Mutagenesis and Biochemical Studies to understand the mechanism of stimulation of the GTPase McrB by the endonuclease McrC

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled **Mutagenesis and Biochemical Studies to understand the mechanism of stimulation of the GTPase McrB by the endonuclease McrC** towards the partial fulfilment of the MS degree at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Sutirtha Bandyopadhyay** at Indian Institute of Science Education and Research under the supervision of **Dr. Saikrishnan Kayarat**, Professor, Department of Biology, during the academic year 2020-2021.

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Declaration

I hereby declare that the matter embodied in the report entitled **Mutagenesis and Biochemical Studies to understand the mechanism of stimulation of the GTPase McrB by the endonuclease McrC** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Saikrishnan Kayarat and the same has not been submitted elsewhere for any other degree.

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Abstract

Modified cytosine recognition B and C (McrBC) complex belongs to the class of bacterial modification-dependent restriction enzymes that recognize and cleave methylated DNA. McrBC is a heterooligomeric complex of fourteen subunits (twelve subunits of McrB and two of McrC). McrB performs sequence-specific DNA binding and GTP hydrolysis, while McrC harbours the endonuclease domain. The GTPase is essential for the endonucleolytic activity. McrB harbours a AAA+ domain that functions as the GTPase. Unlike most AAA+ proteins that are stimulated by their substrate, the GTPase activity of McrB is stimulated by the partner protein McrC. Though the interaction between McrB and McrC is important for functional activities, the molecular basis of the interaction remains unknown. As part of the research work, we plan to dissect out the interaction between McrB and McrC using site-directed mutagenesis and biochemical studies. Our study shows that four selected arginine point mutant and a loop deletion mutant of McrC have no effect in stimulation of GTPase activity of McrB. Biochemical assays with wild type proteins (McrBΔN and McrC) also indicates that how much McrC can stimulates the basal GTPase activity of McrB.

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Chapter 1

Modification dependent restriction enzyme McrBC

1.1 General Introduction:

Bacteriophages are the most abundant organisms in the biosphere, posing a constant threat to their bacterial preys[1]. To counter phage attacks, bacteria employ a number of different strategies, such as blocking the attachment and entry of phage DNA, cleaving phage nucleic acid or using abortive infection systems which cause the death of infected cells to protect the bacterial population[2]. Amongst these strategies, bacteria in general harbour several specialized restriction modification systems that detect and destroy foreign DNA. As the name suggests, the RM systems comprise of two components- restriction and modification. The majority of these enzymes recognize certain unmethylated sequences, which means that these sites must be protected in the bacterial DNA by the action of cognate methyltransferases. Based on their complexity, cofactor requirement and cleavage position with respect to the recognition site, RM enzymes were primarily classified into three classes - Type I, Type II and Type III[3]. Out of the three, Type II RM systems are extensively studied and also an integral part of various laboratory techniques in the field of molecular biology. These type II RM systems differ from the other systems as their cognate methyltransferase functions mutually exclusively and they don't require NTP for their DNA cleavage activity.

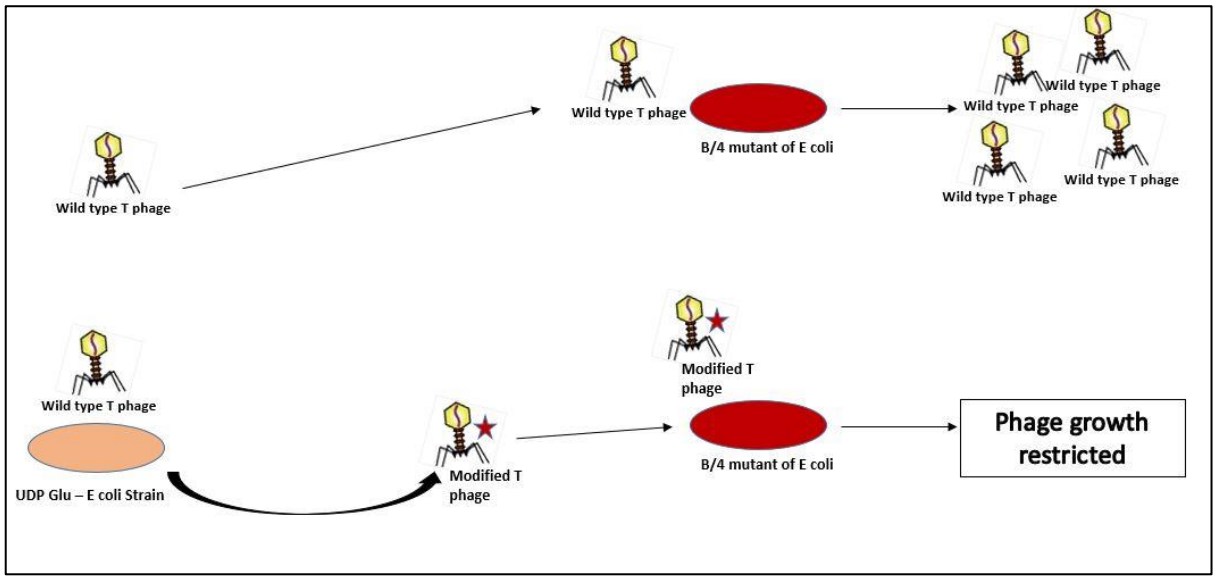
Due to the course of evolution, bacteriophages also acquired methods to methylate their genome in order to evade bacterial restriction and bacteria acquired another type of restriction system to cleave methylated DNA called Modification Dependent Restriction (MDR) system[4,5]. In comparison to the classical RM systems, MDR enzymes lack the cognate methyltransferase activity. Except for Type II, all other RM system's activity is nucleotide dependent[6].

After the discovery of the new classes of restriction enzymes (MDR system), it was difficult to classify them under existing classes and thus were classified as Type IV RM enzymes[5]. My work in this thesis describes studies on one of such NTP-dependent restriction enzyme McrBC which belongs to the MDR family of Type IV restriction systems.

1.2 McrBC: A Type IV MDR Restriction System:

1.2.1 Historical Background:

The first restriction activity was reported by Luria and Human in 1952 in order to study host induced modification[7]. Their study reported that, some mutants of E. coli strain B/4 were able to induce modification in T even phages such that the modified phages were unable to subsequently infect the B strain and its mutants. A similar study was reported by Bertani and Weigle in the year 1953.



This defect in the ability to infect was later explained as the consequence of lack of glucosylation of phage DNA that replicated in these strains, as the E. coli B strain and its mutants were deficient in the enzyme uridine diphosphoglucose pyrophosphorylase (UDPG-PPase)[8]. These modifications on phage DNA were found to be host specific and non-hereditary as pathogenicity was regained upon replicating the phage in a non-modifying host. When the non-glucosylated nucleic acid of T-even phages was injected in E. coli K12 or B4, it was found to be cleaved into small fragments[9].

Later, Revel and group identified two independent restriction systems that were causing the restriction of non-glucosylated T-even phages and they were named as RglA and RglB[10]. It was also observed that these endonucleases specifically cleaved 5'-methyl-cytosine containing DNA and hence the Mcr nomenclature for Modified Cytosine Restriction was adopted in which the RglA and RglB corresponds to McrA and McrBC respectively[11].

1.2.2 *McrBC* operon:

The mcrbc gene was located in E. coli K12 genome which consists of a 14 kbp Immigration Control Region (ICR), a densely packed region with genes encoding different restriction systems[12].In this region Type I RM enzyme EcoKI is flanked by the MDR enzymes McrBC and Mrr, displaying a checkpoint for foreign DNA invasion(Figure-1.1).

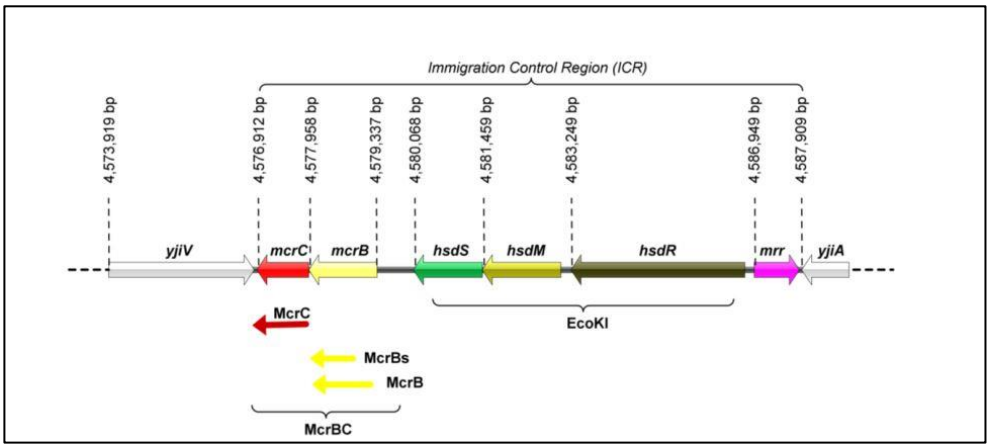


Figure 1.1: The *mcrbc* locus in E. coli genome

In vivo experiments showed that the *mcrB* gene produced two polypeptides of molecular weight 54 kDa and 33 kDa. Due to the presence of an in-frame translation initiation site in the *mcrB* gene the shorter 33kDa peptide is produced which is also termed as $McrB_s$ lacking the 161 N-terminal amino acid residues[13]. The region is flanked by potential transcription termination dyads with high G+C content also having an evolutionary significance.

1.2.2.1: McrB:

McrB is a 54 kDa polypeptide with distinct N-terminal domain for DNA and nucleotide binding and hydrolysing C-terminal domain[14]. As mentioned previously, the McrBC operon transcribes three proteins- McrB, $McrB_s$ and McrC. Maxicell analysis of translated proteins showed that both the full-length McrB and the truncated $McrB_s$ were produced in equimolar amounts.

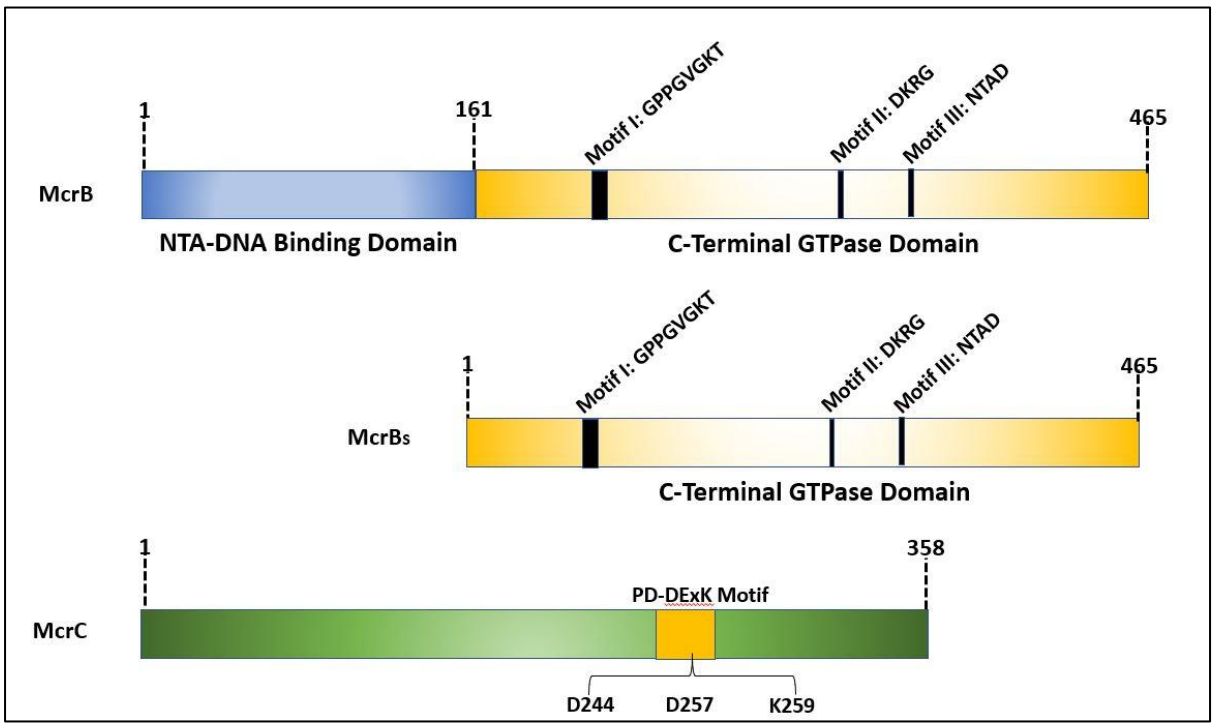
Since McrBC is a GTPase, initial amino acid sequence characterization was carried out to identify the core GTP binding and hydrolysis motifs in the protein. Through sequence alignment and mutational analysis Motif I of McrB was identified as GPPGVGKT (201-

208), Motif II as DKRG (300-303) and Motif III as NTAD (333-336)[14,15]. In subsequent studies, the GTPase domain of McrB was identified and classified as a AAA+ domain[16].

Several truncations of McrB were produced namely - $McrB_{1-190}$, $McrB_{1-122}$, $McrB_{1-137}$, $McrB_{1-162}$, $McrB_{1-170}$, $McrB_{169-465}$ and $McrB_{189-465}$. The truncations were made at predicated loop region in order to minimize the damage caused to the structure of the protein[14]. All these deletion constructs were made with N-terminal GST fusion tags. The tag was previously demonstrated to not interfere with the DNA binding nor hydrolysis activity of the enzyme[15]. The GTP hydrolysis behaviour as well as DNA cleavage activity was tested for the constructs except for $McrB_{1-122}$, $McrB_{1-137}$, $McrB_{189-465}$ as they yielded as insoluble protein, and that led to the conclusion that the protein has two independent functional domains with distinct roles[14]. McrB has a basal GTPase activity which is stimulated about 30-fold in the presence of McrC.

1.2.2.2: <u>$McrB_S$</u>:

$McrB_S$ is a 33 kDa protein which is supposed to have a regulatory role in the activity of McrBC complex. The protein lacks the N-terminal 161 amino acid residues of McrB[17]. $McrB_S$ cannot lead to the cleavage of substrate even in the presence of McrC, as it lacks the DNA binding domain. It was demonstrated both in vivo and in vitro that the optimal cleavage of DNA by McrBC can be modulated by the presence of $McrB_S$ even though it is not required for cleavage. $McrB_S$ binds to and sequesters excess McrC thereby preventing suboptimal cleavage. At limiting concentrations of McrB, $McrB_S$ can compete with the former protein and lead to limited cleavage. These observations also indicates that the optimal ratio of McrB and McrC is crucial for the function of the hetero-protein complex. It has been showed that the optimal ratio of 3-5 McrB for 1 McrC was required for efficient DNA cleavage[13]. $McrB_S$ shows GTPase and oligomeric properties with McrC similar to full length McrB.

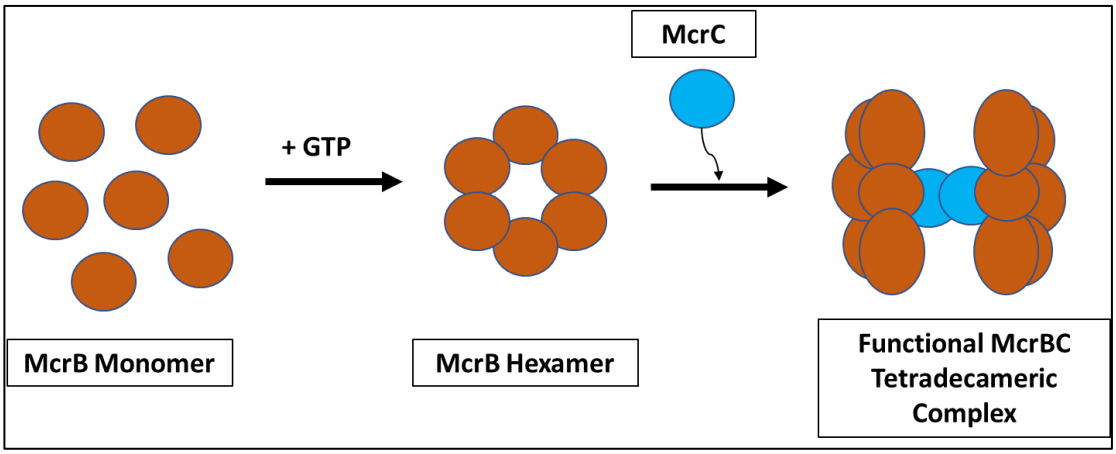


1.2.2.3: McrC:

McrC is a 39 kDa polypeptide which harbours the nucleolytic residues for DNA cleavage activity[18]. The basal GTPase activity of McrB gets stimulated by 30-fold in presence of McrC. Sequence alignment with six homologous proteins combined with mutational analysis showed that McrC harbours the nucleolytic centre TD244...D257AK. This is a variant of the well conserved PD...D/EXK motif of several nucleases including Type II restriction enzymes. Point mutations in the catalytic centre which takes away the proteins ability to cleave did not affect the stimulation of GTPase nor the formation of higher order complexes with its partner[19]. McrC, in presence of GTP, increases the DNA affinity of McrB and promotes the formation of high molecular mass complexes.

1.2.3: Structural assembly of McrBC complex:

The functional form of McrBC is a tetradecameric complex consisting of two McrB hexamers bridged together by a dimer of McrC[20,21]. The assembly is dependent on the presence of nucleotide and the complex can be disassembled by washing off GTP. It has been observed by several groups that McrB like other AAA+ (ATPase Associated with various cellular Activities) protein can form higher order oligomers in a concentration dependent manner even in the absence of GTP. In the presence of GTP, McrB exists predominantly as a hexamer in solution. McrC exist as dimer in solution and does not form higher oligomers in the presence of nucleotide. It has been suggested that the formation of the McrBC complex precedes GTP hydrolysis and hence cleavage of substrate DNA[20,21].



1.2.4: GTP Hydrolysis Activity:

McrBC is unique among AAA+ protein as it hydrolyses GTP rather than ATP. The GTP binding and hydrolysis centre of the complex is the polypeptide McrB. McrB has a basal GTPase activity which is triggered almost 30-fold in the presence of McrC[15]. Unlike other NTPase restriction enzymes, the nucleotide hydrolysis rate of this protein is not enhanced in the presence of substrate DNA. Alanine scanning mutations on C-terminal domain of McrB led to the identification of a mutant McrB^{R337A} that has ten-fold higher GTPase activity as compared to wild type McrB. This mutant was suggested to have conformation similar to that induced upon interaction with McrC[22].

1.2.5: Nucleolytic Activity of McrBC complex:

The MDR enzyme McrBC binds and cleaves DNA in a GTP hydrolysis-dependent manner. It cleaves DNA containing at least two methylated cytosine separated by a distance not less than 30 bp and not beyond 3000 bp[23,24]. Two sites with distance less than 30 bp are not cleaved[11]. This could be due to the footprint of McrBC complex. Successful cleavage takes place in presence of GTP and Mg2+ with cleavage taking place close to one of the two recognition sites. The molecular mechanism of cleavage by McrBC is not yet clear. The prevalent hypothesis in the field is that two hetero-protein complexes bound at the two distinct restriction sites utilizes energy of GTP hydrolysis to translocate on DNA and collide into each other leading to cleavage. Successful cleavage of single-site circular DNA and that of single-site linear substrate with bound lac repressors supports this mode of restriction[24].

1.3 Scope of the Thesis:

McrBC is a type IV restriction enzyme which recognizes and cleaves methylated substrate using the energy derived from GTP hydrolysis. The polypeptide complex was discovered due to the challenges faced while cloning heterologous protein in laboratory strains of *E. coli* K12. The enzyme complex has technological significance, they are used in the field of epigenetics as a tool - for example for profiling CpG islands. Moreover, McrBC serves as a simple model system to understand the mechanism of other AAA+ proteins, in particular, those that translocate on dsDNA utilizing energy obtained from NTP hydrolysis. The functionally active form of the restriction enzyme is a tetradecameric complex containing 12 subunits of McrB and two subunits of McrC[20,21].

McrBC is a unique AAA+ protein, which hydrolyses GTP instead of ATP to translocate along the DNA which is essential for such nucleotide-dependent restriction enzymes to communicate or collide with another such enzyme bound at recognition sequence far away in order to catalyse nucleolytic cleavage[24]. Also, interestingly, unlike most other DNA binding AAA+ motors, the stimulation of the McrB GTPase has been shown to be dependent on complexation with the nuclease McrC rather than substrate DNA[15].

As a step towards understanding the interaction between the two subunits of this complex which results in GTPase stimulation of McrB, I have carried out biochemical, and mutational studies. These studies were also aimed at understanding the domain wise characterisation McrC. Thus, I created point and deletion mutants of McrC to dissect out the regions of McrC that are in contact with McrB through deletion mutagenesis which will help us to get a better understanding of the two components of such a large multimeric complex.

Besides this I also have carried out biochemical assays like GDP Exchange Assay (GEF) and GTPase Activating Assay (GAP) which can give us a distinct picture of how McrC stimulates the basal GTPase activity of the GTPase McrB.

References:

1. Clokie, M. R. J., Millard, A. D., Letarov, A. V. & Heaphy, S. Phages in nature. *Bacteriophage* **1**, 31–45 (2011).
2. Labrie, S. J., Samson, J. E. & Moineau, S. Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* **8**, 317–327 (2010).
3. Boyer, H. W. DNA Restriction and Modification Mechanisms in Bacteria. *Annu. Rev. Microbiol.* **25**, 153–176 (1971).
4. Roberts, R. J. *et al.* SURVEY AND SUMMARY A nomenclature for restriction enzymes , DNA methyltransferases , homing endonucleases and their genes. **31**, (2003).
5. Bickle, T. A. & Kruger, D. H. Biology of DNA restriction. *Microbiol Rev* **57**, 434–450 (1993).
6. Murphy, M., Schmid Nuoffer, S. & Bickle, T. A. Lack of regulation of the modification-dependent restriction enzyme McrBC in Escherichia coli. *J. Bacteriol.* **184**, 1794–1795 (2002).
7. LURIA, S. E. & HUMAN, M. L. A nonhereditary, host-induced variation of bacterial viruses. *J. Bacteriol.* **64**, 557–569 (1952).
8. HATTMAN, S. & FUKASAWA, T. HOST-INDUCED MODIFICATION OF T-EVEN PHAGES DUE TO DEFECTIVE. *Proc. Natl. Acad. Sci. United States* **50**, 297–300 (1963).
9. Hattman, S. The functioning of T-even phages with unglucosylated DNA in restricting Escherichia coli host cells. *Virology* **24**, 333–348 (1964).
10. Hattman, S., Revel, H. R. & Luria, S. E. Enzyme synthesis directed by nonglucosylated T-even bacteriophages in restrictive hosts. *Virology* **30**, 427–438 (1966).
11. Sutherland, E., Coe, L. & Raleigh, E. A. McrBC: a multisubunit GTP-dependent restriction endonuclease. *J. Mol. Biol.* **225**, 327–348 (1992).
12. Sutherland, E., Coe, L. & Raleigh, E. A. McrBC: a multisubunit GTP-dependent restriction endonuclease. *J. Mol. Biol.* **225**, 327–348 (1992).
13. Panne, D., Raleigh, E. A. & Bickle, T. A. McrB(s), a modulator peptide for McrBC activity. *EMBO J.* **17**, 5477–5483 (1998).
14. U., P., T., S., D.H., G., F.-U., G. & A., P. The GTP-binding domain of McrB: More than just a variation on a common theme? *J. Mol. Biol.* **292**, 547–556 (1999).
15. Pieper, U., Brinkmann, T., Krüger, T., Noyer-Weidner, M. & Pingoud, A. Characterization of the interaction between the restriction endonuclease McrBC from E. coli and its cofactor GTP. *J. Mol. Biol.* **272**, 190–199 (1997).
16. Neuwald, A. F. The hexamerization domain of N-ethylmaleimide-sensitive factor: Structural clues to chaperone function. *Structure* vol. 7 (1999).
17. Ross, T. K., Achberger, E. C. & Braymer, H. D. Nucleotide sequence of the McrB

region of Escherichia coli K-12 and evidence for two independent translational initiation sites at the mcrB locus. *J. Bacteriol.* **171**, 1974–1981 (1989).

18. Pieper, U. & Pingoud, A. A mutational analysis of the PD...D/EXK motif suggests that McrC harbors the catalytic center for DNA cleavage by the GTP-dependent restriction enzyme McrBC from Escherichia coli. *Biochemistry* **41**, 5236–5244 (2002).

19. Pieper, U. *et al.* The GTP-dependent restriction enzyme McrBC from Escherichia coli forms high-molecular mass complexes with DNA and produces a cleavage pattern with a characteristic 10-base pair repeat. *Biochemistry* **41**, 5245–5254 (2002).

20. Nirwan, N. *et al.* Hexameric assembly of the AAA+ protein McrB is necessary for GTPase activity. *Nucleic Acids Res.* **47**, 868–882 (2019).

21. Nirwan, N. *et al.* Structure-based mechanism for activation of the AAA+ GTPase McrB by the endonuclease McrC. *Nat. Commun.* **10**, 1–9 (2019).

22. Pieper, U., Schweitzer, T., Groll, D. H., Gast, F. U. & Pingoud, A. The GTP-binding domain of McrB: More than just a variation on a common theme? *J. Mol. Biol.* **292**, 547–556 (1999).

23. Krüger, T., Wild, C. & Noyer-Weidner, M. McrB: A prokaryotic protein specifically recognizing DNA containing modified cytosine residues. *EMBO J.* **14**, 2661–2669 (1995).

24. Panne, D., Raleigh, E. A. & Bickle, T. A. The McrBC endonuclease translocates DNA in a reaction dependent on GTP hydrolysis. *J. Mol. Biol.* **290**, 49–60 (1999).

25. Van Den Ent, F. & Löwe, J. RF cloning: A restriction-free method for inserting target genes into plasmids. *J. Biochem. Biophys. Methods* **67**, 67–74 (2006).

26. Ingerman, E. & Nunnari, J. A continuous, regenerative coupled GTPase assay for dynamin-related proteins. *Methods Enzymol.* **404**, 611–619 (2005).

27. Vetter, I. R. & Wittingho dimensions. *Science* vol. 294 1299–1304 (2001).

Chapter 2

Mutagenesis studies to understand the mechanism of stimulation of the GTPase McrB by the endonuclease McrC

2.1 Materials and Methods:

2.1.1 Introduction:

As a step towards understanding the interaction between the two subunits of this complex which results in GTPase stimulation of McrB, we have carried out mutational studies of McrC. These studies were also aimed at understanding the domain wise characterisation McrC.
Recently our lab has reported a 3.6Å structure of a tetradecameric McrBC complex which is GTP active but DNA binding deficient. By analysing the structure, we have selected some specific residues and region of McrC which are in close proximity of McrB (Figure 2.1.1). These residues or region may play a major role in GTPase stimulation of McrB.

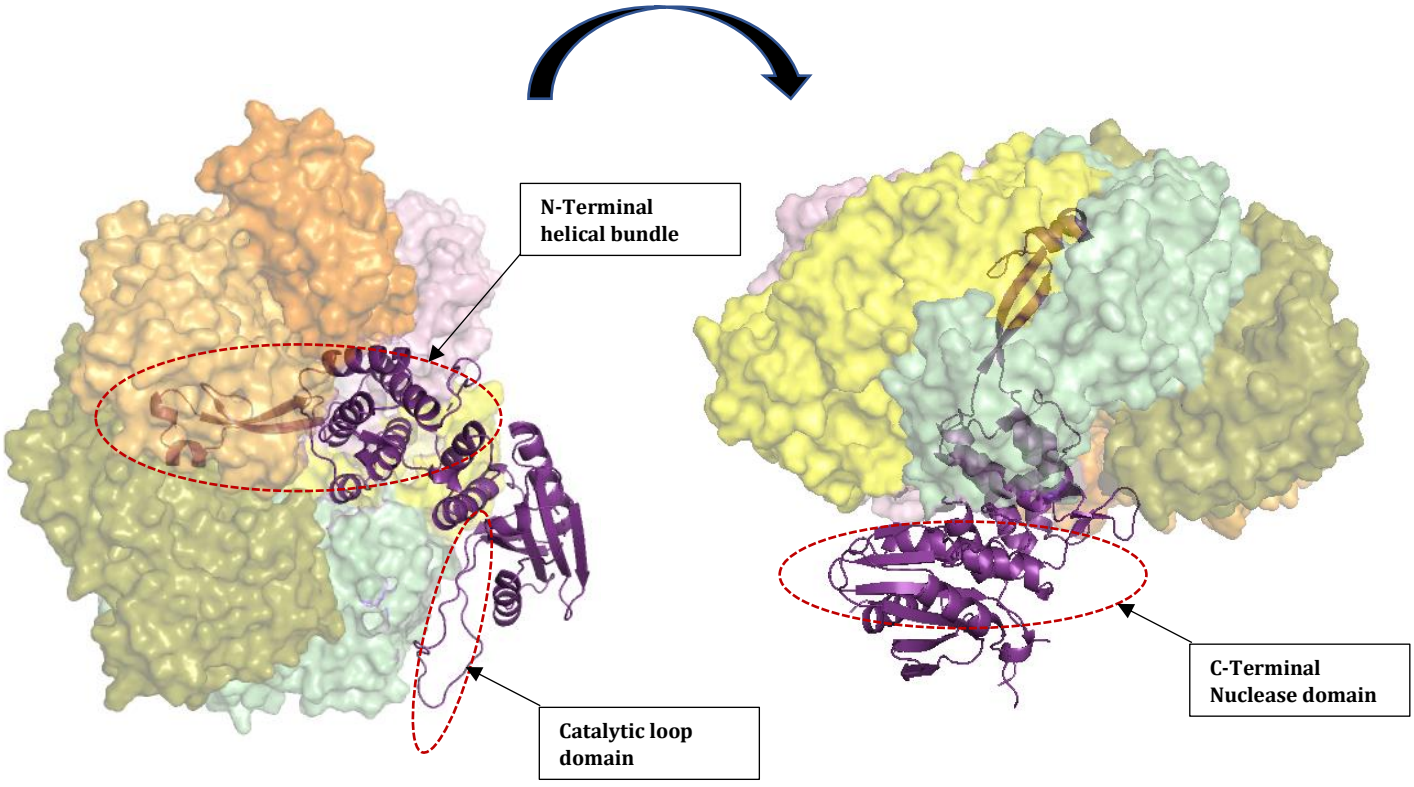


Figure 2.1.1: Structure of McrBC Complex showing interaction between different regions of McrC (shown in purple) with the GTPase McrB hexamer

We deleted the loop region of McrC (222-242nd residue) namely McrC$^{\Delta CatLoop}$ (as this loop is present just before one of the catalytic residues responsible for DNA cleavage) which is close to one of the McrB protomer (Figure 2.1.2).

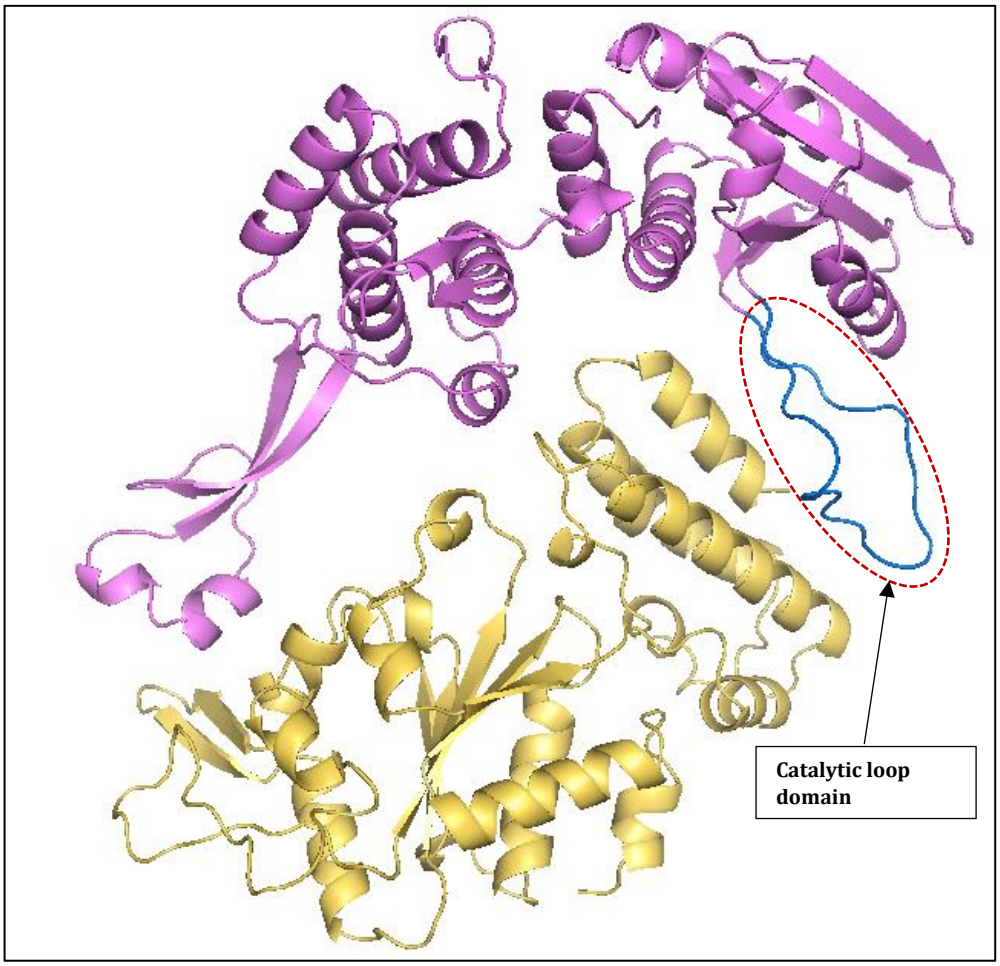


Figure 2.1.2: The catalytic loop (shown in blue) of McrC (shown in purple) in close proximity to one of the protomers of McrB (shown in yellow)

We have also selected four arginine residues present at the N-terminal helical domain of McrC which make multiple interactions with the GTPase McrB and mutated them into alanine by site directed mutagenesis. These residues are R55, R83, R131 and R160; out of them R83 is present at shaft region of McrC (Figure 2.1.3).

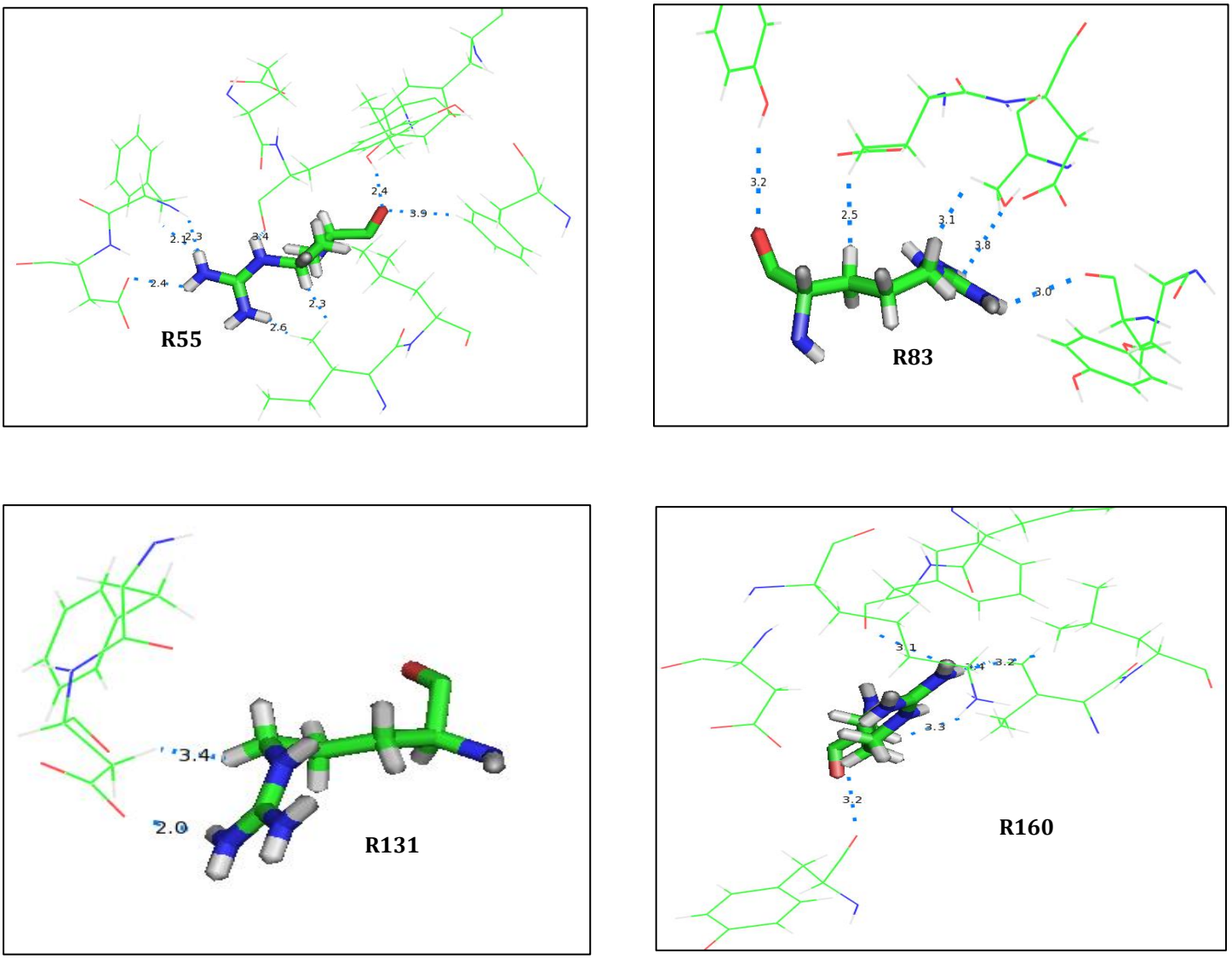


Figure 2.1.3: Possible interactions between different arginine residues (shown as sticks) of McrC N-terminal helical bundle region with the McrB (shown as lines)

2.1.2 Cloning:

All the point mutants and deletion construct were cloned using the restriction-free (RF) method of cloning. The gene of interest was amplified from pHIS-McrC (plasmid containing wild type *mcrc* gene) using the appropriate combination of primers.

The list of primers used for each clone is listed below in table 2.1.1:

Table 2.1.1: Primers used for cloning of McrC mutants:

Constructs	Forward Primer (5'-3')	Reverse Primer (5'-3')	GC content	Theoretical T_m
$McrC^{R55A}$	CAGCTTTCAGCGCGAGGGCTTGAG	GCTAGTTATTGCTCAGCGG	61.9 %	61°C
$McrC^{R83A}$	GCTAAAACAATAGCGGGCTTCCATCTTAATC	GCTAGTTATTGCTCAGCGG	40 %	62.3°C
$McrC^{R131A}$	GATGAAGCTGCGTCACTTTATAGA	GCTAGTTATTGCTCAGCGG	35.7 %	54.7°C
$McrC^{R160A}$	GGAAAAAATACGGCGTATTATAAATTC	GCTAGTTATTGCTCAGCGG	29.6 %	51.5°C
$McrC^{\Delta Catloop}$	GCAAACACAACCCGCTCTGGAGGAACTGACATCACCATTCGC	GCTAGTTATTGCTCAGCGG	56%	87.6°C

2.1.2.1 Restriction Free (RF) Cloning:

The underlying principle of RF cloning is to use a PCR amplified gene of interest as a primer (megaprimer) for the linear amplification of a circular plasmid[25]. Restriction free method of cloning involved two cycles of polymerase chain reaction (PCR). In the first cycle, the region of interest was amplified using synthetic oligonucleotides. The amplicon from the first cycle was purified using QIAquick® PCR purification kit (Qiagen) to be used as a megaprimer against pHISMcrC plasmid – which was pHIS17 vector carrying mcrC gene between NdeI and BamHI restriction sites. Pfu polymerase was used for the amplification. Conditions for PCR:

- **First PCR Components:**

10X Pfu Buffer: 5 µL

Template: 100 ng

Forward primer (10µM): 2.5µL (0.4µM)

Reverse primer (10µM): 2.5µL (0.4µM)

dNTPs (2.5 mM each): 2 µL (100 µM each)

Pfu Polymerase: 0.8 µ

- **Second PCR Components:**

Components	**Control**	**Test**
10x Pfu Buffer	5µL	5µL
Template	100ng	100ng
Megaprimer	**--**	600ng
dNTPs (2.5 mM each)	2 µL (100 µM each)	2 µL (100 µM each)
Pfu Polymerase	0.8 µL	0.8 µL

The reaction volume was made up to 50 µL using MilliQ.

PCR cycle:

Initial denaturation: 95°C for 2’

Denaturation: 95°C for 30’ **35 cycles**

Annealing: 55°C for 30’

Extension: 72°C for 1’

Final extension: 72°C for 10’

The reaction was stored at 4°C once completed.

The template plasmid was digested by DpnI before using this reaction mix for transformation. 0.5 µL of DpnI was added to 9.5µL of the reaction mix for the same.

2.1.2.2: Electroporation:

NEB®Turbo electro-competent cells (*E. coli*) were transformed either with ligation mix or Dpn1 treated RF reaction mix. 10 µL of a solution containing recombinant plasmid was added to approximately 100 µL of competent cells. The cells were incubated on ice for 10 minutes before transferring them into a chilled electroporation cuvette. Cells were given an electric pulse of 2.5 kV using Bio-Rad® Gene Pulser Xcell™. The cells were revived by incubating them at 37°C for about 45 minutes after the addition of 100 µL of 2X LB. The cells were later plated on appropriate antibiotic containing plate. The plates were incubated at 37°C for 8-12 hours.

2.1.2.3: Clone Check:

A single colony from plates incubated overnight was used to inoculate 5 mL of LB. The culture was grown at 37°C in an incubator under shaking condition overnight post which the cells were pelleted down using a table top centrifuge (Eppendorf) at 13000 rpm. The plasmid was extracted from the cell pellet using alkaline hydrolysis method of plasmid extraction as described in the table (2.1.2) below.

Table 2.1.2: Solutions for plasmid extraction:

Solution	Components	Function
Solution I	50 mM Tris pH 8 10 mM EDTA 100 µg/mL RNase A	maintains optimum pH, chelates divalent cations, degrades RNA in the cell lysate
Solution II	0.2 M NaOH 1% SDS	solubilization and disruption of the cell membrane, denature proteins in the cell lysate, denature both genomic and plasmid DNA
Solution III	3 M Potassium Acetate (pH 5.5)	Brings down alkalinity of the cell resuspension allowing smaller plasmid DNA to re-anneal

The cell pellet was resuspended in 250 µL of solution I following which solution II was added. The solution was homogenized by inverting the tube 5-6 times. Finally, solution III was added and the tube was inverted 5-6 times until a white precipitate was seen. The solution was spun at 4°C for 10 minutes following which the supernatant was transferred into a fresh tube. 900 µL of chilled absolute ethanol was added to the supernatant and spun at 4°C for 20 minutes. Addition of ethanol causes the precipitation of DNA in the solution. The supernatant was discarded and pellet was washed with chilled 70% ethanol. The pellet was dried and later resuspended in 50 µL of MilliQ.

The plasmid hence extracted was double digested using restriction enzymes NdeI and BamHI using quick digestion protocol. The typical reaction involves heating of 10 µL reaction containing 1 µL of each of the restriction enzymes and 1 µg DNA in a microwave oven for 30 seconds. The digested DNA fragments were visualized on 1% agarose gel. The plasmids thus verified were sent for sequencing.

2.1.3: <u>Culturing and Protein Expression:</u>

The expression system of choice was *E. coli* BL21(AI) (Invitrogen®) cells, as the overexpression of the wild type protein was optimized in this strain. The bacterial strain was transformed with pHIS17 vector containing the gene of interest following the heat shock transformation protocol. The cells were plated on LB agar plates containing 100 µg/ml Ampicillin. Colonies from the plate incubated at 37°C for 9 hours were used to inoculate 10 mL of LB broth containing the same antibiotic. Once the primary culture reached the mid-log phase, 4 mL of this culture was used to inoculate 1 L of LB broth containing 100 µg/ml Ampicillin. The secondary culture grown at 37°C in an incubator-shaker was induced with 0.02% w/v of L-arabinose at optical density (OD) at 600 nm [OD_{600}] = 0.6. Post induction the culture was left to grow at 18°C overnight. The 1 L culture was pelleted down by centrifugation at 5000 rpm and 4°C.

An expression test was done for each mutant before proceeding to large-scale bacterial cultures. For the same, 10 mL culture was pelleted down in a tabletop centrifuge (Eppendorf) at 13000 rpm which was later resuspended in lysis buffer [50 mM Tris (pH 8), 500 mM NaCl, 25 mM Imidazole, 5 mM $MgCl_2$, 10 % glycerol]. The cell suspension was lysed by using a probe sonicator and SONICS Vibra Cell™ instrument (amplitude 60%, with a pulse of 1s ON, 3s OFF for a total of 1 minute). The cell lysate was spun at 40C in a tabletop centrifuge (Eppendorf) at 15000 rpm for 10 minutes to separate the cell debris as a pellet from the soluble fraction as the supernatant. The proteins in the supernatant represent the fraction of soluble proteins in the cytosol. The sample before centrifugation and post centrifugation was mixed with 2X SDS dye in separate tubes and heated at 99^0C for 10 minutes and spun at 15000 rpm in a tabletop centrifuge (Eppendorf) for 10 minutes before loading in a 12% SDS PAGE gel. The PAGE gel was run at 230 V in an electrophoresis unit (Bio-Rad®), post which the protein bands were visualized by Coomassie blue staining method.

2.1.4: Protein Purification:

The table (2.1.3) below lists the composition of various buffers used for purification of the mutants.

Table 2.1.3: Different Buffer compositions for purification of wild type and mutant proteins:

Buffer	Tris pH 8.0 (mM)	NaCl (mM)	Glycerol (%)	$MgCl_2$ (mM)	Imidazole (mM)	DTT (mM)
Lysis Buffer	50	500	10	5	25	1
Buffer A	50	500	0	0	25	1
Buffer B	50	500	0	0	500	1
B_{250}	50	250	0	0	0	1

2.1.4.1: Resuspension and ultracentrifugation:

The 1 L cell pellet expressing the protein of interest was resuspended in the 50 mL of lysis buffer. For all the mutants 0.04%(w/v) CHAPS was added to the cell suspension. The cells were lysed by sonication on ice (amplitude 60%, with a pulse of 1s ON, 3s OFF for a total of 3 minutes). The cell lysate was clarified by ultracentrifugation at 4°C and 37000 rpm for 45 minutes in an OptimaTM XE Ultracentrifuge (Beckman Coulter). The supernatant after the centrifugation was used as load for the subsequent purification steps.

2.1.4.2: Affinity Chromatography:

The clarified supernatant after ultracentrifugation was loaded onto a 5 mL His Trap HP Ni-NTA column (GE Healthcare) which was pre-equilibrated with Buffer A. The column had a matrix of highly cross-linked agarose beads pre-charged with Ni^{2+} through chelating groups coupled to it. Histidine is known to form complex with Ni^{2+} and hence will stay bound to the column. The bound protein was eluted from the column by washing it with increasing concentrations of imidazole that competes with histidine for binding to Ni^{2+}. An average of 5 fractions (5 mL each) were collected for a concentration of Buffer B ranging from 5% to 100%. The purity of the fractions was checked by loading samples from each fraction onto a 12% SDS PAGE gel. The purest fractions were pooled and concentrated using Vivaspin 2® centrifugal concentrator (Sartorius) with a membrane having a 10 kDa molecular weight cut off. Desalting and buffer exchange was also performed using Vivaspin 2® in lieu of dialysis as the protein precipitated heavily. Approximately 30 mL of Ni-NTA eluate was concentrated to 1 mL and then washed with 10 mL of buffer B_{250}. The diluted protein was again concentrated to 1 mL. All the processes mentioned above was carried out at 4°C. The concentration of the protein was estimated using Nanodrop. The protein was divided into aliquots of appropriate volume and all the aliquots were flash frozen in liquid N_2 and stored at -80°C until further use.

2.1.5: Cleavage Assay:

Substrate DNA was PCR amplified T7-Forward (5'-TAATACGACTCACTATAGGG-3') and T7-Reverse (5'-GCTAGTTATTGCTCAGCGG-3') as primers against pHISMcrBΔN plasmid. 5-methyl-deoxycytidine instead of deoxycytidine was used in the dNTP mix to obtain a 1127 bp long fully-methylated substrate. The substrate was then purified using the Qiagen PCR Purification Kit. Nuclease activity of 50nM McrBC and the mutants was assessed at 37 °C. 75ng of the substrate DNA was incubated with protein complex for 3600s in presence of 10 mM Tris-Cl pH 8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 1 mM GTP (Jena bioscience). The reaction was then quenched using 2 µL 6× STES buffer (40% Sucrose, 0.2 M Tris-Cl pH 7.5, 40 mM EDTA, 1% SDS) followed by heating at 65°C for 10 min. The cleaved substrate was resolved at 110 V for 45 min on a 0.8% agarose gel containing 2 µg/mL ethidium bromide (EDTA). The resolved DNA was imaged on E-Gel™ Imager System (Invitrogen Life Technologies).

2.1.6: NADH-coupled GTPase Assay:

The stimulation of GTPase activity of McrB in the presence of McrC mutants was assayed using a continuous coupled NADH assay. The enzymatic activity of McrBC complex is coupled with the activity of the enzymes i.e., pyruvate kinase and lactate dehydrogenase. McrBC hydrolyses GTP into GDP and Pi. The enzyme pyruvate kinase takes up the GDP released and converts Phospho(enol)pyruvate (PEP) to pyruvate. The pyruvate hence formed is reduced to lactate by the action of the enzyme lactate dehydrogenase, while converting NADH to NAD^+. The depletion of NADH is directly proportional to GTP hydrolysis. The rate of hydrolysis is thus qualitatively estimated by measuring the absorbance of NADH at 340 nm[26].

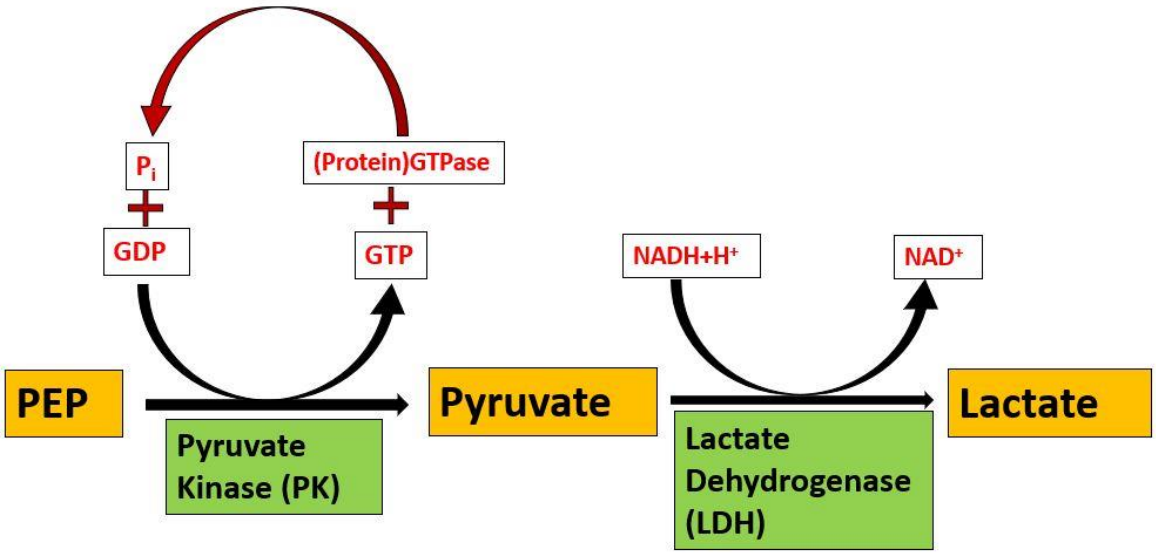


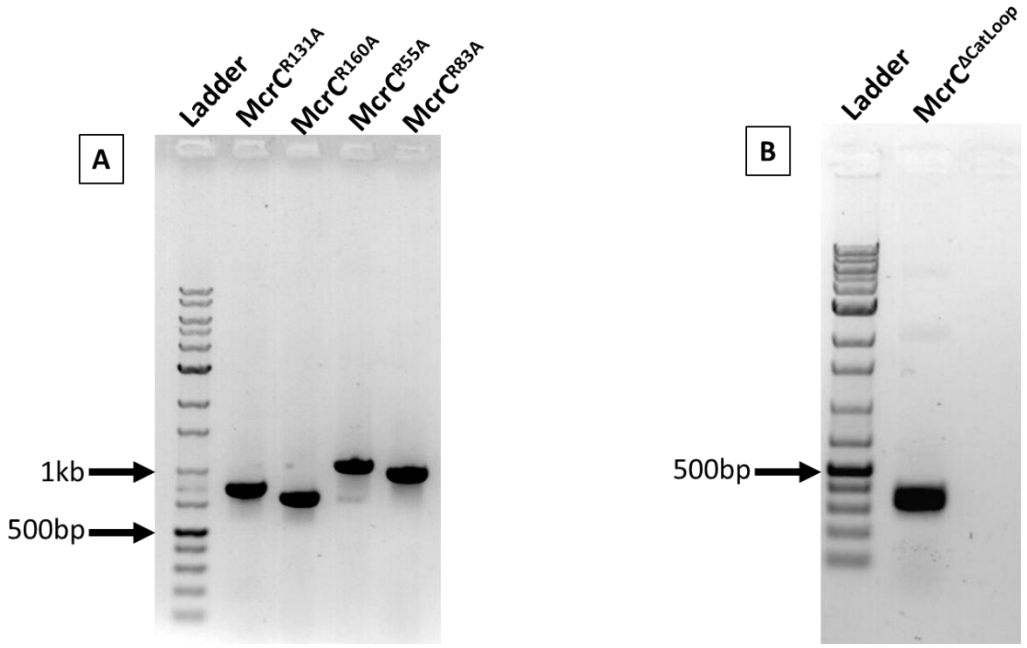
Figure 2.1.4: General outline of NADH-coupled GTPase Assay

The reaction mix consists of McrB and McrC (wild type/ mutants) mixed in a ratio 4:1, 600 µM NADH, 1mM Phospho (enol) Pyruvate (PEP), PK/LDH and 10mM $MgCl_2$ mixed with 1mM GTP (Jena Biosciences) in a total reaction volume of 200 µL. The reactions were transferred into a 96-well flat bottom plate (Corning Costar). The reaction was monitored for 1 hour at 340 nM in a Varioskan Flash™ (Thermoscientific). A 200 µL reaction containing only McrB was used as a negative control whereas the one with a 1:4 mixture of McrB and McrC (both wildtype) was used as positive control. Each reaction was performed in triplicates.

2.2 Results & Discussion:

2.2.1: Cloning of McrC Mutants:

All the four arginine point mutants and the catalytic loop deletion mutant, were cloned using the restriction-free method of cloning. The gene of interest was amplified from pHISMcrC using the appropriate combination of primers. The amplicon of the first PCR (size approximately 900kb for $McrC^{R131A}$, $McrC^{R160A}$; 400bp for $McrC^{\Delta CatLoop}$; 1kb for $McrC^{R55A}$ and $McrC^{R83A}$; Figure:2.2.1) was used as the primer for the second PCR step against pHISMcrC as the template. The recombinant plasmid extracted from transformed colonies were double digested with NdeI and BamHI restriction enzymes in order to test the outcome of RF cloning.



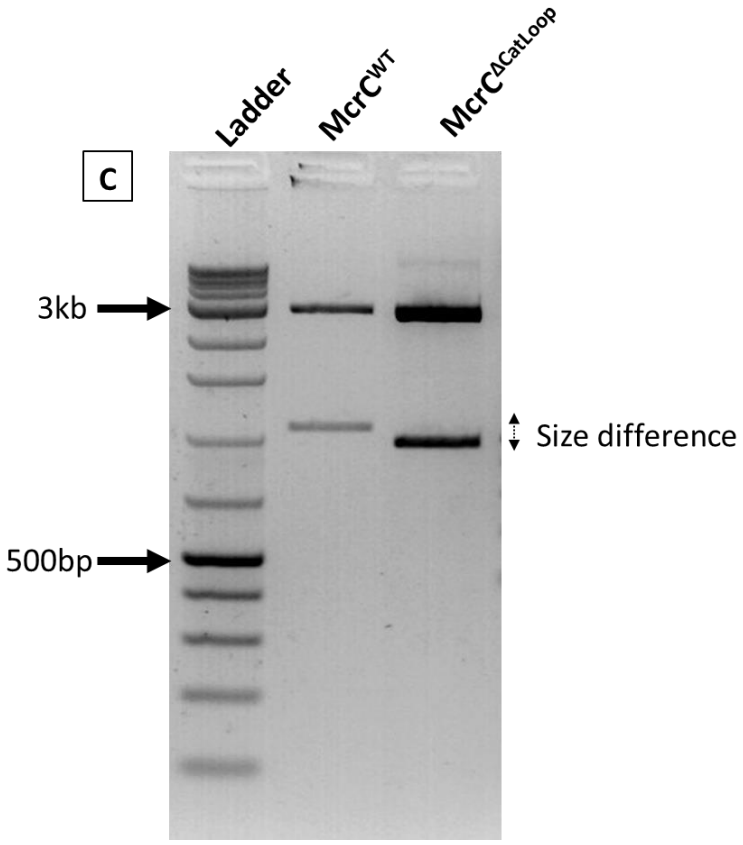


Figure 2.2.1: [A] PCR 1 amplicon of McrC arginine mutants; [B] PCR 1 amplicon of McrC$^{\Delta CatLoop}$; [C] Size difference observed between double digested (BamHI &NdeI) McrC^{WT} and McrC$^{\Delta CatLoop}$ construct

2.2.2: <u>Expression and Purification of Protein</u>:

The clones that were sequenced and verified to have the mutations and deletion were tested for their level of protein expression. All the mutant proteins are purified as described in 2.1.4 previously. The purified fractions of each point and deletion mutants are showed in 12% SDS-PAGE gels separately (Figure: 2.2.2).

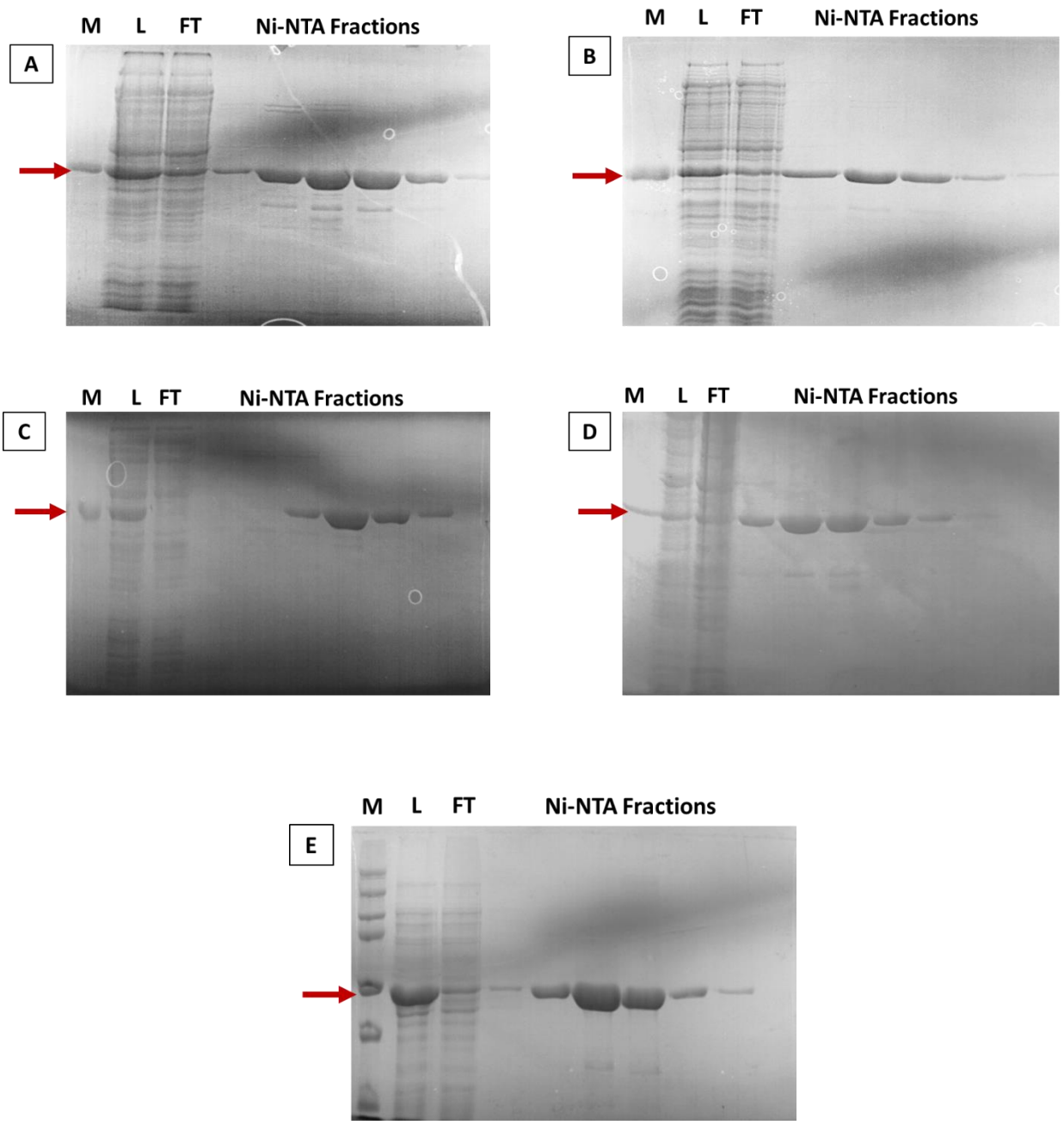


Figure 2.2.2: [A]-[E] Ni-NTA purification gels (alternate fraction samples) for McrC^{R55A}, McrC^{R83A}, McrC^{R131A}, McrC^{R160A} and McrC$^{\Delta CatLoop}$ respectively. The red arrows indicate the region on the SDS PAGE gel where the mutant protein was expected to overexpress. [Key: M=Marker/Ladder, L= Load, FT= Flow through]

2.2.3: Characterization of Arginine Mutants:

All the arginine mutants were successfully expressed and purified. We performed cleavage assay of all the point mutant proteins and compare the nucleolytic activity with the wild type protein. Details of cleavage assay was described in 2.1.5 previously.

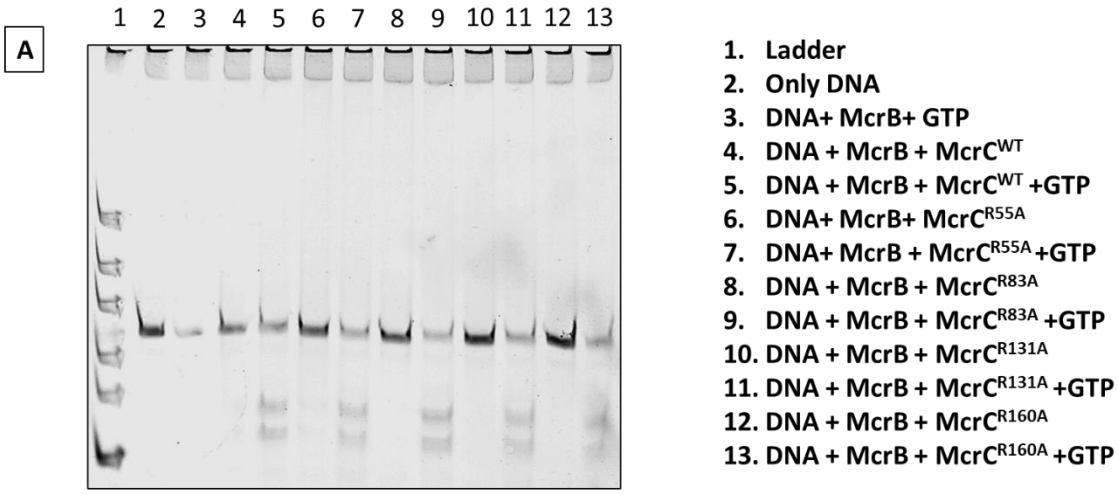


Figure 2.2.3: [A] Nuclease activity check of McrC and its mutants ($McrC^{R55A}$, $McrC^{R83A}$, $McrC^{R131A}$ and $McrC^{R160A}$ in complex with McrB. [B] Comparison of GTP hydrolysis by McrB in presence of wild type and different mutants of McrC.

Also, these mutants are subjected to NADH coupled GTPase assay where they showed almost similar activity that of the wild type (Figure 2.2.3B).

2.2.4 Characterization of Loop Mutant:

We also deleted the loop region of McrC (222-242nd residue) namely McrC$^{\Delta CatLoop}$ (as this loop is present just before one of the catalytic residues responsible for DNA cleavage) which is close to one of the McrB protomer. But this construct also showed comparable GTPase activity (Figure 2.2.4B) to that of the wild type and it was also able to cleave the DNA (Figure 2.2.4A).

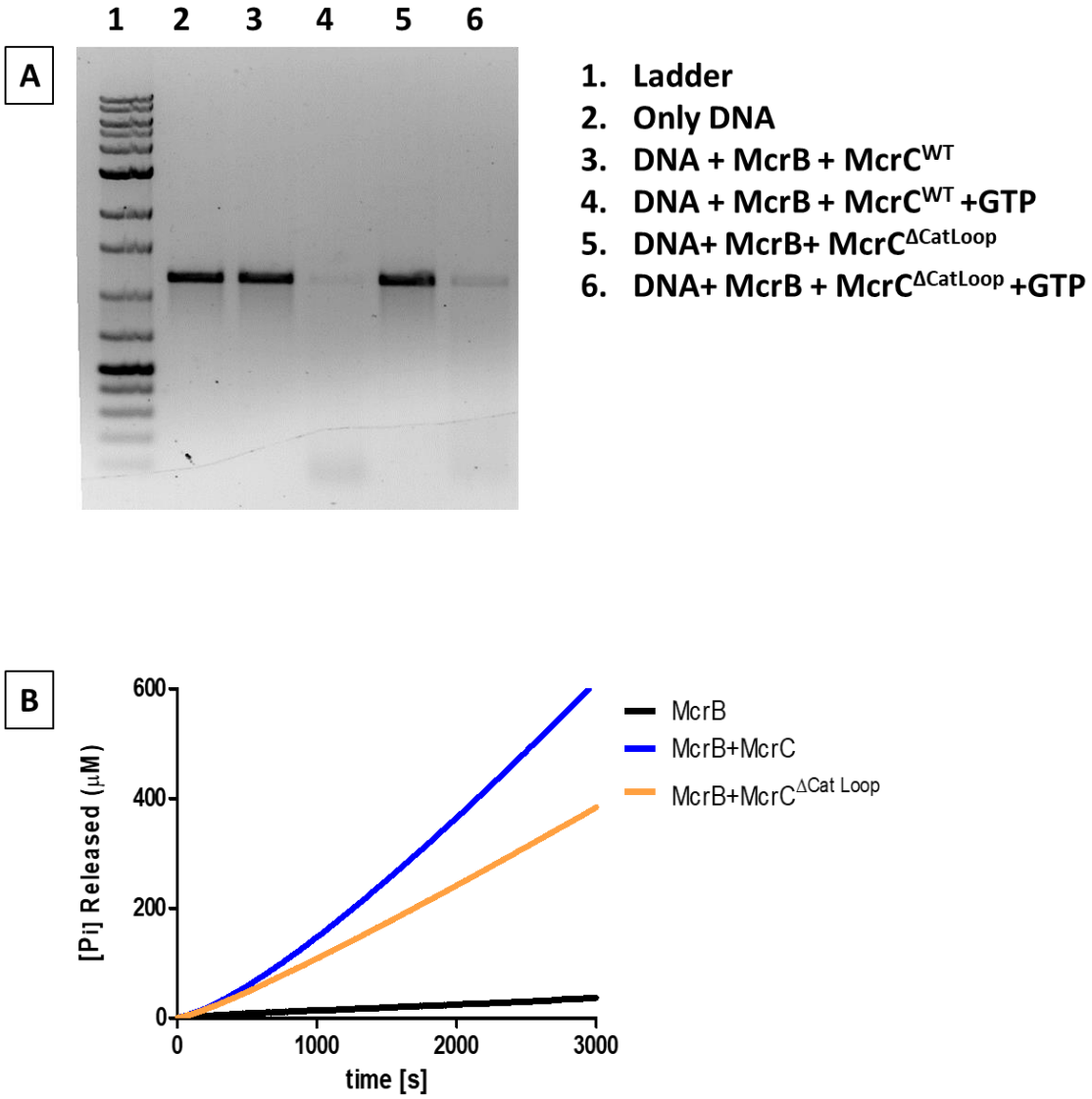


Figure 2.2.4: [A] Nuclease activity check of McrC and its catalytic loop mutant (McrC$^{\Delta CatLoop}$), in complex with McrB. [B] Comparison of GTP hydrolysis by McrB in presence of wild type and catalytic loop deletion mutant of McrC.

2.3 Discussion:

McrBC is a unique AAA+ protein, which hydrolyses GTP instead of ATP to translocate along the DNA which is essential for such nucleotide-dependent restriction enzymes to communicate or collide with another such enzyme bound at recognition sequence far away in order to catalyse nucleolytic cleavage. Also, interestingly, unlike most other DNA binding AAA+ motors, the stimulation of the McrB GTPase has been shown to be dependent on complexation with the nuclease McrC rather than substrate DNA.

The main aim of this study was to delineate the region of McrC critical for interaction with its partner McrB. As a step towards understanding this interaction, we selected, successfully created and purified four arginine point mutants ($McrC^{R55A}$, $McrC^{R83A}$, $McrC^{R131A}$ and $McrC^{R160A}$). We performed cleavage assay of all the point mutant proteins and compared the nucleolytic activity with the wild type protein. It was found that the nucleolytic activity of the mutant proteins were comparable to that of the wild type (Figure 2.2.3A). Also, the GTP hydrolysis rate of McrB in presence of these mutants were found similar (Figure 2.2.3B).

Similarly, we also deleted the loop region of McrC (222-242nd residue) namely $McrC^{\Delta CatLoop}$ (as this loop is present just before one of the catalytic residues responsible for DNA cleavage) which is close to one of the McrB protomer. But this construct also showed comparable GTPase activity (Figure 2.2.4B) to that of the wild type and it was also able to cleave the DNA (Figure 2.2.4A).

The results obtained from the above-mentioned experiments suggest that all the arginine mutants present in the N-terminal helical bundle of McrC, doesn't play a role in the GTPase stimulation activity of McrB as the GTP hydrolysis activity of the mutants are comparable to that of the wild type protein. Similarly, the catalytic loop deletion mutant ($McrC^{\Delta CatLoop}$) also being close to one of the McrB protomer, doesn't affect the GTPase stimulation or the nucleolytic activity of McrC. Simultaneous experiments done by our lab also shows that the deletion of the loop region present in the N-terminal helical domain (60-100th residue) of McrC affects the GTP stimulation as well as the nucleolytic activity of McrC. So, point mutation of residues present in this loop region will give us a better understanding of interaction between the two subunits of this macromolecular complex.

References:

1. Nirwan, N. *et al.* Structure-based mechanism for activation of the AAA+ GTPase McrB by the endonuclease McrC. *Nat. Commun.* **10**, 1–9 (2019).
2. Van Den Ent, F. & Löwe, J. RF cloning: A restriction-free method for inserting target genes into plasmids. *J. Biochem. Biophys. Methods* **67**, 67–74 (2006).
3. Ingerman, E. & Nunnari, J. A continuous, regenerative coupled GTPase assay for dynamin-related proteins. *Methods Enzymol.* **404**, 611–619 (2005).
4. Panne, D., Raleigh, E. A. & Bickle, T. A. The McrBC endonuclease translocates DNA in a reaction dependent on GTP hydrolysis. *J. Mol. Biol.* **290**, 49–60 (1999).
5. Pieper, U., Brinkmann, T., Krüger, T., Noyer-Weidner, M. & Pingoud, A. Characterization of the interaction between the restriction endonuclease McrBC from E. coli and its cofactor GTP. *J. Mol. Biol.* **272**, 190–199 (1997).

Chapter 3

Biochemical Analysis to understand stimulation of GTPase activity of McrB by McrC

3.1 General Introduction:

Small guanine nucleotide binding proteins (G-proteins) control various cellular activities by interacting with effector proteins which further controls different cell signalling pathways. A large number of these GTPases (G-Proteins) require multiple GTPase-activating proteins (GAP) and Guanine nucleotide exchange factors (GEF) for regulating its activity.

GAPs and GEFs are multidomain proteins that act as molecular switches in intracellular signalling pathways. The affinity for GTP/GDP is in picomolar range for most of the G-proteins, thus it has slower dissociation rate due to high affinity[1]. GEFs accelerate the exchange reaction by several orders of magnitude by binding to the inactive (GDP-bound) GTPase and catalyse the GDP dissociation[2]. Once the GDP molecule dissociates from the GTPase a GTP molecule binds to the GTPase to activate it and result in the release of GEF. Whereas, a GAP protein acts as an antagonist to GEF. The GAP proteins increase the intrinsic GTP hydrolysis activity of the GTPase and usually switch the GTPase off as it becomes GDP-bound after hydrolysis (Figure 3.1.1).

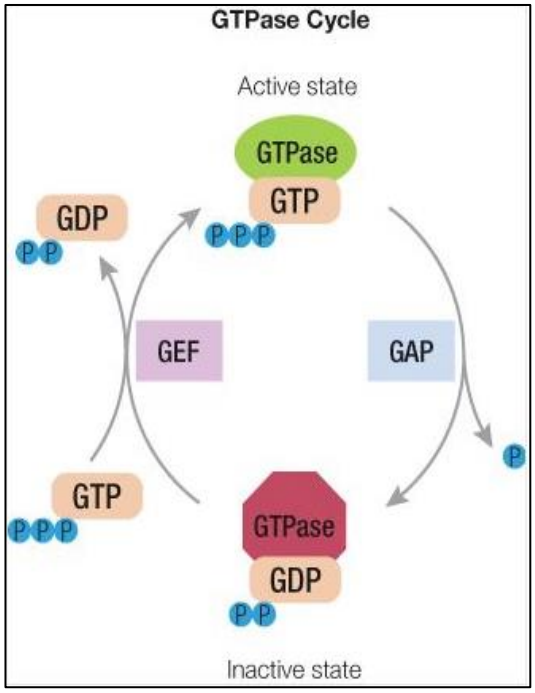


Figure 3.1.1: Regulation of activity of G-Proteins by GAPs and GEFs (Klink et. Al. ,2015)

Though McrB belongs to AAA+ family it binds and hydrolyses GTP like G-proteins and the rate of hydrolysis gets stimulated by 30 folds in presence of its endonuclease partner McrC whereas substrate DNA fails to stimulate the GTPase activity of McrB[3,4]. So, we were interested to find out how McrC stimulates the GTPase, i.e., whether it stimulates the catalysis of McrB by activating GTPase (GAP) or help in exchange of nucleotides by acting as a nucleotide exchange factor (GEF) or a combined effect of all two possibilities.

3.2 Materials and Methods:

3.2.1 Purification of McrBΔN:

3.2.1.1 Growing of culture:

McrBΔN was expressed with a tag of six histidine at C-terminus by overexpression of pHISMcrBΔN plasmids in *E. coli* BL21 (AI) (Invitrogen®) cells. The cultures were grown in 2 L LB media containing 100 µg/ml ampicillin in an incubator-shaker at 37°C until OD reached 0.3 at 600 nm. The temperature of incubator-shaker was then reduced to 18°C and cultures were induced with 0.06% w/v L-Arabinose. The cultures were grown further overnight (15-16 hours) at 18°C. Cells were pelleted by centrifugation at 4°C and 3,315 g for 15 minutes.

3.2.1.2 Resuspension and ultracentrifugation:

The pellet was resuspended in 50 ml lysis buffer (50 mM Tris-Cl pH 8, 25 mM imidazole, 500 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.04% CHAPS). The cells were lysed by sonication at 4°C using Vibra-Cell™ system set at 60% amplitude, pulsing in 1 sec ON and 3 sec OFF mode for 3 minutes. The cycle was repeated after 10 minutes to allow heat dissipation. The cell lysate was then clarified by ultracentrifugation at 4°C and 159,200 g for 40 minutes. McrBΔN was first purified by affinity chromatography using an identical strategy that of wild type McrB.

3.2.1.3 Affinity Chromatography:

The clarified supernatant of the cell lysate was loaded onto a 5 ml Ni-NTA column (GE Life Sciences) equilibrated with Buffer A (50 mM Tris-Cl pH 8, 25 mM imidazole, 500 mM NaCl). The protein was eluted using Buffer A and Buffer B (50 mM Tris-Cl pH 8, 500 mM Imidazole, 500 mM NaCl) by a step gradient from 5% to 100% at intervals of 20%.

3.2.1.4 Dialysis:

The purest of the Ni-NTA fractions were dialyzed against 2 L dialysis buffer (50 mM Tris-Cl pH 8, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT) at 4°C for 2 hours. After dialysis the fractions were pooled into C14 tubes and spun at 18,000 rpm for 20 mins.

3.2.1.5 Ion-exchange Chromatography:

Dialysed McrBΔN was loaded onto an 8 ml MonoQ 10/100 GL column (GE Life Sciences) equilibrated with Buffer B50 (50 mM Tris-Cl pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT). 2 ml fractions were collected in 20 column volumes over a linear gradient of 0% to 50% buffer using B50 and B1000 (50 mM Tris-Cl pH 8, 1000 mM NaCl, 1 mM EDTA, 1 mM DTT). The pure fractions were pooled and concentrated using a 2 ml 10 kDa vivaspin2 concentrator (GE Life Sciences).

3.2.1.6 Size exclusion Chromatography:

Concentrated sample (500 µL) was washed with 5 ml buffer B100 (50 mM Tris-Cl pH 8, 100 mM NaCl and 1 mM DTT) to remove EDTA. The concentrated protein was then centrifuged at 21,000 g before loading onto 24 ml Superdex200 10/300 GL column (GE Life Sciences), equilibrated with buffer B100. Pure fractions were pooled and concentrated using a 2 ml 10 kDa Vivaspin2 concentrator (GE Life Sciences). The concentrated protein was stored in storage buffer at -80°C after checking the protein concentration using NanoDrop®.

3.2.2 Purification of McrC:

McrC was purified with a method very similar way to that of McrBΔN purification.

3.2.2.1 Growing of culture:

McrC, without a histidine tag, in pHISMcrC, was overexpressed in *E. coli* BL21 (AI) (Invitrogen®) cells. The cultures were grown in 2 L LB media containing 100 µg/ml ampicillin in an incubator-shaker at 37°C until OD reached 0.6 at 600 nm. The temperature of incubator-shaker was then reduced to 18°C and cultures were induced with 0.06% w/v L-Arabinose. The cultures were grown further overnight (15-16 hours) at 18°C. Cells were pelleted by centrifugation at 4°C and 3,315 g for 15 minutes.

3.2.2.2 Resuspension and ultracentrifugation:

The pellet was resuspended in 50 ml lysis buffer and lysed by sonication at 4°C. The cell lysate was then clarified by ultracentrifugation at 4°C and 159,200 g for 40 minutes.

3.2.2.3 Affinity Chromatography:

The clarified supernatant was loaded onto a 5 ml Ni-NTA column (GE Life Sciences) equilibrated with Buffer A. The protein was eluted using Buffer A and Buffer B by a step gradient from 5% to 100%.

3.2.2.4 Dialysis:

The purest of the Ni-NTA fractions were dialyzed against 2 L dialysis buffer at room temperature for 45 minutes. After 45 minutes precipitates were observed. So, the fractions were pooled into C14 tubes and centrifuged at 18,000 rpm for 20 mins to sediment the precipitate.

3.2.2.5 Ion-Exchange Chromatography:

Dialysed protein was loaded onto an 8 ml MonoS 10/100 GL column (GE Life Sciences) equilibrated with Buffer B50. 2 ml fractions were collected in 20 column volumes over a linear gradient of 0% to 50% buffer using B50 and B1000.

3.2.3 Nucleotide Exchange Assay:

Determination of nucleotide exchange by only McrBΔN and complex of McrBΔN and McrC were performed using N'-methylanthraniloyl (mant) labelled guanine nucleotide analogue (mantGDP).

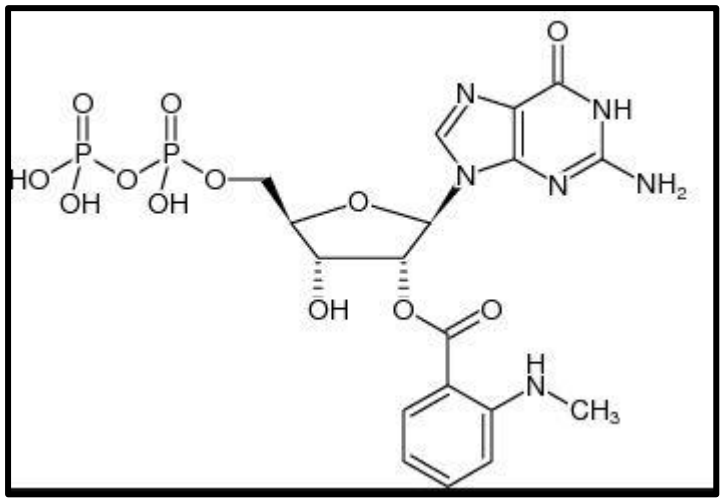


Figure: 3.2.1: Fluorescent 2′- (or 3′-) O-(Nmethylanthraniloyl) (MANT) nucleotide analogues

The maximal excitation and emission wavelength of mantGDP is found to be 360 and 440 nm respectively. All kinetic measurements were performed at 25 °C in 1.5 ml cuvette in a buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM KCL, 5 mM MgCl2 and 1 mM DTT. The emission spectra of mant-nucleotide were recorded from 390 to 490 nm and the fluorescence signal (S1/R1) was measured at 440 nm with a slit width of 2 nm using FluoroMax®-4 (HORIBA Scientific) spectrofluorometer. For each reaction the mant nucleotide (mant GDP) concentration was set to be 400 nM which was displaced using unlabelled GDP/GTP concentration varying from 0.5uM to 240uM. Protein (McrBΔN and McrC) concentrations were determined by UV absorbance at 280nm and the ratio of McrBΔN to McrC was kept 4:1 in all the reactions. For kinetic study purposes, mantGDP was incubated in 1X buffer and the basal fluorescence was recorded for 300 s followed by addition of protein (4uM McrBΔN/ 4uM McrBΔN +1uM McrC). Thereafter, unlabelled nucleotide was added at 800 s for displacement of bound mant-nucleotide and the signal was recorded till 1500 s.

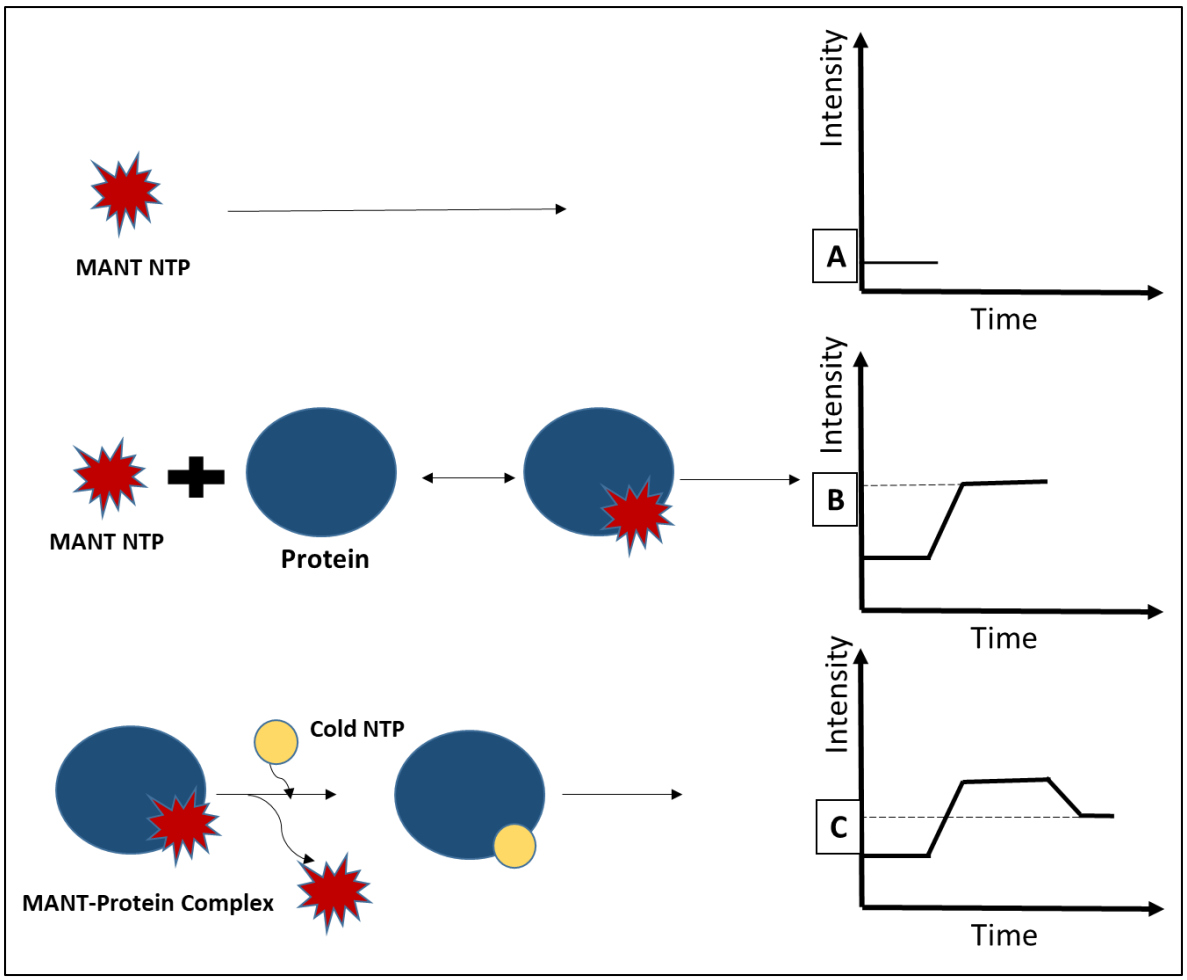


Figure 3.2.2: Schematic of nucleotide exchange assay

Collective data from three independent experiments were plotted using Prism® version 5.0. The percentage of bound mantGDP was calculated using the following formula:

$$\% \textbf{ Bound mantGDP} = \left[\frac{\mathbf{C - A}}{\mathbf{B - A}}\right] * \mathbf{100}$$

Where A, B and C are Y intercept values obtained from linear fits for basal fluorescence intensity of mantGDP, shifted fluorescence intensity after addition of protein and shifted fluorescence intensity after addition of cold nucleotide (GTP) respectively (Figure 3.1.2).

3.2.4: NADH-coupled GTPase Assay:

As we were interested in hydrolysis stimulation of McrB by McrC, we also wanted to have a look on the steady-state GTPase activity of McrBΔNC complex via NADH-coupled GTPase assay.

The GTPase activity was qualitatively measured by monitoring release of phosphate ion (Pi) using NADH coupled assay. A master mix containing NADH, PK/LDH, RB 20X (20 mM PEP, 100 mM MgCl2), protein (McrBΔNC) in hydrolysis buffer (10 mM Tris-Cl (pH 8), 50 mM KCl, 5 mM MgCl2, 1 mM DTT) was made and incubated. Protein (McrBΔN and McrC) concentrations were determined by UV absorbance at 280nm and the ratio of McrBΔN to McrC was kept 4:1 in all the reactions. Varying concentration of GTP (0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 4, 8, 12, 16, 24, 32, 48, 96, 192, 384 and 768 µM) was added to different wells on a 96 well flat bottom corning plate and reaction was shaken to remove air bubbles from the well for 30 seconds before measurements were taken. Absorbance at 340 nm was recorded every 10 seconds for 3600 seconds at 25˚C using Varioskan® plate reader. GDP standards was performed with each set of experiment.

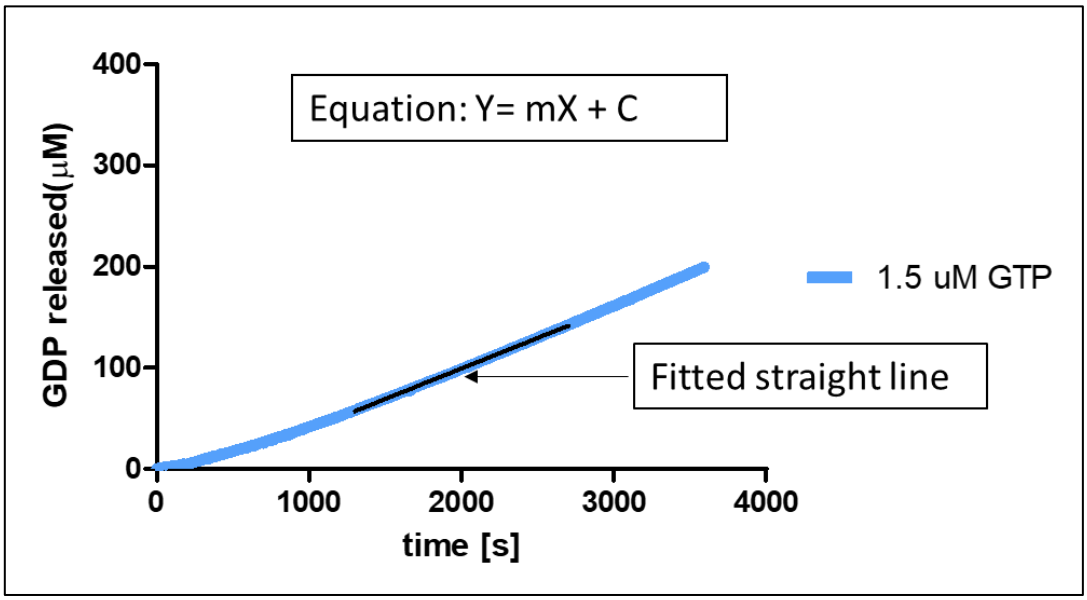


Figure 3.2.3: Calculation of rate of GTP hydrolysed at a particular GTP concentration (In this case, at 1.5µM GTP)

The concentration of GTP hydrolysed was calculated at each time interval using a line equation Y=mX + C (where Y = absorbance, m=slope, X= concentration of GDP produced or GTP hydrolysed and C= intercept on Y axis) obtained from standard plot with different GTP concentrations (Figure 3.1.3). Rates at each GTP concentration was calculated using straight line fit. Graph plotting and rate calculation were done using Prism® version 5.0.

3.2.5 GTPase Activating Assay:

Each reaction mixture contained McrBΔN, McrC in cleavage buffer [10 mM Tris (pH-8),50 mM KCl,5mM MgCl2 1 mM DTT]. Addition of γ^{32}GTP started the reaction. The protein: nucleotide ratio was kept as 10:1. Reaction was incubated at 37°C for definite time. γ^{32} P_i from organic phosphate was separated by charcoal precipitation assay. Aliquots of different reactions was spotted directly onto a Whatman filter paper. Amount of radiolabeled γ^{32} P_i was quantified using a phosphor imager.

3.2.5.1 Washing Activated Charcoal:

The activated charcoal used in this assay for separation of γ^{32} P_i from organic phosphate, was firstly given an acid wash. 200µl of 1:1 mixture of HCl and MQ was added to 10mg of activated charcoal. The mixture was mixed well and centrifuged at 12000g for 5 mins at 4°C. The supernatant was discarded. Then similarly a MQ wash was given by adding 200µl of MQ to resuspend the charcoal and centrifuged. This process was repeated for three times.

The components of the reaction mixture are given below in table 3.1:

	GTP (mix 1)	**McrBΔN (mix 2)**	**McrBΔN (mix 3)**
GTP (not to be premixed)	100nM	100nM	100nM
Protein	----	100µM McrBΔN	100µM McrBΔN+25 µM McrC
10X cleavage buffer	5µL	5µL	5µL

The volume in each case is made up to 50 µL by adding MQ.

3.2.5.2 Mixture for 0th Time Point:

4.5 µL of reaction mix (without GTP) was added to 2 µL 25mM EDTA containing vial and then 0.5 µL of 500nM of GTP-γ^{32} is added to it. To this 25 µL of 5% activated charcoal was added and mixed properly.

3.2.5.3 Mixture for Later Time Points:

4.5 µL of GTP-γ^{32} was added to the reaction mix to start the reaction. At each time point (i.e., 20s, 40s, 60s, 2 mins, 4 mins, 8 mins, 16 mins and 30 mins), 5 µL of reaction mix was taken and added to 2 µL 25mM EDTA containing vial. To this 25 µL of 5% activated charcoal was added and mixed properly. Then the mix was centrifuged at 12000g for 5 mins at 4°C and 20 µL of supernatant was taken. This supernatant was again centrifuged and 10 µL of supernatant was transferred to a new vial. 8 µL of this supernatant was then loaded on Whatman paper in batches of 2 µL.

3.3 Results and Discussion:

3.3.1 Purification of Proteins:

Both McrBΔN and McrC are purified as previously described in 3.2.2 and 3.2.3 respectively. The purified fractions of each purification are showed in 12% SDS-PAGE gels separately (Figure 3.3.1).

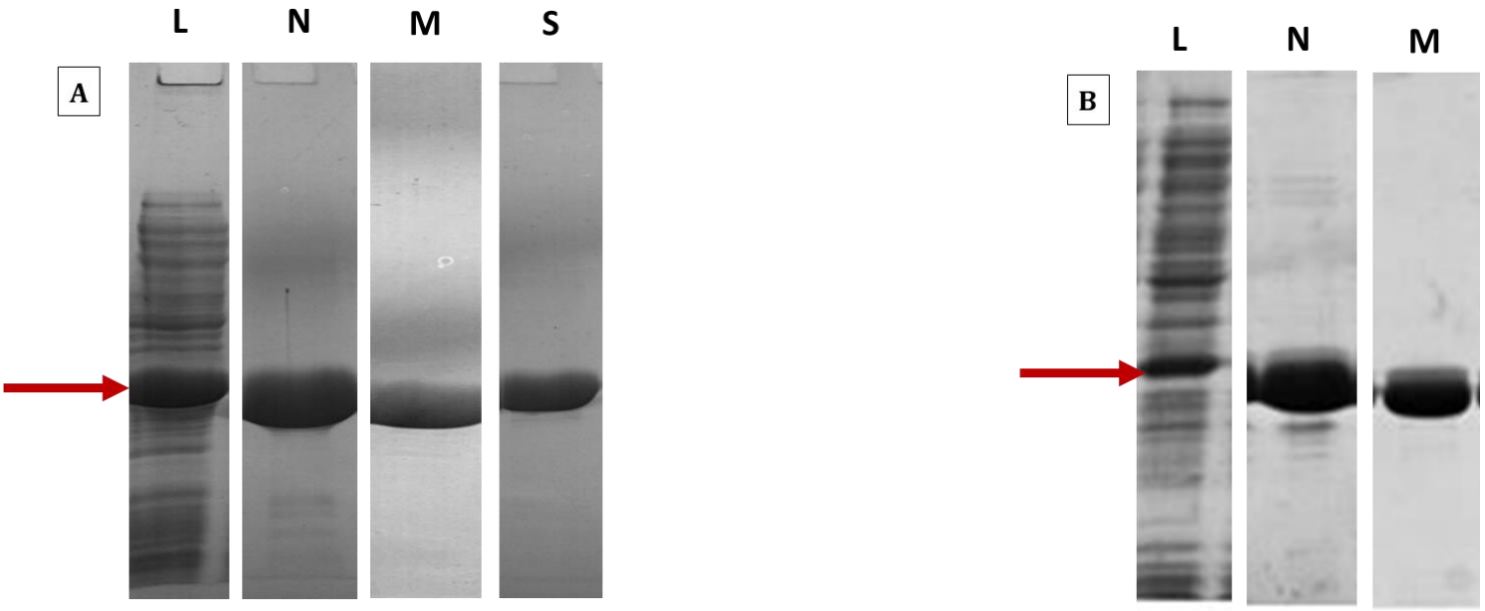


Figure 3.3.1: [A] Purification gel fractions of McrBΔN; [B] Purification gel fractions of McrC. The red arrows indicate the region on the SDS PAGE gel where the mutant protein was expected to overexpress. [Key: L= Lysate, N= Ni-NTA fraction, M= MonoQ/ MonoS fraction, S= Superdex fraction].

3.3.2 Nucleotide Exchange Assay:

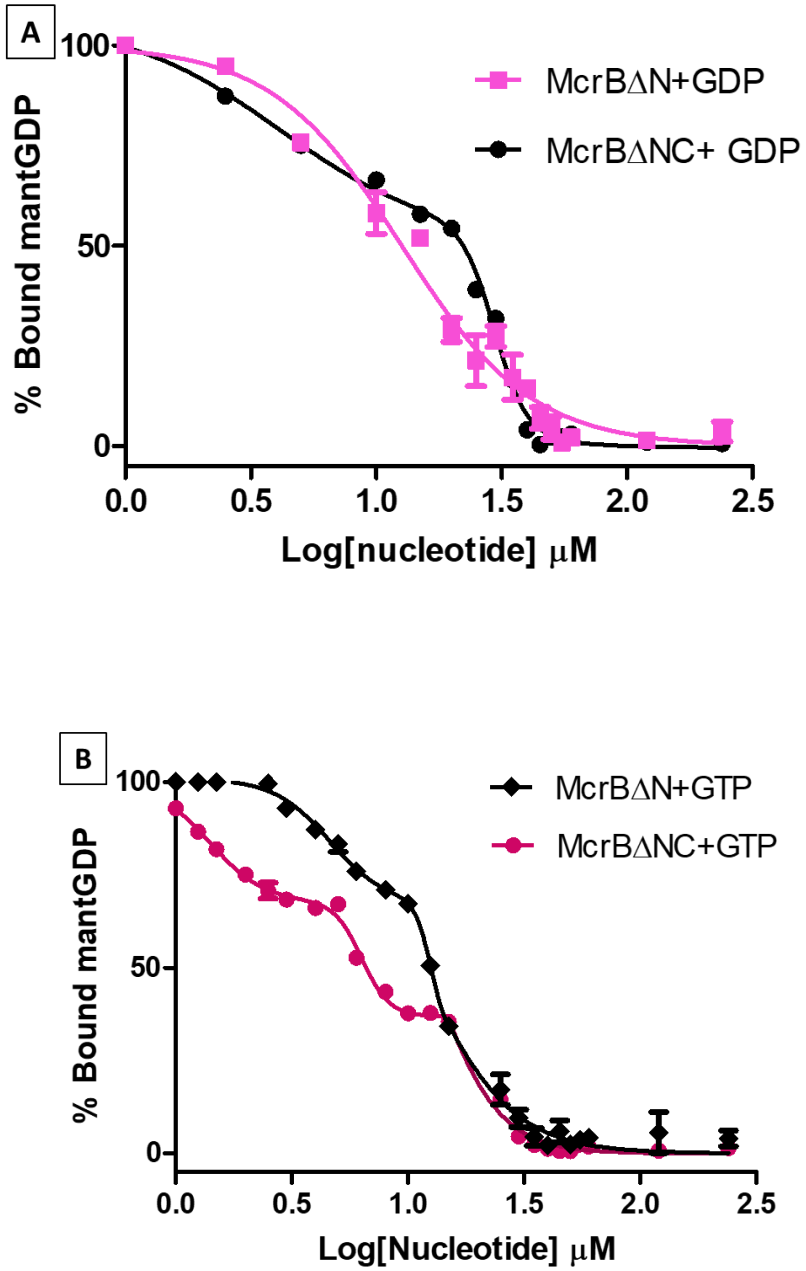


Figure 3.3.2: [A} Percentage of bound mantGDP is fitted to a biphasic equation and plotted against the log of concentration of unlabelled nucleotide for both McrBΔN and McrBΔNC (Where mantGDP is replaced by unlabelled GDP); **[B] Percentage of bound mantGDP is fitted to a biphasic equation and plotted against the log of concentration of unlabelled nucleotide for both McrBΔN and McrBΔNC** (Where mantGDP is replaced by unlabelled GTP) using Prism® version 5.0.

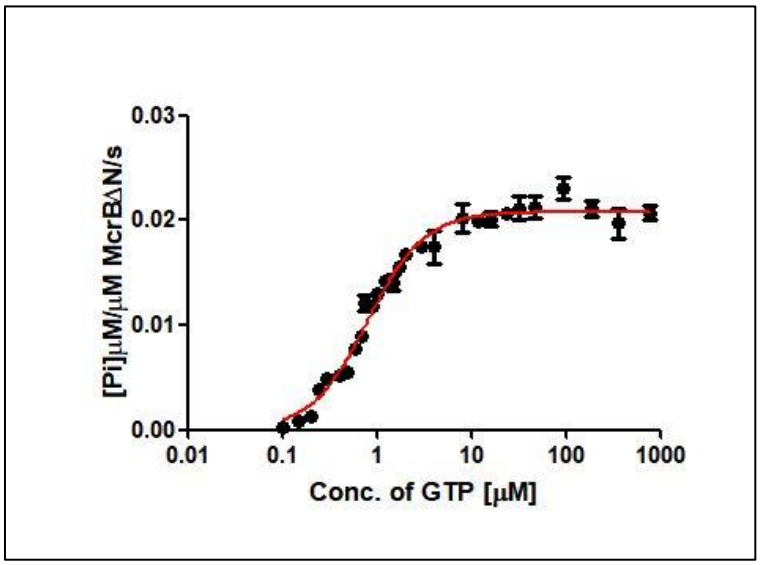
We have done a very preliminary analysis which gives us apparent Kd values at high and low affinity binding sites for both McrBΔN and McrBΔNC which are as shown in the table below:

	Apparent K_d (high affinity binding sites) [µMolar]	**Apparent Kd (low affinity binding sites) [µMolar]**
McrBΔN	0.6±0.031; 1.1±0.010	12.6±0.024
McrBΔNC	0.1±0.056; 0.8±0.012	12.2±0.009

In case of G-proteins the binding to GDP is stronger than to GTP due to which they require a GEF (Guanine nucleotide Exchange Factor) to bind to GTP. But earlier studies showed that, McrB has 50-fold higher affinity for GTP than GDP and hence it has been proposed that it does not require a GEF for its activity[3]. However, our preliminary data indicates that McrC assists in nucleotide exchange at higher binding affinity sites.

3.3.3 NADH-Coupled GTPase Assay:

As we were interested in GTP hydrolysis stimulation of McrB by McrC, we also wanted to have a look on the steady-state GTPase activity of McrBΔNC complex via NADH-coupled GTPase assay.



Equation:
Y= Vmax*X^h/(Kprime + X^h)

Kinetic Parameters:

V_{max} = 0.02078 µM/sec
h =1.460
K_{prime} = 0.7191

Figure 3.3.3: Steady-state GTPase activity of McrBC monitored by NADH coupled GTPase assay

The graph was plotted with rates on Y-axis and GTP concentration on X-axis and fitted to Allosteric-Sigmoidal equation to calculate the kinetics parameters i.e., V_{max}, k_{prime} and h (Figure: 3.3.3) using Prism® version 5.0.

3.3.4 GTPase Activating Assay:

Single turnover reaction with radiolabeled nucleotide probe ($\gamma^{32}P_i$) is a good strategy to show GTPase activation. In this assay, aliquots of different reactions are spotted directly onto a Whatman filter paper as shown in Figure 3.2.4.

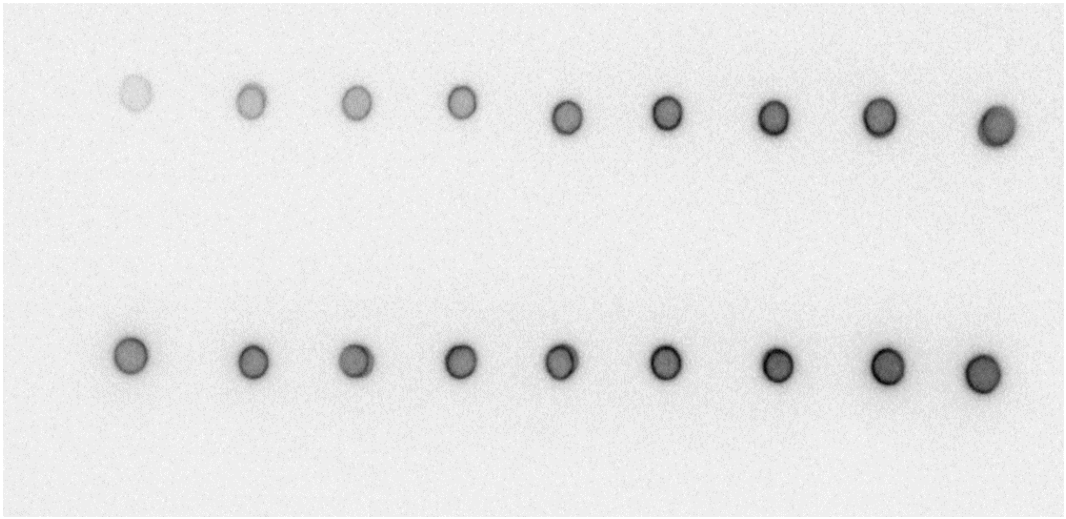


Figure 3.3.4: Different reactions are loaded into Whatman paper and imaged via a phosphor imager

The mean intensity per unit area (which indicates the amount of $\gamma^{32}P_i$) is subtracted from the background noise and plotted against time (Figure 3.2.5).

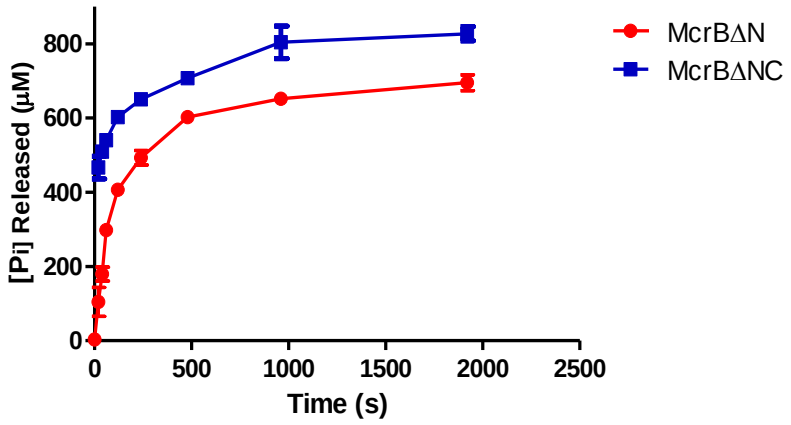


Figure 3.3.5: Amount of $\gamma 32P_i$ (µM) released is plotted against time (s) in X-axis

From the plot it has been observed that McrC assists in activating GTPase activity of McrB.

3.4 Discussion:

McrBC is a unique AAA+ protein, which hydrolyses GTP and unlike most other DNA binding AAA+ motors, the stimulation of the McrB GTPase has been shown to be dependent on the partner endonuclease McrC rather than substrate DNA. In presence of McrC the GTP hydrolysis rate of McrB increases almost by 30 folds. So, it was very intriguing to dissect out how McrC stimulates the GTPase activity of McrB i.e., whether it stimulates the catalysis of McrB by activating GTPase (acting as a GAP) or help in exchange of nucleotides by acting as a nucleotide exchange factor (acting as a GEF) or a combined effect of all two possibilities.

The biochemical assays performed by our lab indicates that McrC assists in nucleotide exchange at higher binding affinity sites (Figure:3.3.2 [B]) which is similar to a GEF action to its GTPase. Also, our results showed that McrC stimulates the intrinsic GTPase activity of McrB by acting as a GAP which further results in GTPase stimulation of McrB (Figure 3.3.5). Still, all our experiments were qualitative in nature which can only give an indication of McrC acting as both GEF and GAP to stimulate the GTPase activity of McrB. Further quantitative methods will help us to understand more about interaction between the two subunits of this macromolecular complex.

References:

1. Bos, J. L., Rehmann, H. & Wittinghofer, A. GEFs and GAPs: Critical Elements in the Control of Small G Proteins (DOI:10.1016/j.cell.2007.05.018). *Cell* **130**, 385 (2007).
2. Vetter, I. R. & Wittinghofer, A. The guanine nucleotide-binding switch in three dimensions. *Science* vol. 294 1299–1304 (2001).
3. Pieper, U., Brinkmann, T., Krüger, T., Noyer-Weidner, M. & Pingoud, A. Characterization of the interaction between the restriction endonuclease McrBC from E. coli and its cofactor GTP. *J. Mol. Biol.* **272**, 190–199 (1997).
4. Panne, D., Raleigh, E. A. & Bickle, T. A. The McrBC endonuclease translocates DNA in a reaction dependent on GTP hydrolysis. *J. Mol. Biol.* **290**, 49–60 (1999).