SAM.

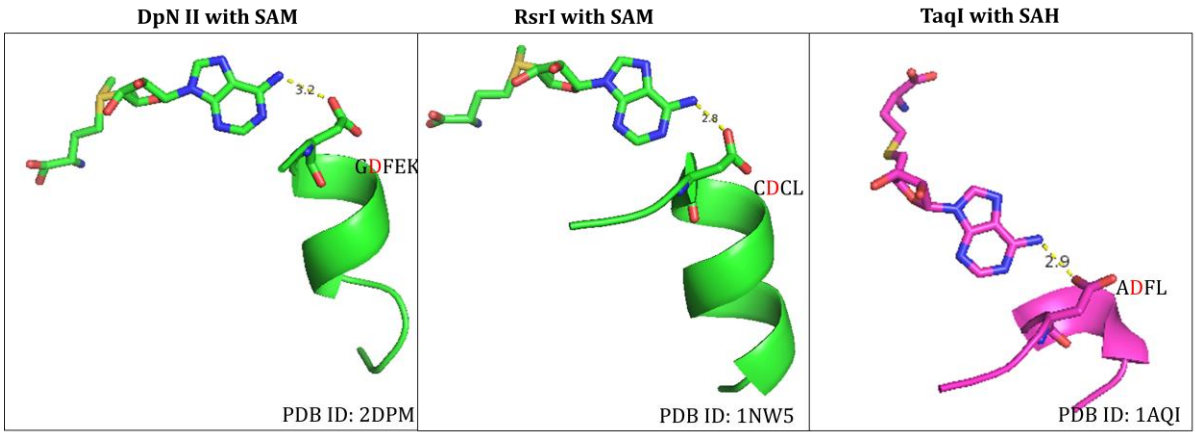

Fig 3.11.1 Interaction of N6-adenine of SAM in N6-adenine methyltransferases

The N6-adenine methyltransferase (DpnII, RsrI, TaqI) has an aspartate residue interacting with the N6-adenine moiety of SAM. In all of these, this aspartate is part of the Motif III GILLADFLLW which is a SAM binding motif.

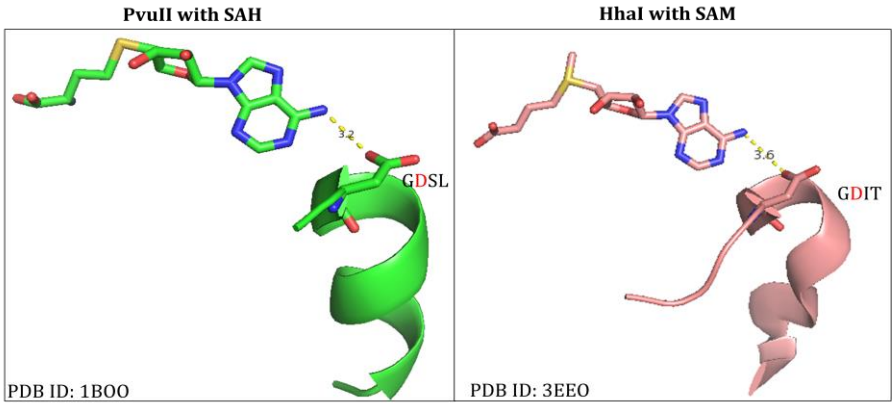

Fig 3.11.2 Interaction of N6-adenine of SAM in Cytosine methyltransferases

The N4-cytosine MTase PvuII (β class) also has GDS, where the aspartate interacts with the N6 of adenine in SAH (S-adenosyl homocysteine). Likewise, C5-cytosine MTase HhaI has an aspartate facing the N6-adenine of SAM. This aspartate when checked in the sequence was part of the Motif III GDIT in HhaI and PvuII. An Asp residue in the motif III is conserved among the DNA methyltransferases and it interacts with N6 of adenine. Hence, we propose that the Motif III in Type III class of enzymes is wrongly defined. Based on the structural analysis, this Asp should belong to the motif III.

Previous structural studies had shown that the SAM binding pocket was conserved in the protein methyltransferase family (Campagna-Slater et al., 2001, Boriack-Sjodin and Swinger, 2015). PMTs too had an interacting residue with the N6 of adenine. We looked at different classes of protein methyltransferase to see if such an interaction with an Asp or an equivalent residue is comparable to DNA MTase.

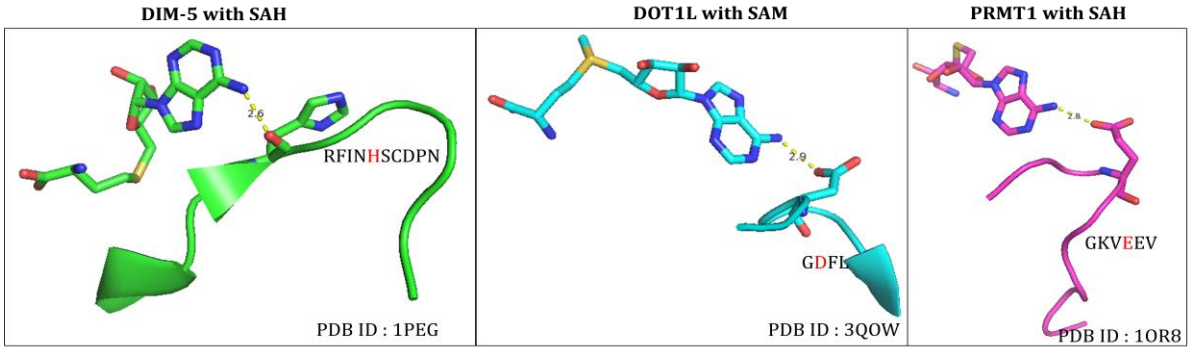

Fig 3.11.3 Interaction of N6-adenine of SAM in protein methyltransferases

DIM-5 protein, a SET domain PKMT, has a histidine in the position of Asp. Histidine interacts with the N6-adenine of SAH through its main chain carbonyl oxygen. This Histidine is part of Motif III RFINHxCxPN in the SET domain. DOT1L, a non SET domain PKMT interacts with the N6-adenine through an aspartate. This aspartate is the residue that precedes the defined motif III F(V/L) in non SET domain PKMT. The PRMT1 enzyme also has the same interaction but through a Glutamic acid residue.

3.12 Methylation assay of MboIII D134A

A methylation assay was done for the MboIII D134A mutant at 100 nM and 100 µM of SAM concentration. The mutant was observed to methylate the DNA as efficiently as the MboIII wt protein (Fig 3.12). This indicated that a change of Asp to alanine does not affect the binding of SAM in its pocket.

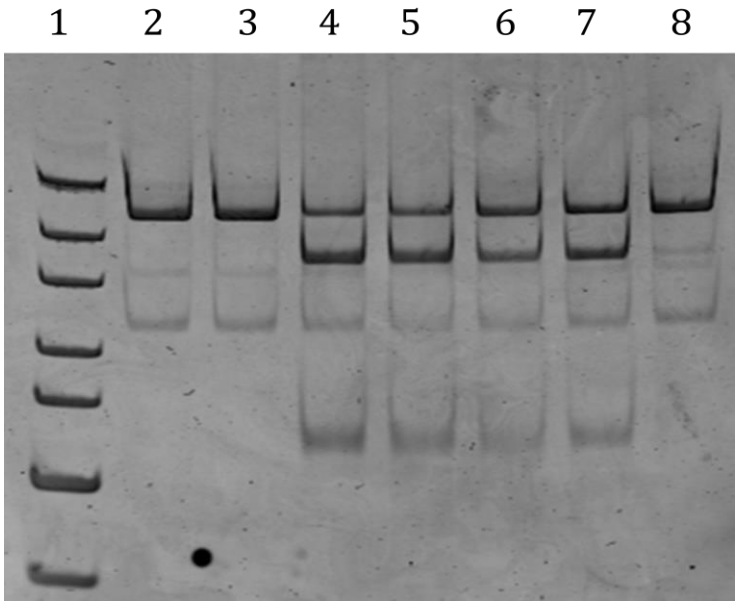

Fig 3.12 Methylation assay of MboIII D134A mutant. Lane 1: DNA Ladder, Lane 2 : DNA Control, Lane 3 : DNA + MboIII wt, Lane 4 : DNA + MboIII wt +ATP, Lane 5 : DNA methylated by MboIII wt at 100 nM SAM + MboIII wt + ATP, Lane 6 : DNA methylated by MboIII wt at 100 µM SAM + MboIII wt + ATP, Lane 7 : DNA methylated by MboIII D134A at 100 nM SAM + MboIII wt + ATP, Lane 8 : DNA methylated by MboIII D134A at 100 µM SAM + MboIII wt + ATP

3.13 Methylation assay of MboIII Loop mutant

MboIII Loop mutant was able to methylate the DNA similar to the methylation pattern of wild-type MboIII. The loop from T128 to S156 was not needed for methylation activity. A disordered loop could also lead to methylation. But mutant was deficient in nuclease activity. Disruption of the loop had affected the activity of the Res subunit. This can be because the oligomerization has affected or a crosstalk between the Mod and Res has affected. The superose6 profile during purification of protein confirms that the complex of Mod and Res has formed. Further cleavage assay had to be carried out to investigate the interaction between mod and res being affected.

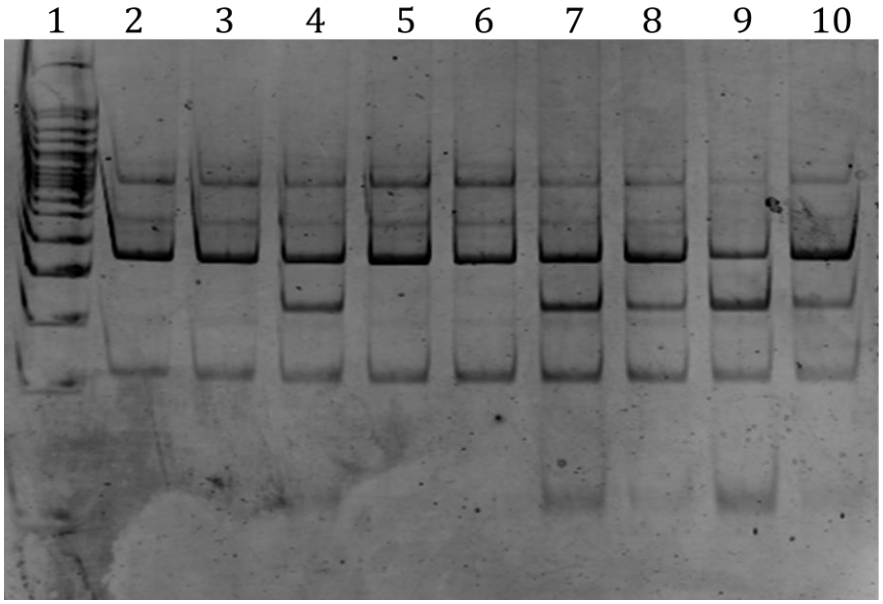

Fig 3.13 Methylation assay of MboIII Loop mutant. Lane 1 : DNA Ladder, Lane 2 : DNA Control, Lane 3 : DNA + MboIII wt, Lane 4 : DNA + MboIII wt + ATP, Lane 5 : DNA + MboIII Loop mutant, Lane 6 : DNA + MboIII Loop mutant +ATP, Lane 7 : DNA methylated by MboIII wt at 1 μM SAM + MboIII wt + ATP, Lane 8 : DNA methylated by MboIII wt at 200 μM SAM + MboIII wt + ATP, Lane 9 : DNA methylated by MboIII Loop mutant at 1 μM SAM + MboIII wt + ATP, Lane 10 : DNA methylated by MboIII Loop mutant at 200 μM SAM + MboIII wt + ATP

3.14 Cleavage assay of MboIII Loop mutant

A cleavage assay was done to find the effect of Loop mutation in the restriction activity. The mutant who was deficient in cleavage could cleave the DNA in presence of SNF. SNF being an analog of SAM, will bind to the pocket of SAM. This binding or the ordered loop seems to be important for the activity of Res. The loop could be acting as an allosteric effector in cleavage. The function of the loop can be replaced by the binding of ligand in the SAM pocket too.

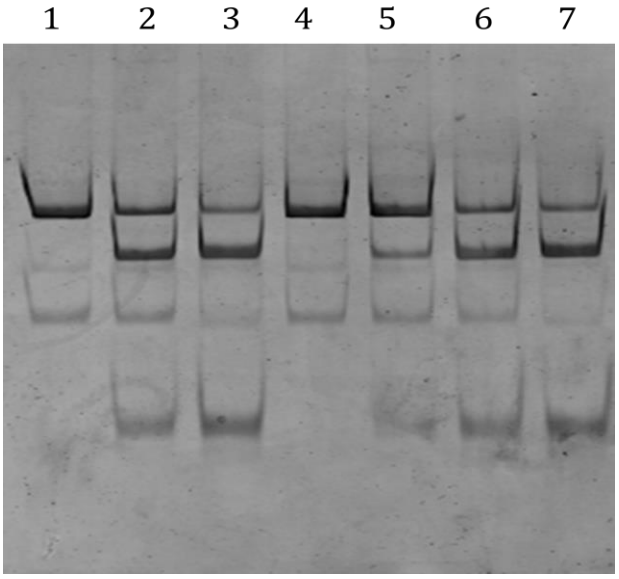

Fig 3.14 Cleavage assay of MboIII Loop mutant. Lane 1 : DNA Control, Lane 2 : DNA + MboIII wt + ATP, Lane 3 : DNA + MboIII wt + SNF + ATP, Lane 4 : DNA + MboIII Loop mutant + ATP, Lane 5 : DNA + MboIII Loop mutant + SNF + ATP, Lane 6 : DNA + MboIII D134A + ATP, Lane 7 : DNA + MboIII D134A + SNF + ATP

DISCUSSION

Cloning of the mutants (EcoP15I D101A, EcoP15I D101N, MboIII D134A & MboIII Loop deletion) was successfully done using restriction digestion method. The recombinant plasmids were confirmed by DNA sequencing and had the desired mutations. Proteins were purified as per the protocol.

Towards finding the significance of a highly conserved motif, GDN in EcoP15I protein, mutagenesis and biochemical assays were carried out. As the mutated residue, Asp101 was found to interact with N6-adenine of SAM, we expected it to be deficient in methylation. It was found that the mutants of Aspartate (D101A & D101N) could methylate the DNA at higher concentrations of SAM but not at lower concentrations. While EcoP15I wild-type methylated DNA completely at 1 μM SAM concentration, EcoP15I D101A & EcoP15I D101N needed 100 μM and 10 μM of SAM respectively to attain the same methylation as that of wild type (Fig 3.9.2 & Fig 3.10). This indicates that the aspartic acid at 101 position is helping in binding of SAM. Since there are redundant interactions for SAM to bind to the pocket, mutation at this position is not effective at higher SAM concentrations. Structural analysis of different methyltransferase was carried out to find the conservation level of this particular stretch. All the enzymes checked had a negative charge, corresponding to the Asp in EcoP15I that interacts with the N6-adenine position of SAM. Also, it was found that the negatively charged residue is part of the motif III in such classes. The motif III of EcoP15I, which is proposed to have SAM binding function was then checked and found to be far from the SAM binding pocket. Hence, we conclude that the Motif III is wrongly assigned in Type III RM enzymes and it should be the GDN stretch. Mutation on this stretch in this study has shown that it is important in SAM binding

Mutations on MboIII gene was introduced to find the relevance of an interaction from loop T128 to S156 in the methylation activity of the protein. We hypothesized that the loop region is responsible for the division of labor observed in Type III RM enzymes. It

was found that the MboIII D134A and MboIII Loop deletion construct could methylate the DNA completely at the concentrations of SAM used (Fig 3.12 & Fig 3.13). The loop from T128 to S156 does not have any significance in the methylation reaction. Interestingly, the loop deletion from T128 to S156 was deficient in nuclease activity. Disruption of the loop has affected the activity of the Res subunit. This is expected to be because the interaction of Mod subunit to the Res subunit is disturbed. A cleavage assay was done to find the effect of Loop mutation in the restriction activity (Fig 3.14). It was observed that the mutant who was deficient in cleavage was able to cleave the DNA in presence of SNF, an analog of SAM. Hence, we propose that the loop is an allosteric effector in the activity of Res subunit.

Conclusion

The mutations and biochemical assays for EcoP15I and MboIII were done as planned.

Identification of GDN as motif III in methyltransferases of Type III

The mutation on EcoP15I was done based on previous identification of a highly conserved motif, GDN. The mutants of GDN motif (D101A and D101N) appeared to be relatively less efficient than the wild type protein in methylating the target DNA. At lower concentrations of the cofactor, the mutant was not able to methylate as efficiently as wild-type. However, on increasing the concentration of the cofactor to 100 μM, the mutant methylated DNA as well as the wild-type protein. This suggested that the aspartate residue is important for binding of SAM rather than for catalysis, as the two mutants displayed efficient methylation at higher concentration of the cofactor. This led us to conclude that GDN motif is a SAM-binding motif. Further quantification of the SAM-binding affinity of the mutants is required to be performed using cleavage assays in the presence of SAM.

Structural analysis of different classes of methyltransferases revealed that the Asp in GDN motif was highly conserved amongst all DNA methyltransferases. Analysis on protein methyltransferase showed the conservation of the same interaction through main chain carbonyl, glutamic acid or aspartic acid residues. This concluded that a presence of a negative charge in the SAM binding pocket facing the NH_2 group in the adenine ring is highly conserved among all methyltransferases. The analysis revealed that the GDN motif was part of motif III of MTases in other classes. The motif III ascribed previously for Type III RM enzymes, which is proposed to be the SAM-binding motif was at the C-terminal end. In the structure of EcoP15I, this region was found to be away from the SAM binding pocket. Hence the Motif III seemed to be wrongly identified

in Type III classes of enzymes. We propose that it is actually the GDN, which is the motif III, which is the first motif that comes in the arrangement towards N-terminal. A previous study had mutated the Asp in Motif III in the enzyme EcoRV (α class of N6-adenine DNA methyltransferase), corresponding to the same mutation done in EcoP15I In this study the authors had concluded that it is not important in SAM binding (Roth et al., 1998). This could have been because a rather higher concentrations of SAM was used (Roth et al., 1998). I noted a similar behavior in case of EcoP15I (D101A and D101N) mutant, which could methylate DNA at higher concentrations of SAM. The effect of the mutation on methylation is evident only at lower concentrations of SAM. This is consistent with my proposal that Motif III is primarily involved in SAM binding.

Regulatory role of loop (128-155) in the restriction-modification enzyme MboIII

Deletion of the loop residues T128 to S156 in MboIII has revealed that the loop is important for restriction activity. This was an unexpected observation as the mutation was on the Mod subunit and hence we expected it to affect the methylation of the enzyme. But the study shows that the loop in Mod modulates the nucleolytic activity of Res subunit. The loss in nuclease activity could be compensated for by addition of SNF. This potentially indicates an allosteric regulation from the Mod subunit to the Res subunit that is needed in the restriction activity. Interestingly, structural analysis of the crystal structure of the dimeric MTase M.MboIII, which is a β-class of N6-adenine methyltransferase, shows a similar interaction mediated by a helix with SNF. However, the same is not conserved in the monomeric MTase Rsr1, another β-class of N6-adenine methyltransferase. This could indicate the relevance of the loop in case of dimeric MTase – MboII and MboIII. How the loop regulates the restriction activity of MboIII, and how SNF binding compensates for the loss of the loop requires further biochemical and structural studies in future.

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