Characterization of short sequence repeats of Type III Restriction Modification Enzyme MboIII

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of Five Year BS-MS Dual Degree Program

Indian Institute of Science Education and Research, Pune

By

Karishma R. Bhagat

20121097

Project Supervisor

Dr. Saikrishnan Kayarat
Associate Professor,
Dept of Biology,
IISER, Pune

Project Advisor

Dr. Jeetendar Chugh
Assistant Professor,
Dept of Chemistry,
Dept of Biology
IISER, Pune

Certificate

This is to certify that this dissertation entitled “Characterization of SSR in Phase Variable TYPE III Restriction Modification Enzyme MboIII” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Karishma Bhagat at IISER Pune under the supervision of Dr.Saikrishnan Kayarat, Associate Professor, Dept. of Biology, during the academic year 2016-2017.

Karishma Bhagat
20121097
5th Year BS-MS,
IISER, Pune

Associate Professor,
Dept of Biology,
IISER, Pune

Declaration

I hereby declare that the matter embodied in the report entitled “Characterization of SSR in Phase Variable Type III Restriction Modification Enzyme MboIII” 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.

Karishma Bhagat
20121097
5th Year BS-MS,
IISER, Pune

Dr. Saikrishnan Kayarat
Associate Professor,
Dept of Biology,
IISER, Pune

Abstract

Short Sequence Repeats (SSR) are a means of achieving phase variation, i.e. turning on/off of genes, in bacteria. Phase variation is known to be important for survival of host-adapted pathogen. However, the effect of SSR on the activity of the gene product is not clear. Type III enzymes are one of four types of Restriction-Modification (RM) enzymes found in bacteria which protects them against foreign DNA invasion. Many Type III enzymes have Short Sequence Repeats (SSR), which have high propensity for phase variation. For example, the Type III enzyme MboIII from the pathogen *Mycoplasma bovis* has 9 AG repeats. A three-dimensional homology model of the enzyme revealed presence of repeat at the interface of the three-subunit protein complex of MboIII. Sequence analysis revealed that the position of the repeats is conserved amongst a number of Type III RM identified in as many as 10 genera. Furthermore, we compared the nuclease and methyltransferase activities of MboIII with and without repeats. Nuclease and methylation assays suggested that MboIII with repeats cleaved and methylated the DNA appreciably better than without repeat. This is a small step towards understanding the plastic nature of Type III enzymes and dual nature of the mod gene. Detailed experiments are required to be carried out to better understand the molecular effect of SSR on enzyme structure and function.

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1. Introduction

Survival is the primary instinct of any living organism. Foreign bodies are constantly invading their hosts to survive. We have immune system which combats these invaders and protects us from them. Bacteria, however, do not have a well-defined immune system which will help them protect against invaders. Viruses and phages constantly attack them, killing the host bacteria. Hence, to protect themselves, bacteria have evolved a very unique strategy, Restriction-Modification Enzymes.

Restriction-Modification enzymes are proteins which cleave foreign DNA entering the cell, protecting its own from restriction by adding methyl groups to its DNA. Based on the sequence specificity, cleavage position, subunit composition and cofactor requirements, R-M systems are divided into four types-Type I, Type II, Type III, Type IV.

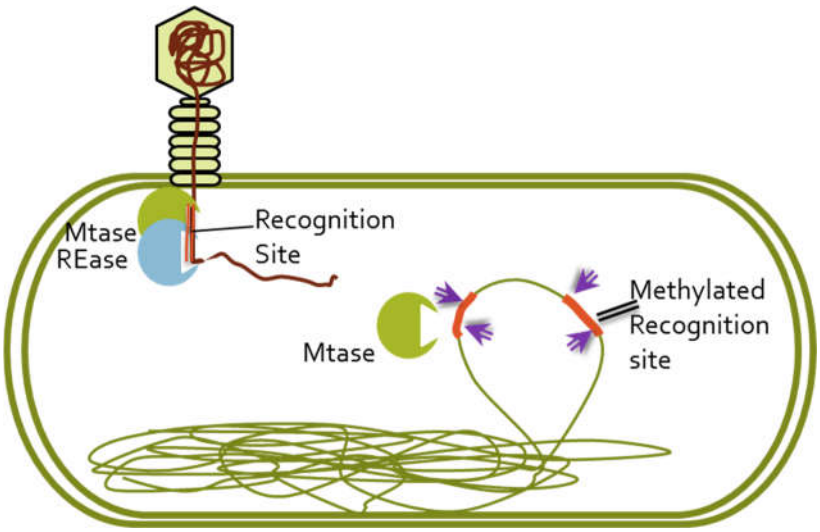

Figure 1.1. Restriction enzymes at work. Methyltransferase carries out methylation while restriction endonuclease cleaves the foreign DNA.

1.1 Classification of R-M systems

Type I enzymes form a hetero-oligomeric complex composed of three subunits: HsdM for methylation, HsdS for specificity, and HsdR for restriction. M_2S (two methylation and

one specificity subunit) methylates the DNA, whereas restriction requires pentameric complex in R_2M_2S form(Bianco et al., 2009). These enzymes recognize a bi-partite sequence. Two enzymes which are bound to DNA in head-to-head orientation are required for cleavage. There is absolute requirement for ATP to translocate and cleave the DNA.

Type II enzymes are the most studies system amongst the four. consists of two separately functioning subunits, Mod for methylation and Res for restriction. Type II enzymes do not require ATP as opposed to Type I, III and IV. These recognize a palindromic sequence and cleave the DNA close to or within the recognition site. A lot of variation has been found within type II R-M enzymes based on which they are further subdivided into Type IIS, IIP, IIE, IIC, IIG, IIT, IIA, IIB, IIC, IIH, IIF, IIM(Pingoud et al., 2014),(Pingoud and Jeltsch, 2001),(Pingoud, 2004).

Type III R-M system comprises of a heterotrimer in R_1M_2 stoichiometry(Gupta et al., 2012). Only a few systems in Type III are studied in detail which includes EcoP1(Kunz et al.),(Ahmad et al., 1995),(Rao et al., 1989), EcoP15(Gupta et al., 2012),(Kunz et al.),(Ahmad et al., 1995),(Möncke-Buchner et al., 2009),(Ecopi, 2015), PstII(Sears et al., 2005),(Sears and Szczelkun, 2005). The Mod subunit belongs to βclass of N6-adenie methyltransferase whereas Res has SF2 helicase motifs(Rao and Bheemanaik, 2013). Type III systems recognize an asymmetric sequence 5-6bp in length. Two enzymes should be oriented in head-to-head direction one of which remains stationary and the other moves in order to cleave the DNA 25-27 bp close to one of the recognition sites (Figure 1). Mod subunit forms a homodimer to methylate its own genome which also prevents cleaving self-DNA. There is division of labor between the two Mod subunits. Mod_A recognizes the target sequence and interacts with the bases in recognition sequence whereas Mod_B methylates the target adenine. Mod in complex with res is required for restriction activity. Adomet provides methyl group and ATP and Mg^{2+} are cofactors involved in cleaving the DNA. A few studies have also suggested the role of Adomet in DNA cleavage(Bist et al., 2001).

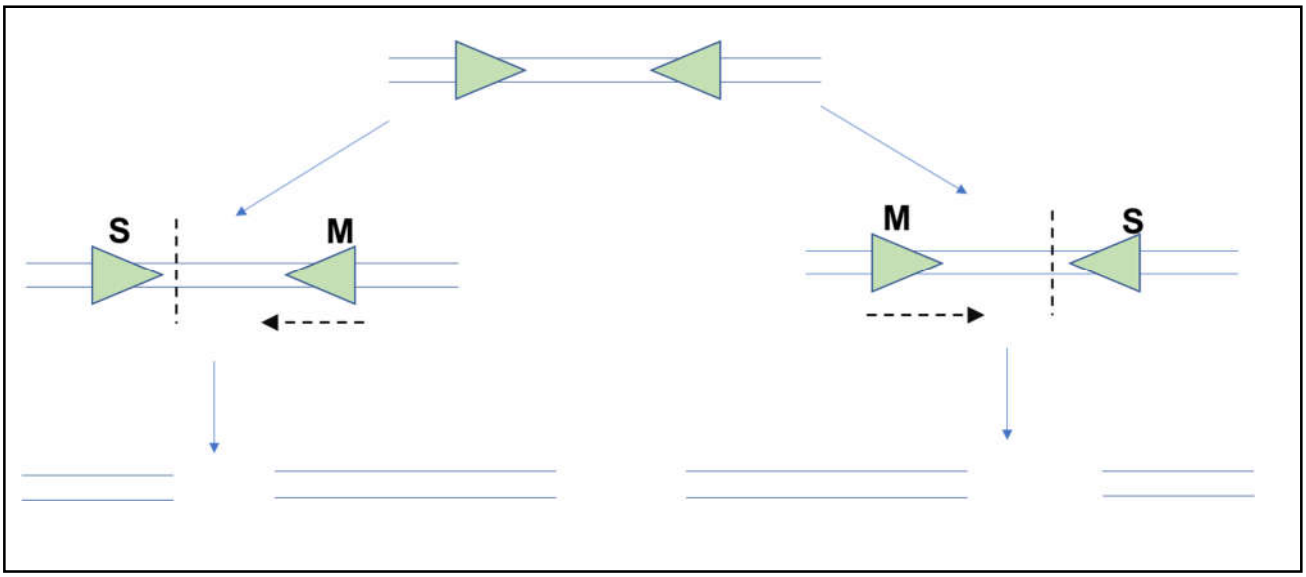

Figure 1.2. Substrate containing two head-to-head oriented recognition sites. The triangles show the position and direction of movement of enzyme. Dashed line represents position of cleavage. Dashed arrow shows direction of movement. S-Stationary, M-Moving enzyme.

Type IV systems have different mode of action than the other three. They are called modification-dependent restriction enzymes, i.e., Type IV enzymes cleave methylated DNA. The recognition sequences are not clearly defined for all Type IV systems. One of the Type IV enzymes studied in depth and highly characterized is McrBC, which recognizes R^mC (R – any purine, C is methylated) (Roberts et al., 2003). These enzymes cleave the methylated DNA in presence of GTP rather than ATP.

Type III systems are of interest to us as protein phase variable MboIII belongs to Type III system. From here onwards we will only discuss about Type III R-M systems.

1.2 Motifs in Mod and Res

Both subunits contain some conserved residues which are characteristic motifs of proteins belonging to methyltransferase and helicases respectively.

1.2.1 Motif assembly of Modification subunit

Malone et. al. described the assembly of motifs in amino-methyltransferases(Malone et al., 1995). 9 motifs were found which rearranged in different types of methyltransferases. Mutational studies shows FXGXG(motif I) accommodates the methyl group from Adomet which acts as a methyl group donor. Mutational studies have been carried out to assess the role of the motifs(Saha et al., 1998). The DPPY(motif IV) is the catalytic motif which accommodates the adenine which is flipped out of the DNA for methylation(Ecopi, 2015). The N- and C-terminal of the protein is conserved. The Target Recognition Domain, however, which lies in the central portion of the gene, is highly diverse and grants variability to the enzyme.

1.2.2 Motif assembly of Restriction subunit

The Res subunit belongs to superfamily II of helicases. SF2 Helicases functions as dsDNA translocases and are responsible for ATP binding hydrolysis(Tuteja and Tuteja, 2004). These consists of seven conserved motifs as shown in Figure 3. Near the N-terminus is the helicase domain which has Walker A/B motif. Walker A binds to ATP and Walker B is responsible for ssDNA binding. A weakly conserved motif PD….D/ExK, not present in SF2 helicases but in Res, which is the endonuclease domain binds Mg^{2+} which is a cofactor required for DNA cleavage. Hence the restriction subunit functions somewhere between helicase and nuclease. The seven conserved motifs are found in one region. N- and C-terminal regions are variable as opposed to Mod.

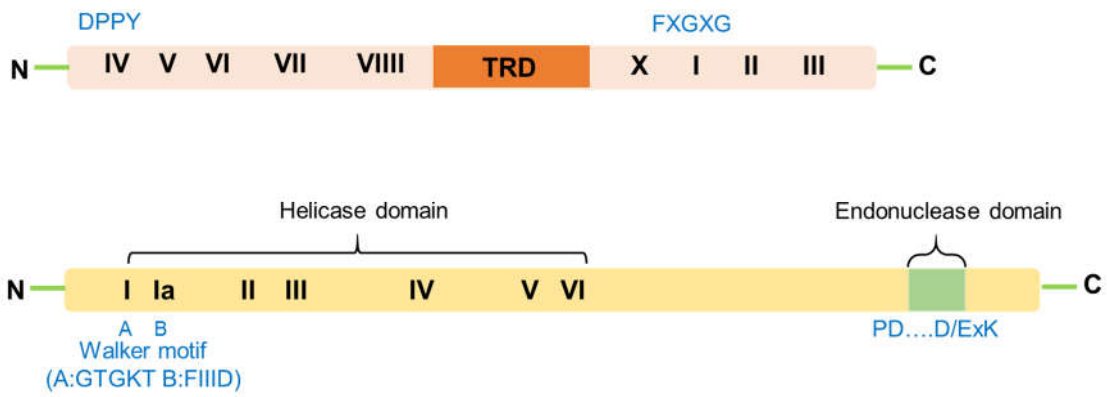

Figure 1.3. Motif Assembly for Mod(pink) and Res(yellow) of Type III enzymes. Roman numerals are different motifs characteristic of Type III. In blue are conserved motif residues which are most important.

1.3 Isolation of Type III system from *Mycoplasma bovis*

Mycoplasma bovis is a pathogen which causes pneumonia, mastitis, arthritis and a lot other diseases in cattle worldwide(Maunsell et al., 2011). Amongst pathogenic mycoplasma species, *M. bovis* is on the top in terms of pathogenicity, leading to high economic loss. It has also been found in sheep, goat, poultry, rabbit, acting as carriers and carrying a risk of being transmitted(Kumar et al., 2011). The type III R-M system present in *Mycoplasma bovis* consists of short AG repeats, which is conserved across the genus, although the number of repeats vary. These short sequence repeats exhibit phase variable regulation.

1.4 Phase variation

Phase variation helps in selection against host immunity. Phase variation is frequent, random and reversible switching on or off of the gene. Phase variation and antigenic variation are sometimes used interchangeable although they are different(van der Woude and Bäumler, 2004). Antigenic variation is when there is presence of various antigen in the genome, and at a particular time, only one or few of them are expressed. What separates phase variation from random mutation is the genetic heritability of

phase variation. A lot of phenomena can lead to phase variation(Henderson et al., 1999) such as:

(a) Genomic rearrangement

DNA inversion or deletion during recombination results in rearrangement of few residues which may switch ON/OFF the gene.

(b) Slipped-strand mispairing

Short sequence repeats (SSRs) may be present in the genome. During replication, due to presence of SSR, hairpin loop may form in the replicating DNA and mispairing could take place which leads to out of frame mutation, which during translation leads to a truncated or different protein which is the OFF state of the gene.

SSRs are involved in synthesis of surface elements such as surface proteins, lipopolysaccharides, pili, fimbriae.

1.4.1 Phase Variation in Type III system

Several pathogens having Type III R-M enzymes contains *mod* gene which is phase variable. These are, however, not involved in surface antigen variation. The gene could contain homopolymeric to pentanucleotide SSR which are phase variable(Srikhanta et al., 2010). The presence of such SSR in *mod* gene must have some significance which is beneficial for the pathogen. Possible roles which are suggested for SSR in Type III enzymes are listed below (Fox et al., 2007a):

(a) Horizontal Gene Transfer

R-M system cleaves foreign DNA and hence act as barrier towards outside DNA. When gene turns in OFF state, this might allow outside DNA and nutrients to be taken inside the cell which is beneficial for the bacteria. This also may serve as one for the mechanism for horizontal gene transfer by alleviating the barrier.

(b) Changes in methylation pattern

Phase variation may lead to differential methylation of the genome. DNA methylation affects gene expression. Hence, differential methylation leads to differential gene expression, thereby allowing gene expression regulation.

There have been extensive studies on phase variation and Type III system individually. There are also few *in vivo* studies which looks at the effect of presence of SSRs on the gene expression(Fox et al., 2007b),(Adamczyk-poplawska and Piekarowicz, 2009). However, there are no current studies which look at the effect of presence of SSRs on the activity of the enzyme *in vitro*. Does presence of repeats increase or decrease the effectivity of the Type III enzyme? Is it a tradeoff between protection from viruses and having flexible conditions that will allow uptake of DNA?

1.5 Objective

Mycoplasma *bovis* consists of Type III system, namely MboIII, which contain SSRs responsible for phase variation. The sequence obtained from database(Roberts et al., 2015) contains $(AG)_{10}$. This means the gene is in OFF state (Figure 4). The recognition sequence for MboIII was identified by Ahmad et al (unpublished data) as YAATC (Y= T/C). So, we have to either add or delete one AG to get the protein. Deletion of one AG gives a functional protein. The *mod* gene without repeat insert was amplified by Ahmad et al. Hence, we inserted $(AG)_9$ in *mod* gene.

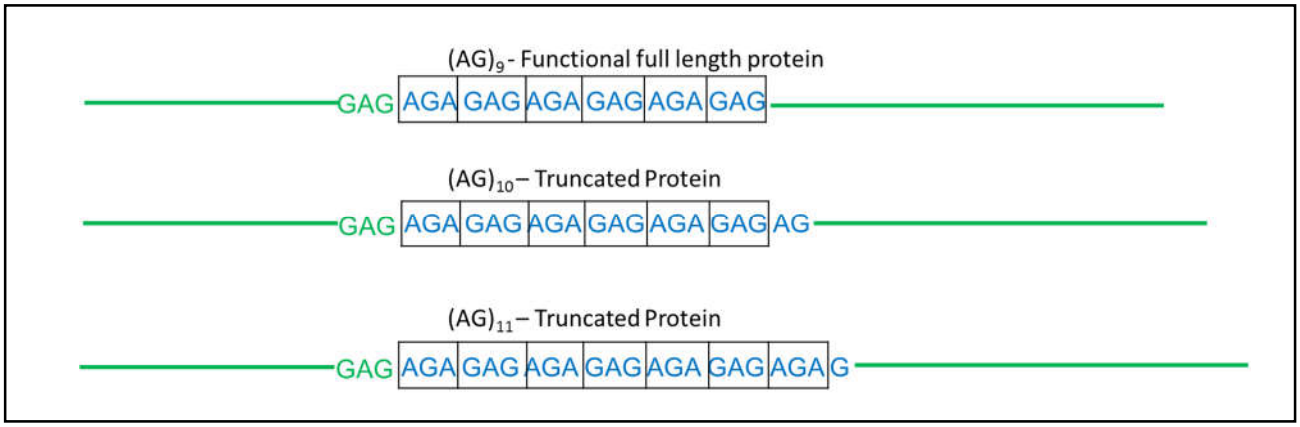

Figure 1.4. Schematic of gene with 9, 10 and 11 AG insertions. Green color represent gene without SSR. GAG in green belongs to the gene without repeat, this is to show that codon starts from start of inserted repeat. Boxes shows codons. Nucleotide in blue outside the box shows codon frame shift.

The **main objective of the project** is to look at effect of the SSRs on the enzymatic activity of MboIII, i.e., using biochemical assays, compare the activity of protein with and without insert.

1.5.1 Specific aims

1) Find the position of repeats with respect to motifs. Check if position is conserved.

2) Model the structure of MboIII from the available Type III enzyme EcoP15I structure to find the position of repeats in structure.

2) To successfully clone the construct of mod gene with AG inserts in pRSF vector.

3) Overexpress the protein and purify the protein (MboIII and MboIII9) in complex form (R_1M_2).

4) Investigate the activity of MboIII9, then compare enzymatic (nuclease and methyltransferase) activities of protein without and with repeats.

2. Materials and Methods

The genes of MboIII were amplified from the genome and purification protocol was standardized. The conditions that were used for carrying out cleavage and methylation assays were already standardized by Ahmad et al. (Unpublished data).

2.1 Structural investigation

The structure of MboIII is not known. Hence, using another Type III R-M enzyme EcoP15I as template (PDB ID:4zcf), the structure of MboIII was modeled with Swiss Model (Biasini et al., 2014; Bordoli et al., 2008; Guex et al., 2009). The position of repeats was visualized using the graphics program PYMOL.

2.2 Bioinformatic Analysis

In *Mycoplasma bovis*, the repeats are positioned between N-terminal and motif IV (DPPY). To check whether the position is conserved, a bioinformatic analysis was conducted. Across *Mycoplasma* genus, the position of repeats was examined. Also, to check the conserved nature across genus, repeat positions in 8 other genus was assessed. The sequences were obtained from the database REBASE(Roberts et al., 2015). The mod gene of Type III enzymes were filtered in search options and for each sequence the position of repeats, if present, was found out manually. For *Mycoplasma* and *Haemophilus*, the residue position and the number of repeats was also analysed.

2.3 Polymerase Chain Reaction

PCR was carried out using Primestar GXL DNA Polymerase to insert 9 AG repeats in Mod gene (Figure 2.1). The template used was *mod* gene without repeats (Ahmad et al., unpublished data). Reaction and amplification conditions are given in Table 2.1 and Table 2.2.

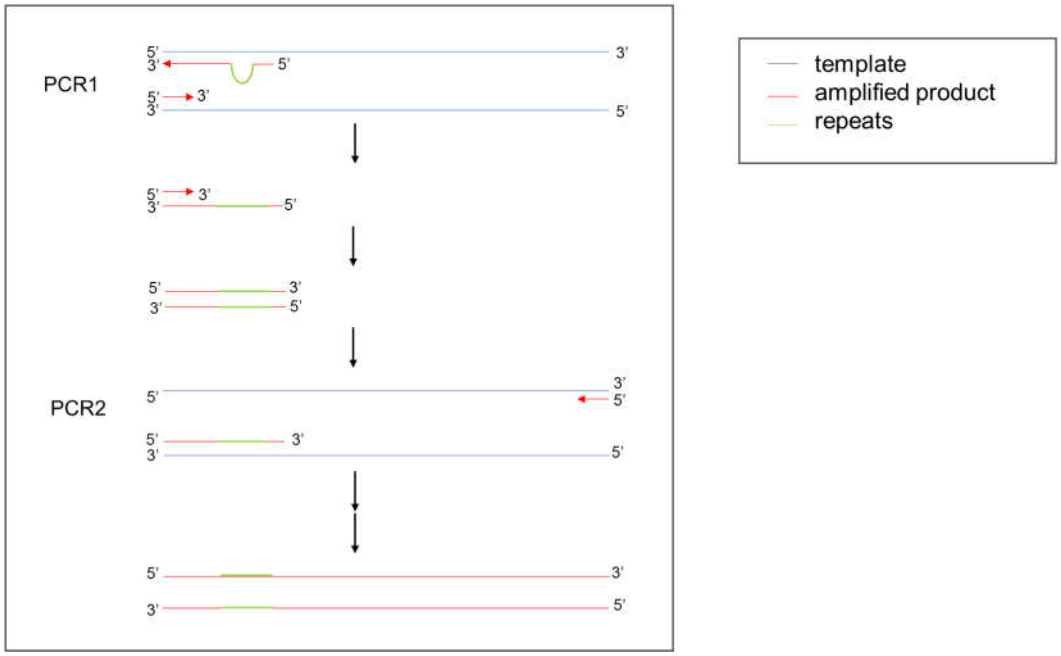

Figure 2.1. Amplification steps to incorporate repeats in mod gene. Two PCR reactions are donw in order to obtain the full gene with repeat insert.

The following primers which shows the repeats were used for PCR:

a. For insertion of $(AG)_9$:

PCR 1:

Forward: GATATACCATGGCACATCACCACCACCATCACATGAATACTATAAAACAG

Reverse: CTCTGACTGTGACTCTCTCTCTCTCTCTCTCTCTCGATCACTATAAG

PCR 2:

Forward: PCR 1 amplified product

Reverse: CGCAGCAGCGGTTTCTTTACCCTCGAGTTACTTAAT

Table 2.1. Standard protocol for PCR amplification

Components	Volume(µL)	Final Concentration
Template	0.5	50ng
Forward primer	2	0.2µM
Reverse primer	2	0.2µM
5X Primestar GXL Buffer	10	1X

dNTPs(2.5mM each)	4	200µM each
MilliQ	31.5	to 50µL
Total	50	

Table 2.2. Amplification conditions to insert repeats $(AG)_9$

Step	Temperature(ºC)	Time	
Denature	98	5 min	
Denature	98	10 sec	30 cycles
Annealing	55	15 sec	
Extension	68	25sec(PCR1)	
		1 min 50 sec(PCR2)	
Extension	68	5 min	

2.4 Cloning

The primers used to amplify the mod gene contained the restriction sites of NcoI and XhoI, which would facilitate ligation of insert and vector. The mod gene and desired vector pRSF were digested with NcoI and XhoI restriction enzmes to generate sticky ends. After double digestion, both inset and vector were gel extracted and ligation were carried out using T4 DNA Ligase. The ligation mixture was then transformed into New England Biolabs Turbo electro-competent cell line and electroporation was carried out using Gene Pulsar Xcell. The cells were spread onto Agar plates containing kanamycin(50µg/ml) and plates were incubated overnight at 37ºC.
Single colony was inoculated in 8 tubes each containing 5ml LB media(50µg/ml kanamycin added) and were incubated overnight at 37ºC. Plasmid purification was carried out using conventional plasmid purification method (solution method). Concentration was measured on nanodrop and positive clones were checked by digesting the plasmid with NcoI and XhoI and loading them on 1% agarose gel.

2.5 Sequencing

To confirm incorporation of the repeat in the clones, the clones were sent for sequencing to Sigma-Aldrich.

2.6 Protein Purification

2.6.1 Purification of Mod/Res

The modification and restriction subunits were separately purified. The plasmid was transformed into BL21DE3 cell line. The cells were spread onto petri plate and incubated overnight. A patch of cells was grown in large scale media at 37ºC. For Mod, induction was performed at 0.6 OD at 18ºC overnight using IPTG. Res was inserted in pHis17 vector and was induced with 0.2% arabinose at 18ºC overnight. The culture was spun at 4500rpm for 20min in Beckman Coulter JLA-8.1000 rotor. Supernatant was discarded and culture pellet was suspended in 200ml cold lysis buffer (50mM Tris pH8, 500mM NaCl, 5mM MgCl2,10% glycerol, 10mM Imidazole) until no clumps seen. The resuspended cells were sonicated (2.5 cycles, each cycle was 3min, 1sec on and 3 sec off, 70% amplitude) . The lysate was centrifuged at 37000rpm in Beckman Coulter 45 Ti rotor for 1hr at 4ºC. The supernatant was collected in a flask and pellet was discarded.

The following three purification steps were carried out to purify the protein from the collected supernatant.

2.6.1.1. Ni-NTA Histidine Tagged Affinity Chromatography

Mod and Res had six Histidine residues on N-terminal and C-terminal respectively, which can bind to the GE Healthcare 5ml HisTrap column. This step was carried out on the AKTA prime system (GE Healthcare Life Sciences). The columns were first washed (3 CV) with Buffer B (50Mm Tris pH8, 500mM NaCl, 500mM Imidazole) to remove any bound impurity to column from earlier purifications followed by wash with Buffer A (50Mm Tris pH8, 500mM NaCl, 15mM Imidazole). The protein was loaded on the column at a rate of 3ml/min for better binding (Max loading rate 5ml/min). A wash step

with buffer A was carried out to remove any non-specifically bound proteins. Elution was carried out by changing Imidazole concentrations via manual instructions (Elution was done at 3%, 8%, 20%, 30%, 50%, 70%, 100%, 100% of Buffer B). Each or alternate fractions were loaded on 10% SDS-PAGE Gel. Most of the impurities were removed in this stage. The fractions containing protein was set up for overnight dialysis at 4ºC (Dialysis Buffer-50mM Tris pH 8, 50mM NaCl, 100mM EDTA, 1mM DTT, membrane had a 10kDa limit).

2.6.1.2. MONOQ - Anion Exchange Chromatography

The dialyzed samples were spun at 17000rpm in JA-25.5 fixed angle rotor. The theoretical Isoelectric point calculated with ProtParam(Gasteiger et al., 2005) of Mod and Res are 5.34 and 6 respectively. The columns were washed with Buffer B1000 (50mM Tris pH8, 1000mM NaCl, 1mM EDTA, 1mM DTT) and then equilibrated with B50 (50mM Tris pH8, 50mM NaCl, 1mM EDTA, 1mM DTT). 1ml fractions were eluted with continuous gradient of increasing %B1000. Fractions with highest UV absorption peak at λ_{max}=280nm were loaded on 10% SDS-PAGE gel. Fractions containing proteins were pooled and concentrated to a volume of upto 200µL in Vivaspin 10,000MWCO centricon. All purification steps were carried out on the AKTA purifier system (GE Healthcare Life Sciences).

2.6.1.3. Gel Filtration Chromatography

Gel filtration was performed using Superose 6 column in the AKTA purifier system. The column was initially equilibrated with Buffer R (10mM Tris, 100mM KCl, 1mM DTT). The protein was concentrated to 500µL and injected into the system. The eluted protein was collected in 0.5ml fractions. The fractions containing protein was concentrated, and the concentration was measured on nano drop after which the aliquots of 10µL protein were flash frozen and stored at -80ºC.

2.6.2 Purification of co-expressed MboIII

Co-expression is expression of mod and res subunits in the same cell. BL21DE3 cells were transformed with the plasmids harboring mod and res. Rest of the purification steps were same as 3.6.1.

2.7 Cleavage Assay

Cleavage assay was performed with the purified protein to assess the activity. Cleavage activity also confirms binding and ATPase activity of the protein. A 235 bp substrate was used having two recognition sites in head to head orientation was used for the assays.

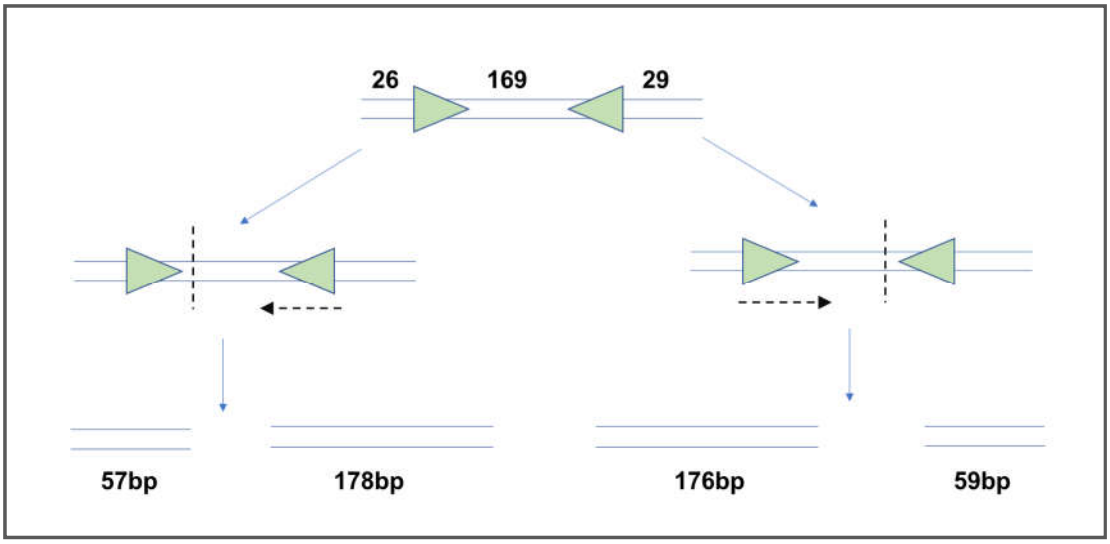

Figure 2.2. 235bp substrate containing two head-to-head oriented recognition sites of MboIII. The 178 bp and 176bp band will run very closely and will be seen as a single band on the gel.

The cleavage reaction consists of 200ng DNA, 1µM protein, 4mM ATP, buffer to make up the volume to 15µL. The reaction mix was incubated at 37ºC for 40min. 0.5X of 2% SDS + STEB (0.1 M Tris–HCl (pH 7.5), 0.2 M EDTA, 40% (w/v) sucrose, 0.4 mg/ml bromophenol blue) was added to stop the reaction. The sample was then loaded on a 15% non-denaturing PAGE gel. The gel was run at a constant voltage of 150V. The gel was stained with 5µL of 10mg/ml Ethidium Bromide and visualized on Typhoon Variable mode Imager system.

Table 2.2.. Composition of 15% non-denaturing PAGE Gel

Component	Volume(ml)
MilliQ	2.9
Acrylamide/Bisacrylamide mix (29:1%w/v)	5
5X TBE	2
10% APS	0.1
TEMED	0.01
Total	10ml

2.8 Methylation Assay

An indirect assay to assess the methylation activity of the protein was performed as shown in Figure 3.3. A 235 bp substrate was used containing two head-to-head oriented sites was used. The methylation reaction consists of 5000ng DNA, 1µL protein, 100µM SAM, Buffer CB to make the final volume of 200µL. The mixture was incubated for 1hr. Then it was PCR purified using Qiagen kit method. The methylated substrate was then cleaved with Mbo_D9 (cleavage conditions same as described in **3.5**).

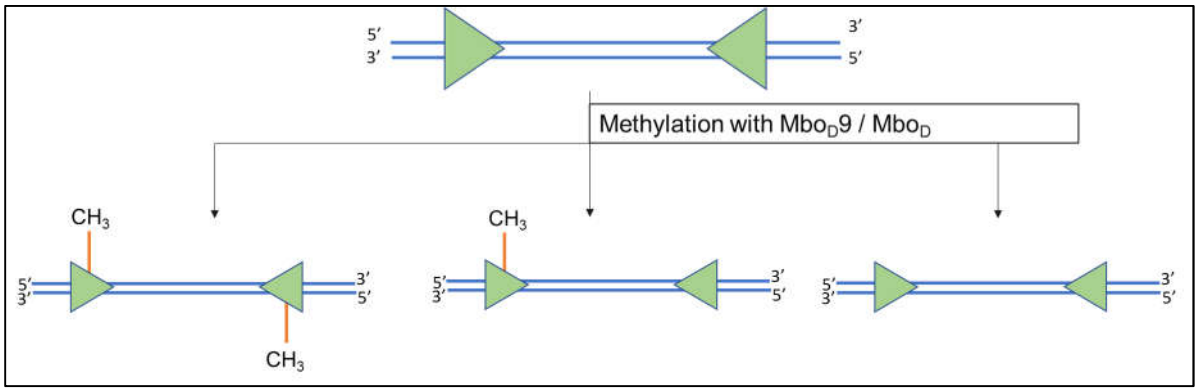

Figure 2.3. Methylation pattern which can be observed in the methylation reaction. Three types of DNA could be observed: methylated, hemi-methylated and non-methylated. Non-methylated DNA in the mix will be cleaved by Mbo_D9.

3 Results and Discussion

3.1 Modeled Structure of MboIII9

The three-dimensional structure of MboIII9 was homology modeled using the available structure of EcoP15I (PDB ID: 4zcf). EcoP15I does not have SSR. Figure 3.1 shows a comparison of the structure of EcoP15I and MboIII. The figure illustrates the position of the AG9 repeats, which is located at the interface of Mod and Res. The figure also shows that the repeats do not perturb the oligomeric structure of MboIII.

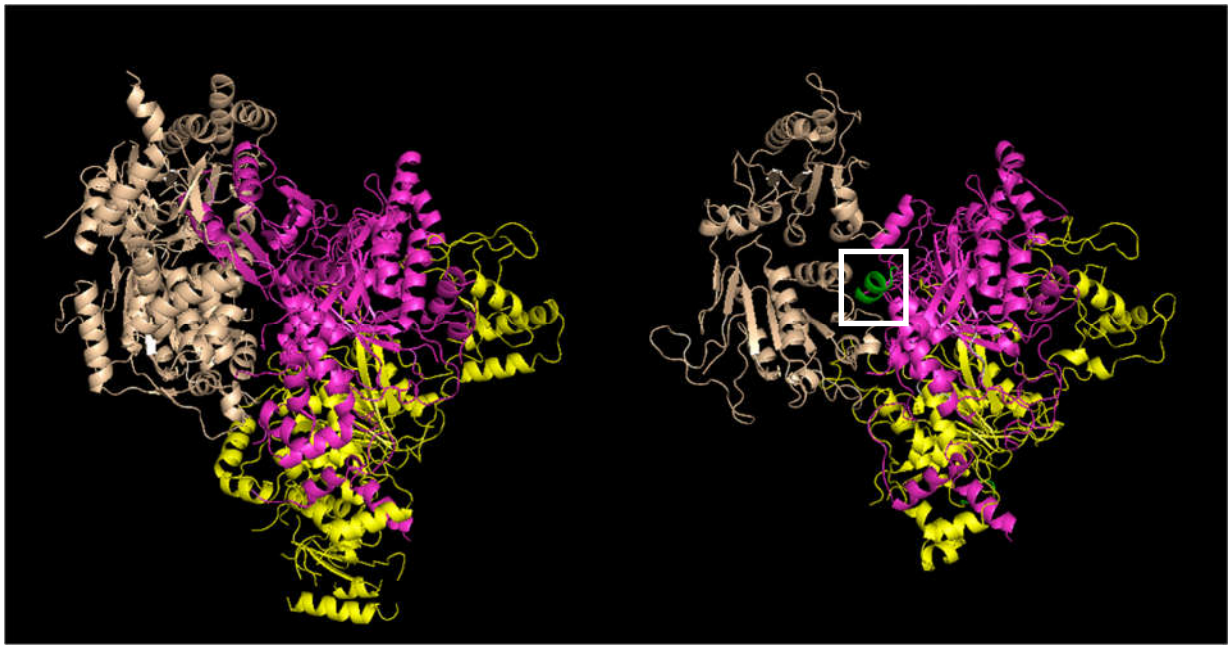

Figure 3.1. Modelled structure of MboIII9. Comparison of structure of EcoP15(left) and repeats position in MboIII9 (right). Wheat – Res (MboIII), magenta – Mod_A, blue shows the position of repeats in the structure which is absent in EcoP15I. White box highlights the position of repeats in the structure

3.2 Bioinformatic Analysis

The above model suggest that the repeats were located at the interface of Res and Mod. We were interested then interested in finding out how may such repeats can be accommodated at the interface. We addressed this question by carrying out a sequence

analysis of all the known Type III enzymes from *Mycoplasma*. It was seen that in *Mycoplasma bovis CQ-W70*, as many as 24 AG repeats were found. The repeats were almost always present between N-terminus and motif IV (**Figure 3.2**). To check if this also holds across different genera, we surveyed the database available for 2265 species across 10 genera namely *Mycoplasma*, *Haemophilus*, *Pasteurella*, *Neisseria*, *Helicobacter*, *Salmonella*, *Mannheimia*, *Streptococcus*, *Moraxella*, *Escherichia* and found that the position of the repeats is highly conserved. Figure 3.2 illustrates the position of the repeats and their distance from motif IV (DPPY)in *Mycoplasma* and *Haemophilus*. The position of repeats varies in both cases.

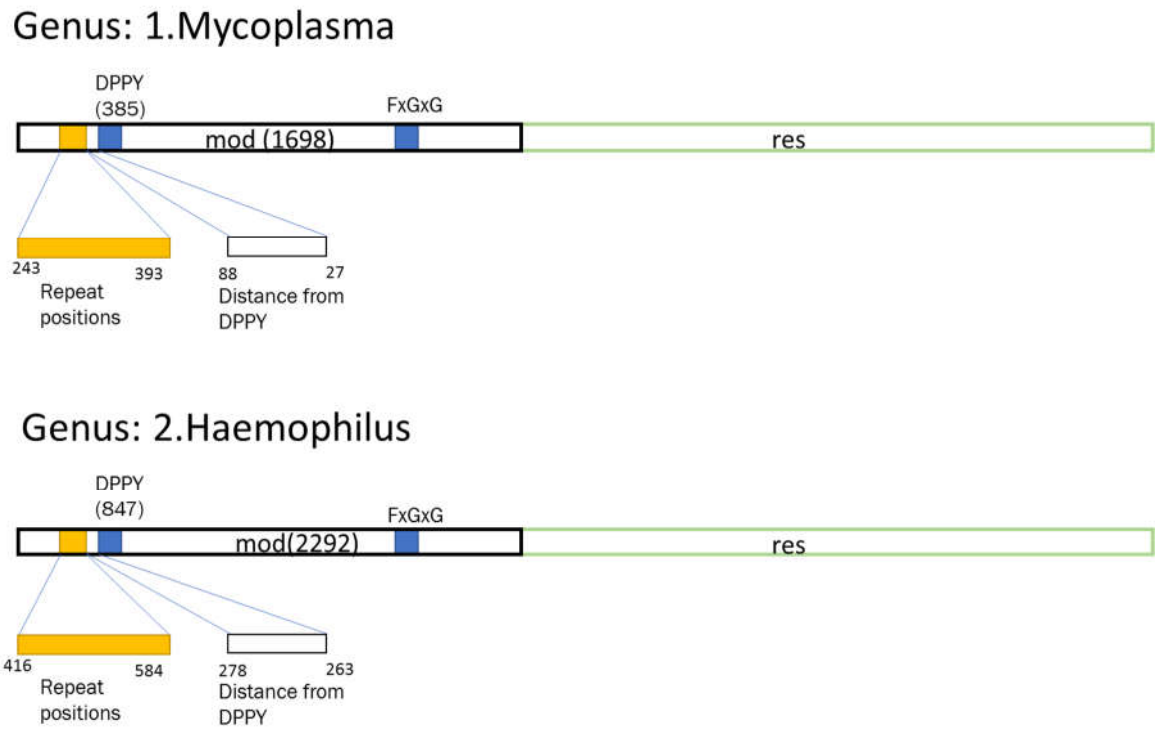

Figure 3.2. Nucleotide position of repeats in mod gene and distance from DPPY motif (motif IV) for *Mycoplasma* and *Haemophilus*. Representative positions of DPPY motif (nucleotide) are mentioned for *Mycoplasma bovis* and *Haemophilus Influenzae*.

3.3 PCR and Cloning

As a first step towards the biochemical studies, 9 AG repeats were inserted into the gene of MboIII Mod, which lacked any repeat. The amplicon was cloned and visualized

on agarose gel, which again confirmed a band of 1.7kb, corresponding to the size of the mod gene, and another of 3.7kb corresponding to the vector (**Figure 3.3. and 3.4**). Clone 2,4 and 8 were selected and sequenced (**Figure 3.4**).

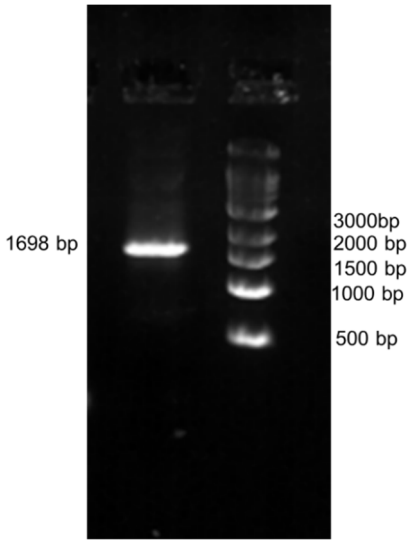

Figure 3.3. ~ 1.7 kb band on the left which shows the amplicon. Ladder is marked on the right.

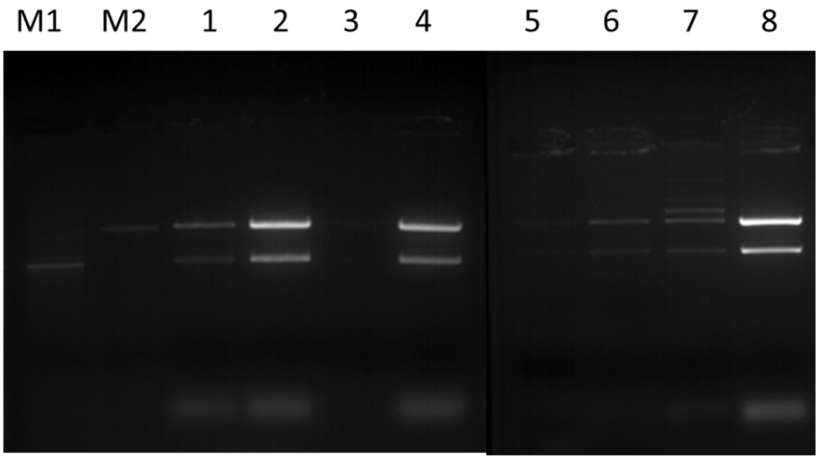

Figure 3.4. Eight clones were digested and run on 1% agarose gel. 2,4 and 8 are prominent and appears positive. M1-1.7kb(for insert), M2-3.7kb(for vector)

3.4 Sequencing

The sequencing results showed successful insertion of 9 AG repeats (**Figure 3.5**). However, there was a mutation at position 159 of the amino acid sequence. This resulted from mutation of an adenine to a guanine. This resulted in the codon GAT(aspartate) being converted to GGT(glycine) which codes for Glycine. We found that the template sequence used for cloning also had the mutation. Based on the analysis of all *mod* gene sequences from *Mycoplasma* in REBASE, we confirmed that aspartate was the correct residue at this position. Hence, GGT was converted back to GAT using PCR and cloning methods and sequenced to confirm the change. The corrected clone was then used for expression of MboIII Mod with repeats (to be referred to as Mod$_D$9).

Figure 3.5. Alignment of sequenced *mod*. Although there is insertion, a mutation is also found in sequenced gene. The mutation was also found in template DNA.

3.5 Expression Test

Before proceeding for large-scale culture, a standardization is necessary to get the highest amount of protein in soluble form. The standard procedure to get protein was earlier done in the lab. Trying the same set of conditions for 5ml culture, protein was seen in soluble form under conditions given in Materials and Methods. Clone 2 was selected for protein expression and purification as it showed highest solubility under the same conditions compared to clone 4 and 8.

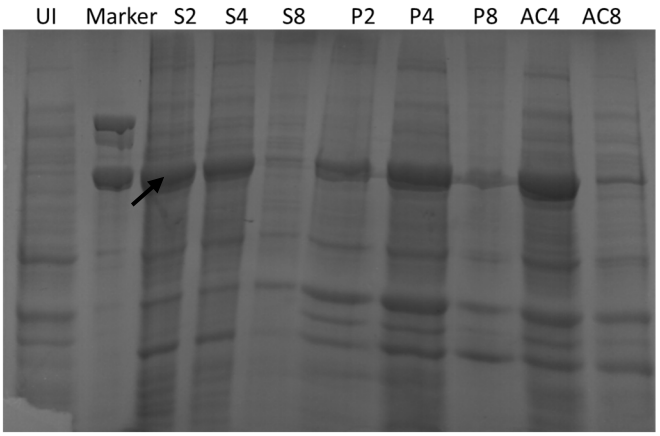

Figure 3.6. Expression test for clone 2,4 and 8. UI - Uninduced, S - Supernatant, P-Pellet, AC - All Cell(S+P). Arrow shows the position of Mod protein in supernatant.

3.6 Purification of *in vitro* complex

Standardized purification protocol (Ahmad et al., unpublished data) was used for purifying mod and res. A three-step purification was done to get as pure protein as possible.

3.6.1 Purification of Modification Subunit

Mod_D9 is a 565 amino acid protein with MW of 66kDa. The overexpressed protein can be seen in the gels. Most of the impurities were removed in Ni NTA, which can be seen on comparing the impurities in load, flow through and fractions collected (**Figure 3.7**). The theoretical isoelectric point (pI) of the protein is 5.34 calculated using ProtParam. Hence, next purification step was anion exchange chromatography which further purified the protein. Last step was gel filtration chromatography. This step also helped predict the oligomeric state and also check if the protein aggregates. The elution volume of Mod suggested it to exist as forms a homodimer(Figure 3.8) which can be seen in the gel filtration chromatography run.

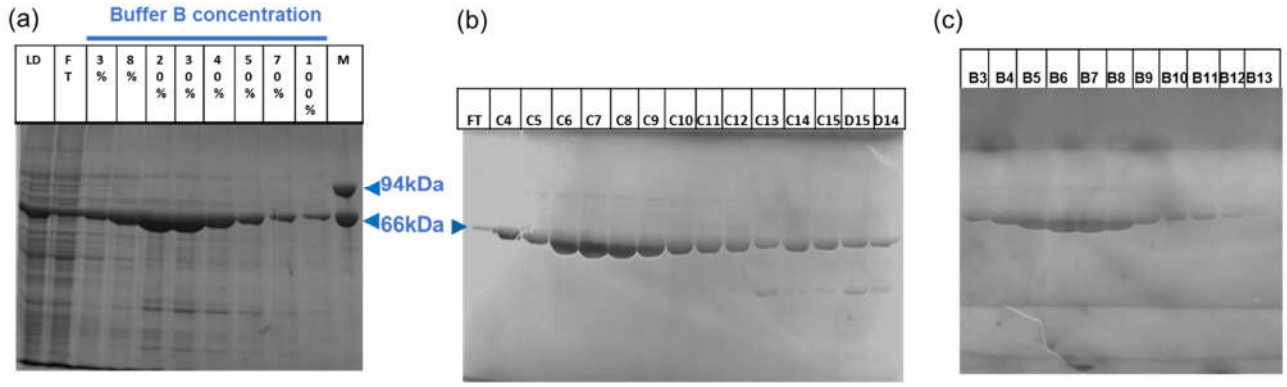

Figure 3.7. Purification of Mod via chromatography. (a) The fractions collected after Affinity chromatography ran on 10% SDS-PAGE gel. LD-Load(Supernatant loaded on gel), FT: Flow through . (b) Anion Exchange Chromatography (c) Gel Filtration Chromatography. On top of each gel, the fraction label is written. The peak of gel filtration run elutes in B7 fraction

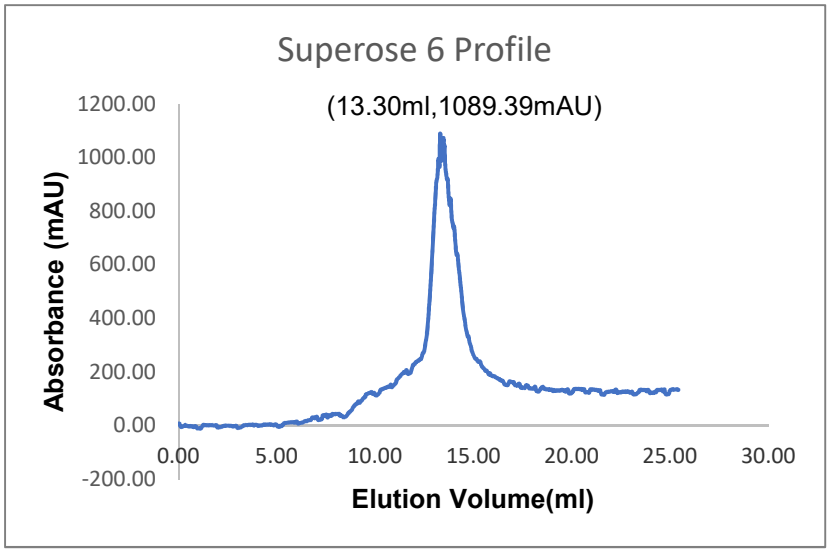

Figure 3.8. Superose 6 run profile for Mod. The peak is where the protein elutes which is at 13.3 ml. The PAGE gel run is shown in Figure 3.6(c).

3.6.2 Purification of Restriction Subunit

Res is 807aa protein with MW 94kDa. Res is purified as a monomer which forms complex with Mod to form the active enzyme. A representative gel for the purification of Res is shown in **Figure 3.9**. Although a lot of impurities are removed as can be seen in flow through, the fractions were still very impure. The isoelectric point of Res is 6 and

the buffer had pH8, hence anion exchange chromatography was performed to purify the protein. Finally, gel filtration was done to estimate the oligomeric state of protein. This study suggested Res to be monomeric.

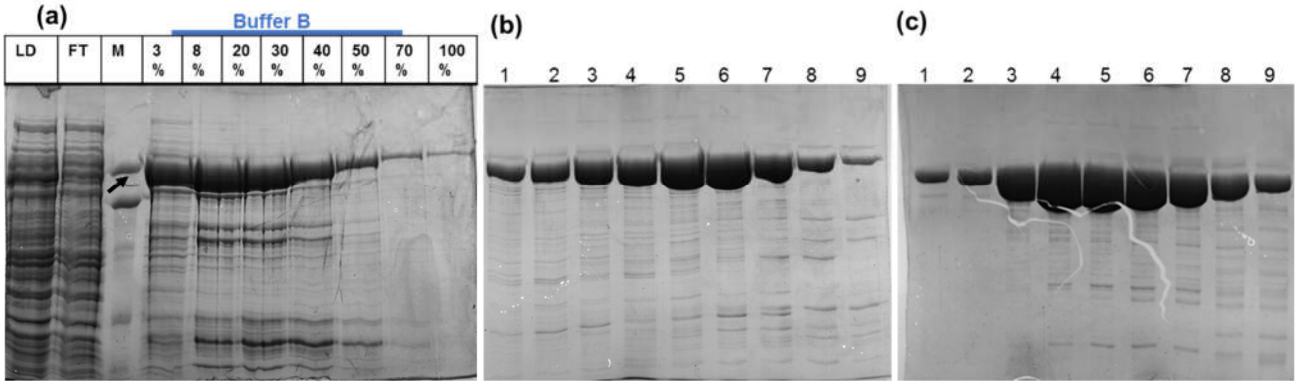

Figure 3.9. Purified Res run on SDS-PAGE gel. (a) Affinity Chromatography. Arrow shows 94kDa Marker. LD-Load, FT-Flow Through, Rest of the lanes are fractions collected by manually changing the Buffer B concentration as % Buffer B. (b) Anion Exchange (c) Gel Filtration.

3.6.3. Formation of R_1M_2 complex

Mod and Res formed a complex in 2:1 ratio with MW 226kDa. Purified Mod and Res were mixed with Mod in excess. In Figure 3.10, A14 to B10 fractions are complex, comparing intensity of bands for mod and res. B9 to B7 represent the excess mod that elutes later.

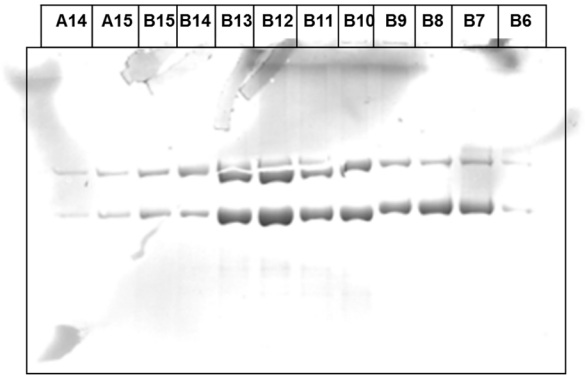

Figure 3.10. Formation of complex and Superose run. The fractions are mentioned on top. The absorption peak is in fraction B12 which contains highest amount of protein.

3.7 Cleavage assay with *in vitro* complex

We purified Mbo_D9 (purified complex of Res and Mod_D9) using the steps mentioned above. Nuclease activity of MboD9 was assessed in Cleavage Buffer (10mM Tris pH8, 30mM NaCl, 5mM MgCl2, 1mM DTT).

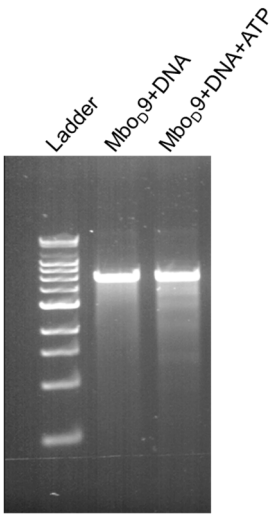

Figure 3.11. Cleavage Assay with Mbo_D9. 4.9 kb substrate is used. >90% DNA is not cleaved. Very light bands are visible for cleaved DNA product.

The efficiency of cleavage was very low for MboD9 (**Figure 3.11**). The reason could be improper or inactive complex formation due to *in vitro* reconstitution. Hence, we tried an alternative method of purification, i.e., co-expression of mod and res. The complex is formed inside the cell and can be directly purified as a complex, which we call *in vivo* complex.

3.8 Purification of $MboIII_D9$ and $MboIII_D$ in vivo complex

In vivo complex directly gives us the protein in complex form. As both Res and Mod had 6X Histidine Tag, the complex was first purified with Ni-NTA affinity chromatography. The theoretical isoelectric point (pI) of M_2R_1 complex is 5.59. Anion exchange chromatography was the next towards further purification of protein. Finally, the complex was passed through a gel filtration column to get purer form of the protein. Identical strategies were used for purification of Mbo_D9 and Mbo_D.

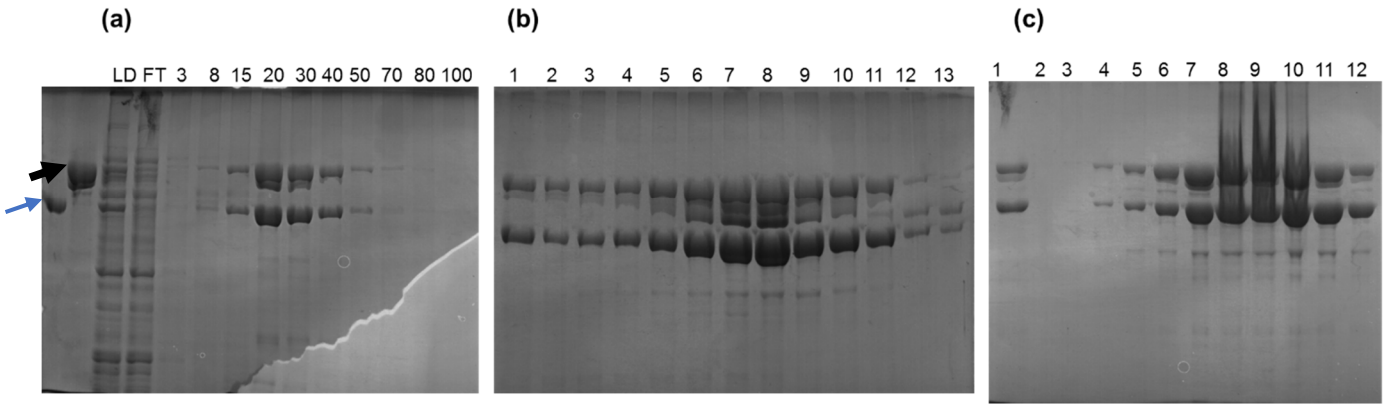

Figure 3.12. Purification of co-expressed Mbo$_D$9 (in vivo). (a) Affinity Chromatography. Blue arrow shows 66kDa marker, Black arrow shows 94kDa marker (b) Anion Exchange Chromatography- Protein is observed in almost every fraction with band intensity 1:1 of Mod:Res. Fraction 2 to 11 pooled. (c) Gel Filtration. First lane is marker for Mod and Res. Fraction 7 to 11 pooled and concentrated.

3.9 Cleavage assay with *in vivo* complex

While the *in vitro* complex of Mbo$_D$9 displayed poor nucleolytic activity (**Figure 3.11**), *in vivo* complex had significantly higher cleavage activity (**Figure 3.13**). We compared the nuclease activities of Mbo$_D$ and Mbo$_D$9 in vivo purified complex. The activities of the two enzymes were significantly higher than that of *in vitro* complex. However, none of the two *in vivo* complexes showed complete cleavage of substrate.

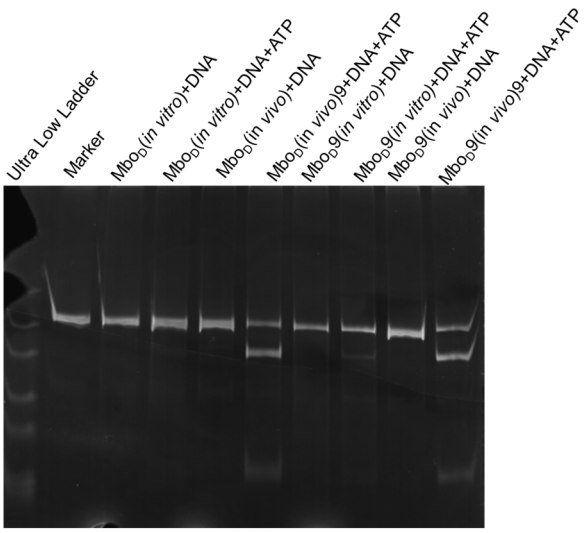

Figure 3.13. Assay with Mbo_D and Mbo_D9 *in vivo* and *in vitro* complexes with short substrate. CB used for assay and recognition site in substrate was TAATC.

The recognition sequence of MboIII is YAATC (Y=T, C). Hence, we compared the activities using substrates having TAATC and CAATC sites.

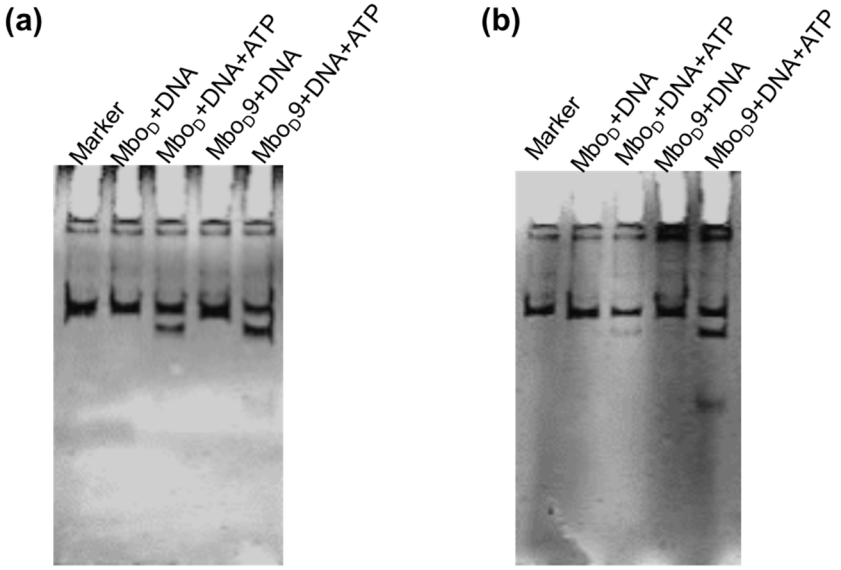

Figure 3.14. Cleavage Assay comparison between Mbo_D9 and Mbo_D with 235bp substrate. The recognition site in DNA is (a) TAATC and TAATC in head-to-head, (b)CAATC and TAATC in head-to-head orientation.

When the two sites are TAATC, the cleavage activities of Mbo_D and Mbo_D9 are comparable, with Mbo_D9 having slightly higher activity (**Figure 3.14(a)**). However, when one of the recognition site is CAATC, Mbo_D clearly showed very less cleavage efficiency in comparison to Mbo_D9 (**Figure 3.14(b)**). Again, in either case, almost 50% or more of the DNA substrate remained uncleaved.

To optimize the cleavage activity, we changed the composition of the buffer. This is described in the next section.

3.9.1 Optimization of protein activity

a. Different Buffers

To begin with, different commercial buffers from New England Biolabs available in the lab were used for the assay. NEB1 and NEB2 buffers showed comparable activity to that of CB buffer. NEB4 showed the maximum amount of DNA cleaved (>70%) (**Figure 3.15**). The major differences between NEB4 in comparison to CB was (1) presence of acetate instead of chloride, and (2) presence of KCl instead of NaCl. Hence, we felt acetate or K+ ions or both could be the reason for the enhanced.

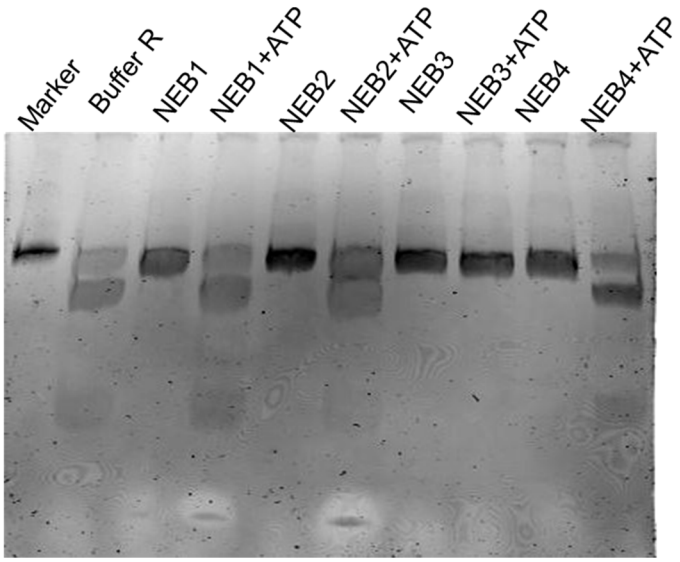

Figure 3.15. Cleavage assay of Mbo_D9 with different buffer. NEB1-4 are commercially available buffers which were used for the reactions. pH of all buffers is 8.

We then compared the activities of Mbo_D and Mbo_D9 in NEB4 buffer. Mbo_D9 cleaved the substrate more than Mbo_D, suggesting, in this particular NEB4 buffer, Mbo_D9 has highler cleavage efficiency (**Figure 3.16**).

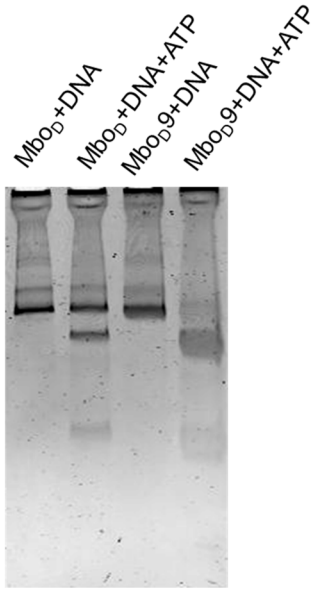

Figure 3.16. Nuclease activity comparison for Mbo_D and Mbo_D9 in NEB4 buffer. Highest activity is observed for Mbo_D9.

b. Variation in Potassium acetate

Changing the concentration of potassium acetate will clarify the role of potassium ions in activity of protein. Potassium acetate was varied from 0mM to 100mM in the buffer and cleavage monitored. With increasing concentration of potassium acetate, the nucleolytic activity was found to decrease (**Figure 3.17**).

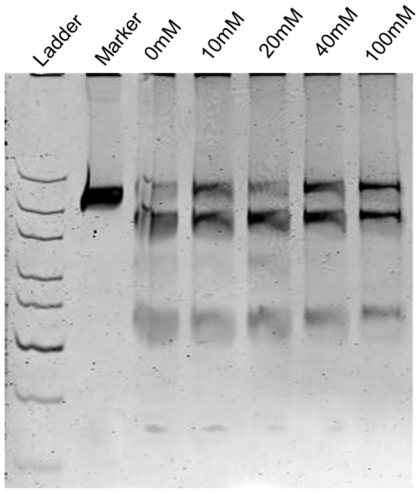

Figure 3.17. Nuclease assay with Mbo_D9. Potassium acetate concentration was varied keeping other components same as in NEB4 buffer.

The buffer used without K+ ion showed maximum efficiency. This means that presence of potassium ions reduces the activity. The new buffer (20mM Tris-Acetate pH 8, 0mM Potassium Acetate, 10mM Magnesium Acetate, 1mM DTT) will be called **Buffer K**.

c. Effect of sinefungin

Sinefungin is an analog of Adomet which does not provide -CH3 for methylation. A few studies have suggested that Adomet is required for cleavage in Type III R-M enzymes(Bist et al., 2001),(Möncke-Buchner et al., 2009). However, presence of Adomet also methylates the DNA. Hence, the analog sinefungin was used. Addition of sinefungin did not enhance the activity.

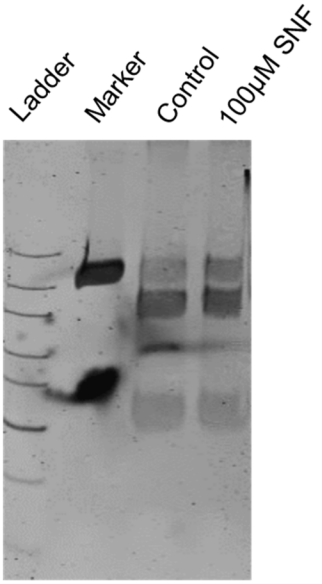

Figure 3.18. Effect of sinefungin, an analog of Adomet, on the cleavage activity of Mbo_D9. Control reaction was carried out in Buffer K. 100µM Sinefungin (SNF) was added.

d. Pre-incubation of DNA-protein complex

DNA and MboIII was pre-incubated for different times, then ATP was added and incubated again for 40 min for cleavage reaction to proceed. This would allow the protein to bind to DNA and may lead to better cleavage efficiency. Surprisingly, control, which was not pre-incubated showed the maximum cleavage efficiency. This shows that initial incubation does not necessarily enhance protein-DNA binding.

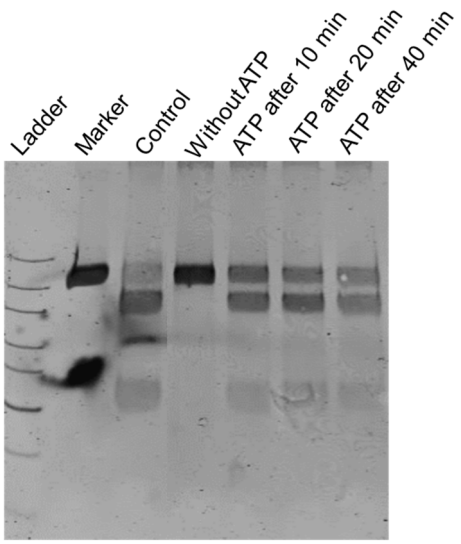

Figure 3.19. Cleavage assay of Mbo_D9 after pre-incubation. Only DNA and Protein was incubated in Buffer K for 10, 20, 40min. ATP was added post incubation and assay was followed through as usual.

e. Magnesium concentration, pH, and Acetate vs chloride

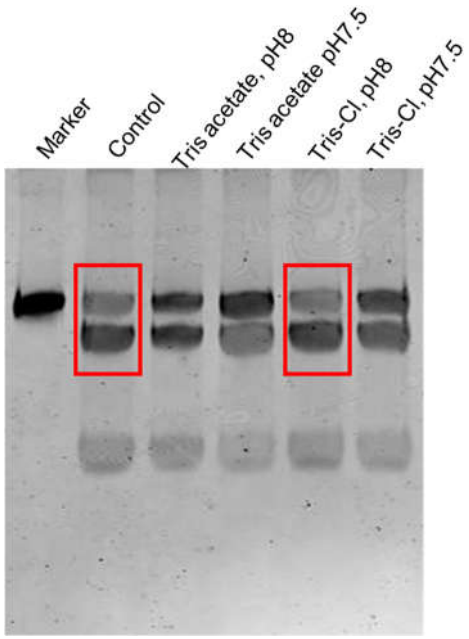

Figure 3.20. Nuclease activity of Mbo_D9 by changing different parameters. Control reaction was performed in Buffer K. Tris Acetate means buffer pH balanced with Glacial acetic acid and for Tris-Cl, HCl was used to maintain the pH.

Next, we changed three parameters simultaneously

1. Magnesium Concentration

Decreasing the concentration of magnesium decreased the efficiency (**Figure 3.20**). Magnesium concentration was decreased as there was already excess of magnesium and Cleavage buffer had less magnesium concentration. Decreasing the concentration of magnesium decreased the efficiency.

2. Tris-acetate buffer and Tris-Cl buffer

Protein in Tris-acetate showed less cleavage compared to Tris-Cl, the concentration of other components being same. This tells us that acetate wasn't enhancing activity but presence of salt ions was reducing the activity. Amongst the cations, the enzyme appears to perform better in presence of potassium than sodium.

3. pH

In both acetate buffer and chloride buffer, the enzyme performs better at pH 8 than pH 7.5.

Overall, in Figure 23, we see that pH and concentration of rest of the components being same, Tris-Cl performs equally better with less amount of Magnesium required.

3.10 Methylation assay

Cleavage activity indirectly confirmed that the purified MboIII complexes had efficient DNA binding and ATPase activity, which are the prerequisites for nucleolytic cleavage to occur. Next, a methylation assay was setup to check whether the Mbo_D and Mbo_D9 methylates DNA, and if so, which methylates better.

To analyze methyltransferase activity, an indirect assay for used. The assay was a nucleolytic assay, which relied on the assumption that methylation inhibits DNA cleavage. Consequently, better the methylation activity of an enzyme on a DNA, poorer would its subsequent cleavage be (see Materials and Methods for details). Mbo_D-methylated DNA shows slightly more cleaved product compared to Mbo_D9-methylated

DNA (**Figure 3.21**). Based on this, we conclude that Mbo_D9 methylates appreciably faster than Mbo_D. However, as this is an indirect and qualitative assay, the results need to be further verified using an assay that directly quantifies methylation.

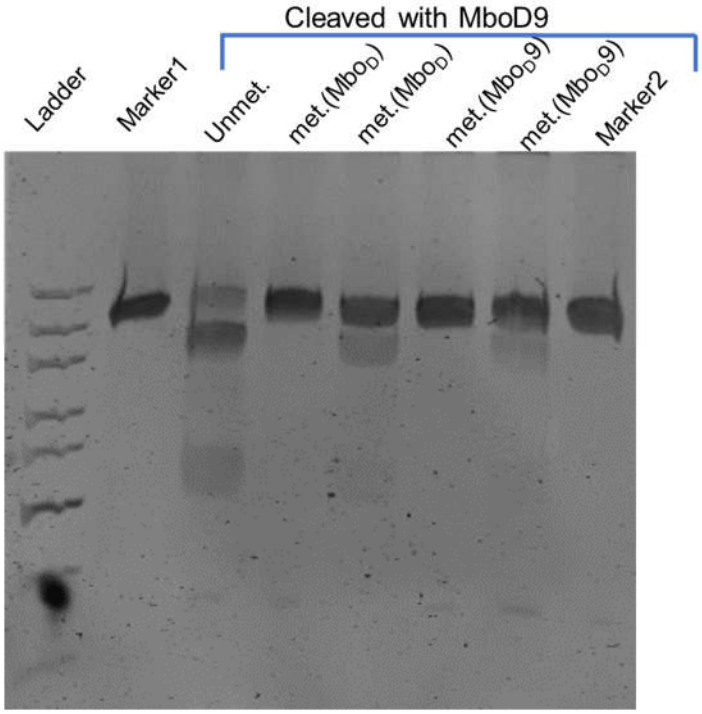

Figure 3.21. Nuclease assay with Mbo_D9 to assess the methylation by Mbo_D and Mbo_D9. Cleavage with a single enzyme helps us to compare the methylation between Mbo_D and Mbo_D9.

4 Conclusion and Future Prospects

The modeled structure of MboIII brings into light the position of repeats which is at the interface of Mod_A and Res. Repeats in Mod_B faces the solvent environment and does not participate in any protein-protein interaction.

Interestingly, bioinformatic analysis revealed that the position of repeats is almost always found between N-terminus and motif IV, the position being closer to motif IV. There are certain exceptions to this, for example, in *Mycoplasma pulmonis*, the repeats are positioned near the C-terminus. In *Mycoplasma*, the number of repeats can be as high as 24. In the analysis of total ten genera, the maximum number of repeats found was 43 in *Haemophilus influenza*e strain RdAW (Rebase accession name: HinAWORF329P). How such long repeats can be accommodated within the framework of the molecular structure of a Type III enzyme is intriguing.

Our studies show that the protein activity is not affected drastically towards accommodating 9 AG repeats. This shows the plasticity of Type III enzymes and that the enzyme activity does not vary a lot upon addition of 6 amino acid residues (resulting from 9AG repeats). Further studies are also being carried out to assess the activity of protein on adding as many as 24 AG repeats (resulting in insertion of 16 amino acids). The repeats are already inserted in gene and cloned (**Figure 4.1,4.2**). In future, the clones will be expressed, and the enzymatic activities of the resulting protein complex will studied.

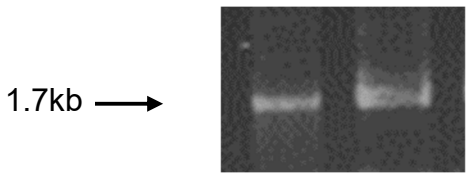

Figure 4.1 . PCR Amplification to insert 24 AG repeat. Left lane shows a 1.7kb marker. Right lane is the PCR amplified product.

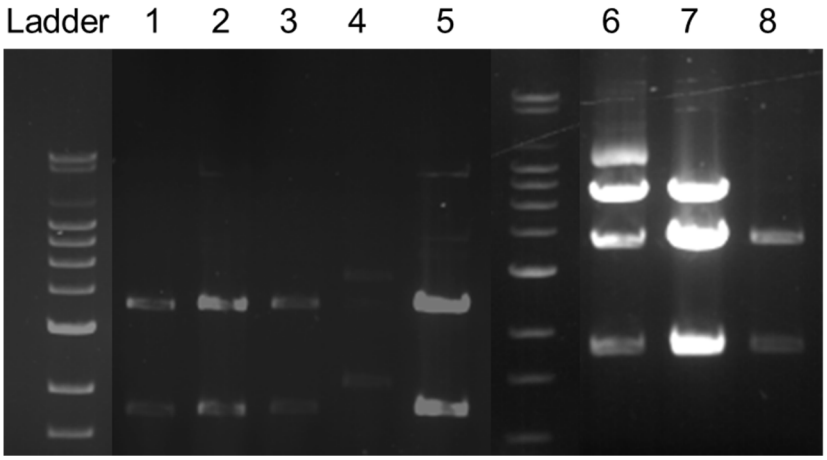

Figure 4.2. 8 clones were made for mod(AG)$_{24}$. 5, 6, 7 were selected for further experiments. Some plasmids are partially digested.

In several neurodegenerative diseases, poly glutamine residues are found which, if are over 40, causes aggregates in neurons due to structural distortion. It is not known if something similar could happen in the case of these restriction enzymes. If such threshold levels of repeats exist, crossing which abolishes activity due to such high number of repeats, we may expect a similar molecular abnormality. Comparing the activity of MboIII24 and MboIII9 will lead us further towards understanding the plasticity and threshold for these types of enzymes.

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