Chapter 3: Characterization of XisA as restriction endonuclease

3.1 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. This recombination event has been studied in *E. coli* by cloning the entire 11kb element including the 11bp recognition sequence in EcoRI site of the pBR 322. To this, a transposon *mini-mu-lac* has been inserted in the 11kb element to create plasmid pMX25, which has been used to study the excision event on the basis of blue-white selection assay (Lammers *et al.*, 1986). During such studies, it was found that along with white colonies, few white sectors appeared in blue colonies. 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 were found to be cured of pMX25 plasmid. It was suggested that the curing of pMX25 was due to abortive deletion events related to the excision of 11kb element. Similar studies were also carried out with another DNA element of 55 kb encoding *xisF* recombinase (Carracso *et al.*, 1994). 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.

Topoisomerase, recombinases and endonucleases can cleave DNA but they do not show any sequence homology (Topal *et al.*, 1995). Restriction endonucleases and recombinases show very good sequence specificity while the topoisomerases are less specific. Discovery of type IIE restriction enzymes suggested an evolutionary relationship between all three types of enzymes. EcoRII, which is a type IIE restriction endonuclease, contains a conserved 30

amino acid region with R-H-R-Y tetrad similar to integrases. Mutation of the conserved tyrosine resulted in the loss of catalytic activity; such mutation has been found to abolish the activity in integrases too. Hence, EcoRII has been considered as a link between endonucleases and recombinases. Another type IIE enzyme NaeI requires two sites for its activity and it induces loops in DNA by binding at the base of the loops, mechanism reminiscent of recombinases. N-terminal region of NaeI contains DNA ligase conserved sequence with leucine instead of catalytically active lysine at 43rd position. Site directed mutagenesis of this leucine to lysine restored its topoisomerase and recombinase activities (Topal *et al.*, 1995). Endonuclease II of coliphage T4 has been shown to possess both endonuclease and recombinase activities (Carlson *et al.*, 1998).

E. coli strains over expressing *xisA* gene do not grow well presumably due to the toxic effects of XisA protein. The inverted repeat $GGAN_4TCC$ was identified in the 11 base pair target sequence (Haselkorn, 1992). Additionally, XisA protein has been shown to belong to Integrase family of proteins (Nunes-Duby *et al.*, 1998). The abortive excision events and the toxicity of XisA protein indicate that XisA may also have restriction endonuclease activity.

In order to check the endonuclease activity of XisA, *E. coli* GN1 strain containing pMX25 and pMC71A was grown for 24h in M9 minimal medium containing chloramphenicol and ampicillin and loss of pMX25 plasmid was monitored. It was observed that viability of the *E. coli* was same on plates without antibiotic and on plates with chloramphenicol but decreased by 100 fold on ampicillin plates (Karunakaran, 2000). Colonies from the chloramphenicol plates failed to grow on ampicillin containing plate, suggesting the lost of pMX25 plasmid specifically, while colonies from ampicillin plate grew on chlormaphenicol proving that pMC71A remains intact since it does not contain the 11 bp target site. pAN207.65 plasmid containing one copy of the 11bp target site was also used to study the endonuclease assay.

In this chapter, characterization of the XisA as endonuclease has been carried out. Substrate plasmids containing one or two copies of the target site were constructed and checked for the endonuclease activity by XisA. In order to determine the specificity of XisA, substrate plasmid has been constructed which contain one copy of the target site and a mutation in one of the base pairs on the inverted repeat in the 11bp sequence.

3.2 Materials and methods:

3.2.1 Culture conditions: Media and culture conditions and antibiotic concentrations were used as described earlier.

3.2.2 Bacterial strains and plasmids used:

Table 1 Plasmids and strains used in the study

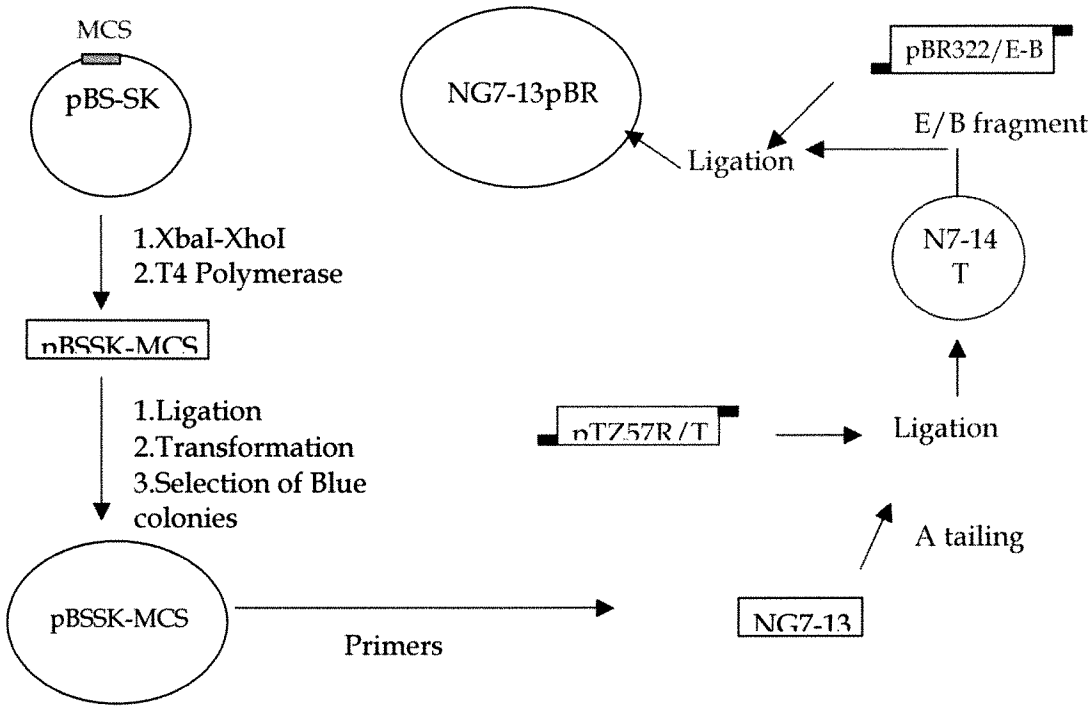
E. coli / Plasmids Strains	Genotype / Features	Source/Reference
JM101	*FΔtraD36lacI^{q}Δ (lacZ)M15 proA$^+$B$^+$/supE `thi Δ(lac-proAB)*	Yanisch-Perron *et al.*, 1985
DH5α	*FΔendA1hsdR17(r_k^- m_k^+)supE44 thi-1 recA1gyrA96(nal^r) relA1(lacZYA-argF)U169deoR (Δ80dlacΔ (lacZ)M15*	Hanahan, 1985
GN1	JM101 (pMX25 and pMC71A)	Karunakaran, 2000
Plasmids		
pMX25	Km^R Amp^R LacZ$^+$ *xisA*$^+$ (*nifD* element vector)	Lammers *et al.*, 1986
pMX32	Km^R Amp^R LacZ$^+$ *xisA*$^-$ (defective *nifD* element vector)	Lammers *et al.*, 1986
pAn207.65	2.3 kb fragment containing *xisA* gene of *Anabaena* PCC 7120 present in pUC18 plasmid, Amp^R	Lammers *et al.*, 1986
pNG7	Contains one 11bp target site,Amp^R	This study
pNG8	G is replaced with T in 11 bp site, Amp^R	This study
pNG9	G3C, Amp^R	This study
pNG10	G10C, Amp^R	This study
pNG11	G11C, Amp^R	This study

pNG12	A4C, Amp^R	This study
pNG13	T10A, Amp^R	This study
pNG1	Contains two 11 bp sites flanking lacZ, Amp^R	This study
pNG2	Contains two 11bp + 8 bps flanking lacZ,Amp^R	This study
p*xis*-gm	Contains *xisA*, Gen^R	This study
p*xis*71A-gm	Contains *xisA* and *nifA* of *Klebsiella pnuemoniae*, Gen^R	This study

3.2.3 Plasmids:

Construction of Substrate plasmids with modified target sites:

Figure 3.1: Strategy used for the construction of substrate plasmids



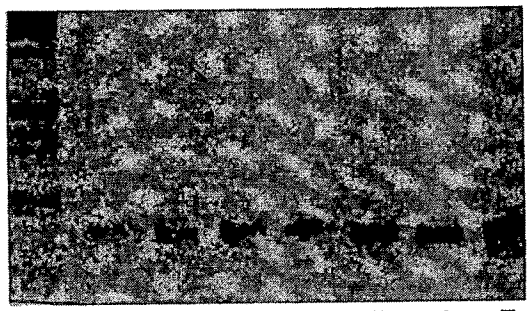
List of primers used:

NG7	TAGTG**C**GGA GTAA TCC ..... 3′
NG8	TAGTG**CT**GA GTAA TCC ..... 3′
NG9	TAGTG **C**G**C**A GTAA TCC ..... 3′
NG10	TAGTG **C**GGA GTAA T**G**C ..... 3′
NG11	TAGTG **C**GGA GTAA TC**G** ..... 3′
NG12	TAGTG **C**GG**C** GTAA TCC ..... 3′
NG13	TAGTG **C**GGA GTAA **A**CC .... 3′

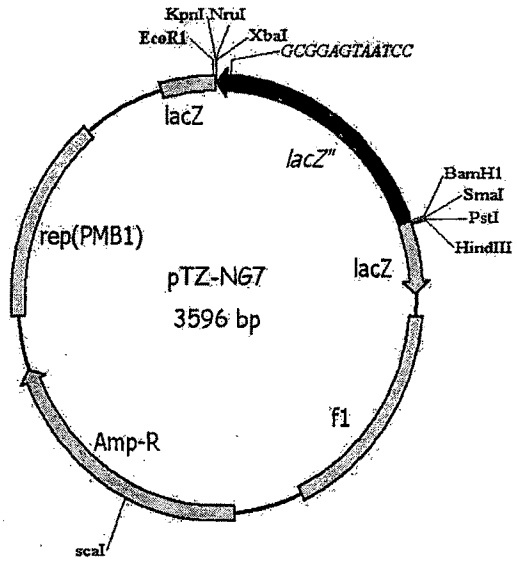
For substrate plasmid construction, set of primers were designed with one base pair modification in the 11bp target site as given in the table , while the unmodified target site containing plasmid was used as control. PCR was done using pBSSK-MCS as template. For this, pBSSK was first digested this XbaI and XhoI and the vector was treated with T4 DNA polymerase as described earlier to make it blunt ended. This DNA was self ligated to generate pBSSK-MCS. The primers 5′ TACGGAGTAATCCGTCAGCG AGTCAGTGA 3′ and 5′ TAGGCG TATCACGAGTAGGATTACTCCGTC 3′ was used to amplify the 750 bp *lacZ′* gene with pBSSK without MCS at template. PCR conditions were used as described earlier with *pfu* polymerase enzyme. Temperature profile - 94°C for 3 mins, (94°C/30 s, 42°C/30 s, 72°C 90 s) for 5 cycles and (94°C/30 s, 62°C/30 s, 72°C 90 s) for 25 cycles. All PCR products were given A tailing using *Taq* polymerase in 10 µl reaction system containing 5mM ATP final concentration at 72°C for 2 hours. These A-tailed products were purified using gel extraction kit and ligated with pTZ57R/T vector from MBI fermentas as described earlier. Recombinants were selected on the basis of blue-white selection to generate pNG7 to pNG14. These plasmids were digested with EcoRI and BamH1 enzyme to release 750bp fragment containing the *lacZ* gene. pBR 322 vector was digested with EcoRI and BamH1 enzyme to linearize it and this vector was ligated with 750bp insert to generate pNG7-BR to pNG14-BR.

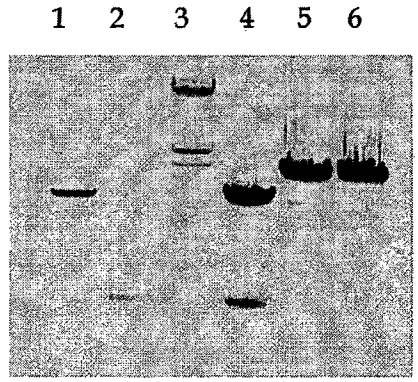
Fig 3.2 PCR amplification products with modified target sites followed by *lacZ*

PCR amplification from NG7-13

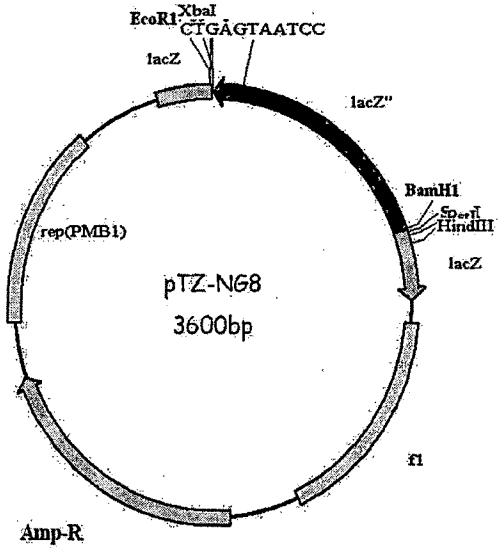


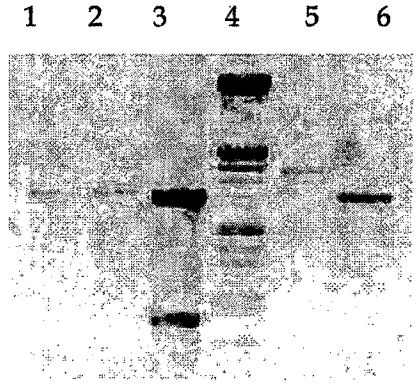
M- λ DNA EcoRI/HindIII double digest
Lane 1 NG7 PCR product
Lane 2 NG8 PCR product
Lane 3 NG9 PCR product
Lane 4 NG10 PCR product
Lane 5 NG11 PCR product
Lane 6 NG12 PCR product
Lane 7 NG13 PCR product





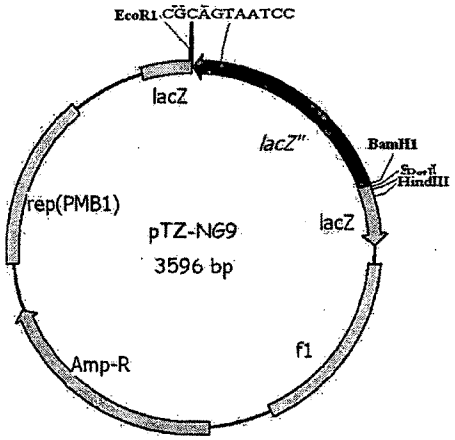
1. T vector digested with EcoRI BamH1
2. NG-7 PCR product
3. λ EcoRI/HindIII double digest marker
4. pTZ-NG7 digested with EcoRI/BamH1

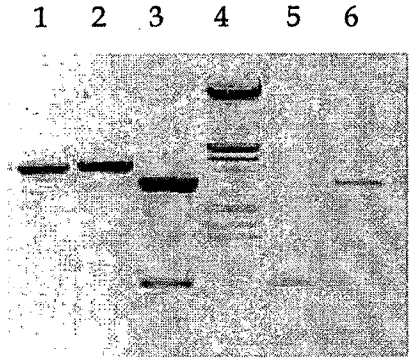




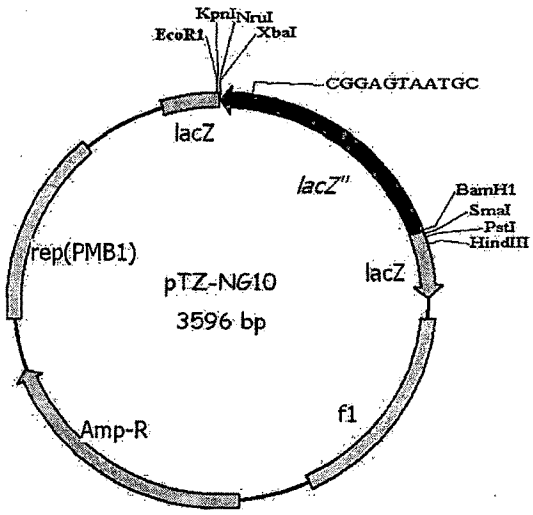
1. T vector digested with EcoRI
2. T vector digested with BamH1
3. pTZ-NG8 digested with EcoRI/BamH1
4. λ EcoRI/HindIII double digest marker
5. pTZ-NG8 digested with EcoRI

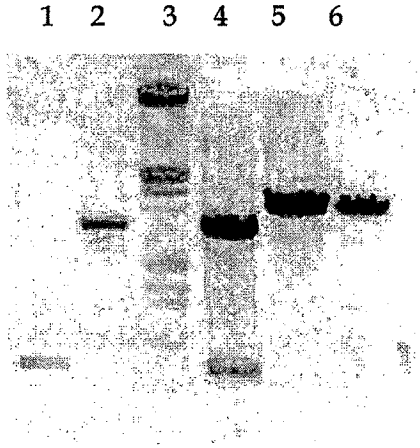
Fig 3.3 Restriction digestion profile of NG7/T and NG8/T plasmids





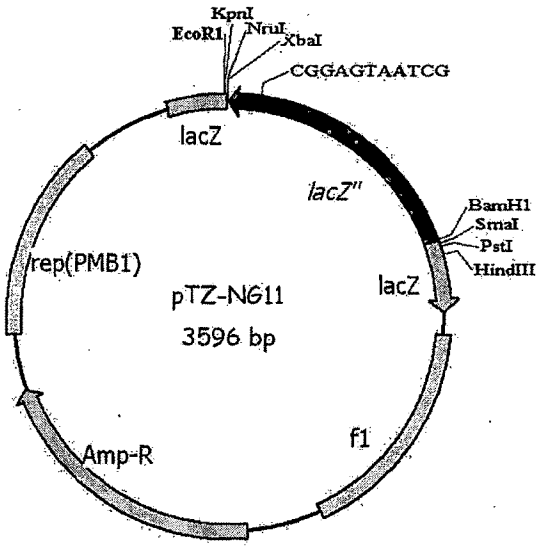
pTZ-NG9 digested with
1. EcoRI
2. BamH1
3. EcoRI/BamH1
4. Marker λ EcoRI/HindIII double digest marker
5. NG-9 PCR product
6. T vector EcoRI/ BamH1 digest

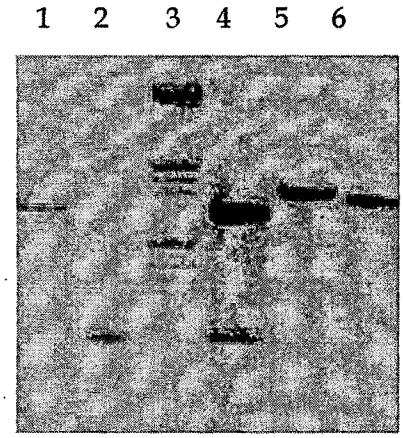




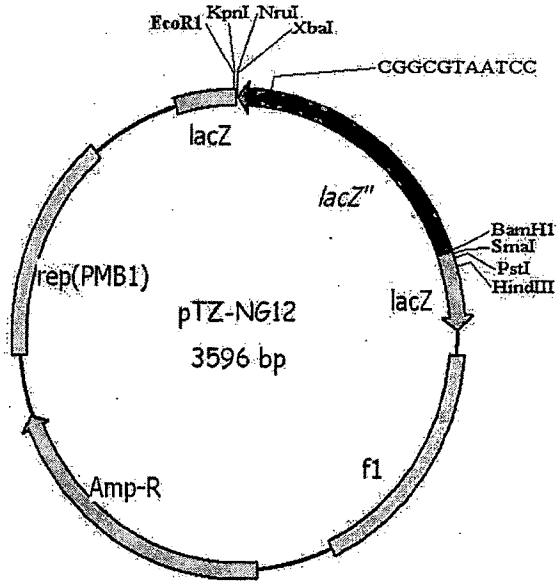
1.NG10 PCR product
2.T vector EcoRI/ BamH1 digest
3. Marker λ EcoRI/HindIII double digest marker
pTZ-NG10 digested with
4. EcoRI/BamHI
5. EcoRI 6. BamHI

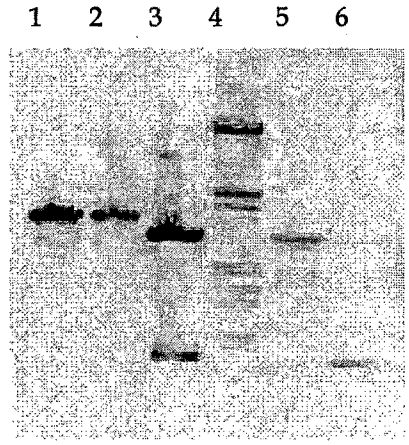
Fig 3.4 Restriction digestion profile of NG9/T & NG10/T plasmids





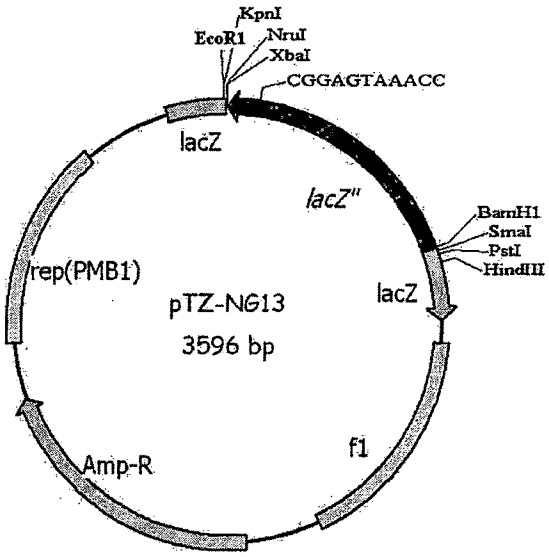
1. T vector EcoRI-BamH1 digest
2. NG-11 PCR product
3. λ EcoRI/HindIII digest marker
4. pTZ-NG11 digested with EcoRI/BamH1
5. pTZ-NG11 digested with EcoRI
6. pTZ-NG11 digested with BamH1

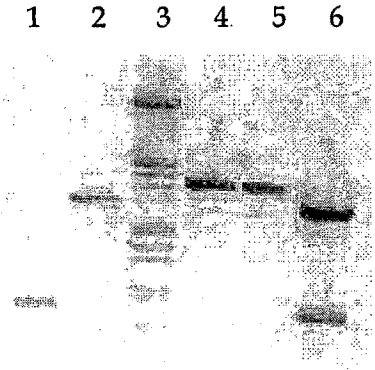




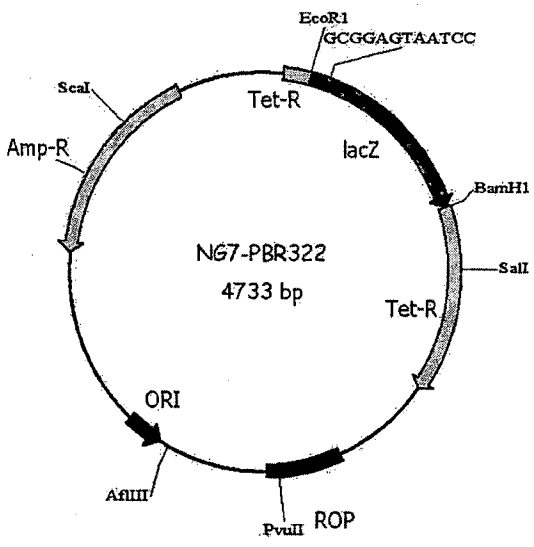
1. pTZ-NG12 digested with BamH1
2. pTZ-NG12 digested with EcoRI
3. T vector digested with EcoRI BamH1
4. λ EcoRI/HindIII double digest marker
5. pTZ-NG12 digested with EcoRI/BamH1
6. NG-12 PCR product

Fig 3.5 Restriction digestion profile of NG11/T and NG12/T plasmids

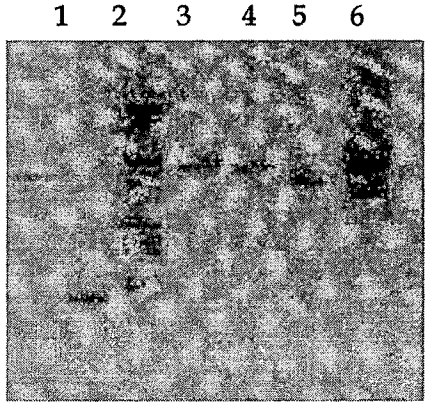




1. NG-13 PCR product
2. T vector EcoRI BamH1 digest
3. λ EcoRI/HindIII double digest marker
4. pTZ-NG13 digested with BamH1
5. pTZ-NG13 digested with EcoRI
6. pTZ-NG13 digested with EcoRI/BamH1

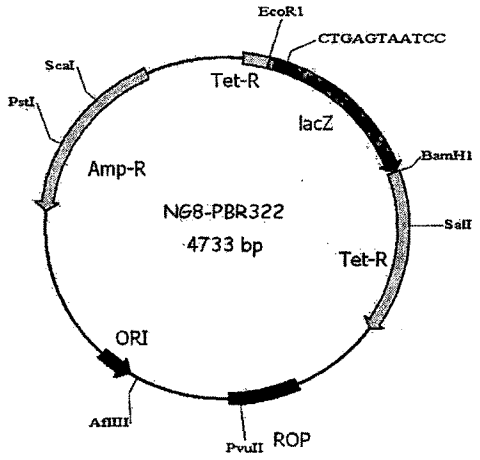


GGAGTAATCC

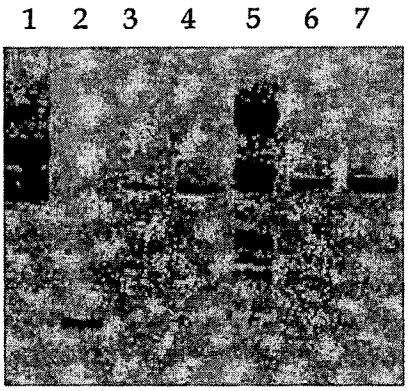


1 pBR322/EcoRI/BamH1
2 Ng7 insert
3 λ EcoRI/HindIII double digest
4 NG7-pBR 322/EcoRI
5 NG7-PBR 322/BamH1
6 NG7pBR322/BamH1/EcoRI
7 Control plasmid

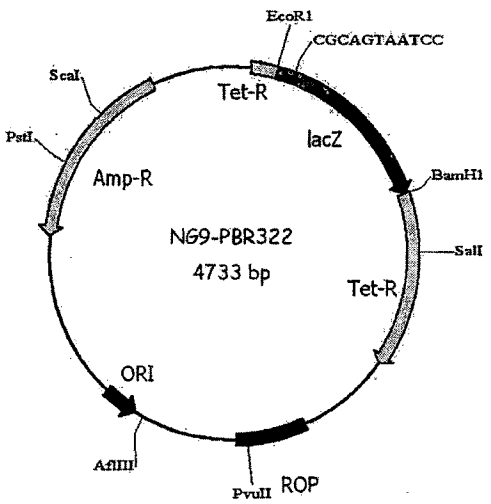
Fig 3.6 Plasmid map and restriction digestion profile of NG-13/T and NG7-BR



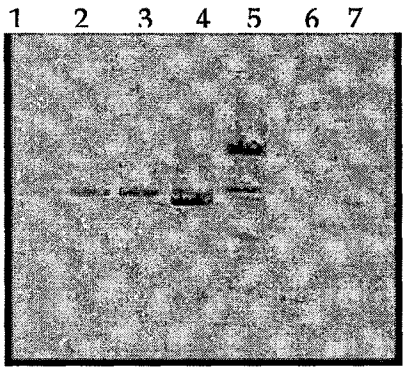
TGAGTAATCC



1 Control plasmid
2 NG 8 insert
3 NG8-pBR 22/BamH1/EcoRI
4 pBR322/EcoRI/BamH1
5 λ EcoRI/HindIII double digest
6 NG8-PBR322/BamH1
7 NG8-pBR 322/EcoRI

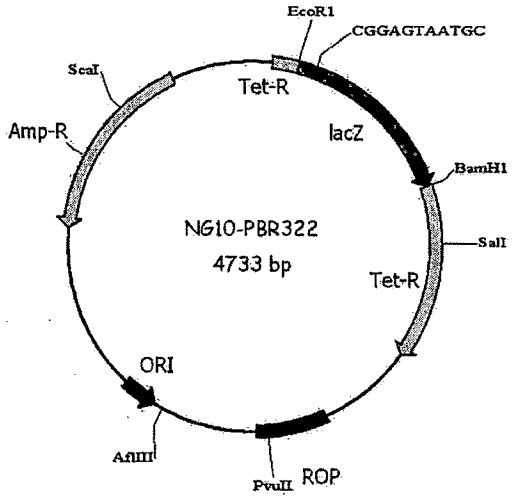


GCAGTAATCC



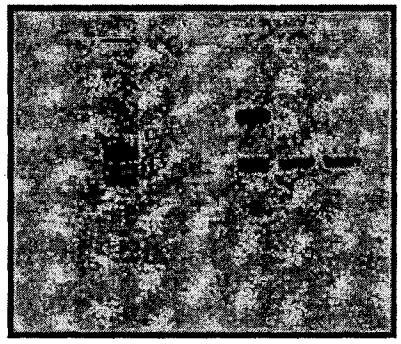
1 Control plasmid
2 NG9-pBR 322/BamH1
3 NG9-pBR 322/EcoRI
4 NG9-pBR322/BamH1/EcoRI
5 λ EcoRI/HindIII double digest
6 NG9 insert
7 pBR322/EcoRI/BamH1

Fig 3.7 Plasmid map and restriction digestion profile of NG8-BR, and NG9-BR plasmids

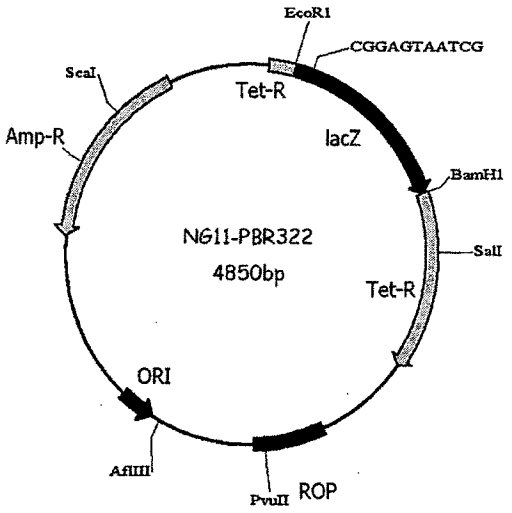


GGAGTAATGC

1 2 3 4 5 6 7

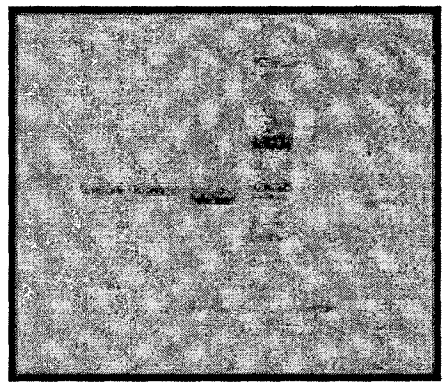


1 pBR322/EcoRI/BamH1
2 NG10 insert
3 Control plasmid
4 NG10-pBR322/BamH1/EcoRI
5 λ EcoRI/HindIII double digest
6 NG10-pBR 322/BamH1
7 NG10-pBR 322/EcoRI



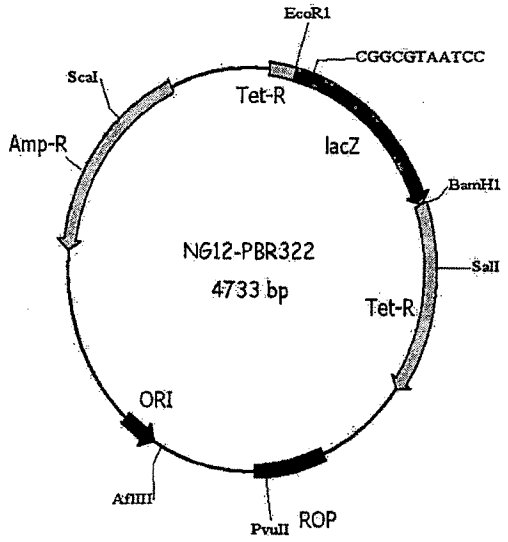
GGAGTAATCG

1

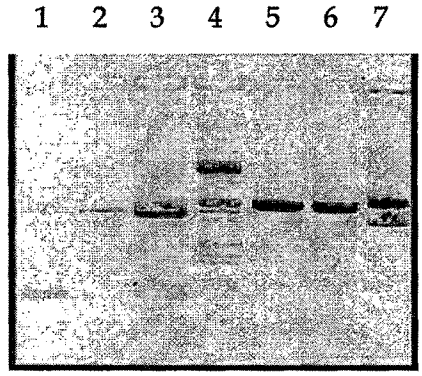


1 Control plasmid
2 NG11-pBR 322/EcoRI
3 NG11-pBR 322/BamH1
4 NG11-pBR 322/BamH1/EcoRI
5 λ EcoRI/HindIII double digest
6 NG11 insert
7 pBR322/EcoRI/BamH1

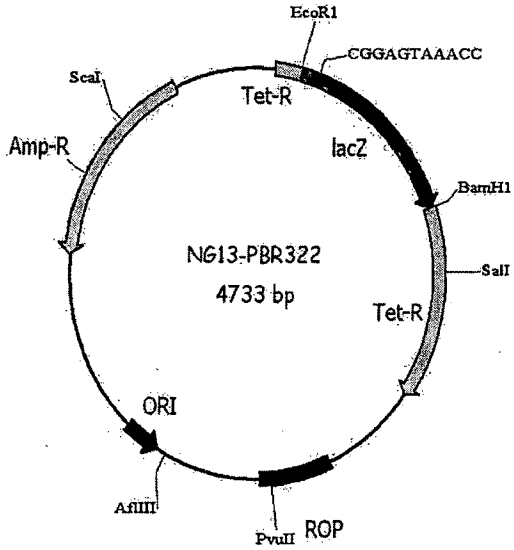
Fig 3.8 Plasmid map and restriction digestion profile of NG10-BR and NG11-BR plasmids

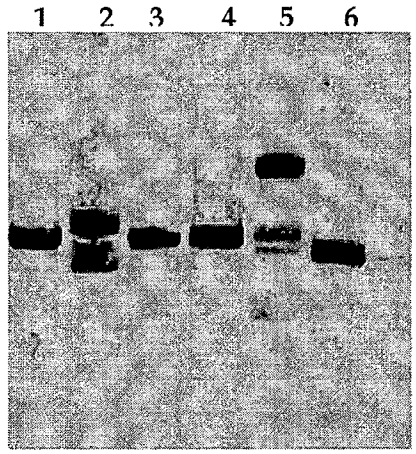


GGCGTAATCC



1 NG12 insert
2 pBR322/EcoRI/BamH1
3 NG12-pBR 322/BamH1/EcoRI
4 λ EcoRI/HindIII double digest
5 NG12-pBR 322/EcoRI
6 NG12-pBR 322/BamH1
7 Control plasmid





1 Control plasmid
2 NG13-pBR 322/EcoRI
3 NG13-pBR 322/BamH1
4 λ EcoRI/HindIII double digest
5 NG13-pBR 322/BamH1/EcoRI
6 pBR322/EcoRI/BamH1

Fig 3.9 Plasmid map and restriction digestion profile of NG12 & NG13-BR plasmid

3.2.4 Cloning of *xisA* :

In order to check its effect on the substrate plasmids, *xisA* gene has been cloned in pACYC184-gm and pMC71Agm plasmid as described earlier. Conformation of the cloning initially in pTZ57R and finally in pACYC184-gm and pMC71Agm was done by restriction digestion analysis [redacted] (Fig. 2.1-2.2).

3.2.5 Construction of substrate plasmid with two copies of the target site:

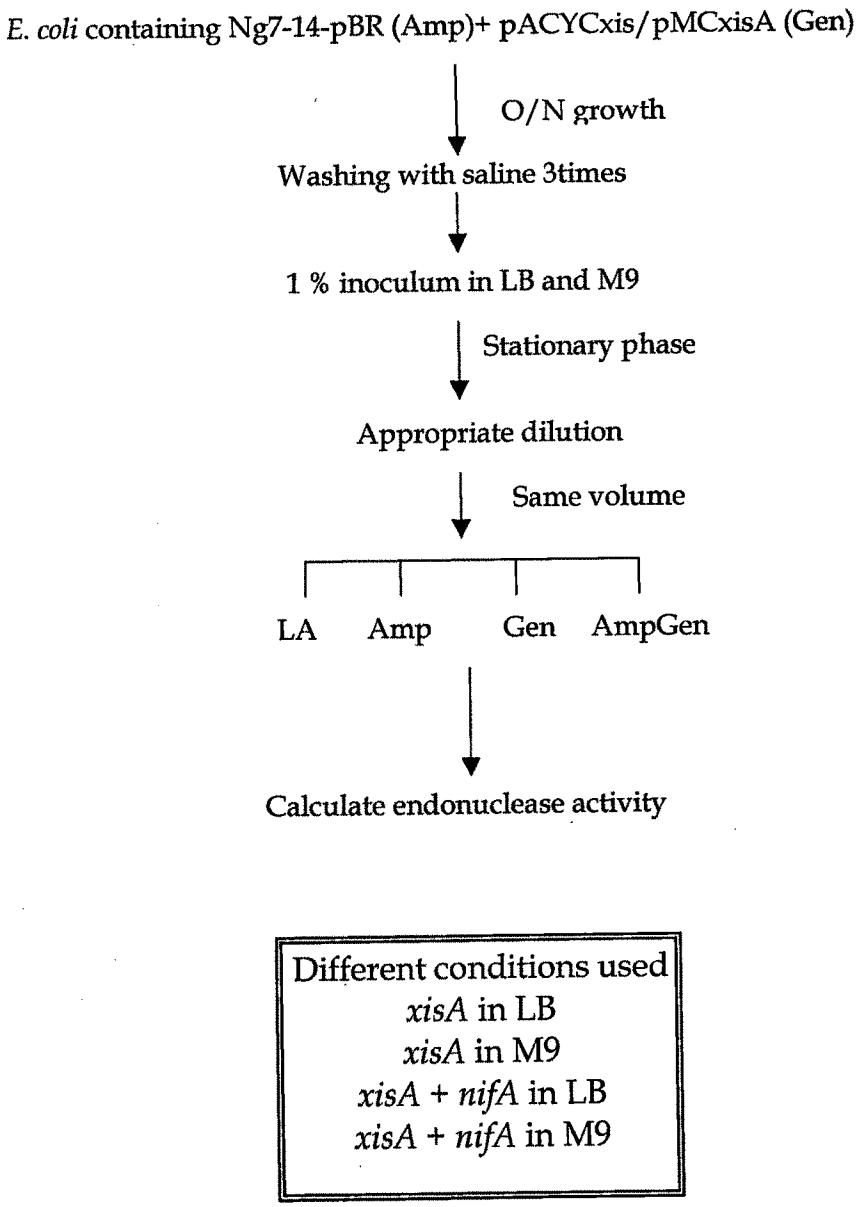
Substrate plasmid with two copies of the target site were constructed with the primers containing the target site which amplify the *lacZ* so as to get *lacZ* flanked by two copies of the 11bp target site. Cloning was done as descried in chapter 2.

3.2.6 Endonuclease activity assay:

E. coli containing *xis* and substrate plasmid was grown over night in Luria broth containing ampicillin and gentamycin at 37°C. 1ml culture was harvested and washed thrice with saline and resuspended in saline. 1% of (100µl) of the cells was reinoculated in LB and M9 minimal medium containing ampicillin and gentamycin. Cells were washed twice with saline and finally diluted and same no. of cells were plated on LA, Ampicillin containing LA, Gentamycin containing LA plate, Ampicillin and gentamycin containing LA plate and endonuclease activity was calculated as :

$$\text{Endonuclease activity (\%)} = \frac{\text{No. of colonies on Amp. Containing plate}}{\text{No. of colonies on LA plate}} \times 100$$

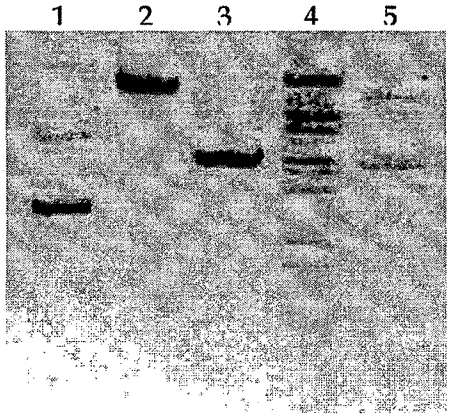
Figure 3.10 Protocol used for endonuclease assay



3.3 Results:

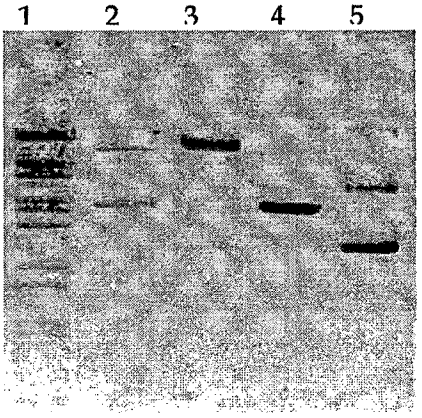
The present work was carried out in order to characterize XisA as restriction endonuclease along with its recombinase activity. Several substrate plasmids were constructed with one or two target site(s) as well as with modified target site in order to determine the specificity of the XisA as endonuclease. *xisA* containing plasmids were transformed with different substrate plasmids in *E. coli* JM101 strain and double transformants were selected on antibiotic markers, confirmed by isolating the plasmid and restriction digestion pattern. These plasmids were used along with different substrate plasmids to check the endonuclease activity. Endonuclease activity was calculated by the formula given earlier. The results are given in the Table .

pMC*xis*-gm and NG7-BR substrate plasmid



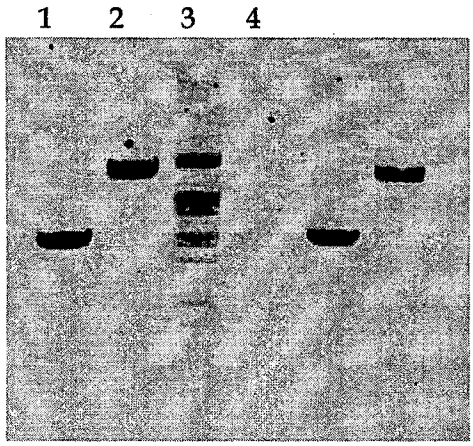
1. Control plasmid
2. pMCxisAGm/EcoRI
3. NG7-pBR/EcoRI
4. Mol wt. Marker
5. NG7-pBR + pMCxisAGm/EcoRI

pMC*xis*-gm and NG8-BR substrate plasmid

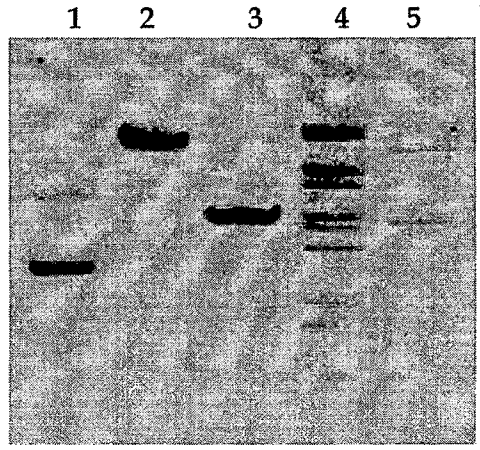


1. Mol wt. Marker
2. NG8-pBR + pMCxisAGm/EcoRI
3. pMCxisgm/EcoRI
4. NG8-pBR/EcoRI
5. Control plasmid

Fig 3.11 Restriction digestion profile of plasmids isolated from *E. coli* containing substrate plasmid and *xisA* plasmid



1. NG9-pBR/EcoRI
2. pMCxisAGm/EcoRI
3. Mol Wt. Marker
4. NG9-pBR + pMCxisAGm/EcoRI



1. Control plasmid
2. pMCxisgm/EcoRI
3. NG10-pBR/EcoRI
4. Mol Wt. Marker
5. NG10-pBR + pMCxisAGm/EcoRI

Fig 3.12 Restriction digestion profile of plasmids isolated from *E. coli* containing substrate plasmid and *xisA* plasmid

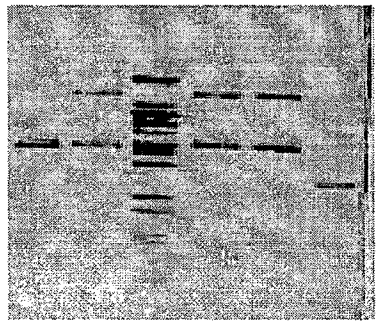


Fig. 3.13 pMC-xis-gm and NG11-13-BR substrate plasmid

1 NG-11/EcoRI digest
2 NG11-pBR+pMCxisgm/EcoRI
3 Mol. Wt. Marker
4 NG12-pBR+pMCxisA.Gm/EcoRI
5 NG13-pBR+pMCxisA.Gm/EcoRI
6 Control Plasmid

Table 3.2: Endonuclease activity of XisA with NG1 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	2453± 29	1593± 146**	35
M9 with 40mM NH_4 Cl/ stationary phase	1067± 67	738± 36*	32
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1710± 146	1007± 110*	41
M9 with 40mM NH_4 Cl/ stationary phase	2140± 30	1573± 118**	37

Table 3.3 Endonuclease activity of XisA with NG2 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	1297±173	1107±214	32
M9 with 40mM NH_4Cl/ stationary phase	1563±31	1023±21***	34
Endonuclease activity in presence of *xisA* in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1588±48	1043±54**	34
M9 with 40mM NH_4 Cl/ stationary phase	2227±139	1523±43**	32

* - P< 0.05, ** - P < 0.01 ***- P < 0.001 n=5

Table 3.4: Endonuclease activity of XisA with NG7 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	1200± 88	1200± 66	0
M9 with 40mM NH_4Cl/ stationary phase	928± 10.9	663± 8.7***	28
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1904± 52	1314± 185**	31
M9 with 40mM NH_4Cl/ stationary phase	1660± 29	1140± 57***	32

Table 3.5: Endonuclease activity of XisA with NG 8 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	630± 49	604± 82	0
M9 with 40mM NH_4Cl/ stationary phase	1400± 115	1420± 81	0
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1234± 22	1202± 57	0
M9 with 40mM NH_4Cl/ stationary phase	892± 38	805± 47	0

* - P< 0.05, ** - P < 0.01 ***- P < 0.001 n=5

Table 3.6: Endonuclease activity of XisA with NG 9 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	630± 13	602± 21	0
M9 with 40mM NH_4Cl/ stationary phase	1183± 28	1147± 28	0
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1234± 22	1202± 57	0
M9 with 40mM NH_4Cl/ stationary phase	1047± 55	950± 85	0

Table 3.7:Endonuclease activity of XisA with NG10 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	2007± 75	1824± 137	0
M9 with 40mM NH_4Cl/ stationary phase	1269± 19	1246± 18	0
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1360± 132	1177± 130	0
M9 with 40mM NH_4Cl/ stationary phase	1645± 51	1570± 68	0

Table 3.8:Endonuclease activity of XisA with NG11 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	1560± 60.9	1600± 89.7	0
M9 with 40mM NH_4Cl/ stationary phase	1243± 26	1210± 20	0
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1530± 40	1577± 3	0
M9 with 40mM NH_4Cl/ stationary phase	626± 9	601± 60	0

Table 3.9:Endonuclease activity of XisA with NG12 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	1375± 118	1420± 163	0
M9 with 40mM NH_4Cl/ stationary phase	1217± 72	680± 69**	44
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1023± 113	499± 26*	51
M9 with 40mM NH_4Cl/ stationary phase	1540± 112	675± 68***	56

* - P< 0.05, ** - P < 0.01 ***- P < 0.001 n=5

Table 3.10 : Endonuclease activity of XisA with NG13 substrate plasmid

Media/ Time (h)	No. of colonies on LA	No. of colonies on LA+Ampicillin	Excision (%)
LB/stationary phase	1460±88	1467±66	0
M9 with 40mM NH_4Cl/ stationary phase	1210±20	1167±52	0
Endonuclease activity in presence of *nifA* of *Klebsiella pneumoniae*			
LB/stationary phase	1173±64	1173±46	0
M9 with 40mM NH_4Cl/ stationary phase	720±12	660±25	0

* - $P < 0.05$, ** - $P < 0.01$ *** - $P < 0.001$ n=5

Table 3.11: Summary of Endonuclease Activity

	NG1		NG2		NG7		NG12	
	LB	M9	LB	M9	LB	M9	LB	M9
XisA	35%	32%	32%	34%	-	28%	-	44%
XisA +NifA	41%	37%	34%	32%	31%	32%	51%	56%

3.4 Discussion

Main objective of this study is to establish XisA protein as a restriction endonuclease besides being a recombinase. Initial experiments with the pMX25 plasmid containing two 11bp target sites resulted in specific loss of this plasmid while pMC71A plasmid without the target site was stably maintained (Karunakaran, 2000). Endonuclease activity was determined by the difference in the number of colonies on antibiotic plates corresponding to each plasmid and total viable cells.

Substrate plasmid containing the 11 base pair target site was specifically lost when XisA protein was provided in *trans*. About 30% restriction endonuclease activity was found with this substrate plasmid which does not possess any other sequences present in pMX25 plasmid. However, the restriction endonuclease activity was much less than that of pMX25. The 11bp target site of the XisA 5′ CGGAGTAATCC 3′ contains an inverted repeat of 3 bp *i.e.* $GGAN_4TCC$, which could be its recognition sequence, as in case of few restriction enzymes where the target sites are of similar pattern e.g. BstXI enzyme target site is $CCAN_6TGG$ (http://rebase.neb.com/rebase/re_base.html).

In order to test whether the inverted repeat sequence within the target site was recognized by XisA, different substrate plasmids were designed with single base mutations in GGA and TCC bases. Endonuclease activity was checked by growing the *E. coli* on LB media, no endonuclease activity was observed even with the NG7 plasmid. Earlier results have showed that the activity of XisA was much lower in rich media like LB, than on M9minimal media probably due to the limiting amount of XisA protein. Previous studies also showed that the expression of *xisA* was higher in stationary phase as compared to logarithmic phase. Hence, endonuclease activity was monitored in both LB and M9 minimal media using *E. coli* cells containing *xisA* and substrate plasmid in stationary phase. Endonucelase activity was 28% in NG7 which contains unmodified target site. Except NG12, all other substrate plasmids, with modified target site did not

show any endonuclease activity. Substrate plasmid NG12 contains A to C mutation in the third position of the 11bp target site. It showed endonuclease activity up to 32 %. However, substrate plasmid NG 13 containing mutation from A to G corresponding base of the inverted repeat at the 8th position did not show any endonuclease activity.

Earlier studies showed that when the *nifA* gene of *Klebsiella* was introduced into *E. coli* along with pMX25 plasmid, it increased the rearrangment frequency in the stationary phase up to 70% in M9 medium and 50% in LB medium (Karunakaran, 2000). The increase in the rearrangment frequency was attributed to the activation of *xisA* gene by NifA through σ^{54} dependent promoter. In order to check the effect of NifA on endonuclease activity of XisA, *xisA* gene was cloned along with *nifA* and this plasmid was used along with different substrate plasmids. In case of NG7, endonuclease activity was 32-34% which was similar to *xisA* alone. While in case of NG12, presence of NifA increased the endonuclease activity from 44 to 55%. Although NifA increased excision very significantly, its influence on the endonuclease activity was much less.

Restriction endonucleases have been classified into different subtypes based on the number and nature of the target sites required for their activity (Table 12). Type IIE and IIF restriction endonucleases require two copies of the target site for their optimal activity. In case of Type IIE, one target site acts as a inducer for the binding and catalysis at the other site but catalysis occurs at both sites in case of IIF. Type IIE enzymes, EcoRII and NaeI, have been linked with the recombinase activity. In case of NaeI, single substitution at 43rd amino acid position resulted in the gain of recombinase activity in addition to its endonuclease activity. EcoRII contains conserved R-H-R-Y signature catalytic tetrad of integrase family, to which XisA belongs, and mutation in tyrosine resulted in the loss of cutting activity but retained the recognition property. XisA substrate plasmids with one target site did now show any endonuclease activity

in LB medium. However, it increased significantly to 35% with two target site containing substrate plasmid. While in M9 minimal medium, single target site containing substrate plasmid showed 28% endonuclease activity, slightly less than plasmid with two target sites which showed 32% activity. However, such increase was not found with *xisA* in the presence of *nifA*. Thus, the presence of additional copy of the target site increased the endonuclease activity similar to EcoRII and NaeI enzymes, which are more closely related to XisA due to their putative recombinase activity.

Table 3.12: Subtypes of type II restriction endonuclease

Subtype	Defining feature	Example	Recognition sequence
A	Asymmetric recognition sequence	FokI	GGATG
B	Cleaves both sides of target on both strands	BcgI	(10/12) CGANNNNNNTGC (12/10)
C	Symmetric or asymmetric target. R and M functions in one polypeptide	GsuI	CTGGAG (16/14)
E	Two targets; one cleaved, one an effector	EcoRII NaeI	CCWGG GCC GGC
F	Two targets, both cleaved coordinately	SgrAI	CR CCGGYG

Subtype	Defining feature	Example	Recognition sequence
G	Symmetric or asymmetric target. Affected by AdoMet	BsgI	GTGCAG (16/14)
H	Symmetric or asymmetric target. Similar to Type I gene structure	AhdI	GACNNN NNGTC
M	IIP or IIA. Require methylated target	DpnI	Gm6 A TC
P	Symmetric target and cleavage sites	EcoRI	G AATTC
S	Asymmetric target and cleavage sites	FokI	GGATG
T	Symmetric or asymmetric target. R genes are heterodimers	Bpu1	CCTNAGC (-5/-2)b

3.5 Summary:

Amongst different substrate plasmids, NG7 and NG12 with A to C mutation at 3rd position retained endonuclease activity while other modifications had no activity. This implies that all these bases are absolutely required for the cleavage by XisA, while the 3rd position is not an absolute requirement and could be replaced by other base. Presence of additional copy of target site marginally increased the endonuclease activity. Thus, XisA appears to belong to Type IIE or IIF restriction endonuclease with the putative target site GGN_5TCC.