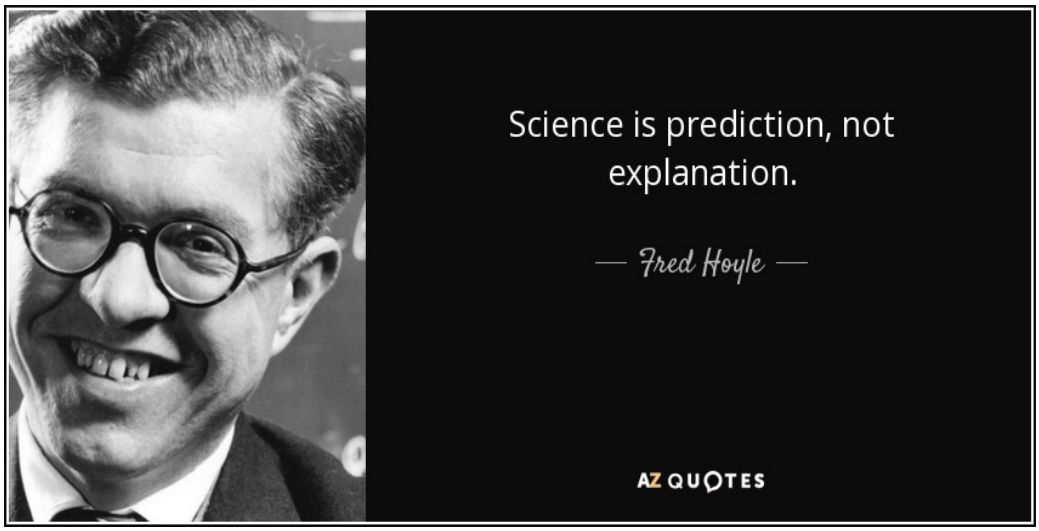

Chapter 6
Role of predicted basic region of leucine zipper of XisA protein in recombinase and endonuclease activities

1.1 Introduction

The leucine zipper is the dimerization domain of the B-ZIP (basic-region leucine zipper) class of eukaryotic transcription factors (Vinson et al., 1989) . The leucine zipper (LZ) motif was first described as a dimerization domain in the yeast transcriptional factor GCN4 and in the oncogenic proteins Fos and Jun (Landschulz et al., 1988; Hurst, 1994). The LZ motif consists of a repetition of 4-5 leucines spaced seven residues apart. The residues of the' motif adopt an a helical secondary structure in which leucines are arranged on the same face of the helix turns, organizing a hydrophobic spine.

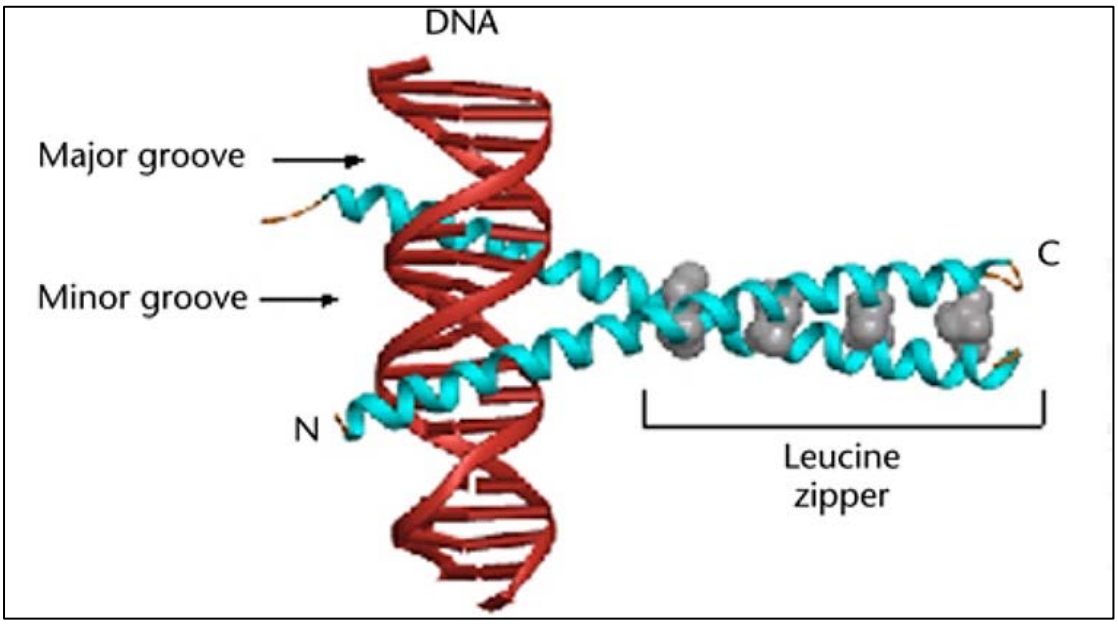

Fig. 6.1 X-ray structure of the B-ZIP dimer GCN4 bound to DNA. The DNAis in red, the a helices are in blue. The leucine position amino acids are shown in grey. The N-terminal and C-terminal parts of the protein are labelled as N and C (Krylov et al., 1998).

1.1.1 Rationale of the present study

Although, leucine zippers were first discovered in eukaryotic system, they are also found in prokaryotes like cyanobacterium *Anabaena* PCC 7120 and is found to be involved in protein-protein interactions (Koksharova and Wolk, 2002). Preliminary bioinformatics analysis suggests the presence of basic region of leucine zipper (bZIP) in XisA protein at extreme C-terminal region **(Fig. 6.2)** and absent in closely related XisC protein.

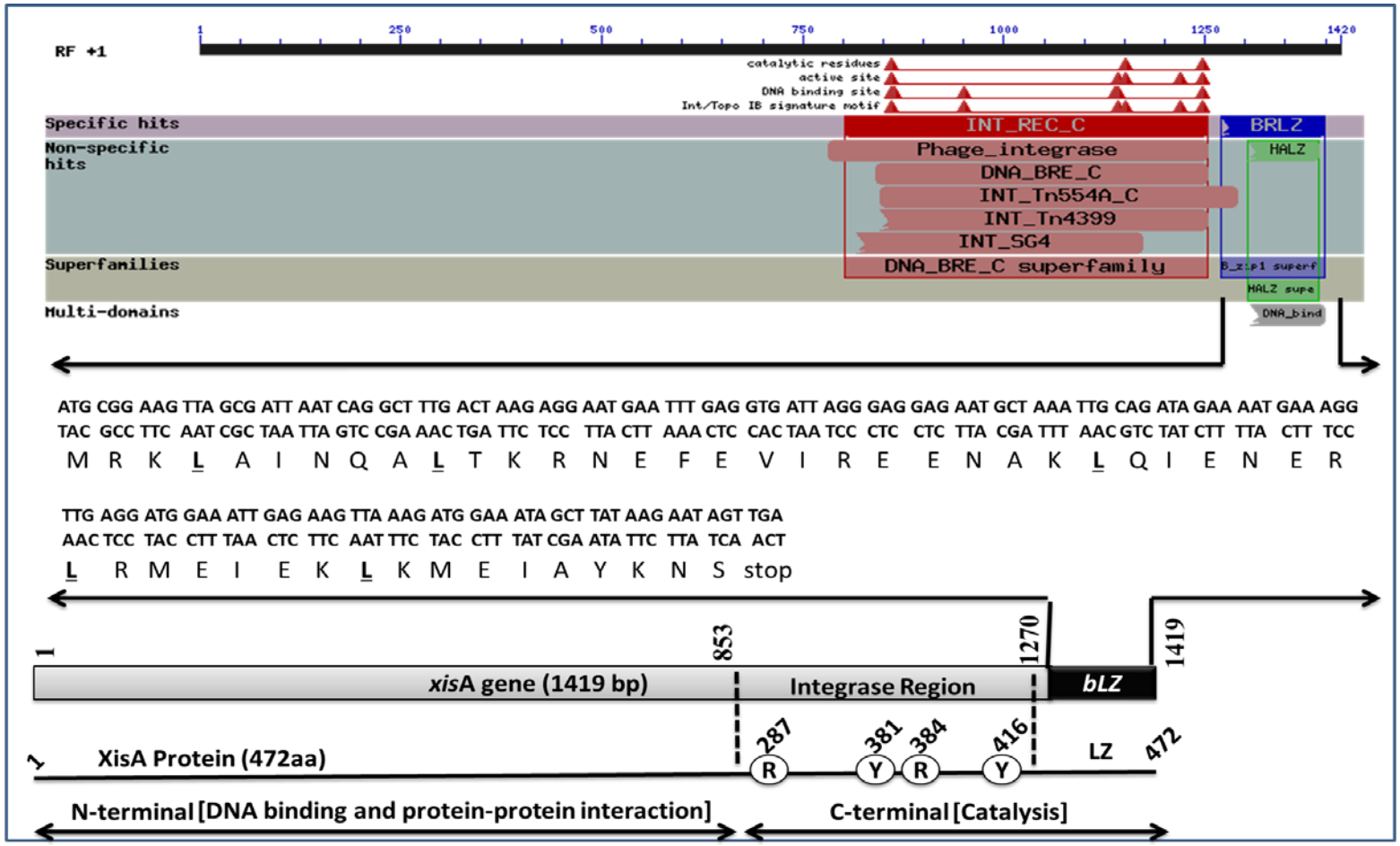

Fig. 6.2 Prediction of basic region of leucine zipper (bZIP) in *xisA* (NCBI conserved domain database).

The primary objective of this study was to determine functionality of XisA in absence of predicted bZIP region and in presence of standard jun leucine zipper jLZ with respect to recombinase and endonuclease activities.

1.2 Materials and methods

1.2.1 *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 6.1** and **Table 6.2,** respectively. Oligonucleotide primers synthesis was out sourced to 1st Base Pvt. ltd (Singapore) and are summarized in **Table 6.3.**

Table 6.1 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*Δ	pJet1.2 containing full length *xisA*Δ gene	This Study
pJET*xisA*1Δ	pJet1.2 containing full length *xisA*1Δ gene	This Study
pJET*xisA*5Δ	pJet1.2 containing full length *xisA*5Δ gene	This Study
pJET*xisA*6Δ	pJet1.2 containing full length *xisA*6Δ gene	This Study
pET28a (+)	T7 promoter based expression vector, Kan^R	EMD Bioscience, Darmstadt.
p*xisA*Δ	pET28a (+) containing 1.2 kb predicted leucine zipper deleted *xisA*Δ gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*1Δ	pET28a (+) containing 1.1 kb predicted leucine zipper deleted *xisA*1Δ gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*5Δ	pET28a (+) containing 0.6 kb predicted leucine zipper deleted *xisA*5Δ gene fragment under pT7 promoter, Kan^R	This Study

p*xisA*6Δ	pET28a (+) containing 0.4 kb predicted leucine zipper deleted *xisA*6Δ gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*j	pET28a (+) containing 1.3 kb *jun* leucine zipper complimented *xisA*j gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*1j	pET28a (+) containing 1.2 kb *jun* leucine zipper complimented *xisA*1j gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*5j	pET28a (+) containing 0.6 kb *jun* leucine zipper complimented *xisA*5j gene fragment under pT7 promoter, Kan^R	This Study
p*xisA*6j	pET28a (+) containing 0.5 kb *jun* leucine zipper complimented *xisA*6j gene fragment under pT7 promoter, Kan^R	This Study

Table 6.2 *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
UN9	BL21 DE3 (p*xisA*Δ+ pAM461)	This Study
UN10	BL21 DE3 (p*xisA*1Δ+ pAM461)	This Study
UN11	BL21 DE3 (p*xisA*5Δ + pAM461)	This Study
UN12	BL21 DE3 (p*xisA*6Δ + pAM461)	This Study
UN13	BL21 DE3 (p*xisA*j + pAM461)	This Study
UN14	BL21 DE3 (p*xisA*1j + pAM461)	This Study
UN15	BL21 DE3 (p*xisA*5j + pAM461)	This Study
UN16	BL21 DE3 (p*xisA*6j + pAM461)	This Study

Table 6.3 Oligonucleotide primers used in the study

Name	Sequence
XisA(F)	5'-**GGATCC**ATGCAAAATCAGGGTCAA-3'
XisA1(F)	5'-**GGATCC**ATGAGAACAAAAGTACTACAAG-3'
XisA5(F)	5'-GC**GGATCC**TGGGCGAGATATAATGCC-3'
XisA6(F)	5'-GC**CATATG**GGTTTACGCCCCAGG-3'
$XisA_{bLZ}^{-}$(R)	5'-GC**GAGCTC**TCAATCTAGCGAGAACCAGC-3'
C(F)	5'- GTATCTCTCTACGCTTGCTGGTTGG-3'
C(R)	5'- ACCACCCACTACATCGATAACGCC-3'
X(R)	5'- TGCCGTCGGTAGATGAAAGTGGC-3'
***jun* LZ**	5'-GC**GAGCTC** CTG CAG GCT GAA ACT GAC CAA CTC GAA GAT GAA AAA ACC GCGCTT CAG ACC GAA ATT GCA AAC CTC CTG AAA GAG AAG GAA AAA CTG **AAGCTT**GC-3'
$XisA_{bLZ}^{-}$(jR)	5'-GC**GAGCTC**ATCTAGCGAGAACCAGC-3'

1.2.2 Strategy to design bLZ deletion products of *xisA* gene

Nucleotide sequence shaded in green corresponding to predicted bZIP region of XisA protein can be seen in **Fig. 6.2.** Color coded arrows represents various start sites used for PCR amplification of corresponding b*LZ* deleted products of *xisA* gene.

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

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

A PCR based approach was utilized to generate *xisA* gene *bLZ* deletion products of *xisA* gene. Nomenclature of *bLZ* deleted *xisA* gene products and encoded proteins with corresponding sizes is given in **Fig. 6.3**. *bLZ* deletion *xisA* gene products were PCR amplified from pMX25 vector as template using various combination of primers **(Table 6.4)**.

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

Cloning of *xisA* gene *bLZ* deletion products *xisA*Δ, *xisA*1Δ and *xisA*5Δ in pET28(a) was carried at *Bam*HI-*Sac*I sites similar to as described in section **3.2.2.1**

ATGCAAAATCAGGGTCAAGACAAATATCAACAAGCCTTTGCAGACTTAGAGCCACTTTCATCTACCGACGGCA
GTTTTCTCGGCTCAAGTCTGCAAGCACAGCAGCAAAGAGAACACATGAGAACAAAAGTACTACAAGACCTAG
ACAAGGTAAATCTGCGTTTGAAGTCTGCAAAGACGAAAGTCTCAGTTCGAGAATCTAACGGAAGTCTGCAATT
ACGAGCAACGTTACCAATTAAACCTGGAGATAAGGACACCAACGGTACAGGCAGAAAGCAATACAATCTCAG
CTTGAATATCCCTGCAAACTTGGATGGACTGAAGACGGCTGAGGAAGAAGCTTATGAATTAGGTAAATTAATC
GCTCGGAAAACCTTTGAATGGAATGATAAATATTTAGGCAAAGAAGCCACTAAAAAAGATTCACAAACAATA
GGTGATTTACTAGAAAAATTTGCAGAAGAGTATTTTAAAACCCATAAACGCACCACTAAAAGCGAACATACCT
TTTTTTACTATTTTTCCCGCACCCAACGATATACCAATTCCAAAGATTTAGCAACGGCGGAAAATCTCATCAATT
CAATTGAGCAAATCGATAAAGAATGGCGCGAGATATAATGCCGCCAGAGCCATATCAGCTTTTTGCATAACATT
CAATATAGAAATTGATTTGTCCCAGTATTCCAAAATGCCTGATCGCAATTCGCGCAACATCCCCACAGATGCAG
AAATACTATCAGGAATTACCAAATTTGAAGACTATCTAGTTACCAGAGGAAATCAAGTTAATGAAGATGTAAA
AGATAGCTGGCAACTTTGGCGCTGGACATATGGAATGTTAGCAGTTTTTGGTTTACGCCCCAGGGAAATTTTT
ATTAACCCTAATATTGATTGGTGGTTAAGCAAAGAGAATATAGACCTCACATGGAAAGTAGACAAAGAATGTA
AAACTGGTGAAAGACAAGCATTACCCTTACATAAAGAATGGATTGATGAGTTTGATTTAAGAAATCCGAAATA
TTTAGAAATGCTGGCAACAGCAATTAGTAAAAAAGATAAAACAAATCATGCTGAAATAACAGCCTTAACTCAG
CGTATTAGTTGGTGGTTTCGGAAAGTCGAATTAGATTTTAAACCCTATGATTTACGTCACGCCTGGGCAATTAG
AGCGCATATTTTAGGCATACCAATCAAAGCGGCGGCTGATAATTTGGGGCATAGTATGCAGGTTCATACACAA
ACCTATCAGCGCTGGTTCTCGCTAGATATGCGGAAGTTAGCGATTAATCAGGCTTTGACTAAGAGGAATGAA
TTTGAGGTGATTAGGGAGGAGAATGCTAAATTGCAGATAGAAAATGAAAGGTTGAGGATGGAAATTGAG
AAGTTAAAGATGGAAATAGCTTATAAGAATAGTTGA

Fig. 6.3: *xisA* nucleotide sequence displaying predicted *bLZ* region (shaded in green).

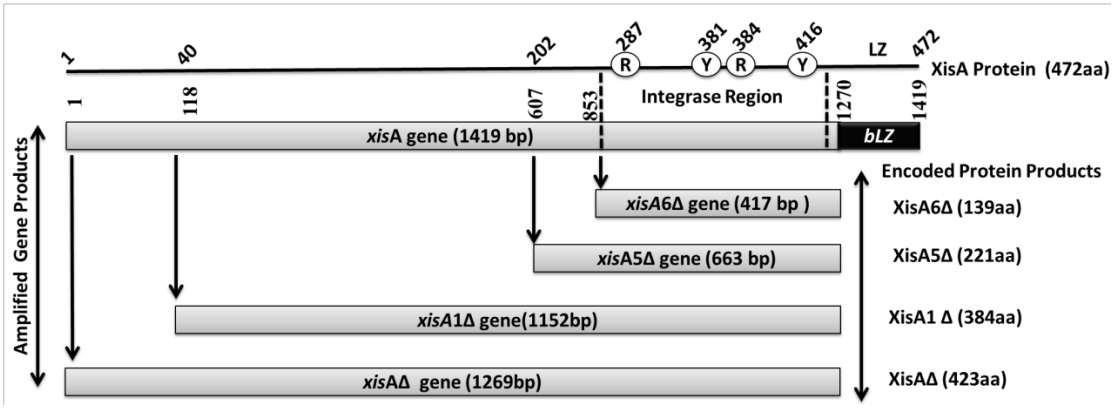

Fig. 6.4: Schematic representation of *xisA* gene *bLZ* deletion products.

Table 6.4: Combinations of primers used in amplification of *xisA bLZ* deletion products.

Gene Name	Name of Forward Primer	Name of Reverse Primer	Size of the amplicons (bp)
***xisA*Δ**	**XisA(F)**	**$XisA_{bLZ}^{-}$(R)**	**1419**
***xisA*1Δ**	**XisA1(F)**		**1302**
***xisA*5Δ**	**XisA5(F)**		**813**
***xisA*6Δ**	**XisA6(F)**		**567**

and 3.2.2.2. *xisA*6Δ was cloned in pET28a in similar fashion at *Nde*I-*Sac*I site **(Fig. 6.5)**.

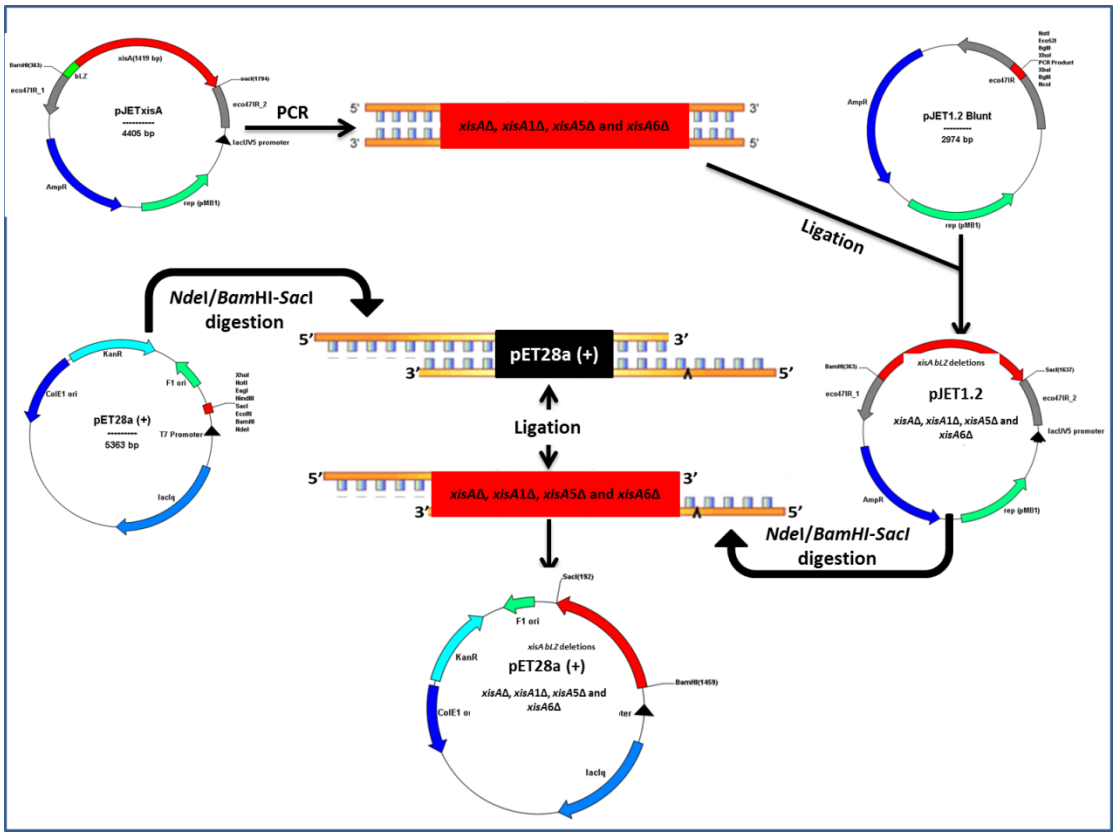

Table 6.5 Schematic representation of cloning *bLZ* deletion products of *xisA* gene in pET28(a).

Nomenclature and sizes of recombinant vectors containing N-terminal truncated *xisA* gene products is given in **Fig.2.2a** and **Table 6.1**.

1.2.4 Construction of expression vectors of jun leucine zipper (*jLZ*) complementation chimer of *xisA* gene *bLZ* deletion products

In order to verify the functionality of bLZ region of XisA, the *bLZ* deleted products of *xisA* gene were complimented with jun lucine zipper sequence (*jLZ*) (**Fig. 6.5)**. Synthesis of double stranded *jLZ* fragment was outsourced to Saffron Lifesciences pvt ltd, Gujarat (India).

1.2.4.1 PCR amplification *bLZ* deletion gene products lacking stop codon

Previously, reverse primer **$XisA_{bLZ}^{-}$(R)** containing stop codon, was used for amplifying *bLZ* deletion mutants of *xisA*. In order to generate chimeric complementation of *jLZ* with *bLZ* deleted *xisA* gene, *bLZ* deletion of *xisA* was performed using reverse primer **$XisA_{bLZ}^{-}$(jR)** was used that lacked stop codon.

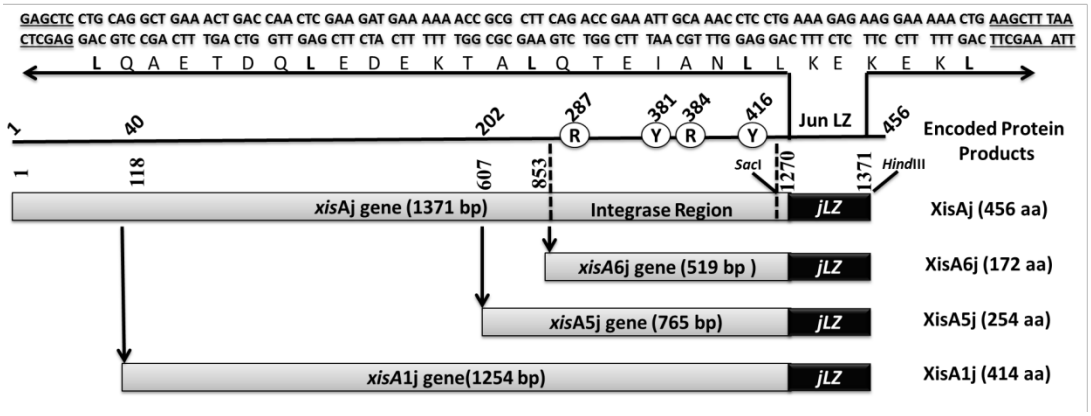

Fig. 6.5 Schematic representation of *jLZ* complemented to *xisA* gene *bLZ* deletion products.

1.2.4.2 Cloning of *jLZ* complemented *bLZ* deleted *xisA* gene products pET28(a)

Schematic representation of cloning *jLZ* complimented *bLZ* deleted *xisA* gene products is depicted in **Fig. 6.6.** Complementation and cloning was done in two steps. Various *bLZ* deleted *xisA* gene products were amplified from pMX25 using forward

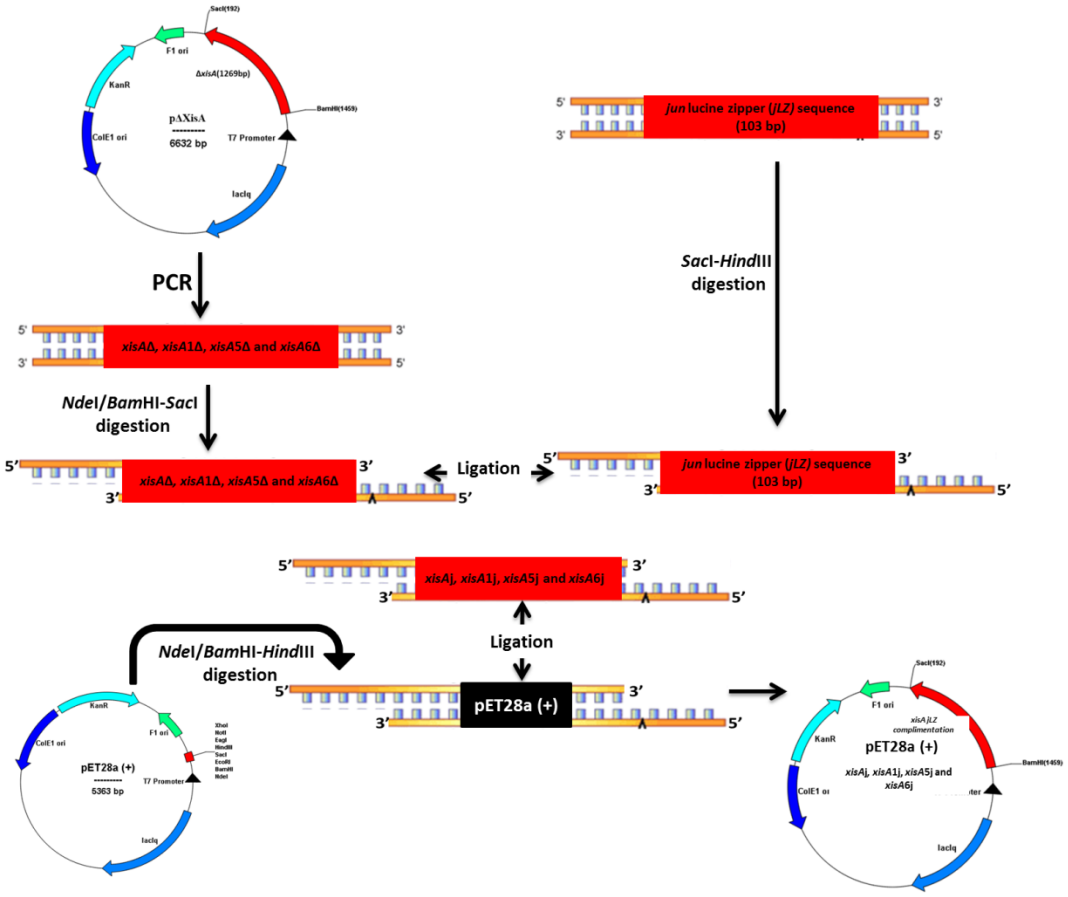

Fig. 6.6 Schematic representation of cloning *jLZ* complemented *bLZ* deleted products of *xisA* gene in pET28(a).

Primers described in **Table 6.4** and **$XisA_{bLZ}^{-}$(jR)** reverse primer. The amplicons were double digested with either *Bam*HI-*Sac*I (for *xisA*Δ, *xisA*1Δ and *xisA*5Δ) or *Nde*I-*Sac*I (for *xisA*6Δ) and ligated with *Sac*I-*Hind*III double digested *jLZ* DNA. Ligated DNA was purified using PCR clean up kit (Thermo Scientific). This chimeric DNA retains *Nde*I/*Bam*HI-*Sac*I staggered ends which were utilized to further ligate in *Nde*I/*Bam*HI-*Sac*I double digested pET28a vector. Nomenclature of the recombinant pET28(a) containing *jLZ* complementation chimers of *bLZ* deleted *xisA* gene products is given in **Section 2.2c.** and **Table 6.1.**

1.2.5 Monitoring recombinase and endonuclease potential of bLZ deleted XisA protein products and their jLZ compliments

Detection and measure of recombinase potential of bLZ deleted XisA protein and their jLZ compliments was performed similar to procedure described in **Section 5.2.5** and **5.2.6**, respectively. Endonuclease potential was determined as described in **Section 5.2.7.**

1.3 Results

1.3.1 Cloning and *bLZ* deletion products of *xisA* gene in pET28a

pMX25 plasmid harboring *nifD* element was used as a template to amplify *bLZ* deletion variants of *xisA* gene **(Fig. 6.7a)**. Further, these variants of *xisA* gene were cloned in pJET1.2 and recombinant constructs were confirmed by restriction analysis by *Bgl*II digestions **(Fig. 6.7b).** 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*Δ, *pxisA*1Δ and p*xisA*5Δ) or *Nde*I-*Sac*I digestion (for *pxisA*6Δ) **(Fig. 6.7c & d).**

1.3.2 Cloning of *jLZ* complimented *bLZ* deleted products of *xisA* gene in pET28a

xisA geneproducts deficient in *bLZ* were amplified using pMX25 template and using **$XisA_{bLZ}^{-}$(jR)** reverse primer which lacks stop codon **(6.7e)**. The chimeric *xisA* gene complements were ligated with *jLZ* and directly cloned in pET28(a) and confirmed by *Sac*I-*Hind*III double digestion **(Fig. 6.7f)**.

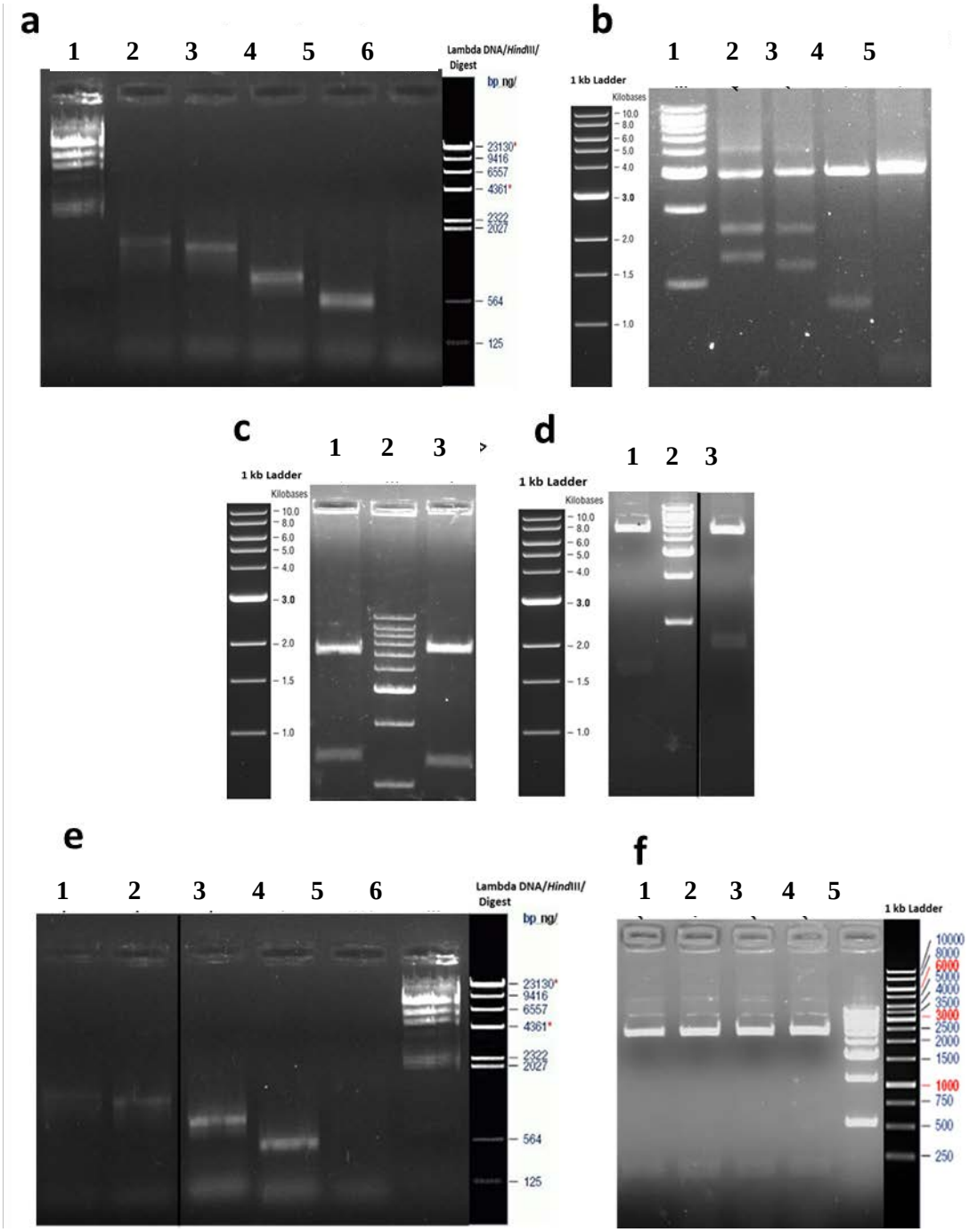

Fig. 6.7 Amplification and cloning of *bLZ* deleted and *jLZ* complimented *xisA* gene products. (a) Agarose gel analysis of amplified *bLZ* deleted *xisA* gene products. Lane 1: Lambda DNA *Hind*III digest; Lane 2-5: *xisA*Δ, *xisA*1Δ, *xisA*5Δ and *xisA*6Δ ; Lane 6: No template control (NTC) (b) *Bgl*II digestion pattern of *bLZ* deleted *xisA* gene products cloned in pJET 1.2. (c) Lane 1: 1 kb ladder; Lane 2-5: *Bgl*II digested pJET*xisA*Δ, pJET*xisA*1Δ, pJET*xisA*5Δ and pJET*xisA*6Δ. (c) Restriction Digestion analysis of *bLZ* deleted *xisA* products in pET28a. Lane 1 and 3: *BamH*I-*Sac*I double digestion of p*xisA*Δ and *pxis*A1Δ; Lane 2: 1 kb ladder *(*d) Restriction Digestion analysis of *bLZ* deleted *xisA* products in pET28a. Lane 1: *Nde*I-*Sac*I double digestion of p*xisA*6Δ. Lane 2: 1 kb ladder; Lane 3: *Bam*HI-*Sac*I digestion of *pxis*A5Δ. (e) Agarose gel analysis of amplified *bLZ* deleted *xisA* gene products without stop codon. Lane 1-4: *xisA*j, *xisA*1j, *xisA*5j and *xisA*6j ; Lane 5: No template control (NTC); Lane 6: Lambda DNA *Hind*III digest. (f) *Sac*I-*Hind*III digestion pattern of *jLZ* complimented to *bLZ* deletion *xisA* gene products cloned in pET28a. Lane 1-4:*Sac*I- *Hind*III digested p*xisA*j, p*xisA*1j, *pxisA*5j and p*xisA*6j; Lane 5: 1 kb ladder.

1.3.3 Effect of *bLZ* deletion and *jLZ* complementation on recombinase and endonuclease activities of XisA.

1.3.3.1 Monitoring recombinase and endonuclease potential of bLZ deleted XisA protein products

PCR based recombinase and antibiotic susceptibility based endonuclease assay of *E. coli* strains synthesizing bLZ deleted products of XisA protein suggests that none of the strains (UN9, UN10, UN11 and UN12) demonstrated either recombinase **(6.8a)** or endonuclease **(Fig. 6.8c)** activities.

1.3.3.2 Monitoring recombinase and endonuclease potential of jLZ complemented XisA protein products deficient in bLZ

PCR based recombinase and antibiotic susceptibility based endonuclease assay of *E. coli* strains synthesizing jLZ complemented XisA protein products deficient in bLZ suggests strains UN13 and UN14 synthesizing proteins XisAj and XisA1j, respectively, were able to demonstrate both the recombinase (**Fig. 6.8b)** and endonuclease **(Fig.6.8c)** activity which are completely absent in their bLZ deletion counterpart XisAΔ and XisA1Δ. However, the activities of XisAj and XisAij were significantly lesser than complete XisA protein. Strains UN15 and UN16 synthesizing protein XisA5j and XisA6j, respectively, lacked recombinase activity but retained detectable endonuclease activity **(Fig. 6.8c)** which was completely absent in their bLZ deleted counterparts XisA5Δ and XisA6Δ.

1.4 Discussion

The leucine zippers are functionally involved in dimerization and DNA binding activity of a number eukaryotic transcription factors (Vinson et al., 1989). Although, first discovered in eukaryotes, a number of leucine zipper like domains are discovered in bacterial systems such as *Anabaena* PCC 7120 (Koksharova and Wolk, 2002) and are reported to be involved in protein-protein interactions. Leucine zipper deletion situated at C-terminal region in tyrosine hydroxylase converted the tetrameric form of the enzyme in to dimer (Vrana et al., 1994) suggesting its crucial role in the functionality of multimeric proteins. Fusion of leucine zipper motifs (bJun and bFos) of the AP-1 to cre fragments (alpha and beta, respectively) increased α

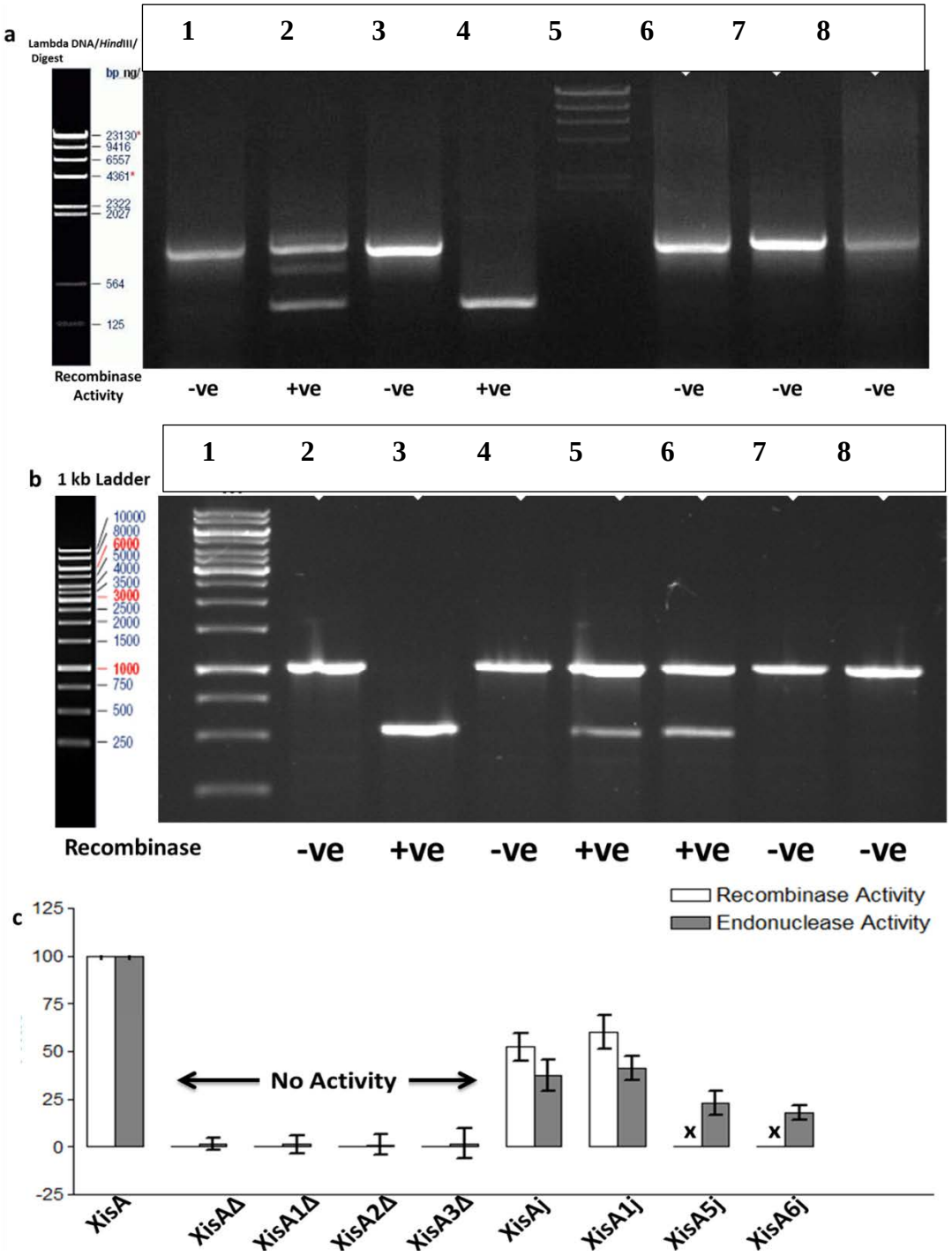

Fig. 6.8 *in vivo* functional characterization of bLZ deleted and jLZ complementation products of XisA protein. (a) Recombinase functionality detection of bLZ deletion products of XisA protein. Lane 1: 1 kb ladder; Lane 2: PCR analysis of UN1 (pET28a vector control); Lane 2 & 3: PCR analysis of UN2 & UN3 (Synthesizing unaltered XisA & XisA1); Lane: 4, 6-8: PCR analysis of strains synthesizing bLZ deleted products of XisA. (b) Recombinase functionality detection of jLZ complimented products of bLZ deleted XisA protein. Lane 1: 1 kb ladder; Lane 2: PCR analysis of UN1 (pET28a vector control); Lane 3: PCR analysis of UN3 (Synthesizing unaltered XisA1); Lane 4: PCR analysis of UN10 (synthesizing bLZ deleted XisA1); Lane 5-8: PCR analysis of strains synthesizing jLZ complimented products of bLZ deleted XisA protein. (c) Graphical representation of recombinase and endonuclease activity of bLZ deleted and jLZ complementation products of XisA protein. Activity by compete XisA protein is normalized to 100. Activity by altered XisA protein products are relative to complete XisA. X- Absence of recombinase activity. Values are expressed as Mean ±SEM.

complementation by facilitating reassociation of the fragments which resulted in two times more efficient recombination system (Seidi et al., 2009).

In chapter 5, we described that both the endonuclease and recombinase activities are present at C-terminal region of XisA. Upon removal of complete N-terminal, XisA protein lost recombinase potential, however retained significant amount of endonuclease activity. Preliminary bioinformatics analysis predicted a leucine zipper like domain at extreme C-terminal region. Hence it was interesting to investigate the functional role of this predicted bLZ in recombinase and endonuclease activities of XisA. bLZ deleted mutants of XisA protein Lacked both endonuclease and recombinase activity, which was regained upon complementation of jun leucine zipper (jLZ) suggesting the role of bLZ in dimer formation of XisA.