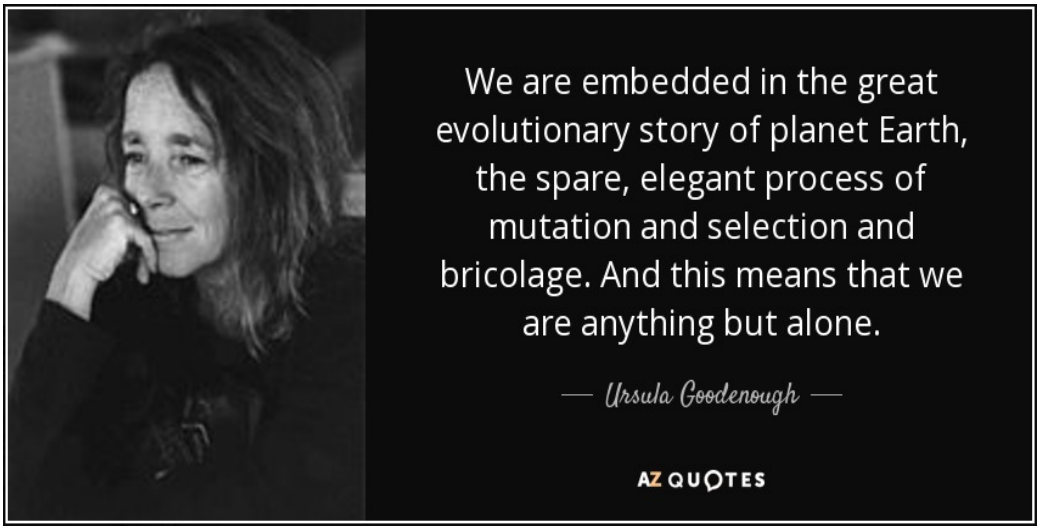

Chapter 7
Role of active site residues in recombinase and endonuclease activities of XisA protein

2.1 Introduction

XisA protein belongs to a member of tyrosine family of recombinases characterized by **R-(X)$_{70-139}$-H/Y-XX-R-(X)$_{29-38}$-Y** catalytic tetrad (Nunes-Duby et al., 1998). Tyrosine recombinases derive their name due to the presence of terminal tyrosine involved in phospho-tyrosine bond formation with sessile phosphate on target DNA during recombination (Grindley et al., 2006). Mutation in this terminal tyrosine results in an catalytically inactive recombinase (Gibb et al., 2010).

A majority of recombinase tyr possess R-H-R-Y active site tetrad. However, in case of *Anabaena* PCC XisA and XisC recombinases, the 2nd active site Histidine is replaced by a tyrosine (Grindley et al., 2006; Henson et al., 2008). Mutational studies with XisC active site residues **(Fig. 7.1)** suggested that mutation of terminal tyrosine (Y433) to phenylalanine or alanine led to loss in the recombinase potential

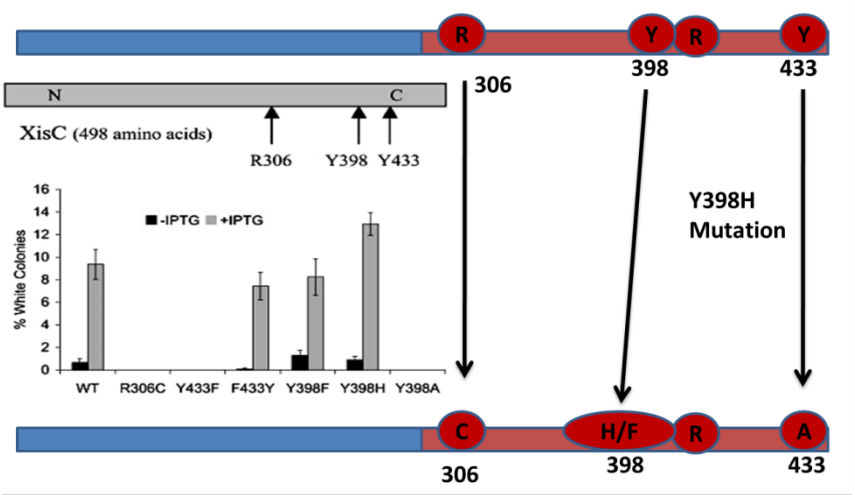

Fig. 7.1 Active site mutagenesis studies of *Anabaena* PCC 7120 XisC. Adapted and modified from Carrasco et al (2005).

of XisC. Mutation of secondary active site tyrosine (Y398) to histidine making it like other tyrosine recombinases, however led to significant increment in the recombinase activity of XisC (Carrasco et al., 2005).

*Eco*RII, a type IIE restriction endonuclease was found to possess **Int** domain of tyrosine recombinase along with PD-(D/E)XK domain shows significant similarity

to tyrosine recombinase members at C-terminal region **(Fig. 7.2)**. Mutagenesis of terminal tyrosine (Y308) to phenylalanine corresponding **Int** domain in *Eco*RII led to loss in the cleavage potential of the enzyme, a phenomenon similar to the tyrosine recombinases (Topal and Conrad, 1993).

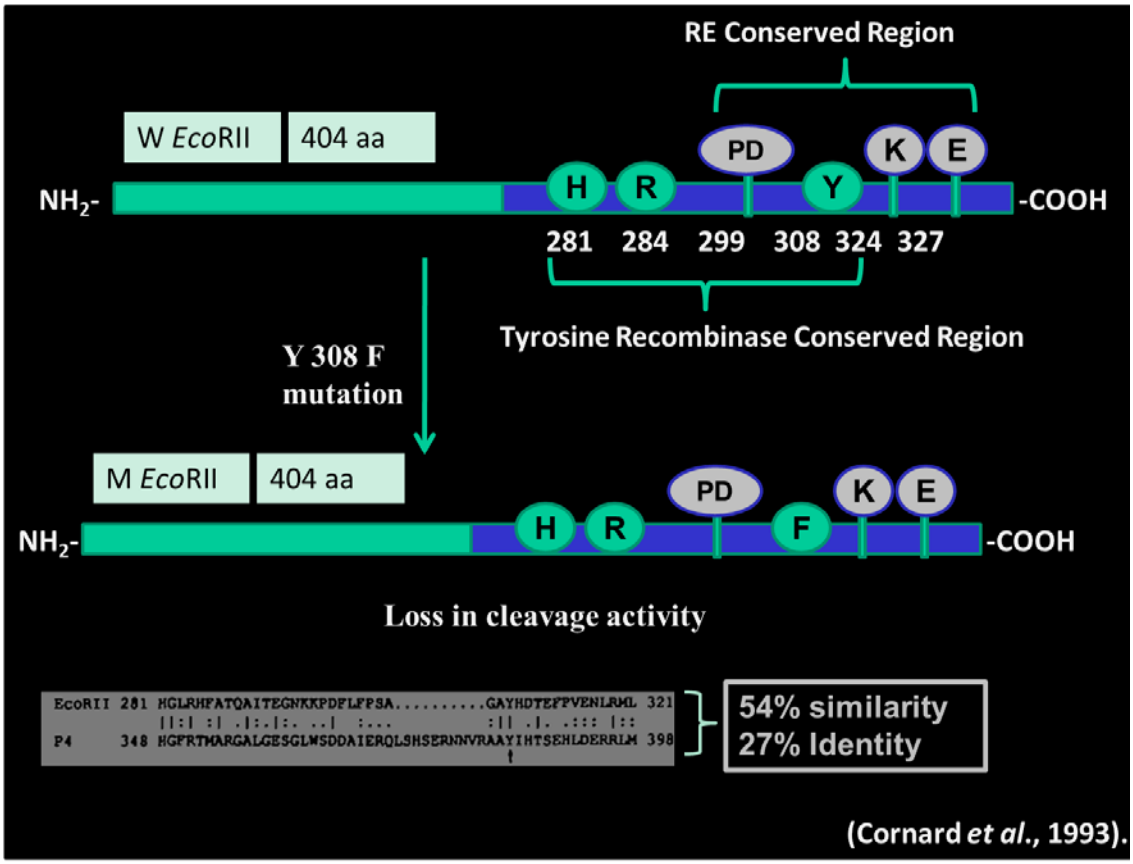

Fig. 7.2 Schematic representation of *Eco*RII Y308F causing loss in the cleavage activity of the enzyme.

Dependence of *Eco*RII cleavage activity on **Int** domain terminal tyrosine suggests it could be an evolutionary intermediate between recombinases and endonucleases.

2.1.1 Rationale of the study

The active site residues of XisA are situated at C-terminal region of the protein. XisA remained elusive for years and hence not been characterized for recombinase activity. Since XisA possesses both site specific recombinase and endonuclease activity (Shah et al., 2007) residing at C-terminal region **(Chapter 5)**, the preliminary aim of this study was to understand the involvement of XisA active site residues in for demonstrating recombinase and endonuclease activities.

2.2 Materials and methods

2.2.1 *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 7.1** and **Table 7.2,** respectively. Oligonucleotide primers synthesis was out sourced to 1st Base Pvt. ltd (Singapore) and are summarized in **Table 7.3.**

Table 7.1 Plasmids used in the study

Name	Characteristics	Reference
p*xisA*1	pET28a (+) containing 1.3 kb N-truncated *xisA*1 gene fragment under pT7 promoter, Kan^R	This Study
pAM461	Contains distal and proximal borders of *nifD* element, Amp_R	(Lammers et al., 1990)
pXisA1R248C	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1R248C, Kan^R	This study
pXisA1Y342H	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y342H, Kan^R	This study
pXisA1Y342F	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y342F, Kan^R	This study
pXisA1Y342A	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y342A, Kan^R	This study
pXisA1R345K	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1R345K, Kan^R	This study
pXisA1R345A	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1R345A, Kan^R	This study
pXisA1Y377F	pET28a (+) containing 1.3 kb mutant *xisA*1 gene fragment encoding for XisA1Y377F, Kan^R	This study

Table 7.2 *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)	Chapter 3

UN2	BL21 DE3 (p*xisA* + pAM461)	Chapter 3
UN3	BL21 DE3 (p*xisA*1 + pAM461)	Chapter 5
UN17	BL21 DE3 (pXisA1R248C + pAM461)	This Study
UN18	BL21 DE3 (pXisA1Y342H + pAM461)	This Study
UN19	BL21 DE3 (pXisA1Y342F + pAM461)	This Study
UN20	BL21 DE3 (pXisA1Y342A + pAM461)	This Study
UN21	BL21 DE3 (pXisA1R345K + pAM461)	This Study
UN22	BL21 DE3 (pXisA1R345A + pAM461)	This Study
UN23	BL21 DE3 (pXisA1Y377F + pAM461)	This Study

Table 7.3 Oligonucleotide primers used in the study.

Name	Sequence
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$_{R345K}$(R)	5'-CGCTCTAATTGCCCAGGCGTG**TTT**TAAATCATAGGGTTTAAAATC-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'

2.2.2 Strategy to design XisA1 protein active site mutants

Measuring recombinase and endonuclease potential of sequentially truncated XisA protein products suggested that XisA1 possessed maximum recombinase activity **(Chapter 5)**. Hence, p*xisA*1 was used as target for mutagenesis of active site residues **(Fig. 7.3).**

MRTKVLQDLDKVNLRLKSAKTKVSVRESNGSLQLRATLPIKP
GDKDTNGTGRKQYNLSLNIPANLDGLKTAEEEAYELGKLIAR
KTFEWNDKYLGKEATKKDSQTIGDLLEKFAEEYFKTHKRTTK
SEHTFFYYFSRTQRYTNSKDLATAENLINSIEQIDKEWARYNA
ARAISAFCITFNIEIDLSQYSKMPDRNSRNIPTDAEILSGITKFED
YLVTRGNQVNEDVKDSWQLWRWTYGMLAVFGLRPREIFINP
NIDWWLSKENIDLTWKVDKECKTGERQALPLHKEWIDEFDLR
NPKYLEMLATAISKKDKTNHAEITALTQRISWWFRKVELDFKP
YDLRHAWAIRAHILGIPIKAAADNLGHSMQVHTQTYQRWFSL
DMRKLAINQALTKRNEFEVIREENAKLQIENERLRMEIEKLKM
EIAYKNS

Fig. 7.3 Amino acid sequence of XisA1 protein with position of active site residues shaded in red.

2.2.3 Construction of vectors synthesizing XisA1 active site mutants

2.2.3.1 Site directed mutagenesis of p*xisA*1

Fig. 7.4 depicts schematic representation of *xisA*1 gene targets for active site mutagenesis of XisA1 protein. Active site mutagenesis of *xisA*1 gene cloned in *pxisA*1 was directly performed on the construct using combination of mutagenic primers as described in **Table 7.4.**

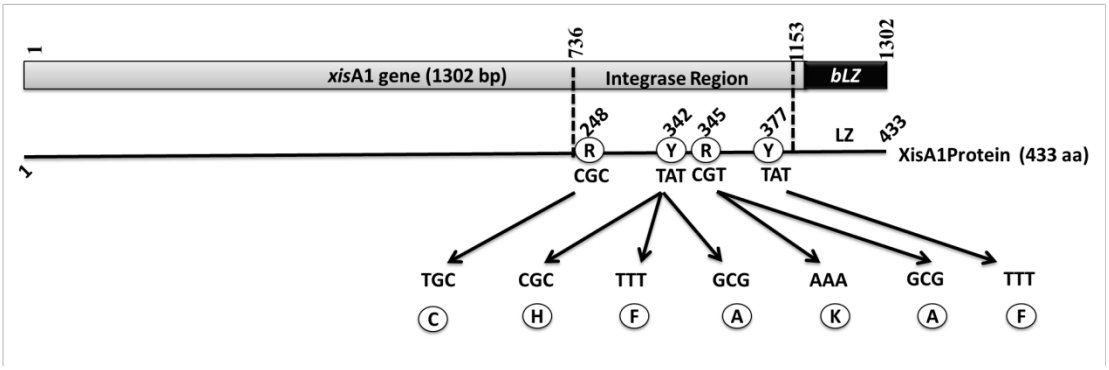

Fig. 7.4 Schematic representation of *xisA*1 gene targets used for active site mutagenesis of XisA1 protein.

Table 7.4 Combination of various mutagenic primers used for mutating p*xisA*1.

Name of Forward Primer	Name of Reverse Primer	PCR Template	PCR Product
XisA1$_{R248C}$(F)	**XisA1$_{R248C}$(R)**	**p*xisA*1**	**p*xisA*1R248C**
XisA1$_{Y342H}$(F)	**XisA1$_{Y342H}$(R)**		**p*xisA*1Y342H**
XisA1$_{Y342F}$(F)	**XisA1$_{Y342F}$(R)**		**p*xisA*1Y342F**
XisA1$_{Y342A}$(F)	**XisA1$_{Y342A}$(R)**		**p*xisA*1Y342A**
XisA1$_{R345K}$(F)	**XisA1$_{R345K}$(R)**		**p*xisA*1R345K**
XisA1$_{R345A}$(F)	**XisA1$_{R345A}$(R)**		**p*xisA*1R345A**
XisA1$_{Y377F}$(F)	**XisA1$_{Y377F}$(R)**		**p*xisA*1Y377F**

2.2.3.2 Mutagenesis of p*xisA*1

Site directed mutagenesis p*xisA*1 was performed according to single step Quick change site directed mutagenesis protocol. **Fig. 7.5** is the schematic representation of strategy used p*xisA*1 site directed mutagenesis. Amplified mutants of p*xisA*1 were analyzed on agarose gel**.** Mutants were confirmed by sequencing out sourced to 1st Base Pvt Ltd. (Singapore).

2.2.4 Expression p*xisA*1 mutants and purification of encoded active site mutants of XisA1 protein.

For the purpose of overexpression and purification, recombinant pET28(a) vectors containing mutant products of *xisA*1 gene were transferred in *E. coli* BL21 (DE3) according to Section **2.3**. Expression of mutant *xisA*1 gene and purification of encoded XisA1 site directed mutant products were carried out as described in **Section 3.2.5** and **3.2.6**.

2.2.5 Monitoring recombinase and endonuclease activities of active site mutants of XisA protein.

Detection and measure of recombinase potential of active site mutants of XisA1 protein 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.**

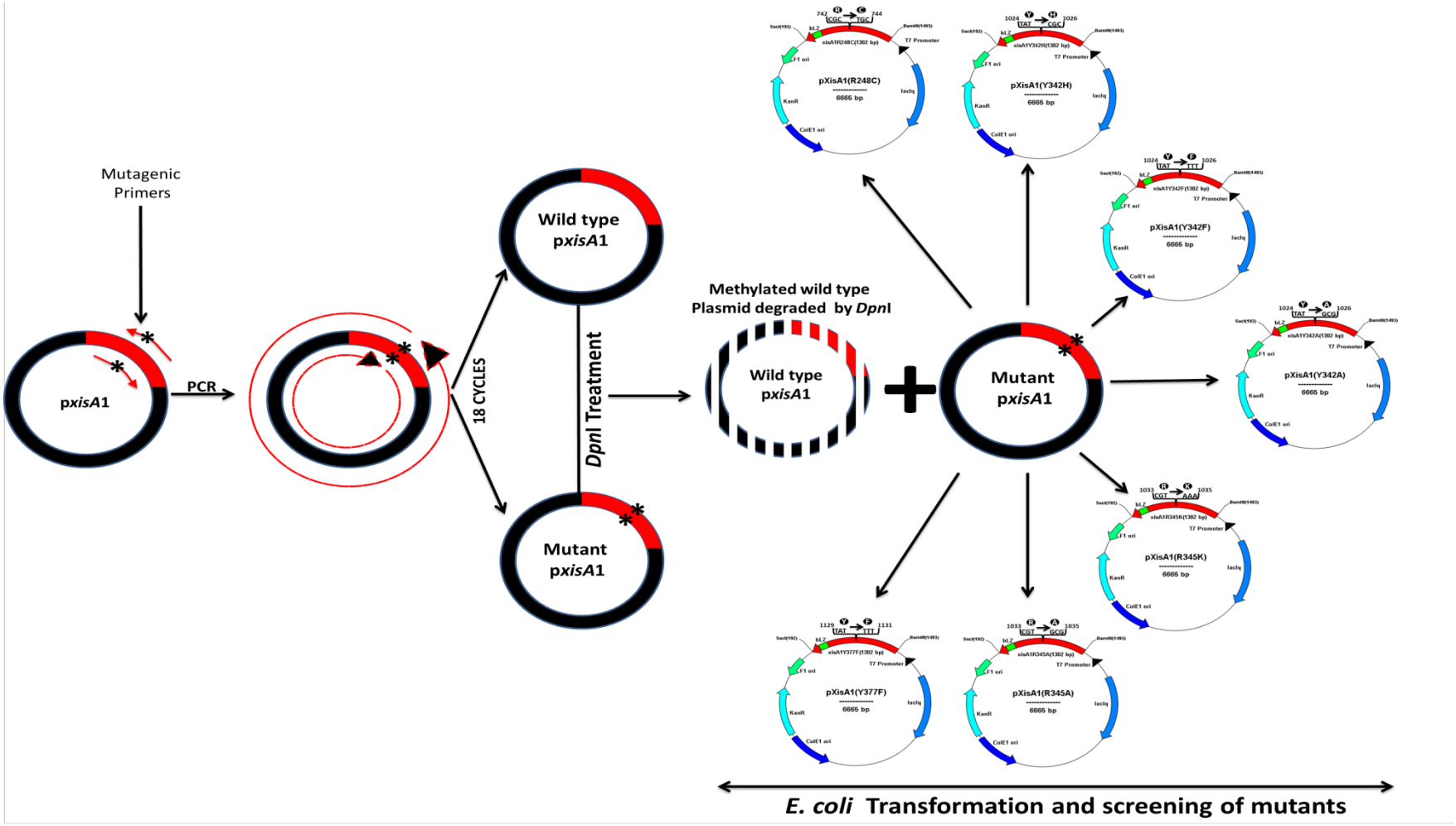

Fig. 7.5 Schematic representation of the strategy used for site directed mutagenesis of p*xisA*1.

2.3 Results

2.3.1 Site directed mutagenesis of XisA protein active site residues

p*xisA*1 was directly used as template for mutagenic PCR for generating mutant products of *xisA*1 gene. Resultant amplicons were analyzed on agarose gel **(Fig. 7.6)**

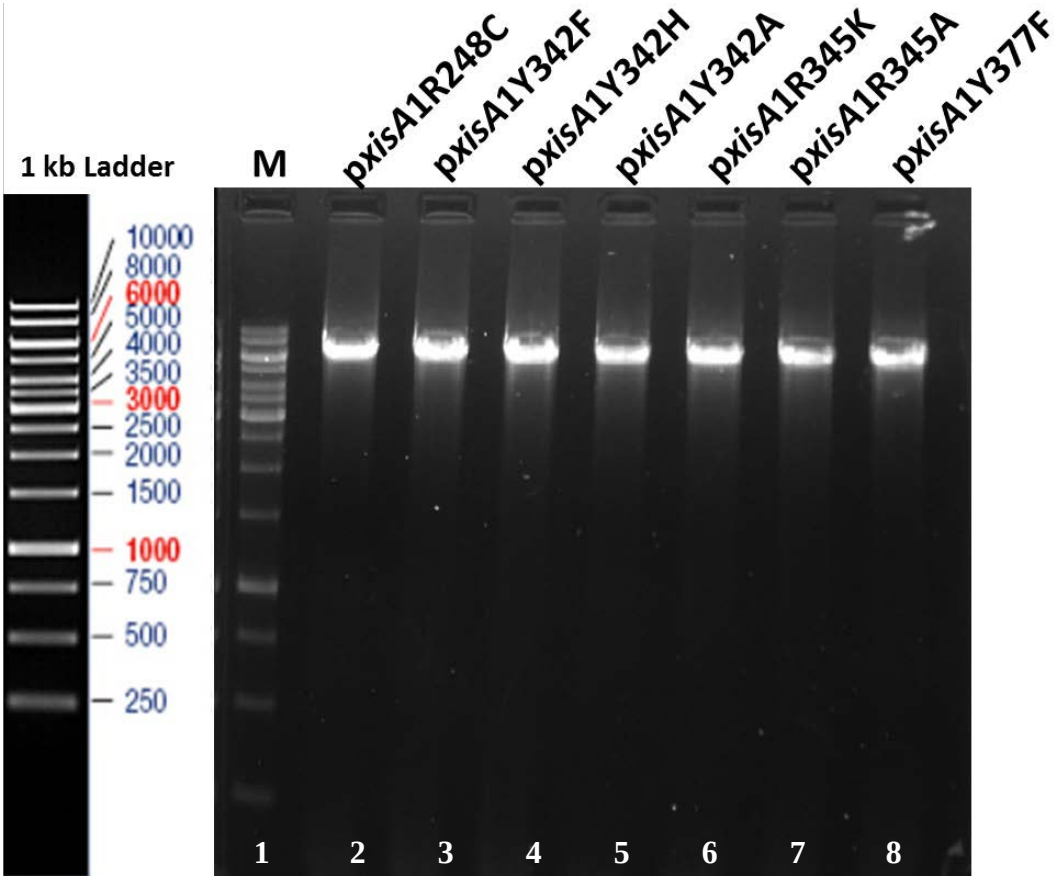

Fig. 7.6 Mutagenic PCR *xisA*1. Lane 1: 1kb ladder; Lanes 2-8: mutants of p*xisA*1

2.3.2 DNA sequencing to confirm *xisA*1 mutants

DNA sequencing of *xisA*1 mutants was outsourced to 1st Base Pvt. Ltd., (Singapore). Results of sequencing were analyzed using Chromas software. The chromatograms of various mutants are given in **Fig. 7.7** which compares wild type *xisA*1 and encoded XisA1 protein with various mutants.

2.3.3 Purification of XisA1 active site mutants

Purification of XisA1 active site mutants was carried out as described in S**ection 3.2.5.** Purified mutant proteins were analyzed on SDS PAGE **(Fig. 7.8).**

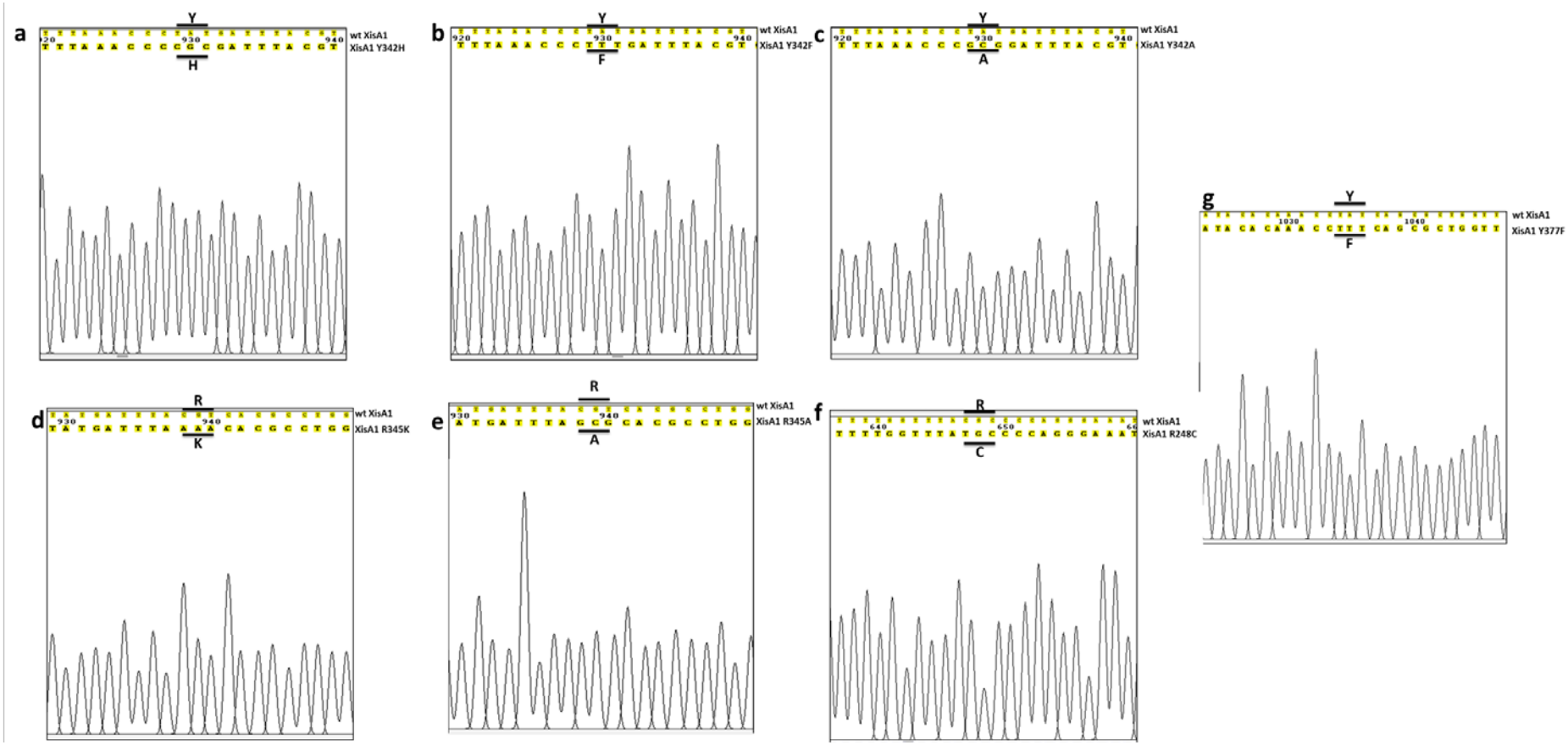

Fig. 7.7: Chromatograms of sequenced *xisA*1gene mutants. (a) *xisA*1Y342H. (b) *xisA*1Y342F. (c) *xisA*1Y342A. (d) *xisA*1R345K. (e) *xisA*1R345A. (f) *xisA*1R345C. (g) *xisA*1Y377F.

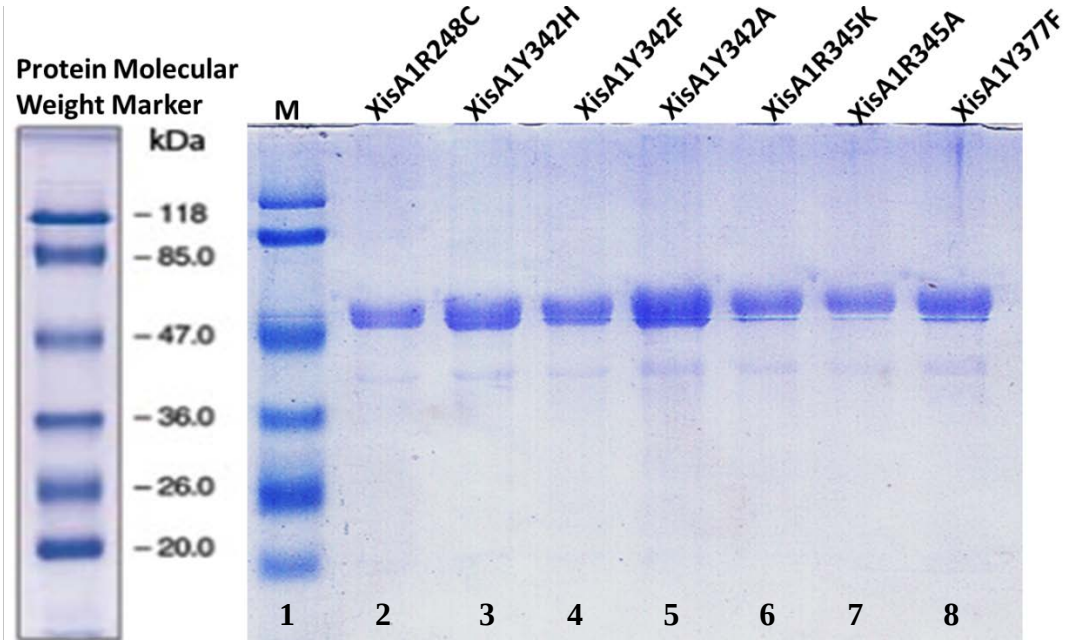

Fig.7.8 SDS PAGE analysis of XisA1 protein active site mutants. Lane 1: Molecular weight marker; Lane 2-8: Purified XisA1 active site mutant proteins.

2.3.4 Effect of active site mutations on recombinase and endonuclease activities of XisA1 protein

2.3.4.1 Monitoring recombinase activity

Qualitative recombinase activity of active site mutants of XisA1 protein was performed as described in **Section 5.2.5**. From functionality assay **(Fig. 7.9, 7.10 a & c),** strains UN18, UN19 and UN21synthesizing proteins XisA1Y342H, XisA1Y342F and XisA1R345K, respectively, showed the presence of recombinase activity.

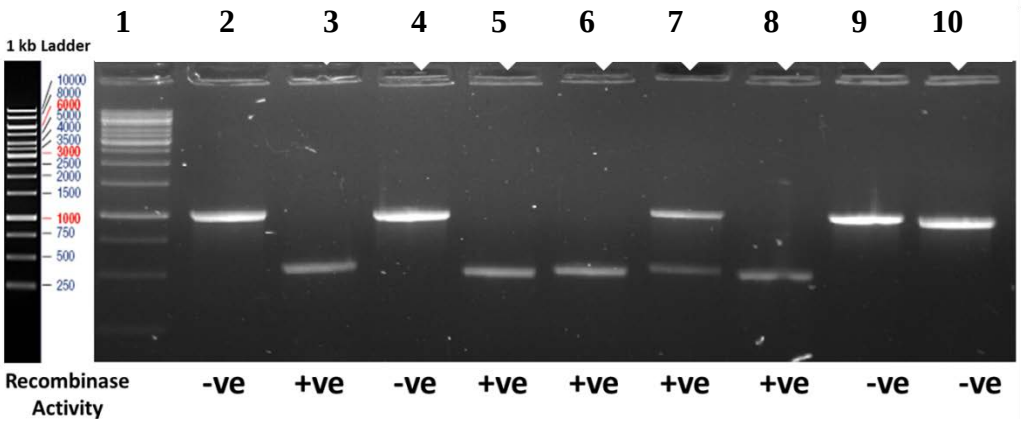

Fig. 7.9 Agarose gel profile displaying PCR based functionality detection of *E. coli* strains synthesising XisA1 mutants. Lane 1: 1 kb ladder; Lane 2: PCR analysis of UN1 (pET28a vector control); Lane 3: PCR analysis of UN3 (Synthesizing wild type XisA1). Lane 4-10: PCR analysis aiding functionality detection of *E. coli* strains (UN17 to UN23) synthesising active site mutants of XisA1 protein.

Activity of strain UN18 was marginally higher than strain UN3 synthesizing unaltered XisA1. Recombinase activity was completely absent in strains UN17, UN22 and UN23 synthesizing protein XisA1R248C, XisA1R345A and XisAY377F, respectively. Strain UN20 synthesizing protein Y342A showed reduced recombinase activity. Strain UN1 strain lacking *xisA* gene was used as vector control.

2.3.4.2 Monitoring endonuclease activity

Endonuclease activity assay of *E. coli* strains synthesizing active site mutants of XisA protein was performed as given in section **5.2.7**. Antibiotic susceptibility assay **(Fig 7.10b & c)** demonstrated endonuclease activity by all strains except UN23 synthesising XisA1Y377F protein. Strain UN18 synthesizing XisA1Y342H protein showed the highest endonuclease activity which was comparable to UN3 and UN19 synthesising XisA1 and XisA1Y342F proteins. Strains UN17 and UN18 showed significant reduction in endonuclease activity compared to UN3 control.

2.4 Discussion

Tyrosine recombinases are characterized by **R-(X)$_{70-139}$-H/Y-XX-R-(X)$_{29-38}$-Y** catalytic tetrad (Nunes-Duby et al., 1998). A majority of tyrosine recombinases possess Histidine as second active site residue, which is, however, occupied by tyrosine XisA and XisC recombinases in *Anabaena* PCC 7120. Biochemical functions of active site residues of a various tyrosine recombinases have been evaluated. Primary and secondary arginine residues of tyrosine recombinases are functionally involved in the transition state formation during recombination (Gibb et al., 2010). Histidine present at second position is known to be involved in general acid base catalysis during Holliday junction formation by tyrosine recombinases (Grindley et al., 2006). Terminal tyrosine acts as a nucleophile and carry out phosph-tyrosine bond formation with sessile phosphate on target DNA to initiate process of recombination. Terminal tyrosine is very sensitive to any mutation which causes loss in the recombinase potential of tyrosine recombinases (Nunes-Duby et al., 1998; Carrasco et al., 2005). Interestingly, *Eco*RII possess **Int** domain along with the endonuclease domain which depends on corresponding terminal tyrosine to display endonuclease activity (Topal and Conrad, 1993). Endonuclease activity of XisA is suggested to be similar to Type IIE restriction endonucleases (Shah et al., 2007).

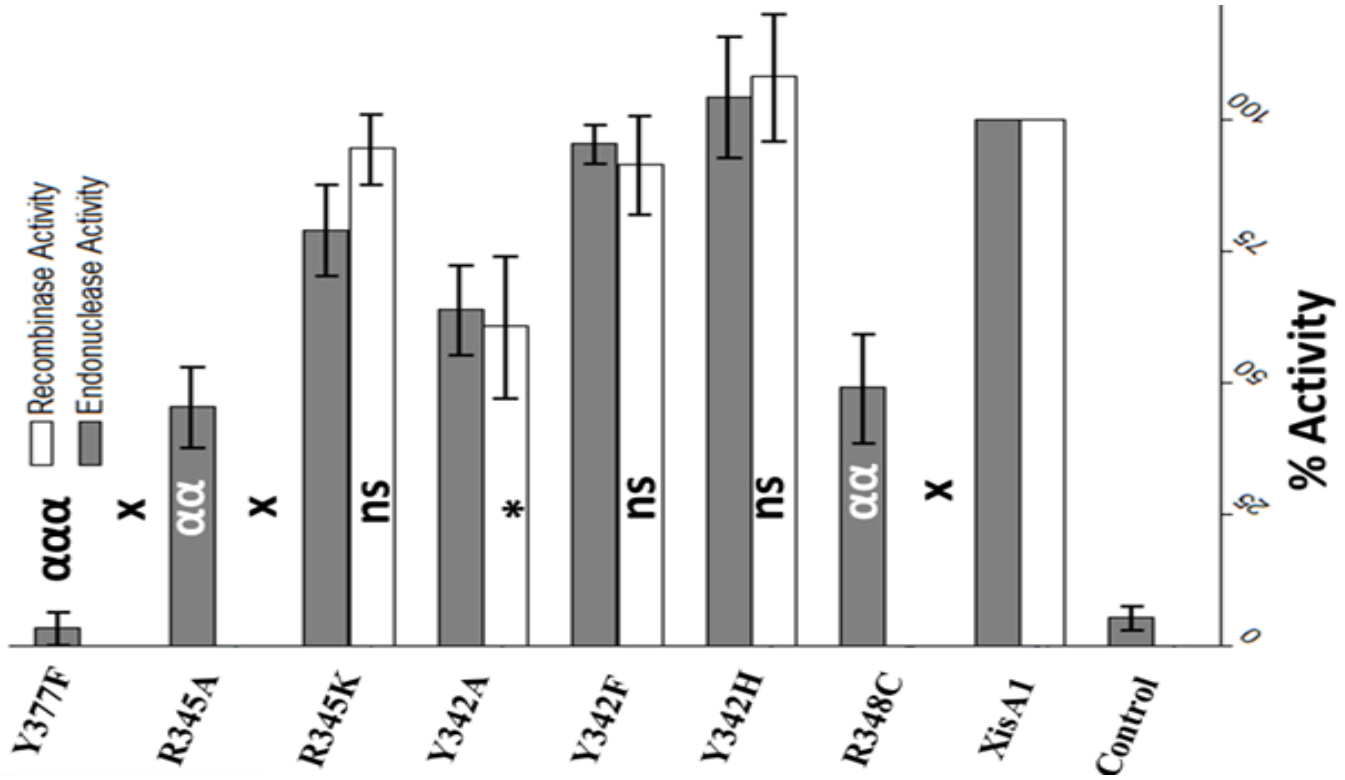

Fig. 7.10: Monitoring *in vivo* recombinase and endonuclease activity of XisA1 protein active site mutants. (a) PCR based recombinase assay. (b) Endonuclease activity by antibiotic susceptibility assay. (c) Graphical representation of recombinase and endonuclease activity of sequential N-terminal truncation products. Activity by complete XisA protein is normalized to 100. Activity by mutant protein are relative to XisA1. Values are expressed as Mean ±SEM (N=3). For endonuclease activity ^{ααα}P-value ≤ 0.001, ^{αα}P-value ≤ 0.01 compared UN 3 and for recombinase activity ns-not significant, *P-value ≤ 0.05 and x-no activity, difference compared to UN2.

Site directed mutagenesis on XisA1 protein revealed that, R248C and R345A XisA mutants lost the recombinase activity but retained significant amount of endonuclease activity as a result of interference in the transition state formation. Y342H mutant demonstrated marginal increase in both the activities compared to wild type XisA1 but Y342F mutation had no significant effect on either of the activities due to similarity in the nature of native and mutant residues. However, Y381A mutation displayed significant loss in both the activities whereas R384K mutant showed only marginal demotion of recombinase activity coupled with significant loss in endonuclease activity due to abortive transition state formation at Holliday junction. Interestingly, Y4377F mutant displayed complete loss in both the activities, indicating a functional similarity to *Eco*RII for endonuclease activity. The presence of such dual activity mediated by common active site is indicative of XisA being an evolutionary intermediate between endonucleases and recombinases.