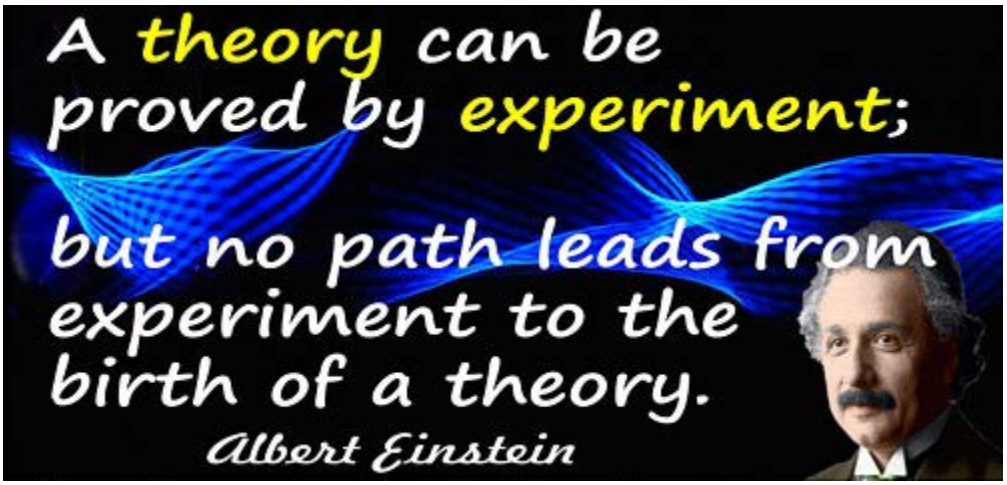


Chapter 2
Materials and Methods

4.1 Bacterial Strains / Plasmids

All the plasmids used in the present study are summarized in **Table 2.1.** Plasmid pMX25 and pAM461 were generous gifts from Dr. James W. Golden, Department of Biological Sciences, University of San Diego (USA) **(Fig. 2.1)**. pJET1.2 was used as intermediate cloning vector and pET28a was used to overexpress wild type and variant *xisA* genes. *E. coli* strains used in the present study are summarized in **Table 2.2.** *E. coli* DH5α was used for all the standard molecular biology experiments wherever required. *E. coli* BL21 DE3, Rosseta and pLysE were used for expression, purification and physiological studies of wild type and variant *xisA* gene constructs **(Fig. 2.2)**. All the oligonucleotide primers used in the study are summarized in **Table 2.3.**

4.2 Culture Condition

All microbiological media were purchased from HIMedia Laboratories, Mumbai, India. *E. coli* strains were routinely grown in Luria Bertani (LB) medium at 37 ^{o}C at 200 rpm, supplemented with appropriate antibiotics as follows: 100µg ml^{-1} Ampicillin, 50µg ml^{-1} Kanamycin, and 34 µg ml^{-1} Chloramphenicol. Bacterial growth was measured as OD_{600}.

Table 2.1 Plasmids used in the study.

Name	Characteristics	Reference
pMX25	*nifD* element with *lacZ* and Kan^R cloned in pBR322, Amp^R	(Lammers, Golden et al. 1986)
pAM461	Contains distal and proximal borders of *nifD* element, Amp_R	(Lammers, McLaughlin 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	Chapter 3

pJET*xisA*2	pJet1.2 containing full length *xisA*2 gene	Chapter 5
pJET*xisA*3	pJet1.2 containing full length *xisA*3 gene	Chapter 5
pJET*xisA*4	pJet1.2 containing full length *xisA*4 gene	Chapter 5
pJET*xisA*5	pJet1.2 containing full length *xisA*5 gene	Chapter 5
pJET*xisA*6	pJet1.2 containing full length *xisA*6 gene	Chapter 5
pJET*xisA*Δ	pJet1.2 containing full length *xisA*Δ gene	Chapter 6
pJET*xisA*1Δ	pJet1.2 containing full length *xisA*1Δ gene	Chapter 6
pJET*xisA*5Δ	pJet1.2 containing full length *xisA*5Δ gene	Chapter 6
pJET*xisA*6Δ	pJet1.2 containing full length *xisA*6Δ gene	Chapter 6
pET28a (+)	T7 promoter based expression vector, Kan^R	EMD Bioscience, Darmstadt.
p*xisA*	pET28a (+) containing full length *xisA* gene under pT7 promoter, Kan^R	Chapter 3 & 5
***pxisA*1**	pET28a (+) containing 1.3 kb N-truncated *xisA*1 gene fragment under pT7 promoter, Kan^R	Chapter 5
p*xisA*2	pET28a (+) containing 1.1 kb N-truncated *xisA*2 gene fragment under pT7 promoter, Kan^R	Chapter 5
p*xisA*3	pET28a (+) containing 1.0 kb N-truncated *xisA*3 gene fragment under pT7 promoter, Kan^R	Chapter 5
p*xisA*4	pET28a (+) containing 0.9 kb N-truncated *xisA*4 gene fragment under pT7 promoter, Kan^R	Chapter 5
p*xisA*5	pET28a (+) containing 0.8 kb N-truncated *xisA*5 gene fragment under pT7 promoter, Kan^R	Chapter 5
p*xisA*6	pET28a (+) containing 0.5 kb N-truncated *xisA*6 gene fragment under pT7 promoter, Kan^R	Chapter 5
p*xisA*Δ	pET28a (+) containing 1.2 kb predicted leucine zipper deleted *xisA*Δ gene fragment under pT7 promoter, Kan^R	Chapter 5
p*xisA*1Δ	pET28a (+) containing 1.1 kb predicted leucine zipper deleted *xisA*1Δ gene fragment under pT7 promoter, Kan^R	Chapter 6
p*xisA*5Δ	pET28a (+) containing 0.6 kb predicted leucine zipper deleted *xisA*5Δ gene fragment under pT7 promoter, Kan^R	Chapter 6

p*xisA*6Δ	pET28a (+) containing 0.4 kb predicted leucine zipper deleted *xisA*6Δ gene fragment under pT7 promoter, Kan^R	Chapter 6
p*xisA*j	pET28a (+) containing 1.3 kb *jun* leucine zipper complimented *xisA*j gene fragment under pT7 promoter, Kan^R	Chapter 6
p*xisA*1j	pET28a (+) containing 1.2 kb *jun* leucine zipper complimented *xisA*1j gene fragment under pT7 promoter, Kan^R	Chapter 6
p*xisA*5j	pET28a (+) containing 0.6 kb *jun* leucine zipper complimented *xisA*5j gene fragment under pT7 promoter, Kan^R	Chapter 6
p*xisA*6j	pET28a (+) containing 0.5 kb *jun* leucine zipper complimented *xisA*6j gene fragment under pT7 promoter, Kan^R	Chapter 6
pXisA1R248C	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1R248C, Kan^R	Chapter 7
pXisA1Y342H	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y342H, Kan^R	Chapter 7
pXisA1Y342F	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y342F, Kan^R	Chapter 7
pXisA1Y342A	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y342A, Kan^R	Chapter 7
pXisA1R345K	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1R345K, Kan^R	Chapter 7
pXisA1R345A	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1R345A, Kan^R	Chapter 7
pXisA1Y377F	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y377F, Kan^R	Chapter 7

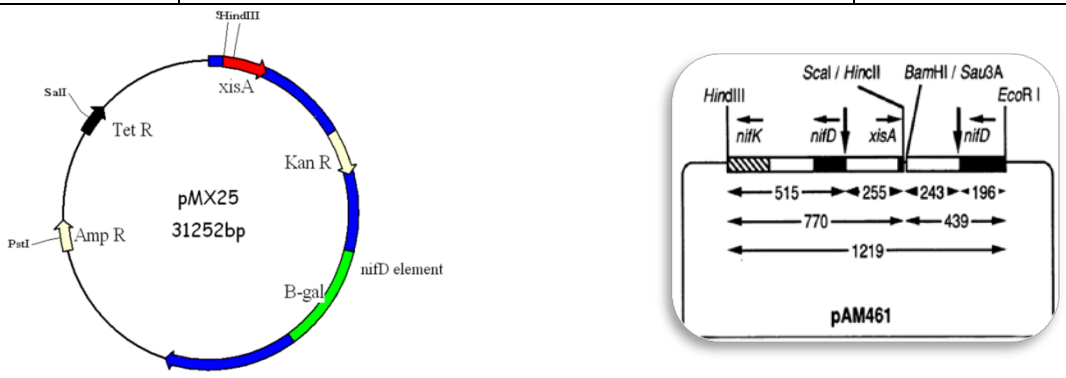
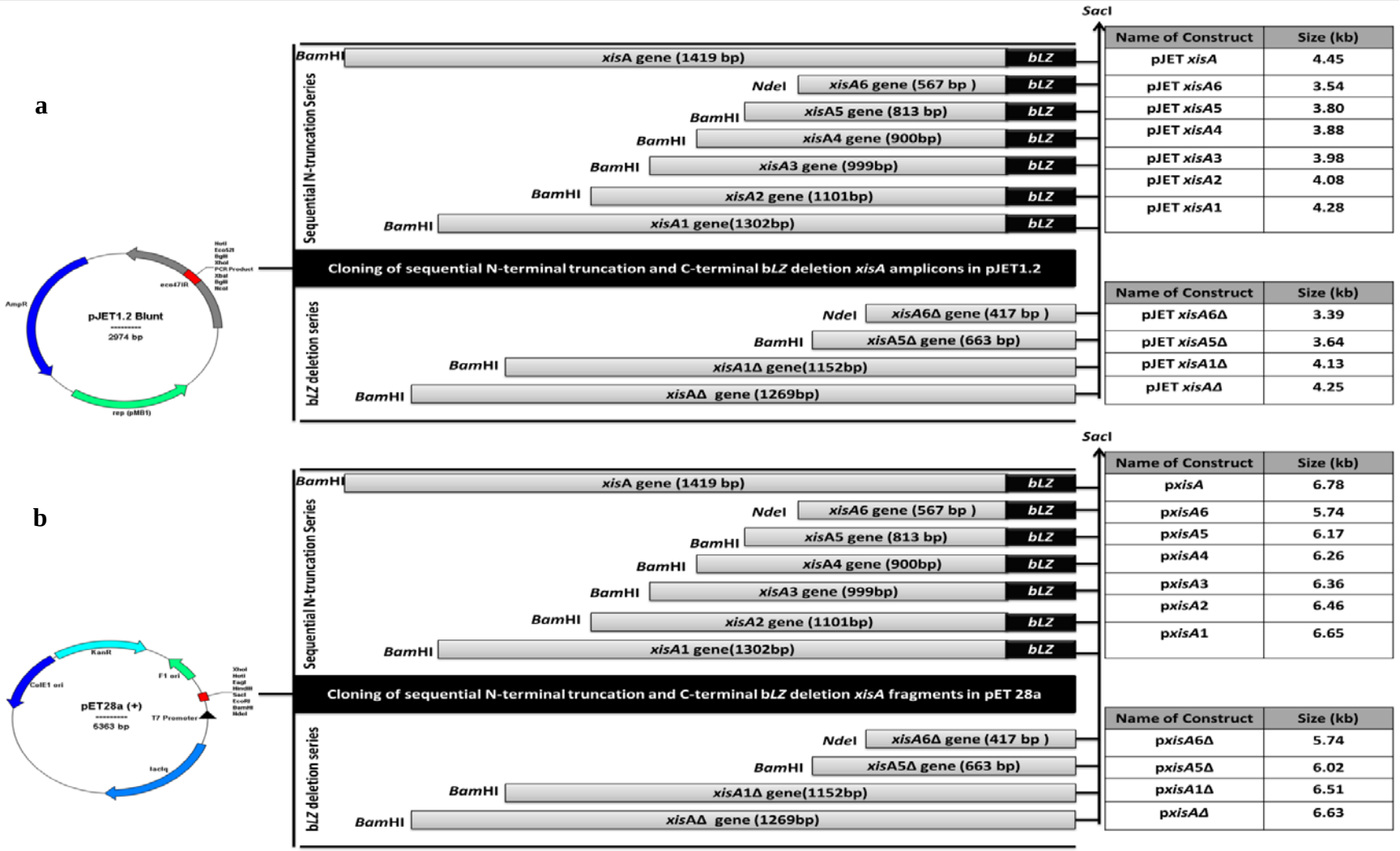
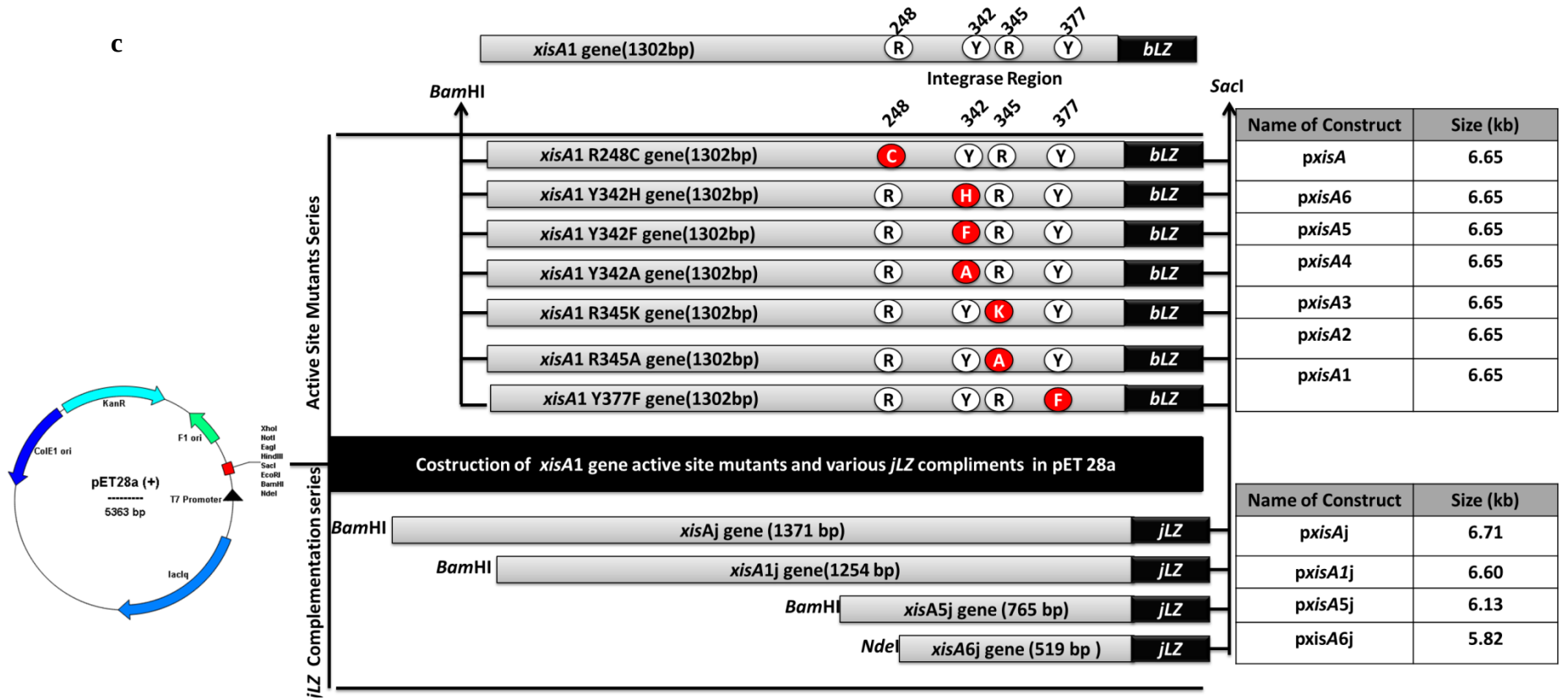


Fig. 2.1 Plasmid gifted by Prof. James. W. Golden, Department of Biological Sciences UC San Diego (USA).


a
pJET1.2 Blunt
2974 bp
AmpR
rep (pMB1)
eco47IR
Sequential N-truncation Series
BamHI
xisA gene (1419 bp)
bLZ
NdeI
xisA6 gene (567 bp)
BamHI
xisA5 gene (813 bp)
BamHI
xisA4 gene (900bp)
BamHI
xisA3 gene (999bp)
BamHI
xisA2 gene (1101bp)
BamHI
xisA1 gene(1302bp)
Cloning of sequential N-terminal truncation and C-terminal bLZ deletion xisA amplicons in pJET1.2
bLZ deletion series
NdeI
xisA6Δ gene (417 bp)
BamHI
xisA5Δ gene (663 bp)
BamHI
xisA1Δ gene(1152bp)
BamHI
xisAΔ gene (1269bp)
SacI
Name of Construct
Size (kb)
pJET xisA
4.45
pJET xisA6
3.54
pJET xisA5
3.80
pJET xisA4
3.88
pJET xisA3
3.98
pJET xisA2
4.08
pJET xisA1
4.28
Name of Construct
Size (kb)
pJET xisA6Δ
3.39
pJET xisA5Δ
3.64
pJET xisA1Δ
4.13
pJET xisAΔ
4.25
b
pET28a (+)
6363 bp
KanR
ColE1 ori
F1 ori
T7 Promoter
lacIq
Sequential N-truncation Series
BamHI
xisA gene (1419 bp)
bLZ
NdeI
xisA6 gene (567 bp)
BamHI
xisA5 gene (813 bp)
BamHI
xisA4 gene (900bp)
BamHI
xisA3 gene (999bp)
BamHI
xisA2 gene (1101bp)
BamHI
xisA1 gene(1302bp)
Cloning of sequential N-terminal truncation and C-terminal bLZ deletion xisA fragments in pET 28a
bLZ deletion series
NdeI
xisA6Δ gene (417 bp)
BamHI
xisA5Δ gene (663 bp)
BamHI
xisA1Δ gene(1152bp)
BamHI
xisAΔ gene (1269bp)
SacI
Name of Construct
Size (kb)
pxisA
6.78
pxisA6
5.74
pxisA5
6.17
pxisA4
6.26
pxisA3
6.36
pxisA2
6.46
pxisA1
6.65
Name of Construct
Size (kb)
pxisA6Δ
5.74
pxisA5Δ
6.02
pxisA1Δ
6.51
pxisAΔ
6.63



Name of Construct	Size (kb)
p*xisA*	6.65
p*xisA6*	6.65
p*xisA5*	6.65
p*xisA4*	6.65
p*xisA3*	6.65
p*xisA2*	6.65
p*xisA1*	6.65

Name of Construct	Size (kb)
p*xisAj*	6.71
p*xisA1j*	6.60
p*xisA5j*	6.13
pxis*A6j*	5.82

Fig. 2.2 Restriction maps of Plasmid used in the study. (a) **pJET1.2 vector and derived sequential N-terminal truncation and C-terminal *bLZ* deletion *xisA* gene constructs;** (b) **pET28a vector and derived sequential N-terminal truncation and C-terminal *bLZ* deletion *xisA* gene constructs;** (c) **pET28a vector derived *xisA*1 gene active site mutants and *j*LZ complementation constructs.**

Table 2.2 *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)
BL21 DE3 pLysE	*F- ompT gal dcm lon hsdSB (rB- mB-) λ(DE3) pLysS(cmR)*	(Moffatt and Studier 1987)
Rosetta(DE3) pLysS	*F⁻ ompT hsdS$_B$(R_B^- m_B^-) gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)*	Novagen
UN1	BL21 DE3 (pET28a + pAM461)	Chapter 5
UN2	BL21 DE3 (p*xisA* + pAM461)	Chapter 5
UN3	BL21 DE3 (p*xisA*1 + pAM461)	Chapter 5
UN4	BL21 DE3 (p*xisA*2 + pAM461)	Chapter 5
UN5	BL21 DE3 (p*xisA*3+ pAM461)	Chapter 5
UN6	BL21 DE3 (p*xisA*4 + pAM461)	Chapter 5
UN7	BL21 DE3 (p*xisA*5+ pAM461)	Chapter 5
UN8	BL21 DE3 (p*xisA*6+ pAM461)	Chapter 5
UN9	BL21 DE3 (p*xisA*Δ+ pAM461)	Chapter 6
UN10	BL21 DE3 (p*xisA*1Δ+ pAM461)	Chapter 6
UN11	BL21 DE3 (p*xisA*5Δ + pAM461)	Chapter 6
UN12	BL21 DE3 (p*xisA*6Δ + pAM461)	Chapter 6
UN13	BL21 DE3 (p*xisA*j + pAM461)	Chapter 6
UN14	BL21 DE3 (p*xisA*1j + pAM461)	Chapter 6
UN15	BL21 DE3 (p*xisA*5j + pAM461)	Chapter 6
UN16	BL21 DE3 (p*xisA*6j + pAM461)	Chapter 6
UN17	BL21 DE3 (pXisA1R248C + pAM461)	Chapter 7
UN18	BL21 DE3 (pXisA1Y342H + pAM461)	Chapter 7
UN19	BL21 DE3 (pXisA1Y342F + pAM461)	Chapter 7

UN20	BL21 DE3 (pXisA1Y342A + pAM461)	Chapter 7
UN21	BL21 DE3 (pXisA1R345K + pAM461)	Chapter 7
UN22	BL21 DE3 (pXisA1R345A + pAM461)	Chapter 7
UN23	BL21 DE3 (pXisA1Y377F + pAM461)	Chapter 7

Table 2.3 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'
XisA$_{bLZ}^{-}$(R)	5'-GC**GAGCTC**TCAATCTAGCGAGAACCAGC-3'
XisA1$_{R248C}$(F)	5'-AGCAGTTTTTGGTTTA**T**GCCCCAGGGAAATTT-3'
XisA1$_{R248C}$(R)	5'-AAATTTCCCTGGGGC**A**TAAACCAAAAACTGCT-3'
XisA1$_{Y342H}$(F)	5'-CGAATTAGATTTTAAACCC**C**ATGATTTACGTCACGCCTG-3'
XisA1$_{Y342H}$(R)	5'-CAGGCGTGACGTAAATCAT**G**GGGTTTAAAATCTAATTCG-3'
XisA1$_{Y342F}$(F)	5'-CGAATTAGATTTTAAACCCT**T**TGATTTACGTCACGCCTGG-3'
XisA1$_{Y342F}$(R)	5' CCAGGCGTGACGTAAATCA**A**AGGGTTTAAAATCTAATTCG-3'
XisA1$_{Y342A}$(F)	5'-GTCGAATTAGATTTTAAACCC**GCG**GATTTACGTCACGCCTGGGC-3'
XisA1$_{Y342A}$(R)	5'-GCCCAGGCGTGACGTAAATC**CGC**GGGTTTAAAATCTAATTCGAC-3'
XisA1$_{R345K}$(F)	5'-GATTTTAAACCCTATGATTTA**AAA**CACGCCTGGGCAATTAGAGCG-3'
XisA1$_{R345A}$(F)	5'-GATTTTAAACCCTATGATTTA**GCG**CACGCCTGGGCAATTAGAGCG-3'
XisA1$_{R345A}$(R)	5'-CGCTCTAATTGCCCAGGCGTG**CGC**TAAATCATAGGGTTTAAAATC-3'
XisA1$_{Y377F}$(F)	5'-GGTTCATACACAAACCT**T**TCAGCGCTGGTTCTCG-3'

XisA1$_{Y377F}$(R)	5'-CGAGAACCAGCGCTGA**A**AGGTTTGTGTATGAACC-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'

4.3 Molecular Biology Tools and Techniques

Routine molecular biology studies were carried out as described in **Table 2.3**. All enzymes for DNA manipulation were purchased from Thermo Fisher Scientific Waltham, Massachusetts, USA. Oligonucleotides were purchased from Integrated DNA Technologies, Coralville, Iowa, USA. Routine chemicals were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

Table 2.4 Routine molecular biology techniques during the present study

Technique	Reference
Polymerase Chain Reaction (PCR)	(Sambrook and Russell 2006)
Agarose Gel Electrophoresis	(Sambrook and Russell 2006)
Gel Elution	Sigma-Aldrich
***E. coli* Transformation**	(Sambrook and Russell 2006)
Plasmid Isolation	(Sambrook and Russell 2006)
Restriction Digestion	(Sambrook and Russell 2006)
Ligation	Thermo Fisher Scientific
SDS PAGE	(Sambrook and Russell 2006)
Protein Purification	GE Healthcare Life Sciences

4.4 DNA Sequencing

The DNA sequencing service was obtained from 1st Base pvt ltd (Singapore) for wild type *xisA* gene and derived variants.

4.5 Online Sequence and Structural Analysis

4.5.1 BLAST

The online computational tool "BLAST" for the DNA sequence alignments and homology search was used for acquiring *xisA* gene and translated XisA protein sequence.

4.5.2 Restriction Analysis

Restriction enzyme analysis for wild type and variant *xisA* genes was performed using NEB Cutter online tool.

4.5.3 Oligonucleotide Sequence Analysis

Integrated DNA Technologies online primer analysis tool was used to screen primers pairs used in amplification of wild type and variant *xisA* genes. Stringent screening parameters were applied to minimize primer-homodimer or heterodimer formation.

4.5.4 Theoretical modeling of XisA protein

Secondary structure prediction and 3D modelling of XisA protein was performed using online bioinformatics tools. To predict the secondary structure of XisA, the amino acid sequence was submitted to PSIPERD tool (Jones 1999; Buchan, Minneci et al. 2013). The domain architecture of XisA was analysed by submitting *xisA* gene nucleotide sequence at Conserved Domain Database tool of National Centre for Biotechnology Information (NCBI) (Marchler-Bauer, Derbyshire et al. 2015). The amino acid sequence of XisA was submitted to I-TASSER server to determine 3D structure (Zhang 2008; Roy, Kucukural et al. 2010; Yang, Yan et al. 2015). The output of I-TASSER was analysed using Pymol program (DeLano 2010). Confidence

score (C-score) and Template Modelling score (TM-score) are the parameters to rank the quality of the modelled structure. A C-score value typically rages from -0.2 to 5 and model with higher C-score describes model with higher confidence. A model with TM score >0.5 is considered to show significant topology with template homolog. XisA protein model with highest C-score and TM-score was selected for structural analysis.

4.6 Site Directed Mutagenesis

XisA1 protein active mutants **(Fig. 2.2c)** using Stratagene's Quick change site directed mutagenesis protocol (Liu and Naismith 2008). Details are described in Chapter 7.

4.7 Physiological Experiments

4.7.1 PCR Based Recombinase Assay

A novel PCR based approach was developed for monitoring wild type and variant XisA protein excisase activity using a substrate plasmid pAM461 containing only the distal and proximal borders of *nifD* element. Details are described in Chapter 4.

4.7.2 Endonuclease Assay

A modified protocol from Shah et al., 2007 was developed to monitor *in vivo* endonuclease activity of wild type and variant XisA proteins. Details are described in Chapter 5.

4.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 3.00 for Windows, GraphPad Software. The statistical significance of the data was determined using one-way analysis of variance (ANOVA). Data are presented as mean ± standard error of the mean (SEM). The results were considered statistically significant at $P<0.05$.