Mutational studies to characterize the interaction between the GTPase McrB and the endonuclease McrC

A thesis submitted in the partial fulfillment of the requirements of BS-MS dual degree program

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Certificate

This is to certify that this dissertation entitled "**Mutational studies to characterize the interaction between the GTPase McrB and the endonuclease McrC**" towards the partial fulfilment of the BSMS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Basila M A at IISER Pune under the supervision of Dr. Saikrishnan Kayarat, Associate professor, IISER Pune during the academic year 2018-19.

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Declaration

I hereby declare that the matter embodied in the report entitled "**Mutational studies to characterize the interaction between the GTPase McrB and the endonuclease McrC**" are the results of the work carried out by me at the Department of Biology, IISER Pune, under the supervision of Dr. Saikrishnan Kayarat and the same has not been submitted elsewhere for any other degree.

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Abstract

Modification dependent restriction (MDR) enzymes evolved as a defense mechanism in bacteria against the attack of phages with modified genome. McrBC is a type IV MDR which binds to a R^mC and cleaves DNA having two such sites using energy derived from GTP hydrolysis. The functional complex of McrBC is a tetradecamer formed by two hexamers of McrB bridged together by a dimer of McrC. McrB, the GTPase, on its own has a very poor GTPase activity, while McrC, the endonuclease, on its own does not cleave DNA. The GTPase activity of McrB and the endonuclease activity of McrC is stimulated when they together form a complex in presence of GTP. The interaction between McrB and McrC is crucial for the functioning of the enzyme, however the mode of the interaction between the two proteins is unknown.

A series of McrC deletion were generated based on the secondary structure prediction from Phyre[2]. The mutants hence obtained were subjected to biochemical characterization using analytical size exclusion chromatography, GTP hydrolysis and nucleolytic cleavage. We identified that the first 192 residues in McrC form an independent domain that can interact with McrB and is sufficient to stimulate GTP hydrolysis. The residues in the region 60-100 which forms an extended loop was found to be crucial for the formation of the complex as well as its activities.

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Acknowledgements

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INTRODUCTION

General Introduction

Bacteria live under a constant threat of attack from bacteriophages as the relative abundance of the virus is ten times more than that of its prey (Bergh et al., 1989). Faced with the tough environment, bacteria evolved several defense mechanisms to cope up with the infection by phages (Labrie et al., 2010).The restriction-modification systems constitute one of the major line of defense in the arsenal of the bacteria. Restriction-modification enzymes have two components, carrying out one of the two functions i.e., restriction or modification. The restriction component recognizes the DNA sequence and cleaves it. The modification component adds certain chemical modifications to the recognition sequence present in the self genome to prevent suicidal effect of the enzyme. In short the bacterial genome is protected by the modification subunit, while a naked foreign genome gets picked up by restriction component and gets cleaved (Boyer, 1971).

The constant interaction between the host and the invader led to the evolution of modified genome in the phage. The classical restriction modification system fails in providing defense against this new class of attackers (Loenen and Raleigh, 2014). Hence bacteria evolved modification-dependent restriction enzymes, which specifically recognizes and cleaves modified genome (Fig 1.1). The various restriction systems present in bacteria can be classified into Type I, Type II, Type III and Type IV based on their NTP utilization, cleavage pattern and mode of action.

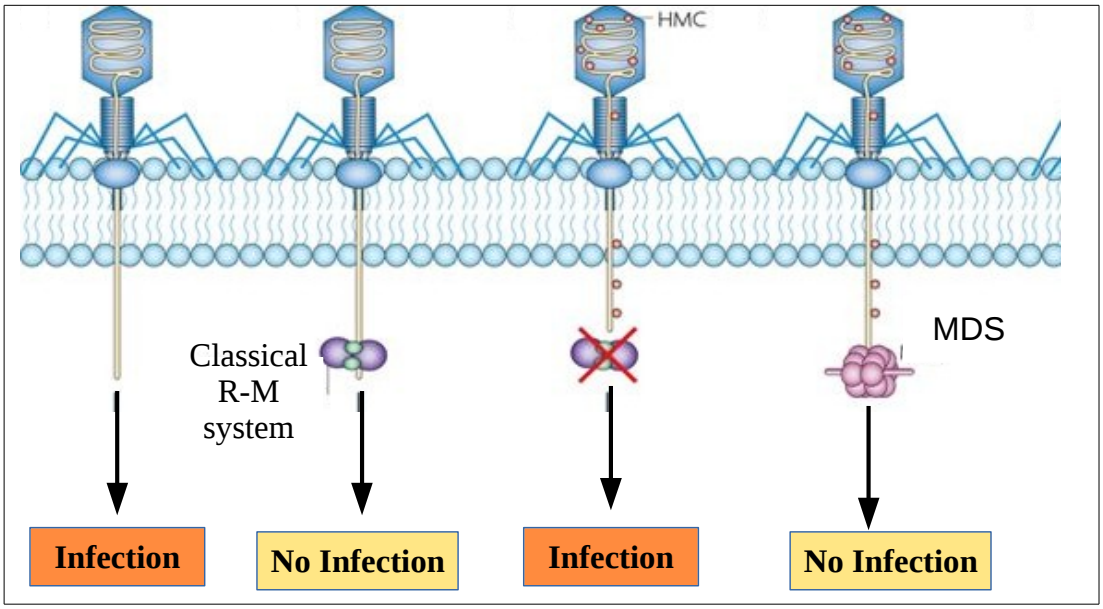


Fig 1.1 RM system vs MDR system Comparison between restriction-modification systems and modification-dependent restriction systems.

Methylated Cytosine Recognition B and C

Luria and Human found that the virulency of a bacteriophage is affected by the genotype of the bacterial host. Phages grown in certain mutant strains of bacteria transiently lost the ability to infect other strains. They hypothesized that the host imparts certain non-heritable modifications on the phage, which affects their ability for further infections (Luria and Human, 1952). This was the first restriction phenomena ever reported and was effected by the activity of McrBC. Revel et al., proposed that phages with non-glucosylated 5-hydroxymethylcytosine gets cleaved by rglA and rglB restriction systems (Revel, 1967). Years later, McrA and McrB systems were discovered which restricts modified genome containing 5-hydroxymethycytosine. The genes regulating the differential restriction of methylated genome was later mapped to be coincident with that of *rglA* and *rglB*. It was established that the restriction system indeed recognizes modified cytosine of the genome and hence the name modified cytosine restriction (Mcr) (Raleigh, 1992), (Noyer-Weidner et al., 1986).

McrBC operon is located in the Immigration control region (ICR) of the *E. coli* K12 genome. The ICR is a 14 kbp long genomic island enriched with multiple restriction systems – namely *eco K, mcrBC and mrr* (Raleigh et al., 1989). The *mcrBC* operon has three distinct reading frames encoding for McrB, $McrB_s$ and McrC. The restriction of methylated cytosine requires the combined activity of both McrB and McrC. The termination codon of *mcrB* and the initiation codon of *mcrC* overlap by one nucleotide, which could lead to translation coupling of the two polypeptides. $McrB_s$ is translated from an ORF within that of *mcrB*. The polypeptide which lacks the N-terminal domain of McrB has been shown to play a regulatory role in the activity of the restriction enzyme both in vivo and in vitro. The relative amount of the translated proteins McrB : McrC : $McrB_s$ was found to be 3:1:3 by maxicell analysis (Ross et al., 1989)

Components of the McrBC complex

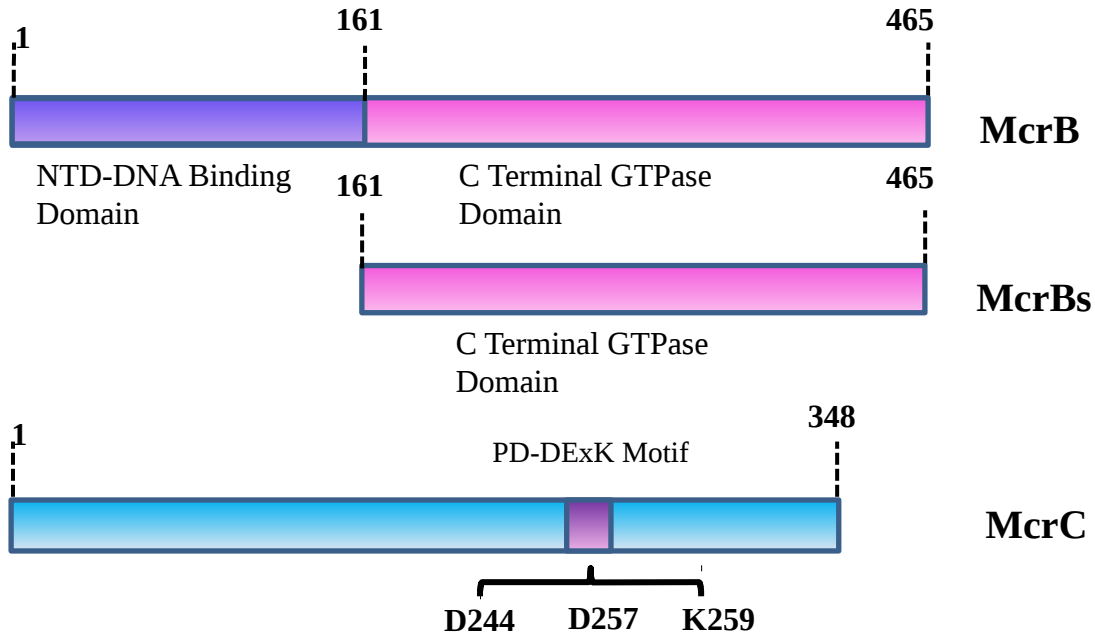


Figure 1.2 Components of McrBC system

McrB

McrB is a 54 kDa polypeptide with distinct DNA binding N-erminal domain and nucleotide binding and hydrolyzing C-terminal domain (Gast et al., 1997). McrB has a basal GTPase activity which is stimulated about 30 fold in the presence of McrC. The domain boundaries of McrB has been assigned through an extensive deletion mutagenesis study guided by secondary structure prediction. Several truncations of McrB was 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 (Pieper et al., 1999a). 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 (Pieper et al., 1997). McrB 1-122, McrB 1-137, McrB 189-465 yielded insoluble protein. The GTP hydrolysis behavior as well as DNA cleavage activity was tested for the remaining constructs, which led to the conclusion that the protein has two independent functional domains with distinct roles (Pieper et al., 1999a).

$McrB_s$

$McrB_s$ 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 (Ross et al., 1989). $McrB_s$ 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 McrBs 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 (Panne et al., 1998).

McrC

McrC is a 39 kDa polypeptide which stimulates the intrinsic GTPase activity of McrB as well as harbors the nucleolytic residues (Ross et al., 1989). For a long time the only function associated with McrC was the formation of higher-order oligomers in the presence of McrB and DNA. Sequence alignment with six homologous proteins combined with mutational analysis showed that McrC harbors the nucleolytic center $TD^{244}...D^{257}AK$. This is a variant of the well conserved PD...D/EXK motif of several nucleases including Type II restriction enzymes. The authors also studied the effect of these catalytic residues on the interaction between McrB and McrC. Point mutations in the catalytic center 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. It has been suggested that the GTPase mutants are defective in cleavage and not vice versa (Pieper and Pingoud, 2002).

Oligomeric status of McrBC

The functional form of McrBC is a tetradecameric complex consisting of two McrB hexamers bridged together by a dimer of McrC. 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+ 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 (Nirwan et al., 2019).

GTP binding and hydrolysis

McrBC is unique among AAA+ protein as it hydrolyzes GTP rather than ATP. The GTP binding and hydrolysis center of the complex is the polypeptide McrB. McrB has a basal GTPase activity which is triggered almost 30 fold in the presence of McrC (Pieper et al., 1997). 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 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 (Pieper et al., 1999b).

Nucleolytic activity of McrBC complex

The MDR enzyme McrBC cleaves DNA containing at least two methylated cytosine separated by a distance not less than 30 bp and not beyond 3000 bp (Stewart and Raleigh, 1998)(Krüger et al., 1995). Successful cleavage takes place in presence of GTP and Mg^{2+} 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 (Panne et al., 1999).

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. In spite of significant progress made in the biochemical characterization of the enzyme complex, molecular details of its function still remain unknown. One of the most interesting open questions is the region in McrC facilitating the interaction with McrB and stimulating its GTPase activity.

The main objective of this thesis is to dissect out the regions of McrC that are in contact with McrB through deletion mutagenesis. I will create a series of deletion mutants of McrC based on the secondary structure prediction and study the mutant enzyme complex with appropriate assays. As a part of this thesis, I have cloned and purified 4 deletion constructs of McrC and studied their oligomerization, GTPase activity and cleavage pattern. I have also included my attempts at cloning multiple N-terminal constructs of McrC, which did not express in *E. coli.*

Materials and Methods

2.1 Cloning

All *mcrC* deletion constructs were generated from pHISMcrC (a plasmid containing the wild type protein) through restriction free (RF) cloning or restriction ligation cloning. The truncation sites were decided based on secondary structure predictions from the software Phyre[2]. The software uses PSIPRED to predict the secondary structure of the protein sequence (Kelley et al., 2015).

The list of primers used for each clone is listed below in table 2.1:

Construct	Forward primer(5' -> 3')	Reverse primer(3' -> 5')
$McrC_{1-192}$	TAATACGACTCACTATAGGG	ATGATGATGATGATGGGATCCTCTTT CAAAATCATAGAAACGG
$McrC_{1-179}$	TAATACGACTCACTATAGGG	ATGATGATGATGATGATGGGATCCTG GAATAGAATTATTGAC
$McrC_{1-183}$	TAATACGACTCACTATAGGG	ATGATGATGATGATGATGGGATCCTT TGTTTTGACCTGG
$McrC_{20-192}$	TAATACGACTCACTATAGGG	ATGATGATGATGATGGGATCCTCTTT CAAAATCATAGAAACGG
$McrC_{20-179}$	AAGAAGGAGATATACATATGTATT TACAGGAAATTAAGCA	ATGATGATGATGATGATGGGATCCTG GAATAGAATTATTGAC
GST-$McrC_{1-192}$	TAATACGACTCACTATAGGG	ATGATGATGATGATGGGATCCTCTTT CAAAATCATAGAAACGG
GST-$McrC_{20-192}$	AAGAAGGAGATATACATATGTATT TACAGGAAATTAAGCA	ATGATGATGATGATGGGATCCTCTTT CAAAATCATAGAAACGG
$McrC_{187-348}$	GAAGGAGATATACATATGGATTTT GAAAGAAACGAAAAAGAG	GATGATGGGATCCTTTGAGATATTCA TCG
McrC L1	CGCCGAGGGCTTGAGGGCGGC AATGAAGACACGCTGGC	GATGATGGGATCCTTTGAGATATTCA TCG
McrC L2	GATTACAATCCTAACGGAGGAGA	GATGATGGGATCCTTTGAGATATTCA

	TATGCTTAATGAAGACAC	TCG
McrC L3	ACCGAGATCATTCCTGGCGGGA AAACCGTCAGT	GATGATGGGATCCTTTGAGATATTCA TCG
McrC L4	CCTGGCATCAAAGGGGGCTTCC ATCTTAAT	GATGATGGGATCCTTTGAGATATTCA TCG

2.1.1 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(Ent and Lowe, 2006). 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:

10X Pfu Buffer	5 µL
Template	100 ng
Forward primer	0.4 µM
Reverse primer	0.4 µM
dNTPs(2.5 mM each)	2 µL(100 µM each)
Pfu Polymerase	0.8 µL

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

First PCR cycle:

Initial denaturation : 95^0C for 2’

Denaturation : 95^0C for 30’

Annealing : 55^0C for 30’

Extension : 72^0C at the rate of 1 kb/min

} 35 cycles

Final extension : 72^0C 10’

The reaction was stored at 4^0C once completed.

Second PCR cycle:

Components	Test	Control
10X Pfu Buffer	5 µL	5 µL
Template	100 ng	100 ng
Megaprimer	600 ng	0
dNTPs(2.5 mM each)	2 µL(100 µM each)	2 µL(100 µM each)
Pfu Polymerase	0.8 µL	0.8 µL

The final volume was made up to 50 µL using MilliQ. The PCR cycle was similar to the one used for the first PCR reaction. 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 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 BioRad Gene Pulser Xcell™. The cells were revived by incubating them at 37^0C 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 37^0C for 8-12 hours.

2.1.4 Clone check

Table 2.2 lists the solutions used 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

A single colony from plates incubated overnight was used to inoculate 5 mL of LB. The culture was grown at 37^{o}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 below.

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^{0}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^{0}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 to Sigma-Aldrich.

2.1.5 Culturing and expression of protein

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 37^0C 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 37^0C in an incubator-shaker was induced with 0.02% w/v of L-arabinose at optical density (OD) at 600 nm = 0.6. Post induction the culture was left to grow at 18^0C overnight. The 1 L culture was pelleted down by centrifugation at 5000 rpm and 4^0C.

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 4^0C 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^{0}C 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 (BioRad), post which the protein bands were visualized by coomassie blue staining method.

2.2 Protein purification

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

Table 2.2 Buffers used in purification

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

2.2.1 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^{0}C and 37000 rpm for 45 minutes in an Optima™ XE Ultracentrifuge (Beckman Coulter). The supernatant after the centrifugation was used as load for the subsequent purification steps.

2.2.2 Affinity Chromatography

The clarified supernatant after ultracentrifugation was loaded onto a 5 mL HisTrap 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 raging 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 (Satarius) 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 4^0C. 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 -80^0C until further use.

2.3 Size Exclusion Chromatography

The nature of the complex formed by wild type McrB with the various McrC mutants were studied by performing analytical size exclusion chromatography on a 24 ml Superose 6 10/300 GL SEC column (GE Life Sciences) in the presence of GTP. The size exclusion column has a matrix packed with fine porous beads of Sepharose. Macromolecules are separated on the basis of their molecular mass and shape on size exclusion column. Large macromolecules which do not get trapped into small pores in the matrix traverse a shorter path on the column as opposed to small macromolecules. The column was equilibrated with B_{250} containing 5 mM $MgCl_2$ and 0.1 mM GTP (SRL). McrB and McrC were mixed in the ratio 4:1 molar ratio in buffer

B_{250} containing 2.5 mM GTP (Jena Bioscience) and 5 mM $MgCl_2$ in a solution of total volume 500 µL. The hetero-protein mix was spun at 15^{0}C in a tabletop centrifuge (Eppendorf) for 15 minutes before injection. The load was injected into the column manually through a 1 mL sample application loop. Samples from eluted protein fractions were loaded on SDS PAGE gel to confirm the identity of proteins present in the complex.

2.4 Substrate for cleavage assay

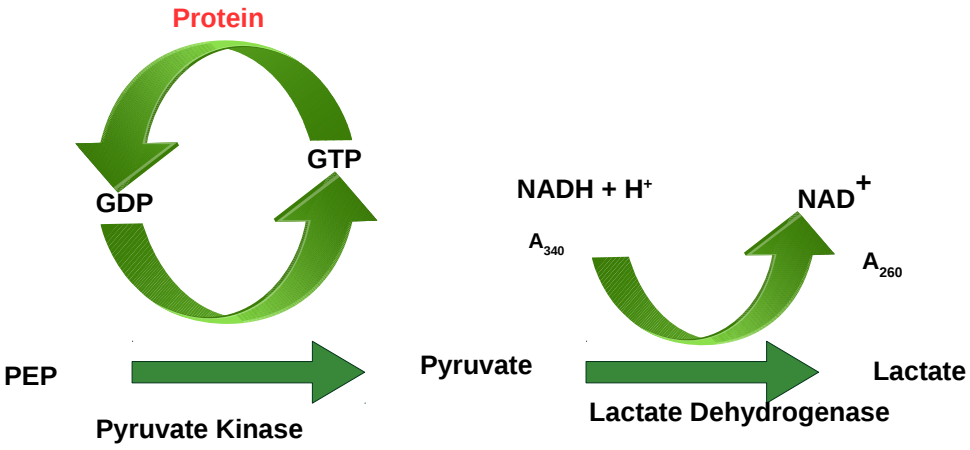
The *mcrB*$_s$ gene with 5-methylcytosine (d^{m}CTP) was used as the substrate for the cleavage assay. The substrate was PCR amplified using the same cycle as described in section 2.1.1 except that d^{m}CTP was used instead of dCTP. The dNTP mix contained 2.5 mM each of dATP, dTTP, dGTP and d^{m}CTP.

Cleavage assay

McrB and McrC (wild type/ mutants) mixed in a molar ratio of 4:1 were incubated with 75 nM 5- ^{m}C McrB$_s$ along with 1 mM GTP (Jena Biosciences) in TMDK buffer (10mM Tris-Cl pH 8, 50mM KCl, 5mM MgCl2, 1mM DTT) for 1 hour at 37^{0}C. The control reaction for these assays to check for any other nuclease contamination was the incubation of the respective mutant McrCs and all the other components, including McrB, but without GTP. At the end of 1 hour, 2 µL 2X STES buffer [40% Sucrose, 0.2 M Tris-Cl pH (7.5), 40 mM EDTA, 1% SDS] was added to stop the reaction. The reaction was incubated at 65^{0}C for 10 minutes before loading on a 0.8% agarose gel. Samples were migrated on gel at 100V for approximately 40 minutes post which it was imaged using E-Gel® Imager (Life Technologies). With every mutant cleavage assay the cleavage by McrBC wild type complex was performed as a control. In addition to that an only DNA control was also used.

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 pyruvate kinase and lactate dehydrogenase. McrBC hydrolyzes 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 (Ingerman and Nunnari, 2006) The reaction taking place can be summarised as follows:



The reaction mix consists of McrB and McrC (wild type/ mutants) mixed in a ratio 4:1, 600 µM NADH, 1mM phosphoenol 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.

3. Results & Discussion

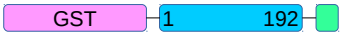
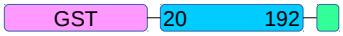
3.1 Secondary Structure prediction of McrC

The strategy we followed in order to identify the regions important for the interaction between McrB and McrC was systematic deletion of certain parts of the latter. This was followed by biochemical characterization of the effect of deletion on the ability of mutant to complex with McrB and then study the activity of the complex formed. Choosing the truncation sites was challenging due to the lack of any structural information of McrC or its close homologues. Hence, the selection of regions for deletion was made based on the secondary structure of McrC that was predicted by the software PhyRe[2] (Fig 3.1). Based on the predicted secondary structure McrC could be divided into two regions – an N-terminal region rich in α helices and a C-terminal region with a mix of α-helices and β-strand. The secondary structure of the C-terminal region appeared to best represent the nuclease catalytic domain, which based on the structures of other nucleases is expected to have an α-β-α fold. This conclusion is also consistent with the observation that the C-terminal region harbors the nuclease catalytic motifs.

The N-terminal region appeared to be a bundle of α-helices. However, we noted a long unstructured stretch of residues between residues 60 to 100 interspersed with three short β-strands and a helix. The placement of two of the strands suggested the possibility of this unstructured region forming a long two-stranded β-sheet. This picture was consistent with a low resolution structure of McrBC determined in the laboratory (Nirwan, 2018). We decided to characterize the importance of each of the regions by generating a series of deletion mutants as listed in table 3.1.

Table 3.1 List of all the deletion constructs

Class	Construct	Design
	$McrC_{1\text{-}192}$	
	$McrC_{1\text{-}179}$	
	$McrC_{1\text{-}183}$	

N-terminal constructs	$McrC_{20-192}$	
	$McrC_{20-179}$	
	GST-$McrC_{1-192}$	
	GST-$McrC_{20-192}$	
C-terminal construct	$McrC_{187-348}$	
Loop deletion constructs	McrC L1	
	McrC L2	
	McrC L3	
	McrC L4	

Key :  denotes GSHHHHHH ; GST (Glutathione-S-transferase)

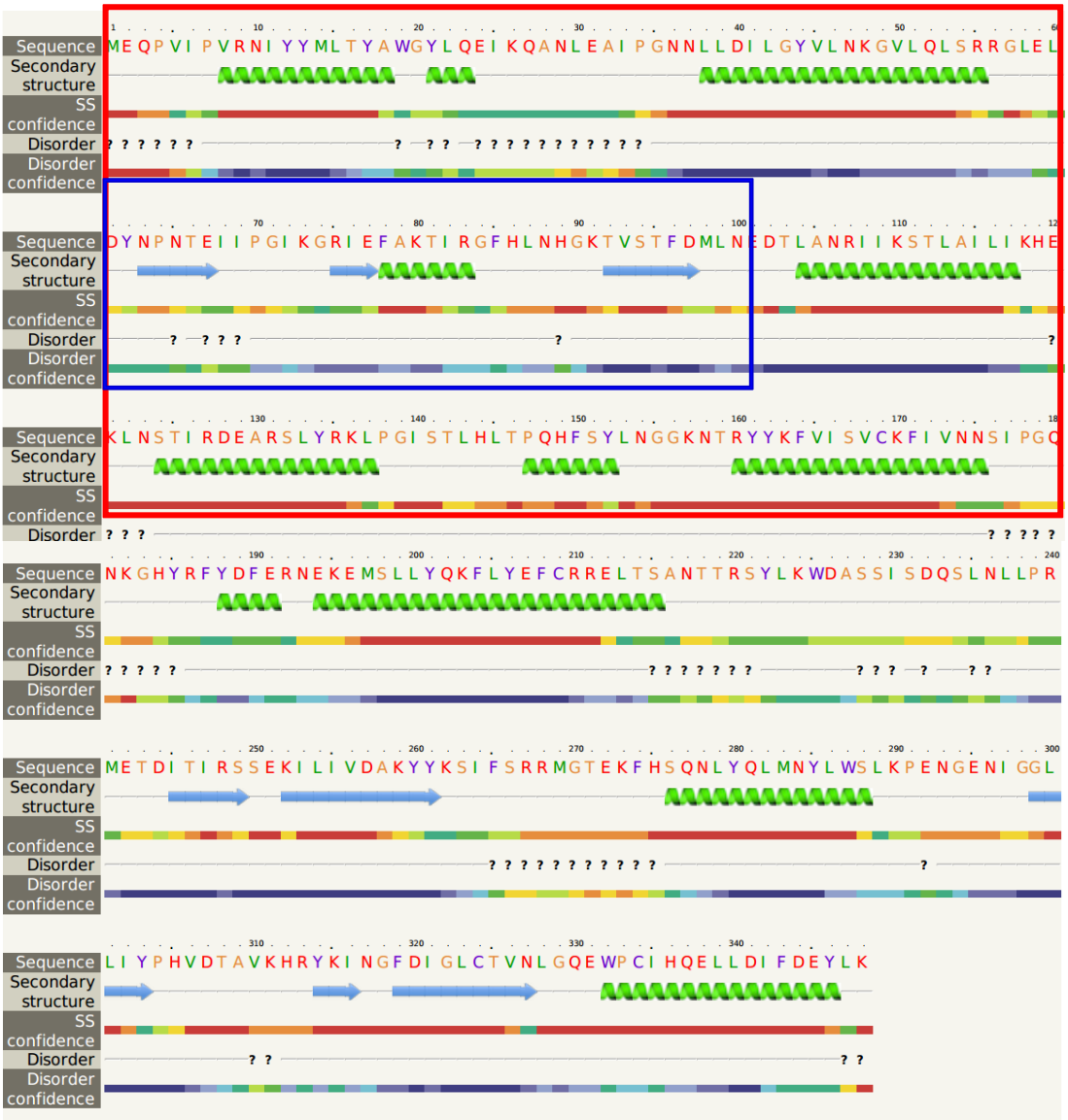


Fig 3.1 Secondary structure prediction of McrC McrC appears to have three distinct secondary structure rich regions. Enclosed in the red box is the alpha helix rich N-terminal domain, highlighted within the blue box is the putative β sheet region and the remaining residues have predominantly conserved α-β-α fold

3.2 Cloning of McrC mutants

All the constructs except those with GST tag 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 500 bp for $McrC_{1-192}$ and $McrC_{187-348}$, 1 kbp for McrC L1) (Fig 3.2 A & 3.2 D) 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. Three colonies were screened for $McrC_{1-192}$ out of which two had the desired deletion, the same case was true for $McrC_{187-348}$. In the case of McrC L1 all the four plasmid screened were positive based on this preliminary clone check (Fig 3.2 B, 3.2 C and 3.2 E).

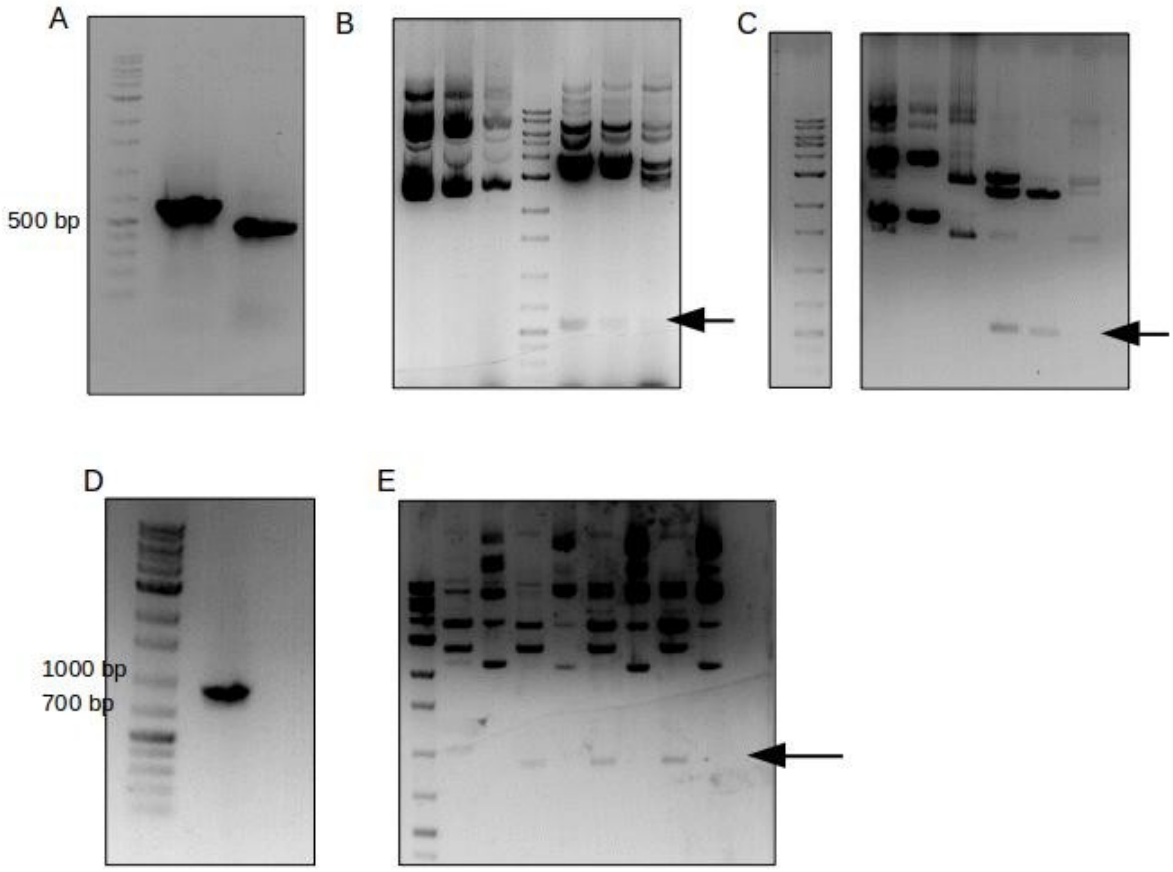


Fig 3.2 Cloning of McrC deletion mutants (A) Amplified product from the first PCR reaction of $McrC_{187-348}$ (lane 2) and $McrC_{1-192}$ (lane 3). (B) Clone check of $McrC_{1-192}$. Lanes 1 to 3 are the plasmid purified from transformed colonies. Lanes 5 to 7 are the corresponding double digested plasmids. (C) Clone check of $McrC_{187-348}$. Lane 2 to 4 are the plasmid purified from transformed colonies. Lanes 5 to 7 are the corresponding double digested plasmids. (D) Amplified product from the first PCR reaction of McrC L1. (B) Clone check of McrCL1. Lanes 1 to 3 are the plasmid purified from transformed colonies. Lanes 4 to 7 are the corresponding double digested plasmids. Alternate lanes contain double digested and undigested plasmids.

3.3 Protein expression check

The clones that were sequenced and verified to have the deletions were tested for their level of protein expression. The results of protein expression test for each class of deletion mutations will be presented separately.

3.3.1 N-terminal constructs

The first McrC N-terminal constructs to be tested for protein expression was $McrC_{1-192}$. The recombinant plasmid was transformed into BL21(AI) as well as BL21(DE3) cells in order to test the level of expression as well as the solubility of the protein (Fig 3.3 A and Fig 3.3 B). No overexpressed band corresponding to the size of the protein was seen on an SDS PAGE gel. To rule out the possibility of degradation of protein over time within the host cell, the level of expression was monitored every 30 minutes post induction up to 4 hours in BL21(AI) cells induced with arabinose at OD at 600 nm = 0.6 (Fig 3.3 C). There was no distinguishable expression of protein at any point in time. Since $McrC_{1-192}$ did not overexpress in any of the expression systems tested, new constructs with variation to the length of the N-terminal domain were engineered (Fig 3.1). This led us to design, $McrC_{1-183}$ and $McrC_{1-179}$. However they did not express in BL21(AI) cells (Fig 3.3 D).

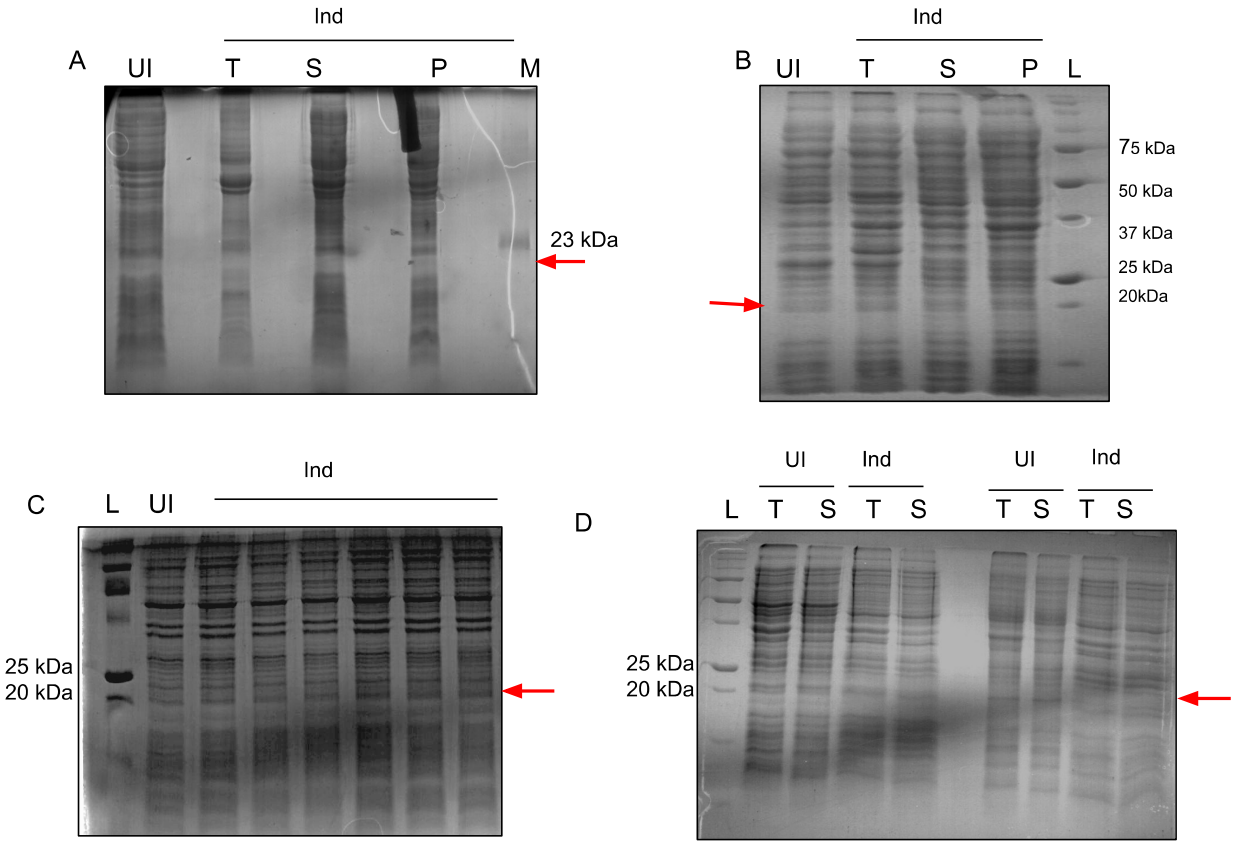


Fig 3.3 Expression test of McrC N-terminal constructs Expression test of $McrC_{1-192}$ in BL21(AI) cells (A) and BL21(DE3) cells (B). (C) Time dependent expression test of $McrC_{1-192}$ in BL21(AI) cells post induction with L-Arabinose at 37^0C. Expression was tested every thirty minute post induction up to 4 hours. (D) Lane 2-5 represent the expression test of $McrC_{1-179}$ and lanes 7-10 represent the expression test of $McrC_{1-183}$. The red arrows points to the region on the SDS PAGE gel where the mutant protein was expected to overexpress. [Key : UI – un induced ; I – induced ; T - total ; S - supernatant ; P - pellet ; L/M – ladder / marker]

GST is routinely used in recombinant cloning as a fusion tags which has been shown to improve expression and solubility of the heterologous proteins in bacterial cells. In addition to this, a previous study which characterized the functional domains of McrB used GST fusion tags to obtain deletion constructs of the protein. They have also shown that GST does not interfere with the functions of McrBC complex i.e., DNA

binding and cleavage. McrC deletion constructs with N-terminal GST tag were designed, however these proteins also did not express in BL21(AI) (Fig 3.4).

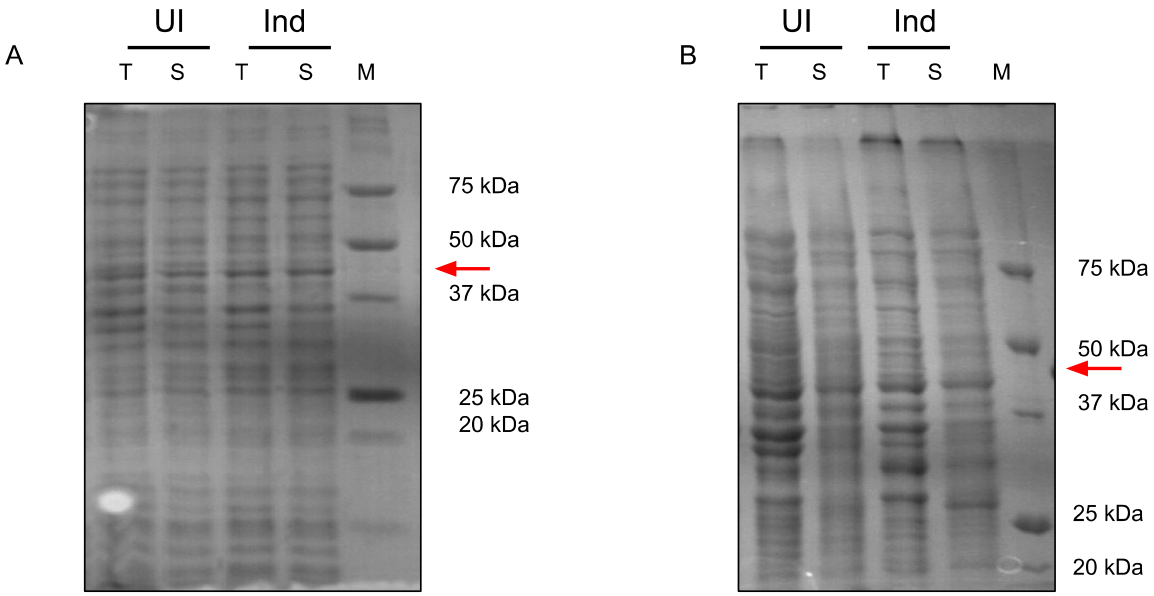


Fig 3.4 Expression test of GST-McrC deletion constructs Expression of (A) $McrC_{1-192}$ and (B) $McrC_{20-192}$ with an N-terminal GST in BL21(AI) cells. The red arrows indicates the region on the SDS PAGE gel where the mutant protein was expected to overexpress.

A closer look at the primary sequence of McrC revealed that the first few amino acids of McrC are highly hydrophobic. Based on the expectation that deletion of these residues will improve the expression of the N-terminal construct in bacterial cells, the construct $McrC_{20-192}$ was designed. The protein expressed and was soluble in BL21(AI) cells (Fig 3.5).

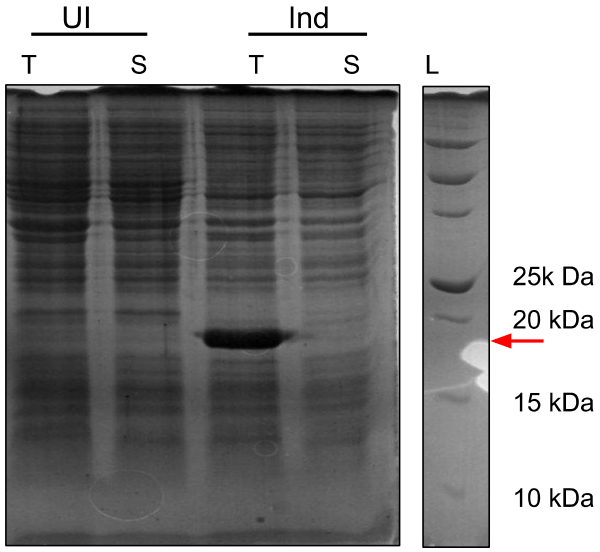


Fig 3.5 Expression test of $McrC_{20-192}$ Expression of $McrC_{20-192}$ in BL21-AI cells. The red arrows indicates the region on the SDS PAGE gel where the mutant protein was expected to overexpress.

3.3.2 C-terminal construct

The only C-terminal constructs to be screened was $McrC_{187-348}$. The protein was expressed well in BL21(AI) cells but was insoluble (Fig 3.6). As we obtained a soluble form of the N-terminal region, I decided to focus on this region and, due to limited time available for the project, did not proceed with further mutation of the C-terminus region to obtain a soluble construct.

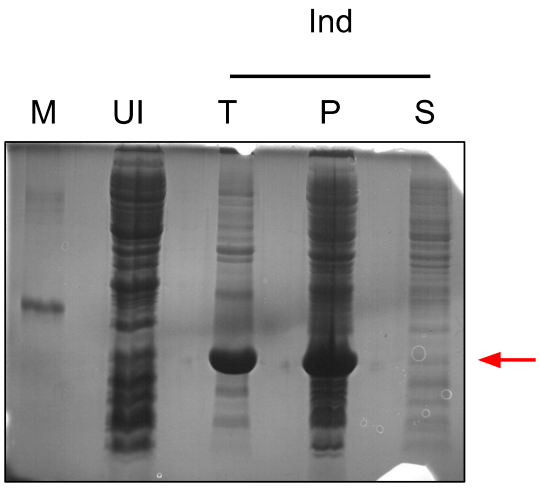


Fig 3.6 Expression test of $McrC_{187-348}$ The expression of $McrC_{187-348}$ was tested in BL21(AI) cells. A 36 kDa protein was used as the marker. The red arrows indicates the region on the SDS PAGE gel where the mutant protein was expected to overexpress.

3.3.3 Loop deletion constructs

The constructs McrC L1, McrC L2, McrC L3 and McrC L4 were all overexpressed and soluble in BL2(AI) cells (Fig 3.7).

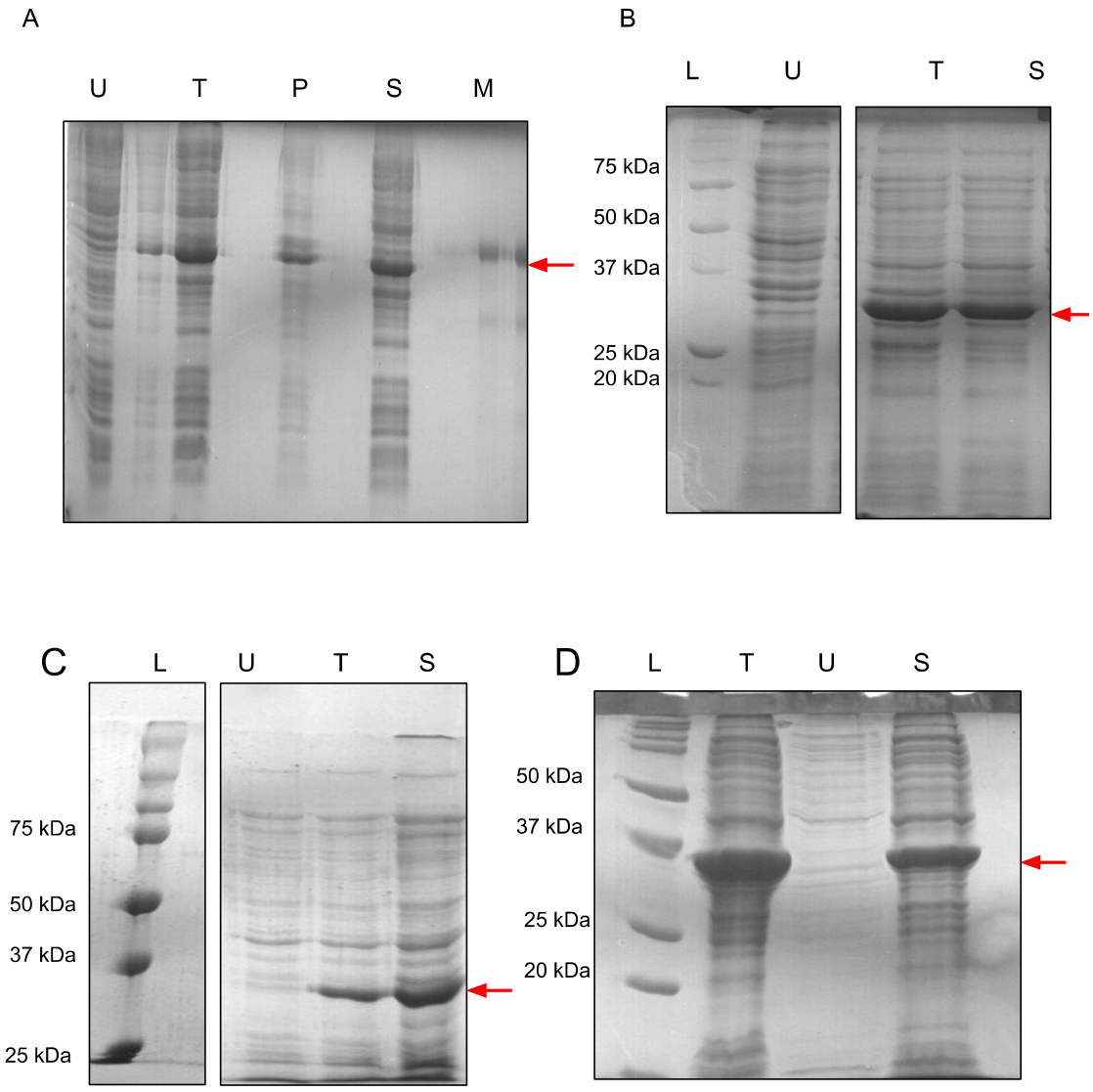


Fig 3.7 Expression test of McrC loop deletion constructs Expression check of McrC L1(A) , McrC L2 (B), McrC L3 (C) and McrC L4 (D) in BL21-AI cells. The red arrows indicates the region on the SDS PAGE gel where the mutant protein was expected to overexpress.

3.4 Overexpression and Purification

After optimization of expression, the constructs $McrC_{20-192}$, McrC L1, McrC L2, McrC L3 and McrC L4 were purified via single-step affinity chromatography. The purest fractions from Ni-NTA as deduced from SDS PAGE gel (Fig 3.8) were pooled and concentrated.

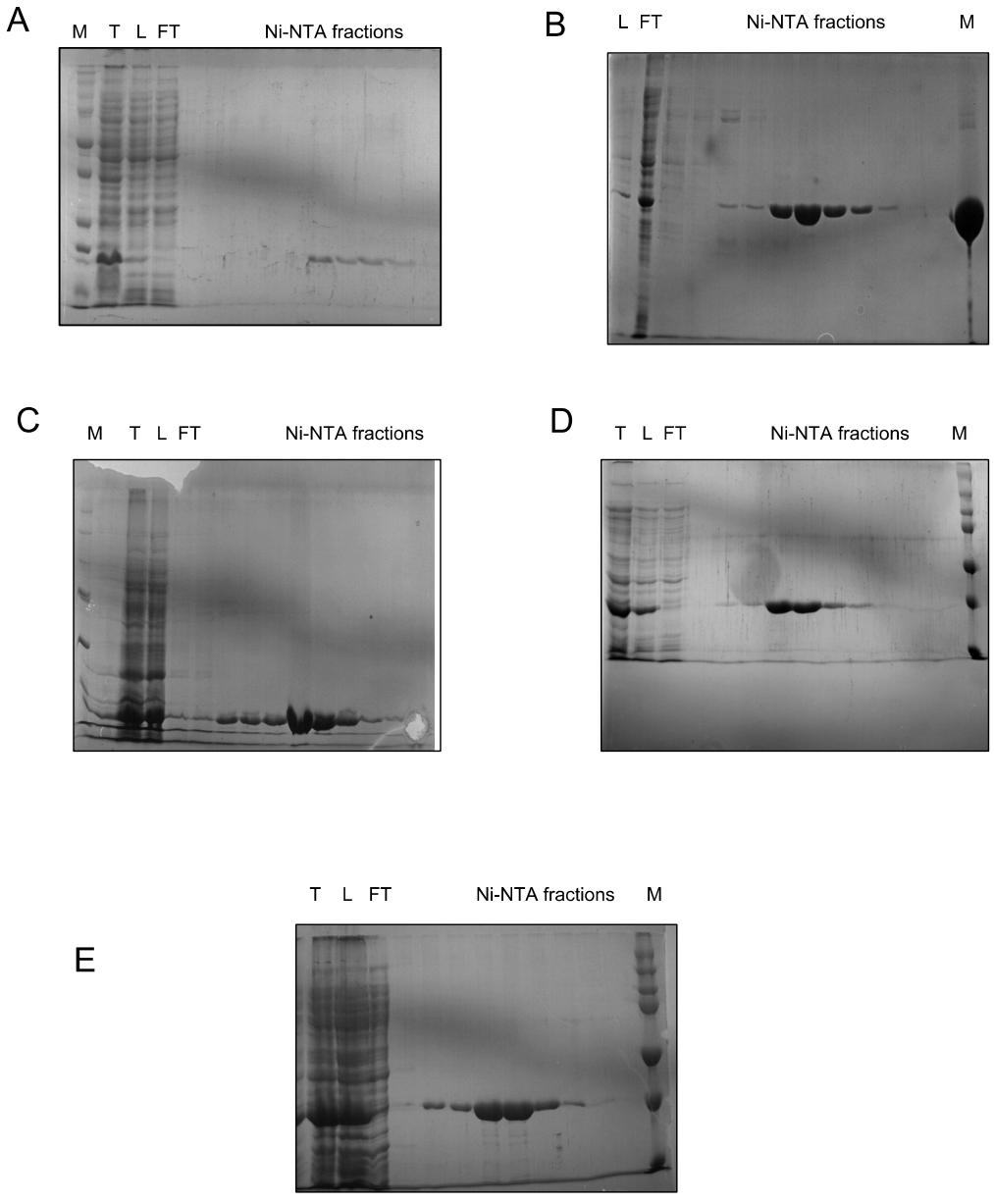


Fig 3.8 Purification of McrC mutants Samples from alternate fractions of Ni-NTA of $McrC_{20-192}$ (A), McrC L1 (B), McrC L2 (C), McrC L3 (D) and McrC L4 (E)

3.5 Characterization of McrC$_{20\text{-}192}$

McrC$_{20\text{-}192}$ was the only N-terminal construct that was successfully expressed and purified. The protein has a predicted molecular weight of 20 kDa. We performed an analytical size exclusion chromatography run of the protein on Superose 6 10/300 GL column, in order to test its ability to form oligomers with McrB. In the wild type McrBC complex, it is proposed that two hexamers of McrB are brought together by a dimer of McrC (Nirwan et al., 2019).

The mutant protein is not expected to form a dimer and hence may not assemble into a tetradecameric complex in the presence of McrB and GTP. The SEC elution profile of McrC$_{20\text{-}192}$ with McrB shows a peak of UV absorbance at 14.8 mL, which aligned with the profile of only McrB in presence of GTP. The elution profile of wild type McrB and McrC showed an elution peak at 13.2 mL. The fractions from SEC column were loaded on an SDS PAGE gel to visualize the proteins present in the complex. The fraction corresponding to elution volume 15 and 16 mL showed a band corresponding to McrB as well as one to that of McrC$_{20\text{-}192}$ (Fig 3.9 A). The GTPase activity of the preformed complex of McrB and McrC$_{20\text{-}192}$ was estimated by performing a continuous coupled NADH assay on the samples from fraction 15 and 16. GTPase stimulation of McrB upon addition of McrC$_{20\text{-}192}$ was estimated by mixing the two protein in 4:1 molar ratio as described in section 2.5. The rate of GTP hydrolysis of the preformed complex as well the mixture of McrB and McrC$_{20\text{-}192}$ was comparable to that of the activity of wild type McrBC (Fig 3.9 B). As the entire catalytic domain with the predicted catalytic region of McrC including the TD^{244}….D^{257}AK motif is absent in the mutant, we expected the protein to be deficient in DNA cleavage. Accordingly, the McrC$_{20\text{-}192}$ did not cleave DNA substrate in the presence of McrB and GTP (Fig 3.9 C).

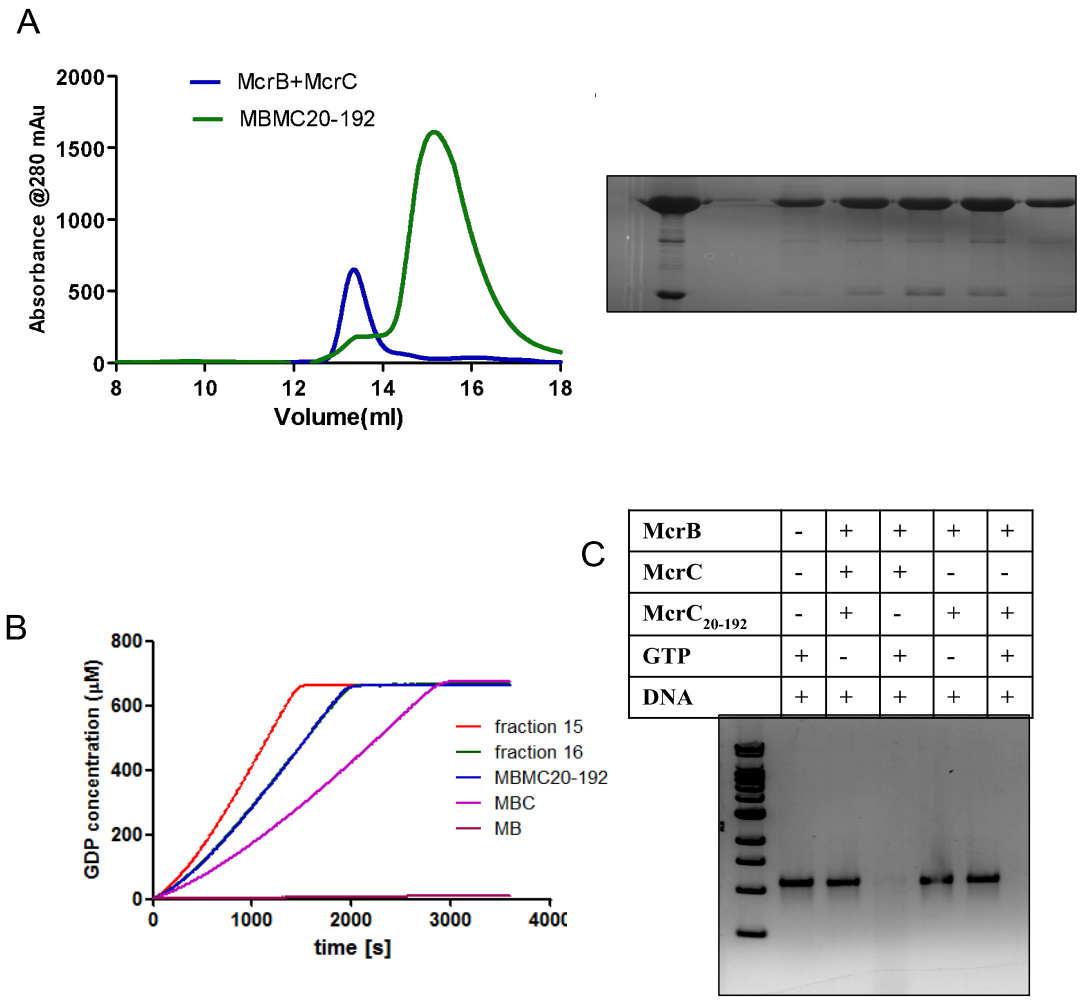


Fig 3.9 Characterization of McrC$_{20\text{-}192}$ (A) The size exclusion profile of McrB and McrC$_{20\text{-}192}$ in presence of GTP performed on Superose 6 10/300 GL (GE Healthcare) column. The fractions 13 to 18 were tested on SDS PAGE gel. (B) Stimulation of GTPase activity of McrB by McrC$_{20\text{-}192}$ [Key : MB- only McrB, MBC - wild type proteins McrB and McrC mixed in 4:1 molar ratio, MBC$_{20\text{-}192}$- wildtype McrB and McrC$_{20\text{-}192}$ mixed in 4:1 molar ratio, fraction 15 and 16 represent preformed complex eluted from SEC column] (C) Cleavage activity of the complex of McrB and McrC$_{20\text{-}192}$.

3.6 Characterization of loop mutants

McrC L1, McrC L2, McrCL3 and McrC L4 were all tested for their oligomeric status, GTPase activity and ability to cleave DNA substrate. The loop mutants were designed by removing an approximately equal number of residues from both ends of the loop. But this was not always possible as the deletions were not recommended within a predicted secondary structure. We kept the deletions close to glycine residues as they are secondary structure breakers. The SEC profile of McrC L1, McrC L2 and McrC L4 suggested that these interacted with McrB to form higher order oligomers. McrC L1 and McrC L4 had an elution peak at around 14 mL, McrC L2 showed an elution peak at 13.6 mL indicating the possibility of a tetradecameric complex. McrC L3 did not interact with McrB to form a higher order complex (Fig 3.10).

Table 3.2 Elution position of the complex of loop mutants with McrB

Input	Elution volume (mL)
McrB + GTP	14.8
McrB + McrC + GTP	13.2
McrB + McrC L1 + GTP	14.1
McrB + McrC L2 + GTP	13.6
McrB + McrC L3 + GTP	15
McrB + McrC L4 + GTP	14.2

Since the McrC mutants McrC L1, McrC L2 and McrC L4 interacted with McrB we decided to characterize the complex for GTPase and cleavage activities. McrC L1 and McrC L2 were not successful in cleavage nor stimulation of GTPase (Fig 3.10 A , Fig 3.10 B). Preliminary characterization of McrC L4 suggest slight activation of the GTPase activity of McrB (the data is not presented here). McrC L3 was not

further characterized functionally as the protein did not interact with McrB to form higher oligomers.

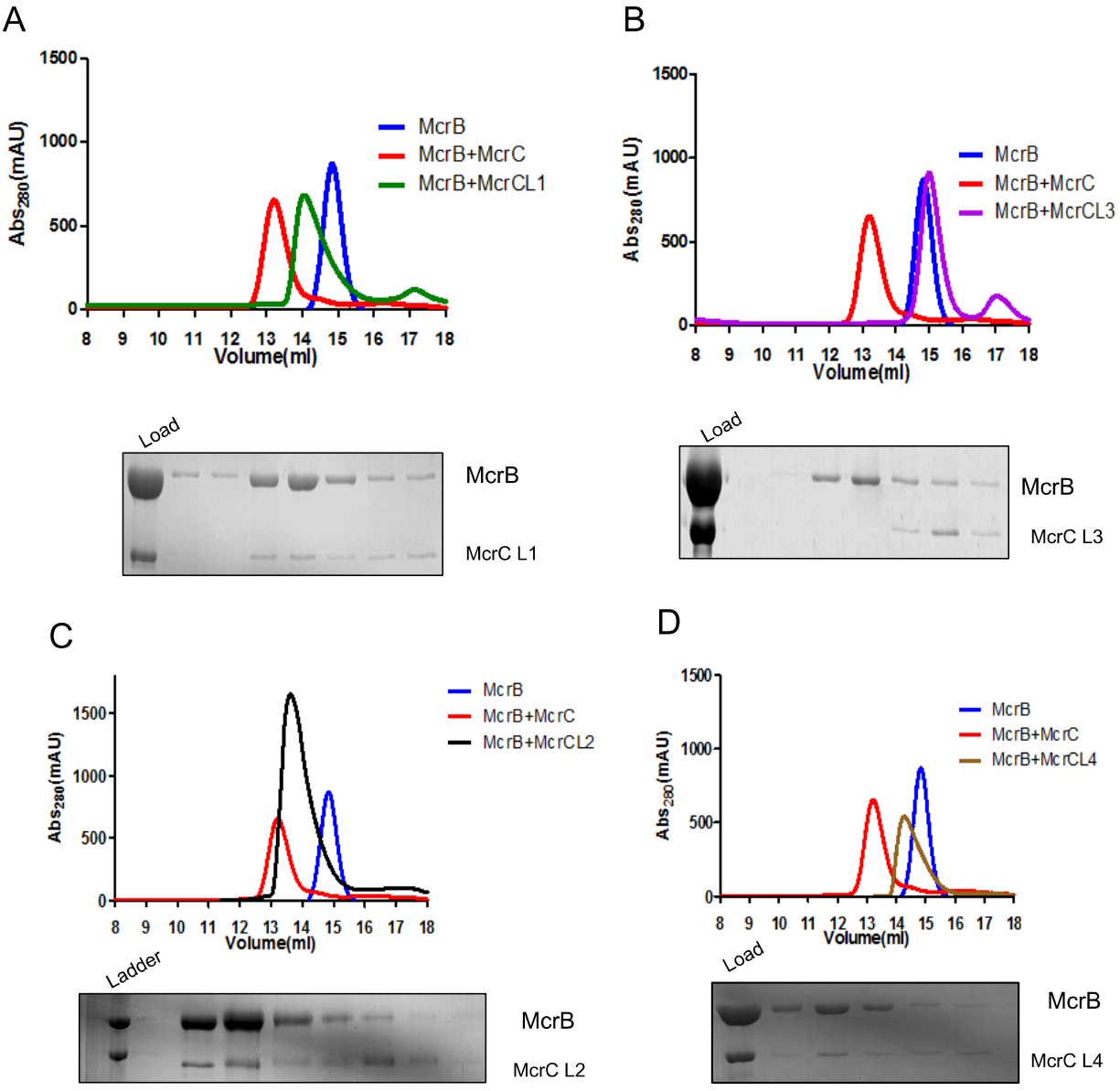


Figure 3.10 SEC profile of McrB along with McrC loop mutants The SEC runs were performed in Superose 6 10/300 GL (GE Healthcare) column. A, B, C and D represent the oligomeric profile of McrB and McrC mutants in presence of 1 mM GTP. The gels show the proteins present in the fraction from 13 to 18 mL. McrC L1 , McrC L2 and McrC L4 co-eluted with McrB, while McrC L3 did not.

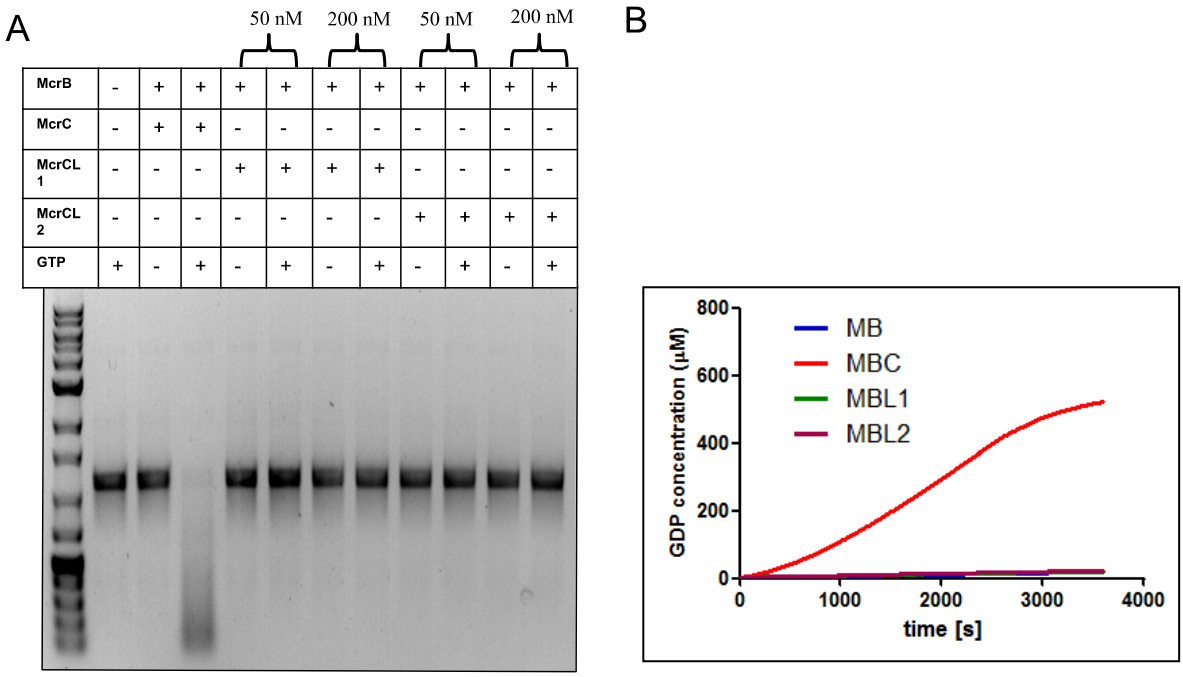


Figure 3.11 Functional characterization of McrC loop mutants (A) The ability of McrC mutants to cleave methylated substrate in presence of McrB and 1 mM GTP was tested. Since no cleavage was observed at 50 nM concentration of the tetradecamer cleavage was also tested at 200 nM concentration of the complex (B) GTPase activity of McrB in the presence of loop mutants McrC L1 (MBL1) and McrC L2 (MBL2).

Discussion

The main aim of this study was to delineate the region of McrC critical for interaction with its partner McrB. A series of deletion mutants of McrC were successfully cloned to this end. The deletions were made within the residues 180 to 200 with the intention of generating independently folded N-terminal and C-terminal domains. The constructs were made by varying the length of the protein by deletion mutagenesis. All the N-terminal constructs of McrC except $McrC_{20\text{-}192}$ failed to express in the bacterial cells. The construct $McrC_{187\text{-}348}$ yielded an insoluble protein in the bacterial expression strain. This behavior of the mutants would indicate that certain deletions were made at the domain boundaries which disrupt the architecture of the polypeptide. The secondary structure prediction suggested that the residues in the region 60 to 100 form a long two stranded β sheet. Four different constructs of McrC were cloned by decreasing the length of the region being deleted. All the loop deletion mutants expressed and were found to be soluble in BL21(AI) cells. The major challenge in handling the loop mutants was that they showed decreased stability and hence an increased rate of precipitation.

The fact that the construct $McrC_{20\text{-}192}$ was successfully expressed and soluble indicated that these residues form an independent domain. The mutant co-eluted with McrB hexamer in presence of GTP from a SEC column at 14.8 mL. $McrC_{20\text{-}192}$ also stimulated the GTPase of McrB as much as full-length McrC does. All these findings taken together suggest that the first 192 amino acid residues form an independently folded functional domain of the protein. These experiments indicated that the amino acids in the N-terminal half of McrC interacts with McrB. The N-terminal half of the protein has no known protein homologue in the database.

The results of the functional and oligomeric characterization of McrC loop deletion constructs indicated that residues in the region 60 to 100 is critical for the interaction between the two proteins. None of the deletion constructs formed a tetradecameric complex with McrB. The elution profile suggested the existence of unstable

complexes formed by weak interaction between the mutant proteins and McrB. Even in the case of the mutants McrC L1 and McrC L2 that appeared to interact with McrB, the activity of the protein was completely lost. The results provide convincing evidence for the importance of the role of the loop in stabilizing the interaction between the two proteins, but there should be further validation of the exact nature of the cross talk. We need to differentiate the role played by the structure of the loop and the biochemical nature of the residues present in this region in stabilizing the complex and their role in stimulating the GTPase activity. In addition, a series of point mutations in this region might help us understand the importance of the amino acids constituting this loop. Though the SEC profile provided convincing evidence for the interaction between McrB and the deletion constructs of McrC, the exact nature of the complex is not clear. The absolute molecular mass of the complex can be studied by SEC coupled to multiple angle light scattering (SEC-MALS).

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