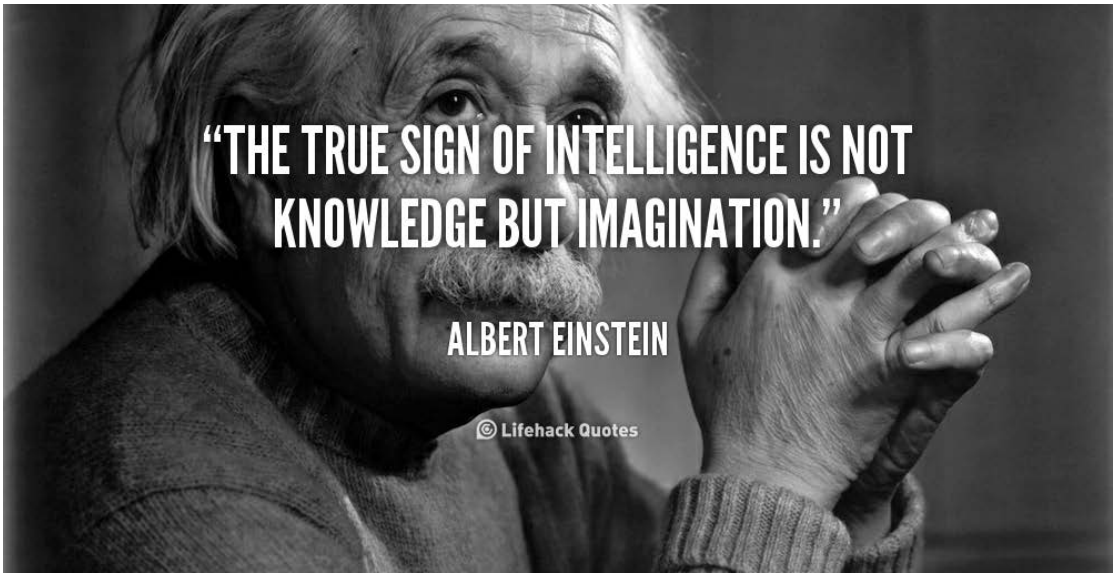

Chapter 5
Determining minimal regions of XisA protein displaying recombinase and endonuclease activities

5.1 Introduction

XisA carries out the excision of *nifD* element by site specific recombination event in the late stages of heterocyst maturation in *Anabaena* PCC 7120 (Lammers et al., 1986; Lammers et al., 1990). Interestingly, precise *nifD* excision was demonstrated in *E. coli*, which is physiologically distinct bacterium from *Anabaena* (Karunakaran et al., 2008). During *nifD* rearrangement studies in *E. coli*, along with white colonies, a few white sectors appeared in blue colonies **(Fig 5.1)**. Cells from such white sectors in blue colonies when restreaked on Ampicillin containing plate, were unable to grow. However, the cells grown on plates not supplemented with any antibiotic found to be cured of pMX25 plasmid.

E. coli strains over expressing *xisA* gene do not grow well presumably due to the toxic effects of XisA protein. The inverted repeat GGAN4TCC was identified in the 11 base pair target sequence (Haselkorn, 1992). Curing of pMX25 was attributed to the endonuclease activity of XisA on the substrate plasmid (Shah et al., 2007). Site directed mutagenesis studies of 11 bp target site **(Fig. 5.1 inset)** suggests that XisA protein belonged to Type IIE family of restriction endonucleases and acted up $GGAN_4TCC$ recognition sequence which is similar another type IIE restriction enzyme *Bst*XI target site $GGAN_4TCC$ (Shah et al., 2007).

Similar studies were also carried out with another DNA element of 55 kb encoding *xisF* recombinase (Carrasco et al., 1995). A few of the *E. coli* cultures started from either blue or white had lost the *xisF* containing plasmid suggested that over expression of *xisF* caused plasmid instability, an effect presumably related to its recombinase activity.

Based on sequence homology, XisA and XisC have been included in integrase family of tyrosine recombinase while XisF shows similarity with resolvase family (Nunes-Duby et al., 1998; Carrasco et al., 2005). Integrase family of recombinases are characterized by conserved catalytic tetrad **R-$(X)_{70-139}$-H/Y-XX-R-$(X)_{29-38}$-Y**. XisA and XisC are 47 % identical and 65 % similar. Our earlier lab studies evaluated the

role of N-and C-terminal regions of XisA and XisC in recombinase and endonuclease activity. N- and C-terminal regions of XisA and XisC were cloned separately as

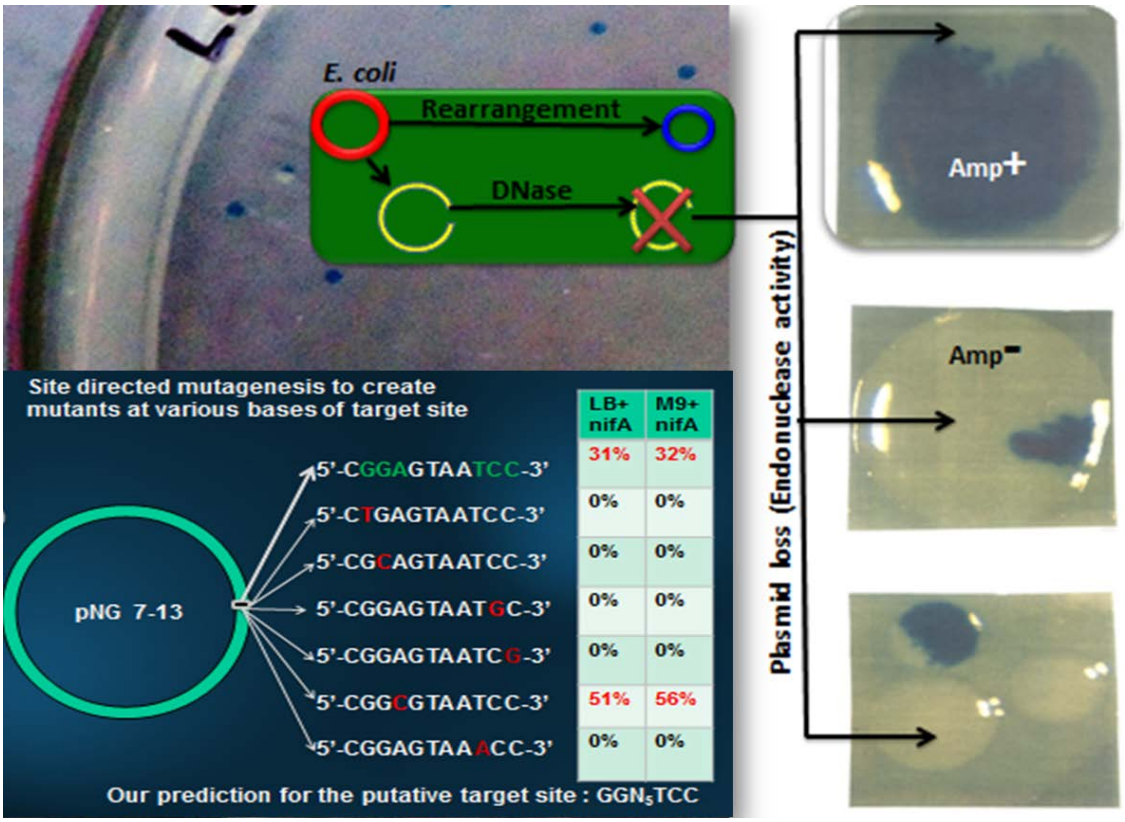

Fig. 5.1 Abortive deletion events arising during *nifD* rearrangement in *E. coli* (pMX25) resulting in blue white sectored colonies indication endonuclease activity if XisA (Karunakaran thesis, 2000). (Inset) Characterization of target site for XisA endonuclease activity (adapted and modified from Shah et al (2007).

shown in **Fig. 5.2**. A chimeric XisAC was also constructed harboring N-terminal and C-terminal (Int domain) of XisC. Despite of significant similarity and identity between XisA and XisC, only XisA demonstrated endonuclease activity which was specific to 11bp target site and lacked cross reactivity with XisC target site. Furthermore, none of the XisA or XisC N-terminal regions demonstrated endonuclease activity on either of the substrate plasmids. However, C-terminal of XisC was found to be catalytically inactive in the absence of N-terminal region and regained the recombinase activity specific to XisC substrate plasmid in presence of XisA N-terminal region as seen in XisAC chimer **(Fig 5.3a & b)**.

This study suggests that endonuclease activity was exclusive property of XisA. Although, active site residues are found on C-terminal region of XisA and XisC, catalytic inability of these recombinases in absence of N-terminal suggests that

N-terminal region is important for recombination and may be involved in multimeric interactions.

5.1.1 Rationale of the present study

Integrase domain for all the members of tyrosine recombinase family is situated at C-terminal region of the proteins (Nunes-Duby et al., 1998; Grindley et al., 2006). XisA protein along with site specific recombinase activity also demonstrates restriction endonuclease activity at its 11bp target site (Shah et al., 2007). However, it is not known which region of XisA protein is involved in endonuclease activity. Interestingly, *Eco*RII, a Type IIE REase displays significant similarity with members of tyrosine recombinase family at C-terminal region in having Int like domain (Topal and Conrad, 1993). It is also interesting to note that upon removal of N-terminal auto regulatory domain of *Eco*RII, enzyme become independent from additional accessory site for cleavage activity (Reuter et al., 1998; Gemmen et al., 2006; Szczepek et al., 2009).

Rationale of our present study was to determine the minimal region demonstrating recombinase and endonuclease activities of XisA. Furthermore, it is also of our interest to determine whether recombinase and endonuclease operated through common region.

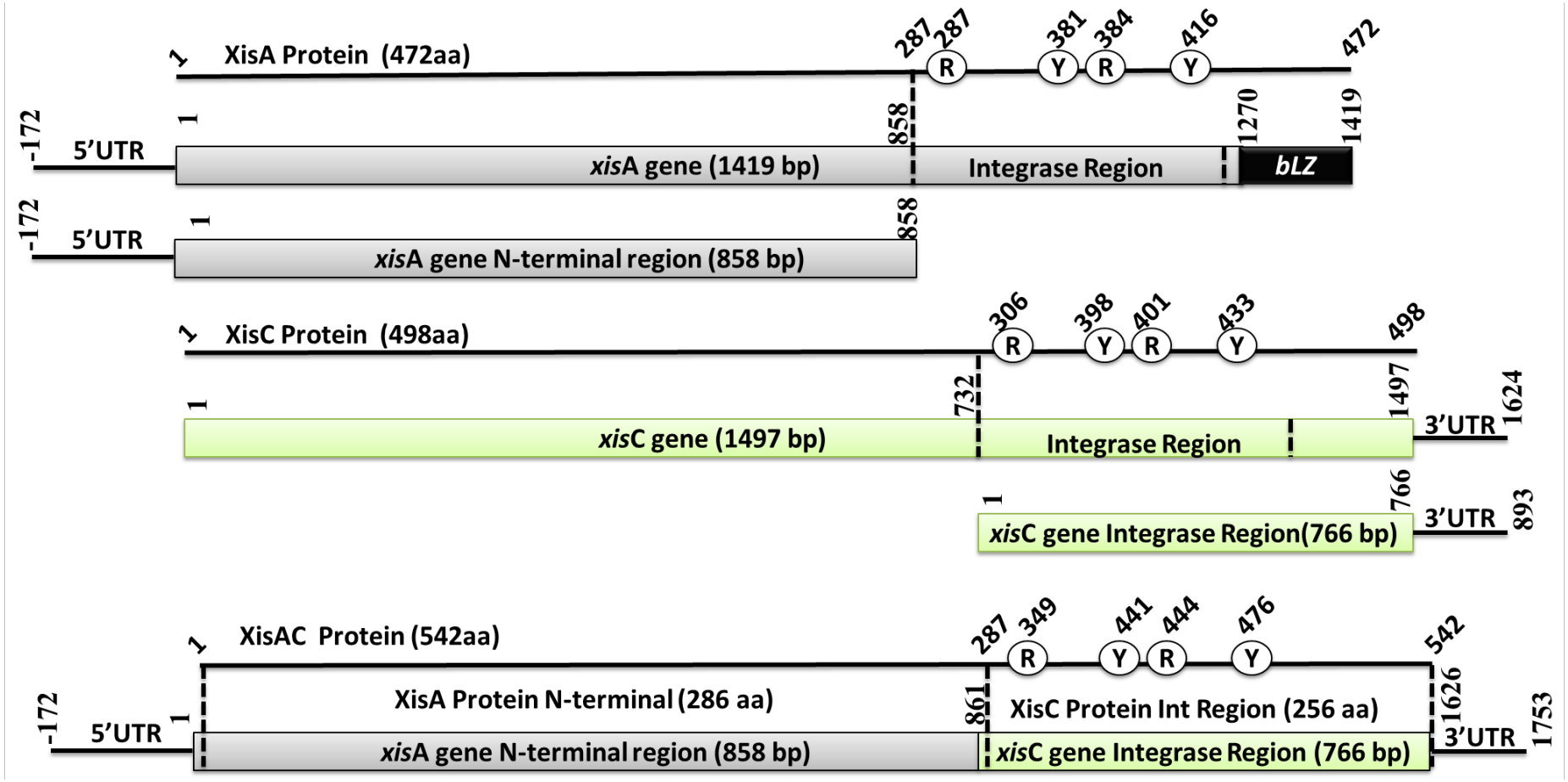

Fig. 5.2 Strategy for constructing chimeric XisAC, harbouring N-terminal of XisA and C-terminal of XisC.

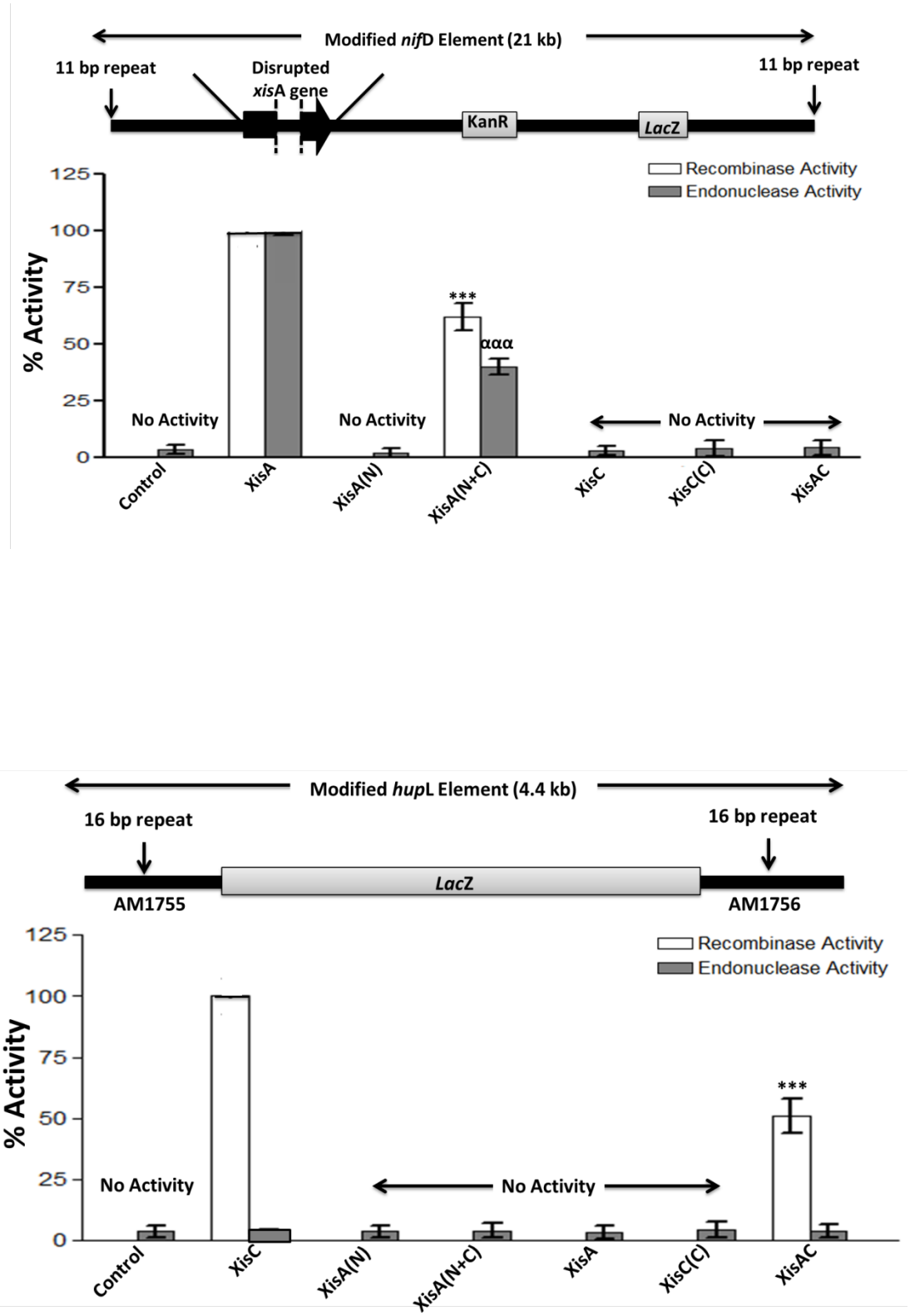

Fig. 5.3 Monitoring recombinase and endonuclease activities of N-terminal, C-terminal and chimer of XisA and XisC. (a)Monitoring activities on XisA substrate (pMX25). (b) Monitoring activities on XisC substrate (pAM1500) plasmid.

5.2 Materials and Methods

5.2.1 *E. coli* Strains, Plasmids and Oligonucleotide Primers Used in the Study

E. coli strains and plasmid used in the present study are summarized in **Table 5.1** and **Table 5.2,** respectively. Oligonucleotide primers synthesis was out sourced to 1st Base Pvt. ltd (Singapore) and are summarized in **Table 5.3.**

Table 5.1 Plasmids used in the study

Name	Characteristics	Reference
pMX25	*nifD* element with *lacZ* and Kan^R cloned in pBR322, Amp^R	(Lammers et al., 1986)
pAM461	Contains distal and proximal borders of *nifD* element, Amp_R	(Lammers et al., 1990)
pJET1.2	pBR322 based positive selection cloning vector with a lethal insert that allows for efficient recovery of blunt-ended PCR products, Amp^R	Thermo Scientific
pJET*xisA*	pJet1.2 containing full length *xisA* gene	This Study
pJET*xisA*2	pJet1.2 containing full length *xisA*2 gene	This Study
pJET*xisA*3	pJet1.2 containing full length *xisA*3 gene	This Study
pJET*xisA*4	pJet1.2 containing full length *xisA*4 gene	This Study
pJET*xisA*5	pJet1.2 containing full length *xisA*5 gene	This Study
pJET*xisA*6	pJet1.2 containing full length *xisA*6 gene	This Study
pET28a (+)	T7 promoter based expression vector, Kan^R	EMD Bioscience, Darmstadt.
p*xisA*	pET28a (+) containing full length *xisA* gene under pT7 promoter, Kan^R	This Study
***pxisA*1**	pET28a (+) containing 1.3 kb N-truncated *xisA*1 gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*2	pET28a (+) containing 1.1 kb N-truncated *xisA*2 gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*3	pET28a (+) containing 1.0 kb N-truncated *xisA*3 gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*4	pET28a (+) containing 0.9 kb N-truncated *xisA*4 gene fragment under pT7 promoter, Kan^R	This Study

p*xisA*5	pET28a (+) containing 0.8 kb N-truncated *xisA*5 gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*6	pET28a (+) containing 0.5 kb N-truncated *xisA*6 gene fragment under pT7 promoter, Kan^R	This Study

Table 5.2 *E. coli* strains used in the study

Name	Genotype	Reference
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	(Hanahan, 1983)
BL21 DE3	*F⁻ ompT gal dcm lon hsdS$_B$(r$_B^-$ m$_B^-$) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	(Studier and Moffatt, 1986)
UN1	BL21 DE3 (pET28a + pAM461)	This Study
UN2	BL21 DE3 (p*xisA* + pAM461)	This Study
UN3	BL21 DE3 (p*xisA*1 + pAM461)	This Study
UN4	BL21 DE3 (p*xisA*2 + pAM461)	This Study
UN5	BL21 DE3 (p*xisA*3+ pAM461)	This Study
UN6	BL21 DE3 (p*xisA*4 + pAM461)	This Study
UN7	BL21 DE3 (p*xisA*5+ pAM461)	This Study
UN8	BL21 DE3 (p*xisA*6+ pAM461)	This Study

Table 5.3 Oligonucleotide primers used in the study

Name	Sequence
XisA(F)	5'-**GGATCC**ATGCAAAATCAGGGTCAA-3'
XisA1(F)	5'-**GGATCC**ATGAGAACAAAAGTACTACAAG-3'
XisA2(F)	5'-**GGATCC**CTGAAGACGGCTGAGGAAGAAG-3'
XisA3(F)	5'-**GGATCC**GAT TCACAA ACAATA GGTGATTT-3'
XisA4(F)	5'-**GGATCC**TTTTCCCGCACCCAACGATATA-3'
XisA5(F)	5'-GC**GGATCC**TGGGCGAGATATAATGCC-3'
XisA6(F)	5'-GC**CATATG**GGTTTACGCCCCAGG-3'
XisA(R)	5'-**GAGCTC**TCAACTATTCTTATAAGCTATTTCCA-3'

C(F)	5'- GTATCTCTCTACGCTTGCTGGTTGG-3'
C(R)	5'- ACCACCCACTACATCGATAACGCC-3'
X(R)	5'- TGCCGTCGGTAGATGAAAGTGGC-3'

5.2.2 Strategy to design sequential N-terminal truncation products of *xisA* gene.

Various target start sites for sequential N-terminal truncations of *xisA* gene are depicted in **Fig. 5.4a** and corresponding sequentially N-terminal truncated protein regions are shown in **Fig. 5.4b**. Selection for various truncation start site was based on *in silico* protein stability analysis of encoded proteins using Expasy-ProtParam tool. The selected targets were chosen on the basis of their high stability, keeping in mind the N-end rule. Preliminary bioinformatics analysis of XisA protein also suggests presence of basic region of Lucine Zipper at the extreme XisA C-terminal **(Fig 5.4b).**

Fig. 5.4 Strategy for selecting targets target for *xisA* gene sequential truncation. (a) *xisA* nucleotide sequence displaying start sites for N-terminal truncation represented with colour coded arrows. (b) XisA protein amino acid sequence displaying start sites of encoded protein fragments (highlighted in black) corresponding to various start sites in *xisA* gene. XisA active site residues are highlighted in red. Grey shaded region represents putative basic region of Leucine Zipper and representative leucine repeats are highlighted in blue.

5.2.3 Construction of vectors expressing sequential N-terminal truncation products of *xisA* gene

5.2.3.1 PCR amplification of *xisA* gene N-terminal truncation products

A PCR based approach was utilized to generate *xisA* gene sequential N-terminal truncation products. Nomenclature of *xisA* gene truncation products and encoded proteins with corresponding sizes is given in **Fig. 5.5**. Sequential N-terminal truncation *xisA* gene products were PCR amplified from pMX25 vector as template using various combination of primers **(Table 5.4)**.

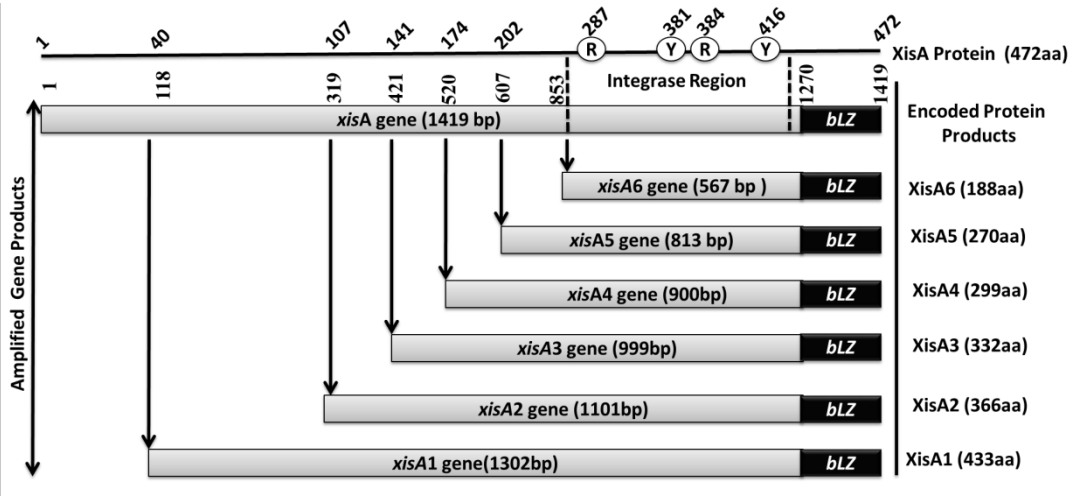

Fig. 5.5 Schematic representation of *xisA* gene sequential N-terminal truncation products.

Table 5.4 Combinations of primers used in amplification of *xisA* N-terminal truncation products.

Gene Name	Name of Forward Primer	Name of Reverse Primer	Size of the amplicons (bp)
xisA	**XisA(F)**	**XisA(R)**	**1419**
***xisA*1**	**XisA1(F)**		**1302**
***xisA*2**	**XisA2(F)**		**1101**
***xisA*3**	**XisA3(F)**		**999**
***xisA*4**	**XisA4(F)**		**900**
***xisA*5**	**XisA5(F)**		**813**
***xisA*6**	**XisA6(F)**		**567**

5.2.3.2 Cloning of various N-terminal truncation products of *xisA* gene in pET28(a)

Cloning of *xisA* gene sequential N-terminal truncation products *xisA*1 to *xisA*5 in pET28(a) was carried at *Bam*HI-*Sac*I sites similar to as described in section **3.2.2.1 and 3.2.2.2**. *xisA*6 was cloned in pET28a in similar fashion at *Nde*I-*Sac*I site **(Fig. 5.6)**.

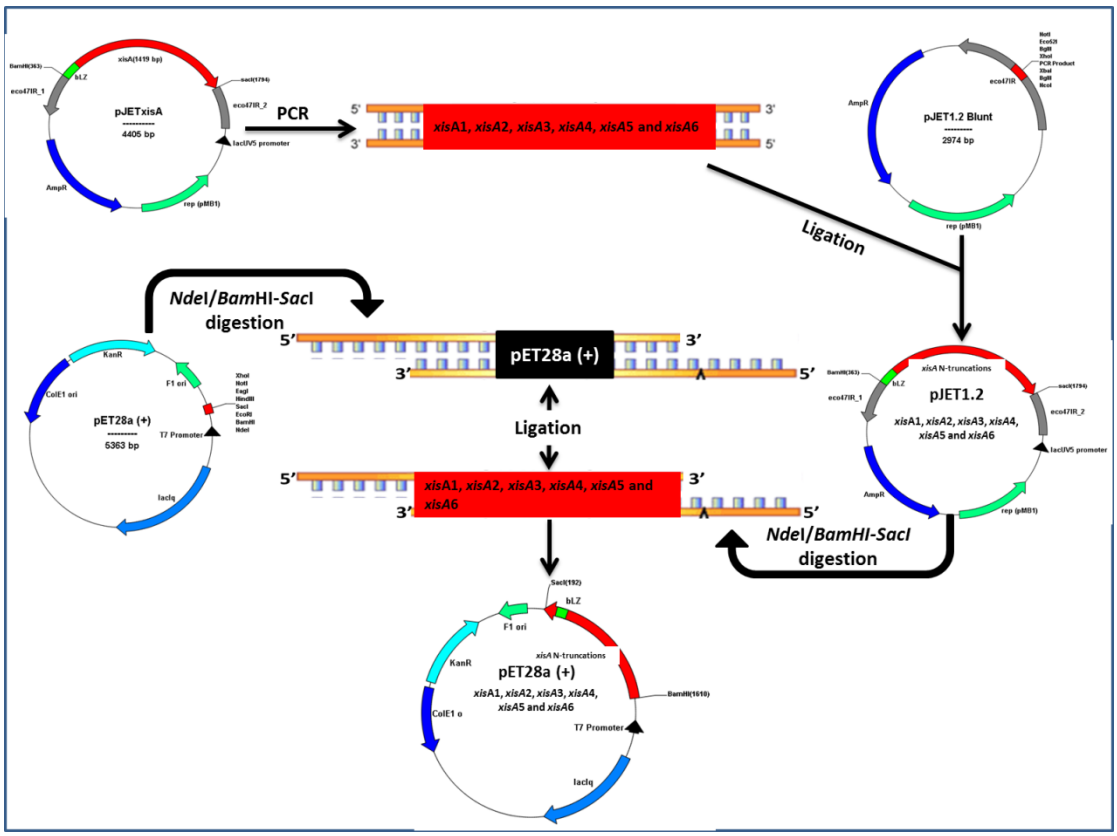

Fig. 5.6 Schematic representation of cloning *xisA* gene sequential N-terminal truncation products in pET28(a).

Nomenclature and sizes of recombinant vectors containing N-terminal truncated *xisA* gene products is given in **Fig.2.2a** and **Table 5.1**.

5.2.4 Heterologous over expression and purification of sequential N-terminal truncation products of *xisA* gene

For the purpose of overexpression and purification, recombinant pET28(a) vectors containing N-terminal truncation fragments of *xisA* gene were transferred in *E. coli* BL21 (DE3) according to Section **2.3**. Expression of N-terminal truncated *xisA*

gene products and corresponding encoded truncated XisA protein products were carried out according to the procedure described in Section **3.2.5**. Expressed and purified proteins were analyzed by SDS PAGE.

5.2.5 Detection of Recombinase activity of N-terminal truncation products of XisA protein

To detect recombinase ability of various N-terminal truncated products of XisA protein, *E. coli* BL21 (DE3) harbouring recombinant pET28(a) vectors containing N-terminal truncation fragments of *xisA* gene was co-transformed with pAM461 to obtain *E. coli* strains UN2 to UN8 (Table 5.2). *E. coli* BL21 (DE3) harbouring pET28(a) and pAM461 (UN1) was used vector control. The obtained *E. coli* strains were allowed to grow over night as described in Section 2.2. The following day, overnight grown culture was used for plasmid mini prep. Isolated plasmid contained mixture of pAM461, ΔpAM461 (rearranged pAM461) and pET28a. 30 ng of isolated plasmid mixture was used for PCR assay using product specific primers (CF-CR). Products of amplification was analyzed by 0.8 % agarose gel electrophoresis. Appearance of only 500 bp band confirms complete rearrangement of pAM461 in to ΔpAM461, while appearance of only 1 kb band suggests no rearrangement. Appearance of both 1 kb and 500 bp band suggests partial rearrangement of pAM461. Rearrangement of pAM461 was detected in uninduced conditions.

5.2.6 Monitoring the recombinase activity of N-terminal truncation products of XisA protein

To monitor the recombinase efficiency of various N-terminal truncation products of XisA protein, *E. coli* BL21 (DE3) strain harboring pAM461 was freshly transformed with recombinant pET28(a) vectors containing N-terminal truncation fragments of *xisA* gene as described in previous section. The following day, a transformed colony was inoculated in 15 ml LB medium supplemented with appropriate antibiotics and was allowed to grow for 12 h in shaking conditions at 200 rpm, 37 ^{o}C. On the next day, overnight grown cells were normalized to OD_{600} =1 and washed with N-saline (three times). 10 µl of washed culture was withdrawn of which, 5 µl of each was used for substrate (with primers CF and XR) and product (with

primers CF and CR) specific PCR. Amplified products were quantified on agarose gel as by measuring IDV values, described in **Fig**. **5.7** Rest of the washed cells were used for monitoring endonuclease activity discussed in next section.

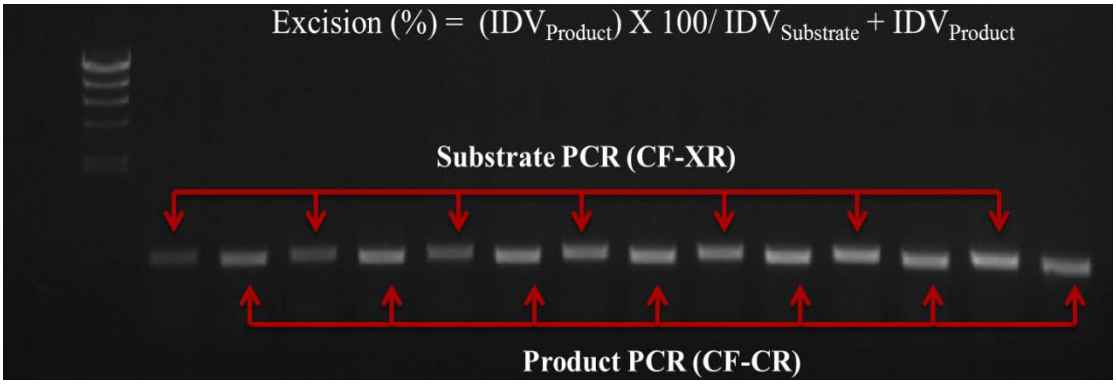

Fig. 5.7 A typical Agarose gel profile of PCR based recombinase assay of XisA

5.2.7 Monitoring endonuclease activity of various sequential N-terminal truncation products of XisA protein by antibiotic sensitivity assay

E. coli BL21 (DE3) strains harbouring pAM461 and recombinant pET28(a) vectors containing N-terminal truncation fragments of *xisA* gene were prepared as described in Section **5.3.8**. The N-saline washed *E. coli* strains were transferred to fresh 15 ml LB medium supplemented with ampicillin and kanamycin and allowed to grow for 6 hr. as described above. After the growth, cells were harvested and washed with N-saline (3 times). Washed cells were serially diluted10^6 times with N-saline out of which 100 µl was used to spread on each Luria agar plate with kanamycin (control) and Luria agar plate with ampicillin and kanamycin. Plates were incubated overnight at 37 ^oC. On the following day, colonies were counted and endonuclease activity was calculated as given in **Fig. 5.8.**

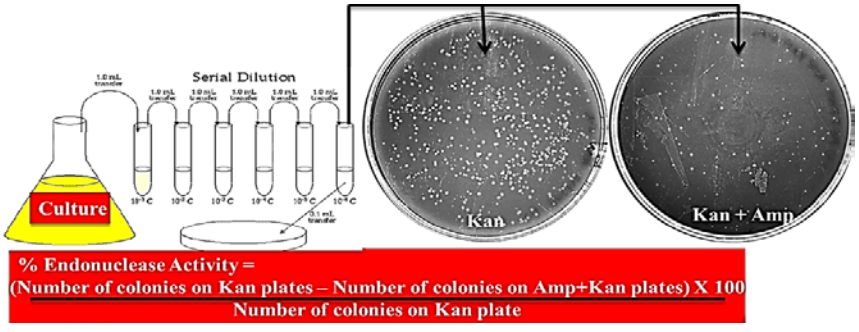

Fig. 5.8 Methodology to monitor endonuclease activity of XisA and derived variants.

5.3 Results

5.3.1 Cloning and heterologous expression of sequential N-terminal truncation products of *xisA* gene

pMX25 plasmid harboring *nifD* element was used as a template to amplify N-terminal truncation variants of *xisA* gene **(Fig. 5.9)**. Further, these variants of *xisA* gene were cloned in pJET1.2 and recombinant constructs were confirmed by restriction analysis by *Bgl*II digestions **(Fig. 5.10).** Variants were further sub-cloned in pET28(a) under strong T7 promoter and recombinant constructs were confirmed by restriction analysis by either *Bam*HI-*Sac*I digestion (for p*xisA* to p*xisA*5) or *Nde*I-*Sac*I digestion (for *pxisA*6) **(Fig. 5.11).**

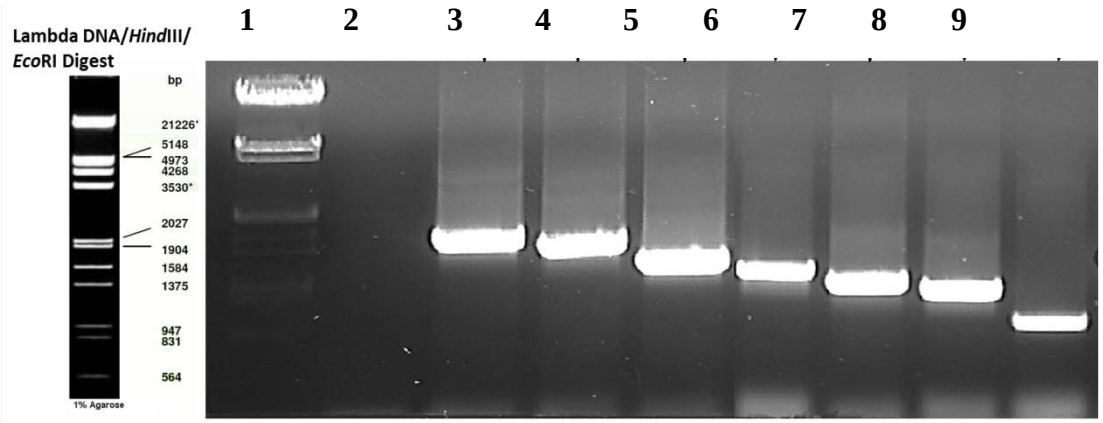

Fig. 5.9 PCR amplification of wild type *xisA* gene and its sequential N-terminal truncation variants. Lane 1: Lambda DNA *Hind*III-*Eco*RI double digest; Lane 2: No template control; Lane 3: 1.4 kb *xisA*; Lane 2: 1.3 kb *xisA*1; Lane 4: 1.1 kb *xisA* 2; Lane 5: 1.0 kb *xisA*3; Lane 6: 0.9 kb *xisA*4; Lane 7: 0.8 kb *xisA*5; Lane 8: 0.5 kb *xisA*6.

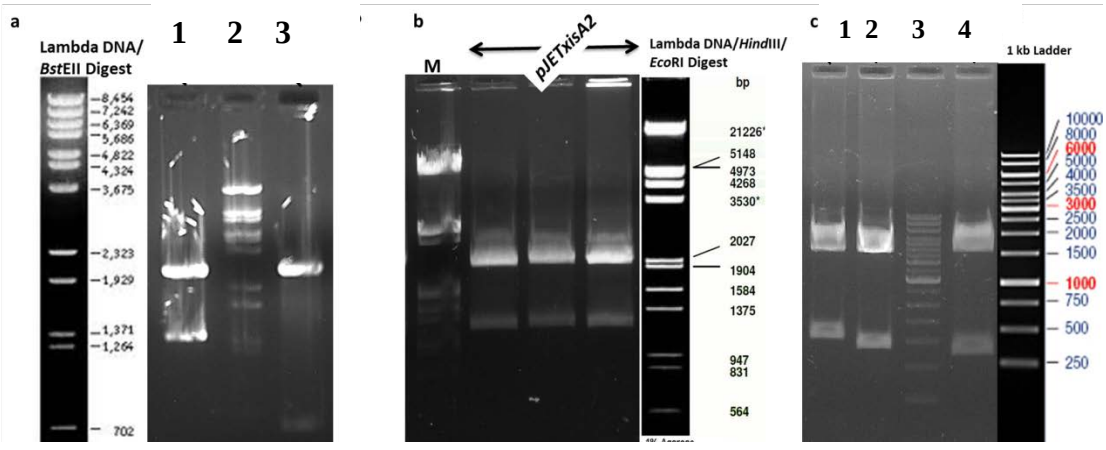

Fig. 5.10 *Bgl*II digestion pattern pJET1.2 containing *xisA* gene sequential N-terminal truncation variants. (a) Clone confirmation of *xisA*1 and *xisA*6 genes. Lane 1, 3: *Bgl*II digested pJET*xisA*1 and pJET*xisA*6 respectively; Lane 2: 1 kb ladder. (b) Clone confirmation of *xisA*2. Lane 1: Lambda DNA *Hind*II-*Eco*RI digest; Lane 2-4: *Bgl*II digested pJET*xisA*6. (C) Clone confirmation

of *xisA*3, *xisA*4 and *xisA*5 genes. Lane 1, 2 and 4: *Bgl*II digested pJET*xisA*3, pJET*xisA*4 and pJET*xisA*5, respectively.

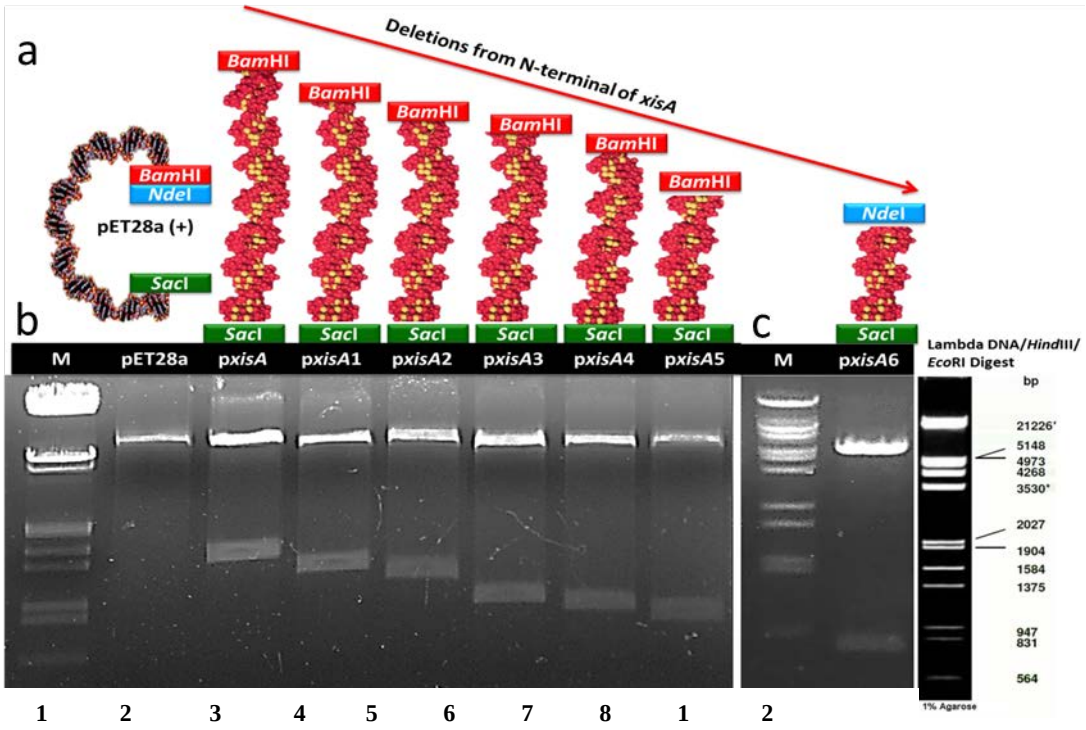

Fig. 5.11 Restriction digestion pattern of pET28(a) containing *xisA* gene sequential N-terminal truncation variants. (a) Schematic representation of various *xisA* gene sequential N-terminal truncation variants and with flanking RE cleavage sites. (b) *Bam*HI-SacI digestion pattern of pET 28(a) containing *xisA* gene sequential N-terminal truncation variants. Lane 1: *Bam*HI-*Sac*I digested pET28(a). Lane 2-6: *BamH*I-*Sac*I digested p*xisA*, p*xisA*1, p*xisA*2, p*xisA*3, p*xisA*4, p*xisA*5. (c) *Nde*I-*Sac*I digestion pattern of p*xisA*6.

Overexpression of N-terminal truncated *xisA* gene products was achieved similar to as described in Section **3.3.5.** Expressed and N-terminal truncation XisA variants were analysed by SDS PAGE **(Fig. 5.12a).** Nomenclature of the encoded protein was shown in **Fig 5.5**.

5.3.2 Purification of sequential N-terminal truncation products of XisA protein

Purification of N-terminal truncation products of XisA protein was achieved in a similar manner as described in Section **3.2.5**. Purified XisA protein varients were analyzed on SDS PAGE **(Fig. 5.12b).**

5.3.3 Determining the minimal region of XisA protein exhibiting recombinase and endonuclease activities.

To determine the functionality of various N-terminal truncated products of XisA proteins, *E. coli* BL21 (DE3) harboring pAM461 were co-transformed with

recombinant pET28a containing various N-terminal deletion products of *xisA* gene. Nomenclature of transformed *E.coli* strains were kept as given in **Table 5.2.**

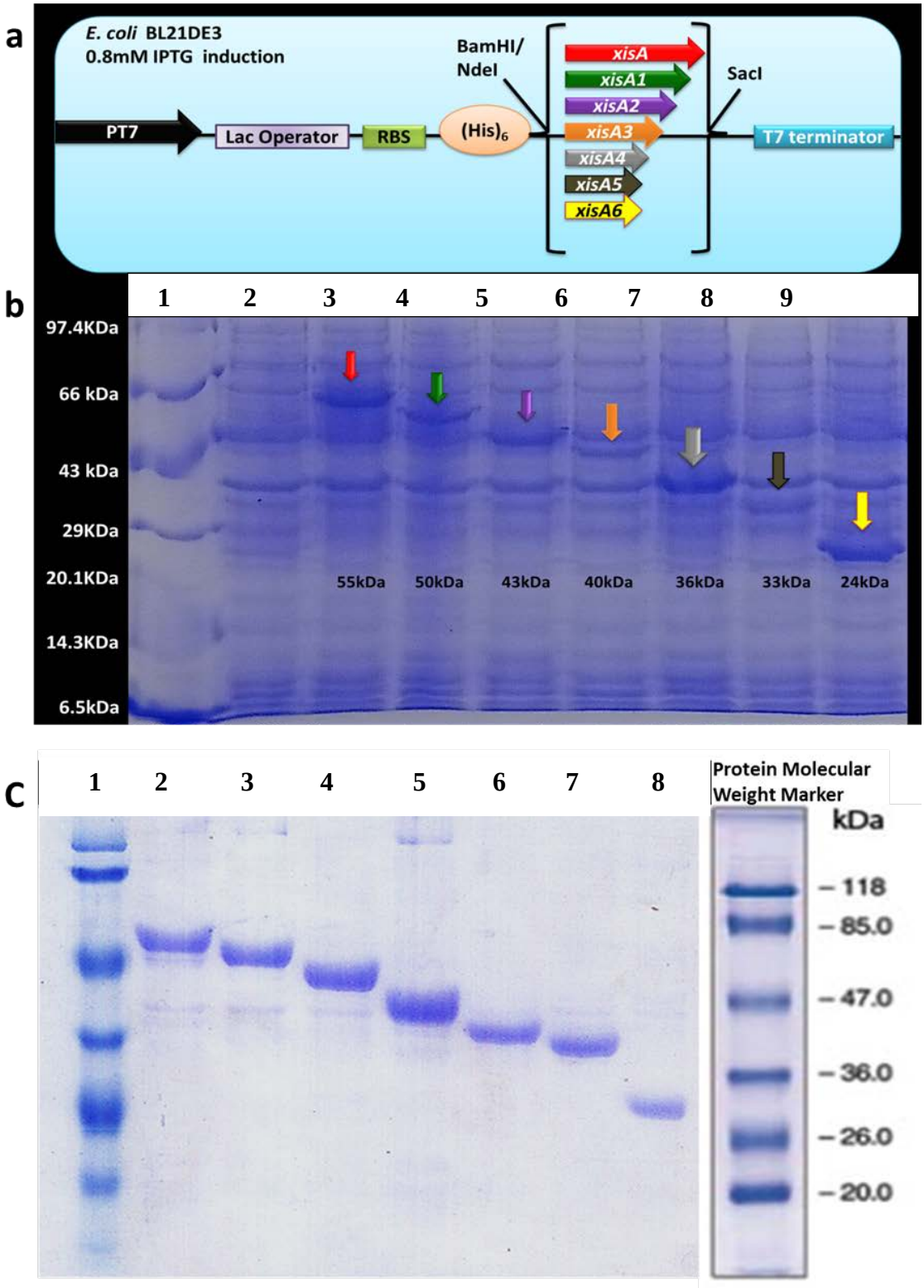

Fig. 5.12 Expression and purification of N-terminal truncation products of XisA protein. (a) Schematic representation of pET28a expression cassette containing various N-terminal truncation products of *xisA* gene. (b) Expressed N-terminal truncated XisA protein variants. Lane 1: Protein molecular weight marker; Lane 2: pET28a vector control; Lane 3-9: Sequential

N-terminal truncation XisA protein variants. (c) Purified N-terminal truncated XisA protein variants. Lane 1: Molecular weight marker; Lane 2-8: Sequential N-terminal truncation XisA protein variants.

5.3.3.1 Monitoring recombinase activity

Qualitative recombinase activity of N-terminal truncation variants of XisA protein was performed as described in **5.2.5**. From functionality assay **(Fig. 5.13),** strains UN2, UN3, UN4 and UN5 synthesizing protein XisA, XisA1, XisA2 and XisA3, respectively, showed the presence of recombinase activity. Recombinase activity was completely absent in strains UN6, UN7 and UN8 synthesizing protein XisA4, XisA5 and XisA6, respectively. UN1 strain lacking *xisA* gene was used as vector control.

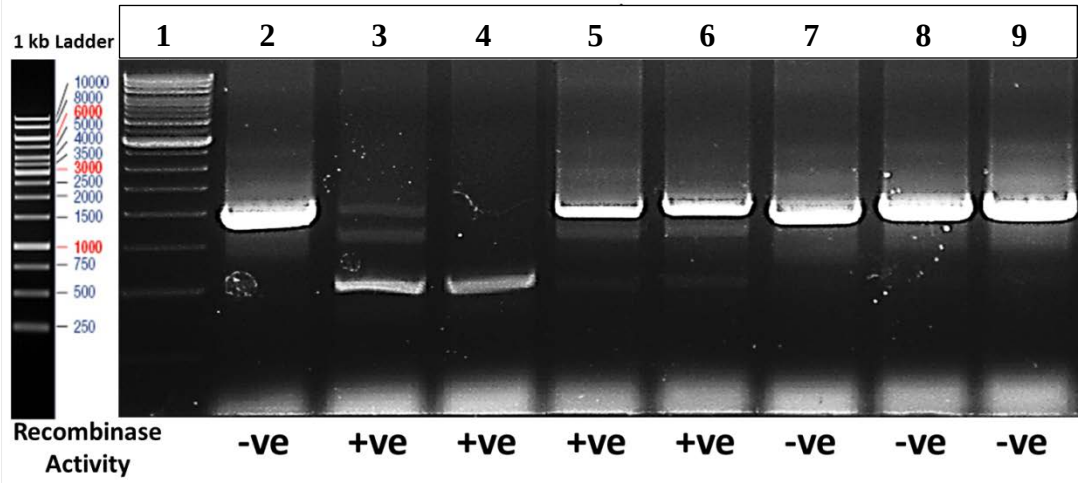

Fig. 5.13 Agarose gel profile displaying PCR based functionality detection of *E. coli* strains synthesising N-terminal truncation products of XisA protein. Presence of Appearance of only 500 bp amplicon band confirms recombinase activity, while appearance of only 1 kb amplicon band suggests no activity. Appearance of both 1 kb and 500 bp band suggests partial recombinase activity. Lane 1: 1 kb ladder; Lane 2: PCR analysis of UN1 (pET28a vector control); Lane 3-8: PCR analysis aiding functionality detection of *E. coli* strains (UN2 to UN8) synthesising N-terminal truncation products of XisA protein.

Quantitative monitoring of recombinase activity of *E. coli* strains synthesizing N-terminal truncation variants of XisA protein was performed as given in section **5.2.6**. Quantitative recombinase assay **(Fig. 5.14a & c)** suggests that, strain UN3 synthesizing XisA1 showed the highest recombination activity and the strain UN5 synthesizing XisA3 retained significant amount of recombinase activity . None of the strains (UN6, UN7 and UN8) synthesizing N-terminal truncation product smaller than XisA3 showed any detectable recombinase activity.

5.3.3.2 Monitoring endonuclease activity

Endonuclease activity assay of *E. coli* strains synthesizing N-terminal truncation variants of XisA protein was performed as given in section **5.2.7**. Antibiotic susceptibility assay (**Fig. 5.14b & c)** demonstrated endonuclease activity by all strains. Strain UN3 synthesizing XisA1 protein showed the highest endonuclease activity while strain UN8 synthesizing XisA6 protein retained significant endonuclease activity compared to control UN1.

5.4 Discussion

XisA protein is essential for excision of *nifD* element during nitrogen starvation condition to reconstitute functional *nifD* gene (Brusca et al., 1990). Sequence homology studies have revealed that XisA protein belongs to the class of the lambda Integrase family of recombinases which is characterized by presence of RHRY tetrad as conserved active site residues situated near the carboxyl terminal of the protein (Esposito and Scocca, 1997). This protein remained elusive for almost three decades and Chapter 3 demonstrated the identification and purification of XisA protein. XisA protein along with recombinase activity also possesses endonuclease activity (Shah et al., 2007) suggesting that this protein could be an evolutionary intermediate between site-specific recombinases and endonucleases.

In order to determine the role of N-terminal and C-terminal regions of XisA and XisC, our previous laboratory work involved cloning of C-terminal and N-terminal domains of these enzymes separately. Chimeric recombinase containing N-terminal of XisA and C-terminal of XisC was also constructed (**Fig. 5.2).** All these plasmids were monitored for the recombination of XisA and XisC substrate plasmids, pMX25 and pAM1500, respectively, as well as for the endonuclease activity. N-terminal of XisA did not show any activity, while the C-terminal alone was found to be sufficient for the recombinase as well as endonuclease activity with specificity similar to XisA. XisC C-terminal region did not show any rearrangement and

endonuclease activities. Chimeric recombinase showed rearrangement of XisC substrate plasmid pAM500 **(Fig. 5.3).**

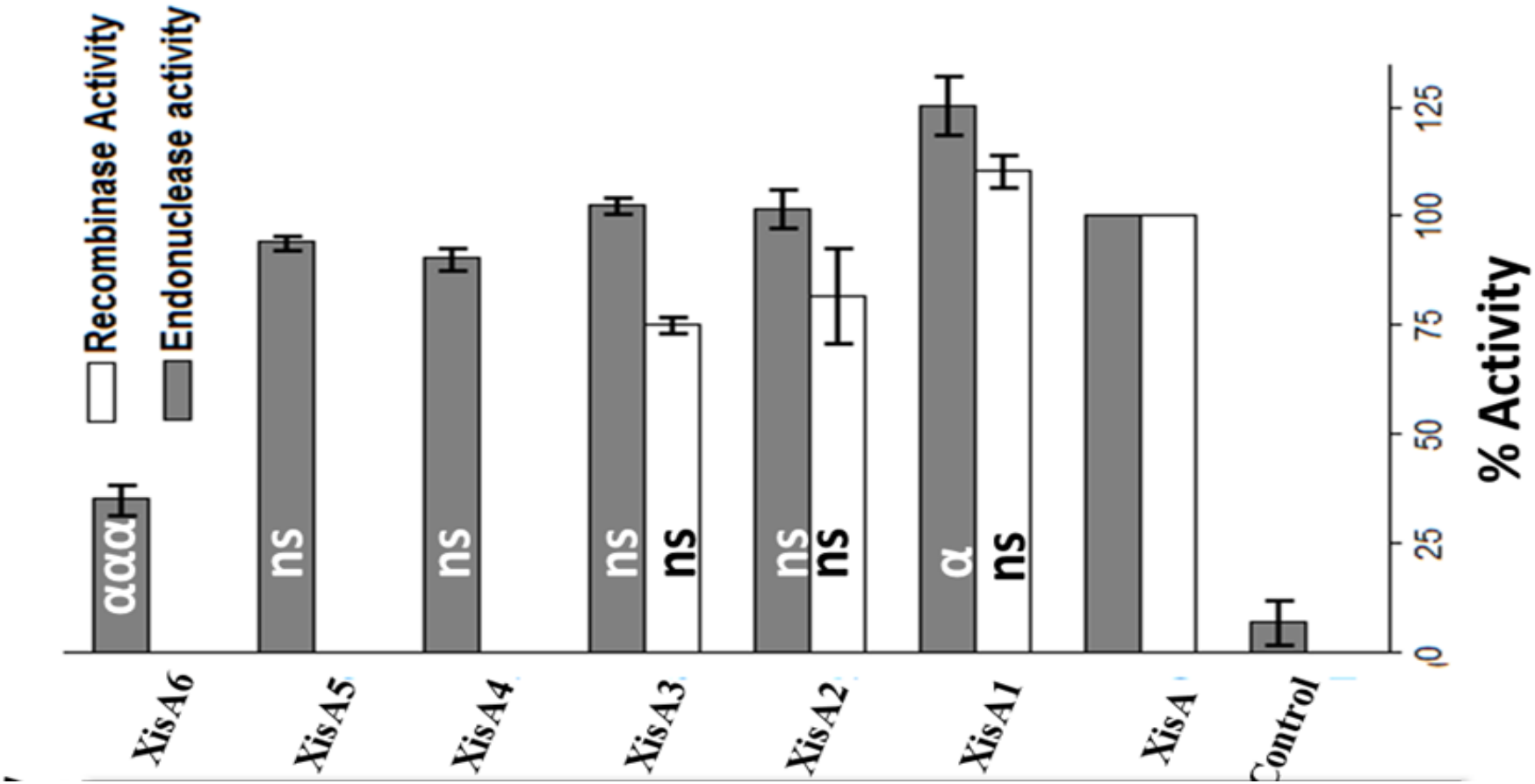

Fig. 5.14 **Graphical representation of recombinase and endonuclease activity of sequential N-terminal truncation products. Activity by compete XisA protein is normalized to 100. Activity by truncated proteins are relative to complete XisA. Values are expressed as Mean ±SEM (N=3). For endonuclease activity $^{\alpha\alpha\alpha}$P-value ≤ 0.001, $^{\alpha}$P-value ≤ 0.05 compared UN 2 and for recombinase activity ns-not significant difference compared to UN2.**

Hence, XisA and XisC are functionally different even though they show very good sequence similarity. This shows that N-terminal of XisA is important for recombinase activity. General structure of tyrosine recombinases suggest that N-terminal of protein is involved in dimer of dimer formation whereas C-terminal has cleavage and relegation activity where lies the active site residues of recombinases. In the present study to validate the role of N-terminal and C-terminal in recombinase and endonuclease activity, we constructed sequential N-terminal deletions of *xisA* and assayed its recombinase and endonuclease potential.

In vivo recombinase and endonuclease assays for sequential N-terminal deletions suggests that XisA1 (40-472aa) possessed both highest recombinase and endonuclease activities whereas XisA3 (141-472aa) was sufficient for both the activities. Int domain of XisA (287-472aa) was unable to carryout recombination but retained significant endonuclease activity. Abolishment of recombinase activity but not endonuclease activity on N-terminal deletions indicates that N-terminal is strictly required for recombinase activity as it may be important for dimer-dimer interaction during excision by XisA **(Fig. 5.14)**. On the other hand, C-terminal of XisA (Int domain) may exist as dimer through its leucine zipper like region and can still bind to DNA and show DNA cutting activity (**Fig. 5.4b and 5.5).**