CHAPTER IV

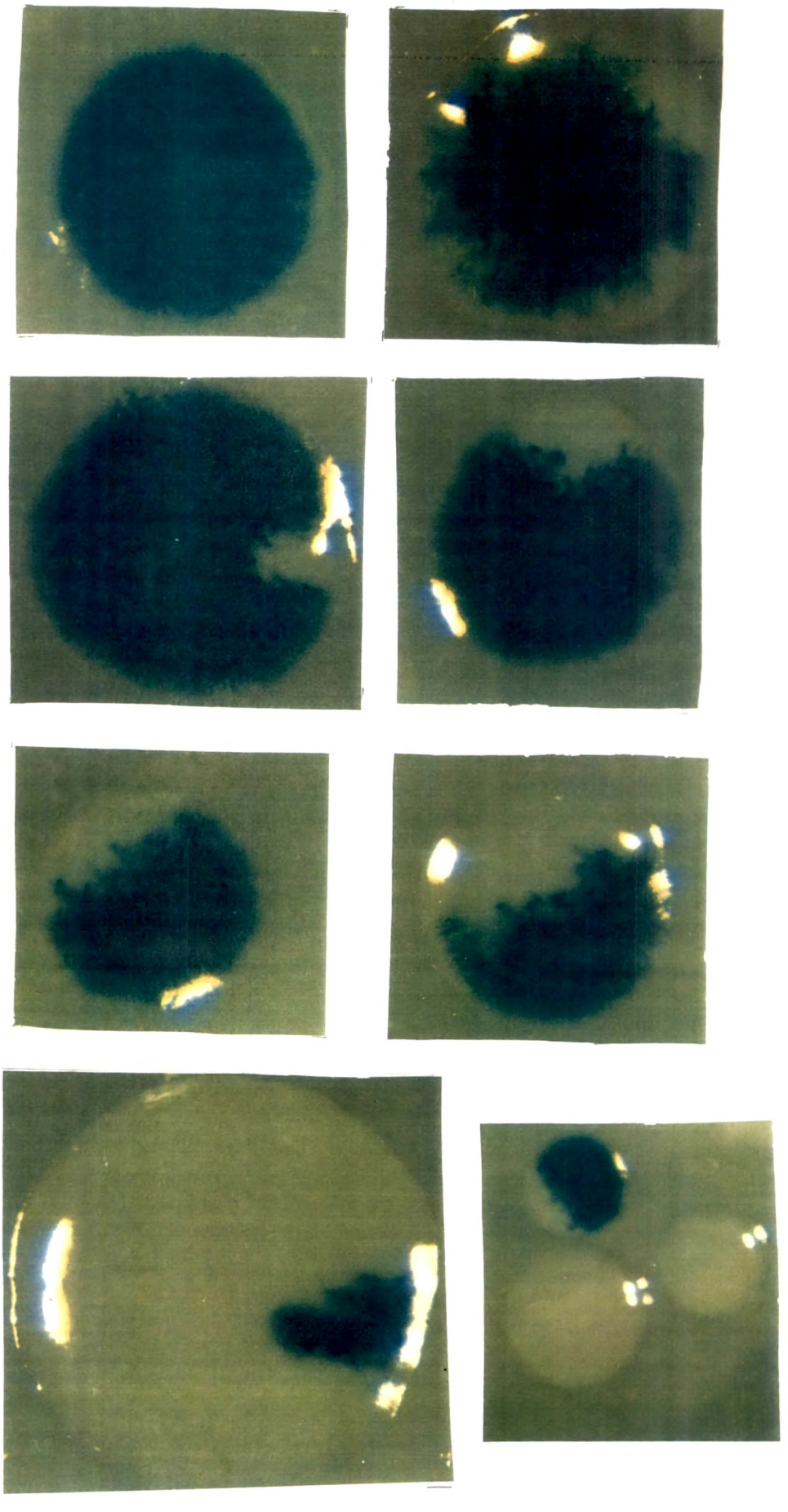
ENDONUCLEASE ACTIVITY OF EXCISASE A (XisA) OF *ANABAENA* PCC 7120.

4.1: INTRODUCTION

Excision of *nifD* element of *Anabaena* PCC 7120 is a site-specific recombination occurring at the 11bp direct repeat (CGGAGTAATCC) sequences which is mediated by Excisase A (XisA). Excision of the *nifD* element in *E. coli* was monitored by Blue/White colony assay using pMX25 plasmid (Lammers *et al.*, 1986). In addition to white colonies, white sectors appeared in large proportions of the blue colonies. Cells from the white sectors failed to grow when replated on ampicillin plates. However, the cells grew on plates not supplemented with any antibiotic, were found to be cured of plasmid pMX25. It was suggested that the curing of pMX25 was due to abortive deletion events related to the excision of 11kb element. Growth of white colonies on ampicillin plates may be due to the secretion of β-lactamase by the neighboring (blue) cells in the colony. Sectored colonies are also observed in this study (Fig 4.1).

A subset of type II restriction endonucleases, type IIe, cleave DNA at specific sites which have dyad symmetry but require two such sites and the two sites can be effective even in *trans* (Jo and Topal, 1995). In addition, type IIe restriction endonucleases require the recognition of a second DNA (effector) sequence to cleave DNA. NaeI (Jo and Topal, 1995) NarI, BspMI, Hpa II, SacII (Oller *et al.*, 1991), EcoRII (Gabbara and Bhagwat, 1992), Atu BI, Sau BMKI, Eco 57I and Ksp 6321 (Reuter *et al.*, 1993) are some of members of this group of type IIe enzymes. Eco RII is homologous to the integrase family of proteins. NaeI induces loops in DNA, with the enzyme binding at the base of the loops. Interestingly, introduction of type IIe restriction endonucleases in eukaryotic cells such as yeast, and mammalian cells induces DNA rearrangements (Schiestl and Petes, 1991). These kind of rearrangements arose due to the random ligation of the fragments made recombinogenic by restriction endonucleases cleavage (Chang and Cohen, 1977). Thus, the presence of endonuclease and ligase activities function together in the same polypeptide might together make a form of recombinase.

Fig 4.1: White sectoring in blue colonies in *E. coli* containing pMX25 plasmid on luria agar containing X-gal IPTG



Coliphage T4 EndoII postulated that it may have both endonuclease and recombinase activities in a single enzyme. This enzyme may be involved in more than one aspect of DNA metabolism and that the pathways are intertwined in the living cell (Carlson and Kosturko, 1998). Homologous recombination is commonly initiated by double stranded breaks. In most cases the nature of endonucleolytic activity is unknown, but in one case its sequence specific endonuclease (Endo-SceI in yeast mitochondria) has shown to be involved (Nakagawa *et al.*, 1992). Sequence-specific endonuclease may stimulate both homologous and illegitimate chromosomal recombination (Haber and Leung, 1996; Sargent *et al.*, 1997) and are found to be involved in gene conversions (a recombination event) which occurs in eukaryotes (Delahodde *et al.*, 1989; Morishima *et al.*, 1993).

E. coli strains with overexpressing *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 excisase A may also have restriction endonuclease activity. Interestingly, Carracso *et al* (1994) found that a few of the *E. coli* cultures started from either blue or white had lost the *xisF* containing plasmid. Loss of *xisF* containing plasmids suggests that overexpression of *xisF* causes plasmid instability an effect presumably related to its recombinase activity. This phenomenon was not studied further.

In this chapter, the excisase's A endonuclease activity was monitored in *E. coli* containing different plasmids under different growth conditions. The parameter used was the antibiotic susceptibility of the cells that results from loss of antibiotic resistant marker as a result of endonuclease action on the target site borne by the plasmid.

4.2: MATERIALS and METHODS

4.2.1: Media

5X M9 Salt/liter (Sambrook *et al.*, 1989)

$Na_2HPO_4.2H_20$: 64g
KH_2PO_4 : 15g
NaCl : 2.5g
NH_4Cl ** : 5g
Distilled water to 1000ml;
After autoclaving add
Glucose : 100mM
1M $CaCl_2.2H_2O$: 0.1ml
1M $MgSO_4$ $7H_2O$: 2ml
Thiamine .HCl : 10mg /Liter

Unless otherwise indicated here M9 minimal medium refers to M9 minimal medium containing 40mM NH_4Cl.

**- used either NH_4Cl or KNO_3

4.2.2: Strains

Strains	Genotype	Reference
GN#1	JM101 (pMX25 and pMC71A)	This study
DH5α	pAn207.65	This study
JM101	pAn207.65	This study
DH5α	pAn256	This study
JM101	pAn256	This study
OK#1	JM101 (pAn207.65 and pMC71A)	This study
OK#2	DH5α (pAn207.65 and pMC71A)	This study

4.2.3: Plasmids

4.2.3.1: pAn207.65 (Lammers *et al.*, 1986)

An 2.4kb KpnI-HincII fragment containing complete *xisA* gene of A*nabaena* PCC 7120 *nifD* element subcloned into pUC19 multiple cloning site. This plasmid has

ampicillin resistance gene and a 11bp target site for excisase A located in the upstream of the promoter region. The size of the pAn207.65 is 5.1kb **(Fig 4.2)**

4.3.2.2: pAn256 (Rice *et al.*, 1982)

An 2.9 kb HindIII fragment of distal region of A*nabaena* PCC 7120 *nifD* element subcloned into pBR322 vector. It has ampicillin and tetracycline resistance genes and a 11 bp target site for excisase at 5' end of the carboxy terminal fragment of *nifD* element The size of the pAn256 is 7.2kb **(Fig 4.3)**.

4.2.4: Endonuclease activity of excisase A with pAn207.65 under aerobic conditions.

E. coli containing pAn207.65 or pAn 256 was grown over night in Luria broth or M9 minimal medium containing ampicillin at 37°C. 1ml culture was harvested and washed twice with either Luria broth or M9 medium and resuspended in 1ml Luria broth or M9 minimal medium. 1% of (150µl) of the cells was reinoculated in M9 minimal medium containing ampicillin. At different time points, the cultures were diluted and plated on Luria agar with and without ampicillin. OD at 620nm monitored. The endonuclease activity was calculated using this formula:

$$\text{Excision (\%)} = \frac{\text{Total number of white colonies}}{\text{Total number of colonies}} \times 100$$

For induction of *xisA* gene from *lac* promoter in plasmid pAn 207.65, 0.1mM IPTG was used.

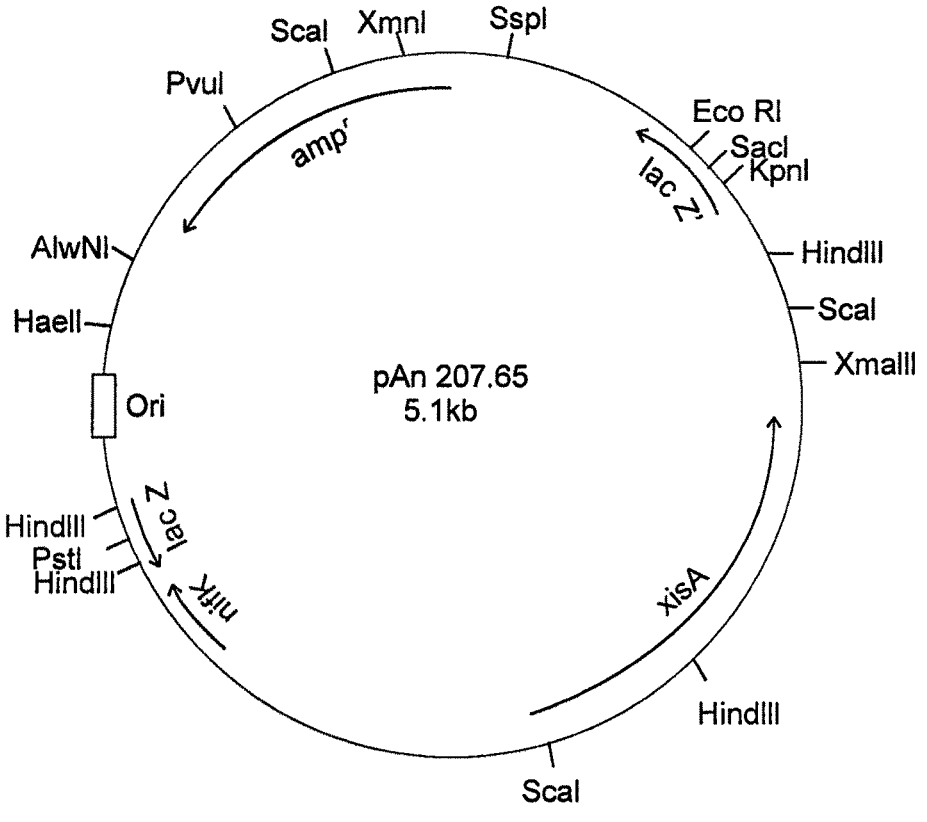


Fig. 4.2 : Restriction map of pAn207.65 plasmid.

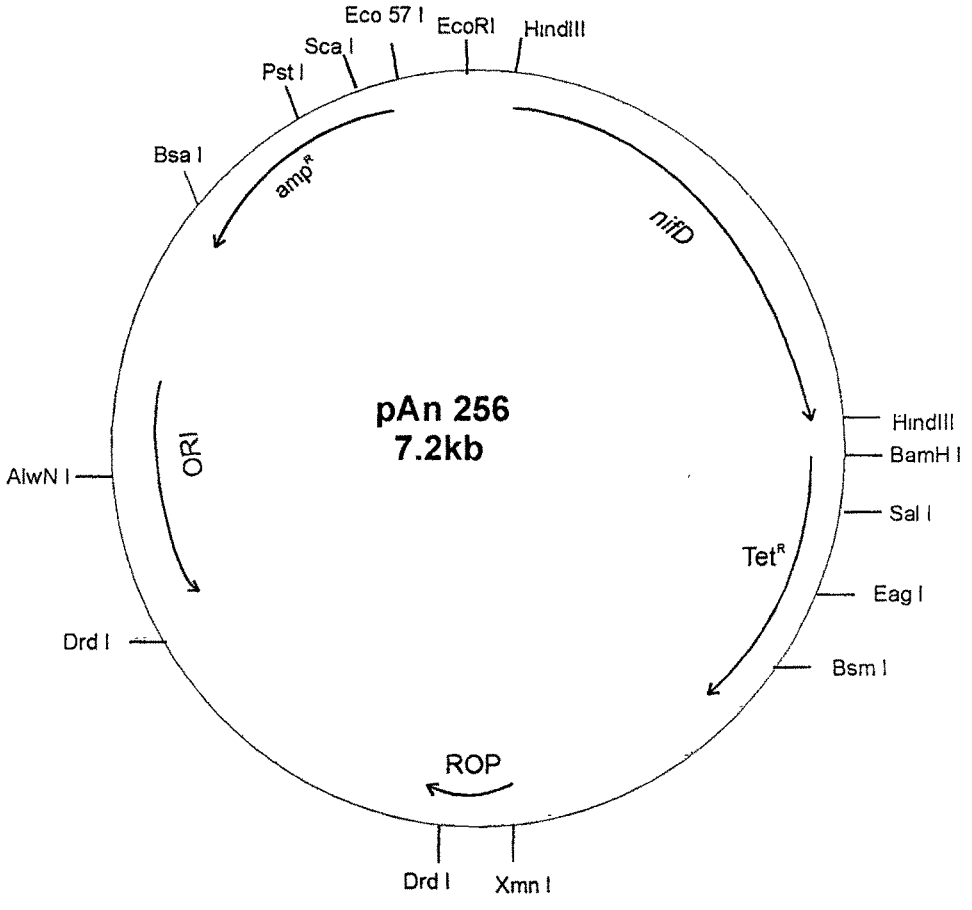


Fig. 4.3 : Restriction map of pAn 256 plasmid

4.2.5: Endonuclease activity of excisase with pAn207.65 under anaerobic conditions.

E. coli containing pAn207.65/pAn256 were grown overnight in Luria Broth or M9 minimal medium /M9 minimal medium containing 20mM KNO_3 at 37°C with ampicillin. 1ml cells were harvested and washed twice with either Luria broth or NH_4Cl / KNO_3 and resuspended in 1ml of respective media. 1% inoculum was reinoculated into M9 containing 40mM NH_4Cl /20mM KNO_3. At different time points of growth, the cells were diluted in saline and plated on Luria agar with and without ampicillin. Anaerobic experiments were carried out by the method of Gerhardt *et al.* (1981). For anaerobic growth experiments, the cells were grown in microfuge or test tubes. For growth in M9 minimal medium 0.025% Cystine HCl and Resazurine were added and sealed with paraffin oil to generate anaerobiosis. The cells were kept in incubator for growth. At different time points, dilutions were made and plated on Luria agar with and without ampicillin. OD was monitored at 620nm. The endonuclease activity was calculated using the formula given above.

4.2.6: Endonuclease activity of excisase A with pMX25 under aerobic conditions.

A single colony of *E. coli* GN#1 was grown overnight at 37°C in 15ml Luria broth with kanamycin, carbenicillin and chloramphenicol. 0.1ml cells from 10^{-4} dilution (diluted in saline) were plated on Luria agar containing ampicillin and chloramphenicol X-Gal. 1.0 ml was spun at 5000 rpm at 4° C for 5min. The pellet was washed twice with M9 minimal medium and suspended in same volume of M9 minimal medium. 1% of (150µl) of the cells were reinoculated into 15ml of M9 minimal medium without any antibiotics. 1% (500 µl) cells were reinoculated in 50ml of medium containing carbenicillin and chloramphenicol and medium lacking antibiotics. The cells were drawn at different time points, from each of the culture,

diluted in saline and plated on Luria agar with X-gal , X-gal carbenicillin, and X-gal chloramphenicol. The number of blue and white colonies were counted.

Growth dependent endonuclease activity experiments were same as above and the viable *E. coli* cells were determined on different antibiotic plates containing X-Gal. Number of white colonies appearing on plates without any antibiotic and on plates containing chloramphenicol (z) were due to either loss of pMX25 by the cells (x) or formation of rearranged pMXΔ25 (y). The value x will indicate the extent of endonuclease activity whereas y the excision frequency. However, the white colonies on ampicillin plates are only due to *E. coli* containing plasmid pMXΔ25 (y). Thus the endonuclease activity x = z-y.

4.2.7 : Time dependent endonuclease activity of *E. coli* GN#1 in Luria broth and M9 minimal medium.

A single colony of *E. coli* GN#1 was grown overnight at 37°C in 15ml Luria broth with kanamycin, carbenicillin and chloramphenicol . Dilutions were made in saline, 10^{-4} 0.05-0.1 ml from 10^{-4} was plated on Luria agar containing carbenicillin and chloramphenicol and on plate without any antibiotic. 1ml culture was spun down and washed twice with Luria broth or M9 minimal medium and resuspended. 1% cells were reinoculated into 50ml Luria broth or 50ml M9 minimal medium. After different time points, the culture was diluted in saline and plated on Luria agar without any antibiotic; Luria agar containing carbenicillin, and Luria agar containing chloramphenicol. The viable count was determined. The endonuclease activity was calculated using the formula given above.

4.2.8: Endonuclease activity of excisase A in the presence of *nifA*.

A single colony of *E. coli* OK#1, was grown overnight in 15ml Luria broth overnight with ampicillin and chloramphenicol. Dilutions were made in saline and plated on Luria agar containing ampicillin and chloramphenicol; Luria agar containing chloramphenicol and Luria agar without any antibiotic. The endonuclease activity was calculated using the formula as described earlier.

4.3: RESULTS

4.3.1: Restriction endonuclease activity of excisase A in the *E. coli* GN#1 grown in M9 minimal medium with ampicillin and chloramphenicol.

Endonuclease activity of excisase A was studied by monitoring the loss of the plasmid pMX25 from the *E. coli* GN#1 after growing for 24h in M9 minimal medium containing chloramphenicol and ampicillin. *E. coli* which had lost the plasmids, failed to grow on ampicillin plates but grew on plates without antibiotic. The results were presented in the **Table 4.1**. 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. Around 1000 white colonies from Luria agar containing chloramphenicol and from Luria agar not containing any antibiotic, were restreaked on Luria agar, with chloramphenicol and with ampicillin and chloramphenicol. All white colonies tested grew on plates without antibiotic and chloramphenicol but not on plates supplemented with ampicillin and with chloramphenicol. However, all blue colonies from the same experiments grew on all plates. These results suggest that only pMX25 plasmid is lost from *E. coli* GN#1 but not pMC71A.

Table 4.1 Viability of *E. coli* GN#1 and PK#1 strains grown in M9 minimal medium containing chloramphenicol and ampicillin.

Strain	Number of blue colonies	Number of white colonies	Total	Viability
GN#1				
No antibiotic	348	16680	17028	$1.7X10^7$
With Chloramphenic	130	26620	26750	$2.7X10^7$
With ampicillin	649	1732	2381	$2.4X10^5$

Strain	Number of blue colonies	Number of white colonies	Total	Viability
PK#1				
No antibiotic	202	16400	16602	$1.6X10^7$
With Chloramphenic	468	23952	24420	$2.4X10^7$
With ampicillin	1198	1998	3196	$3.2X10^5$

Results are an average of three independent experiments.

4.3.2: Growth dependent antibiotic susceptibility of *E. coli* GN#1 grown in the presence of ampicillin and chloramphenicol.

E. coli GN#1 was grown in Luria broth containing chloramphenicol and ampicillin and the viability was monitored at different time points. The observations were presented in the **Table 4.2.** At all time points, no significant change in the viability of *E. coli* was found between chloramphenicol plates and plates without any antibiotic. It was seen that at 0h, the viability of pMX25 plasmid containing cells on ampicillin plates was almost equal to that on plates without any antibiotic (118%) but steadily decreased to about 26% by 12h. No further decrease was found in the viability at later time points.

Table 4.2 : Growth dependent antibiotic susceptibility of *E. coli* GN#1 grown in Luria broth containing chloramphenicol and ampicillin.

Time (h)	OD 620nm	Without antibiotic	+ Chloram phenicol	+ Ampicillin	% Viability on Ampicillin Plate
0	0.01± 0.00	1.6x 10^5 ±0.15	2.2 x 10^5 ±0.28	1.9 x 10^5 ±0.30	118±17
3	0.10± 0.00	3.4 x 10^7 ±0.55	3.6 x 10^7 ±0.21	3.6 x 10^7 ±0.16	105±16
6	0.49± 0.01	1.6 x 10^8 ±0.36	1.6 x 10^8 ±0.42	1.4 x 10^8 ±0.23	88±8
9	0.72 ±0.01	2.1 x 10^8±0.02	2.2 x 10^8 ±0.24	1.5 x 10^8 ±0.49	67±10
12	0.73 ±0.02	3.4 x 10^8 ±0.62	3.6 x 10^8 ±0.42	9.0 x 10^7 ±0.30	26±7
24	0.63 ±0.03	4.2 x 10^8 ±0.28	4.2 x 10^8 ±0.56	1.0 x 10^8 ±0.02	24±4

Results are of three independent experiments.

Similar experiments were also done with M9 minimal medium using *E. coli* GN#1. The results were presented in the **Table 4.3**. It was found that at 0h the viability of pMX25 plasmid on Luria agar containing ampicillin plates compared to that on Luria agar lacking any antibiotic and the value was about 95%. Viability decreased with time rapidly on ampicillin plates in M9 minimal medium but viability loss was less for Luria broth grown cells. After 6h of growth, about 38% *E. coli* were viable on ampicillin plates and this value decreased to about 5% at 24h. Even when grown in M9 minimal medium, there was no difference in the cell number of *E. coli* GN#1 on Luria agar containing chloramphenicol or the plates lacking antibiotics.

Table 4.3: Growth dependent antibiotic susceptibility of *E.coli* GN#1 in M9 minimal medium in the presence of chloramphenicol and ampicillin.

Time (h)	OD 620nm	Without antibiotic	+Chloram phenicol	+ Ampicillin	% Viability on Ampicillin Plate
0	0.06±0.01	$2.4x\ 10^6$ ±0.40	$2.3 x\ 10^6$ ±0.23	$2.3 x\ 10^6$ ±0.26	95±9
6	0.06 ±0.01	$2.3 x\ 10^6$ ±0.30	$2.3 x\ 10^6$ ±0.35	$0.9 x\ 10^6$ ±0.11	38±5
12	0.11±0.01	$1.5 x\ 10^7$ ±0.15	$1.5 x\ 10^7$ ±0.17	$3.4 x\ 10^6$ ±0.43	23±4
18	0.30 ±0.10	$2.7 x\ 10^8$±0.27	$2.9 x\ 10^8$ ±0.21	$3.1 x\ 10^7$ ±0.02	12±2
24	0.91 ±0.02	$1.2 x\ 10^9$ ±0.30	$1.2 x\ 10^9$ ±0.14	$6.0 x\ 10^7$ ±0.17	5±2

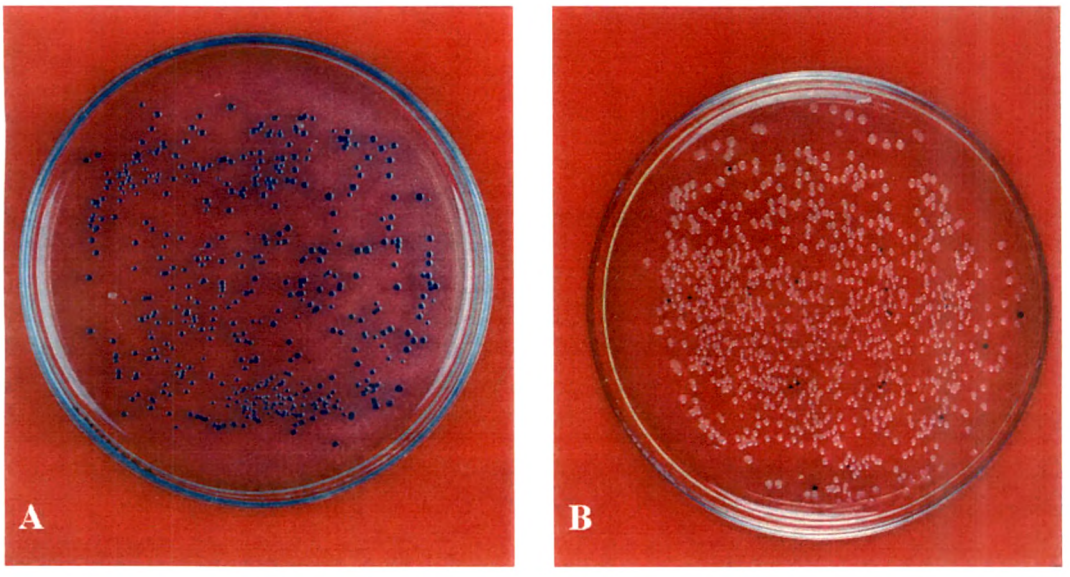
Results are of three independent experiments.

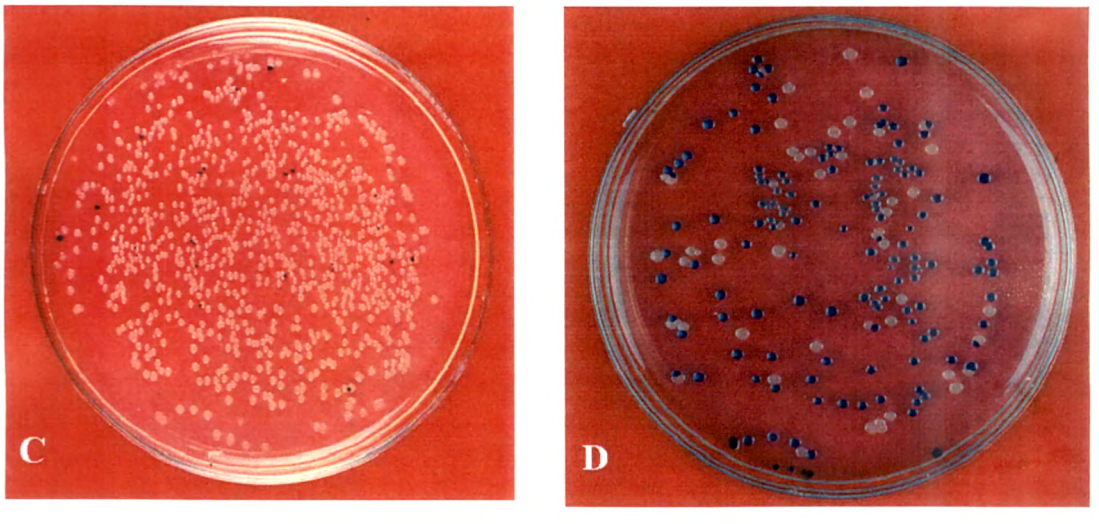
4.3.3: Growth dependent restriction endonuclease activity of excisase A in GN#1 grown in the absence of antibiotics.

E. coli GN#1 cells cured of plasmid pMX25 cannot grow on the plates in the presence of ampicillin and chloramphenicol. In order to determine growth dependent restriction endonuclease activity of excisase A, the antibiotic susceptibility was determined with *E. coli* GN#1 which was grown in M9 minimal medium in the absence of antibiotics. The results were shown in the **Table 4.4A** and **Fig. 4.4.** X-Gal was added in the plates to also monitor blue and white colonies. The 0h plating showed no white colonies (rearranged) **Fig 4.4A**. Viability of the cells when grown in M9 minimal medium in the absence of antibiotics was found to be similar to that of *E. coli* GN#1 grown with both antibiotics (**Table 4.4B**). But the number of white colonies on plates containing carbenicillin and plates without antibiotic were very high and majority of the colonies were white after 12h. Surprisingly, white colonies reached a maximum of 25% by 24 h. Blue and white colonies were picked from

Fig 4.4: Antibiotic susceptibility of *E. coli* GN#1 on minimal medium

A: 0h
B: 24h M9 minimal medium without Antibiotic
C: 24h M9 minimal medium with chloramphenicol
D: 24h M9 minimal medium with carbenicillin and chloramphenicol


A
B


C
D

plates lacking antibiotics and restreaked on Luria agar X-Gal; carbenicillin and chloramphenicol plates. All blue colonies grew on all three plates but white colonies arising at all time points except 0h could not survive on Luria agar containing carbenicillin plates (Data not shown).

Table 4.4A: Growth dependent excision of the *nifD* element of *Anabaena* PCC 7120 and the antibiotic resistance of *E. coli* containing *nifA* gene of *K. pneumoniae* grown under M9 minimal medium in the absence of antibiotics.

Time (h)	X-Gal No Antibiotic		X-Gal + Chloramphenicol		X-Gal + Carbenicillin	
	Blue	**White**	**Blue**	**White**	**Blue**	**White**
0	5.1×10^7	2.3×10^6	2.8×10^7	4.0×10^6	7.9×10^7	-
4	3.0×10^6	4.6×10^6	3.8×10^6	3.2×10^6	1.5×10^6	6.4×10^4
8	4.0×10^6	6.0×10^6	4.2×10^6	7.2×10^6	2.2×10^6	2.5×10^5
12	1.0×10^6	2.0×10^7	1.2×10^6	3.4×10^7	3.9×10^6	5.9×10^5
16	2.2×10^7	6.9×10^7	1.9×10^7	8.5×10^7	4.2×10^6	6.0×10^5
24	1.5×10^7	1.0×10^8	1.3×10^7	1.1×10^8	5.0×10^6	1.7×10^6
48	1.9×10^7	1.6×10^8	6.8×10^8	1.2×10^8	8.5×10^6	6.0×10^5

Results are of three independent experiments.

White colonies on Luria agar containing carbenicillin plates are a result of the excision of pMX25 plasmid whereas the white colonies appearing on plates containing chloramphenicol and on plates without antibiotic will also be due to the *E. coli* GN#1 loosing the pMX25 plasmid by endonuclease activity. The endonuclease activity calculated from this data shows, that it rapidly increases to about 41% at 4h and steadily increases to about 80% at 48h **(Table 4.4 C).**

Table 4.4B : Viability of *E. coli* GN1 grown under minimal medium conditions.

Time (h)	Total number cfu			Viability of Cells (%)	
	No Antibiotic	Chloramphen-icol	Carbenicillin	Chlora mphen icol*	Carben icillin **
0	$5.3 \times 10^7 \pm 0.60$	$3.2 \times 10^7 \pm 0.20$	$7.9 \times 10^7 \pm 0.49$	60	150
4	$7.6 \times 10^6 \pm 0.80$	$7.0 \times 10^6 \pm 0.60$	$1.6 \times 10^6 \pm 0.40$	92	21
8	$1.0 \times 10^7 \pm 0.10$	$1.1 \times 10^7 \pm 0.18$	$2.2 \times 10^6 \pm 0.24$	110	25
12	$2.1 \times 10^7 \pm 0.30$	$3.5 \times 10^7 \pm 0.42$	$2.5 \times 10^6 \pm 0.18$	166	21
16	$9.1 \times 10^7 \pm 0.63$	$1.0 \times 10^8 \pm 0.09$	$4.5 \times 10^6 \pm 0.12$	110	5.3
24	$1.0 \times 10^8 \pm 0.36$	$1.2 \times 10^8 \pm 0.21$	$6.7 \times 10^6 \pm 0.15$	120	6.7
48	$1.8 \times 10^8 \pm 0.30$	$1.2 \times 10^8 \pm 0.14$	$9.1 \times 10^6 \pm 0.33$	66	5.07

* Chl/-Ab x 100, ** Carb / -Ab x 100
Results are of three independent experiments.

Table 4.4C: Viability and excision of *E .coli* GN#1 grown under M9 minimal medium conditions:

	Percentage of white colonies			
Time (h)	X-Gal no antibiotic (z1)	X-Gal + chloramphenicol (z2)	X-Gal carbenicillin	Endonuc-lease activity (x)
0	4.3	12.5	0.0	4.3* / 12.7**
4	60.5	45.7	4.0	56.5 / 41.7
8	60.0	65.5	10.	50.0 / 55.5
12	95.2	97	13.1	82.1 / 84.0
16	75.6	85	12.5	63.1 / 72.5
24	100	91.7	25.4	74.6 / 66.3
48	88.8	100	6.6	82.2 / 93.4

z1-x = *
z2- x = **

4.3.4: Growth dependent antibiotic susceptibility of *E. coli* JM101 (pAn207.65) in M9 minimal medium.

To study the endonuclease activity of *xisA* on plasmids containing single target site, the *E. coli* containing the plasmid pAn207.65 was grown in M9 minimal medium in the presence of ampicillin. The results were presented in the Table 4.5. For these experiments, the cells were first grown overnight in Luria broth and reinoculated in M9 minimal medium along with ampicillin. At 0h the viability of pAn207.65 on Luria agar containing ampicillin was 110%. After 6h of growth, the viability was reduced drastically to 14% after which there was no significant change in viability at longer periods.

Table 4.5 : Growth Dependent antibiotic susceptibility of *E. coli* JM101 containing pAn207.65 plasmid in M9 minimal medium.

Media/ Time (h)	OD 620nm	Without Ampicillin	With Ampicillin	% Viability on Ampicillin Plate
Luria broth/0	0.30±0.01	6.7×10^7 ±0.9	7.2×10^7 ±0.98	110±19.27
M9minimal medium/6	0.29 ±0.01	2.5×10^7 ±0.05	3.4×10^6 ±0.17	14±0.60
12	0.44±0.05	3.3×10^7 ±0.8	2.8×10^6 ±0.70	8.5±2.15
16	0.64 ±0.14	2.0×10^7 ±0.70	2.0×10^6 ±0.60	10±3.45
24	1.20 ±0.00	1.0×10^8 ±0.10	1.0×10^7 ±0.10	10±0.64

Luria broth grown cells were transferred to M9 minimal medium.
Results are of three independent experiments.

4.3.5 : Endonuclease activity of *E. coli* JM101 (pAn207.65) grown under different nitrogen sources in the absence of antibiotics.

E. coli JM101 cells containing pAn207 65 grown over night in M9 minimal medium and ampicillin was used to inoculate M9 minimal medium, containing different N-sources. The culture were grown both aerobically and anaerobically. The results of growth and endonuclease activity of excisase presented in the **Table 4.6**, indicated that the viability of ampicillin resistant cells at 0h was 12%. The viability decreased to a low of 1% after 24h under aerobic conditions of growth in the presence of ammonium chloride. But under anaerobic growth conditions, when supplemented with ammonium chloride, the viability decreased to 3% and 0.6% at 12h and 24h of growth respectively. Similar results were obtained when grown in anaerobic conditions with nitrate as the N source.

4.3.6 : Viability of *E. coli* containing pAn256 under different N-conditions.

The plasmid pAn256 contains proximal end the *nifD* element of *Anabaena* PCC 7120 and contains only one 11bp direct repeat sequence but does not have excisase A **(Fig 4.3).** The viability of plasmid (pAn256) harboring cells was compared to viability of cells containing pMX25 or pAn207.65, each of the latter two plasmids included in addition to 11bp target DNA , and the *xisA* coding sequence. The experiment was designed to test if the loss of viability of the cells harboring pMX25 or pAn207.65 or is a result of expression of *xisA* gene on the context of target site, Viability was monitored at different time points for aerobically and/or anaerobically grown cells under different N source supplementation conditions. The results presented in Table 4.7 suggested no difference in the viability of the culture containing pAn256 under different growth conditions. as against the **dramatic loss of viability** of cells harboring pAn 207.65 or pMX25 **(Table 4.4 & Table 4.6).**

Table 4.6 : Ampicillin susceptibility of *E. coli* JM101 containing pAn207.65 in M9 minimal medium under different growth conditions.

Time (h)	O.D. 620nm.	Total number of cells in the medium.	Number of cells medium with Ampicillin resistance	% Viability on Ampicillin Plate
0	0.13± 0.05	$3.4 \times 10^7 \pm 0.35$	$4.2 \times 10^6 \pm 0.35$	12.4±0.14
		Aerobic 40mM NH_4Cl		
12	0.41± 0.20	$7.2 \times 10^7 \pm 0.80$	$5.6 \times 10^6 \pm 1.1$	8.8±4.11
24	0.98± 0.13	$3.8 \times 10^7 \pm 0.40$	$3.9 \times 10^5 \pm 0.20$	1±0.80
36	1.03± 0.12	$3.9 \times 10^8 \pm 0.50$	$6.0 \times 10^6 \pm 0.60$	1.4±0.14
48	1.06± 0.10	$3.3 \times 10^8 \pm 1$	$2.0 \times 10^6 \pm 0.60$	0.6±0.16
		Anaerobic 40mM NH_4Cl		
12	0.19± 0.10	$6.0 \times 10^5 \pm 1.1$	$2.0 \times 10^4 \pm 0.10$	3.0±0.16
24	0.40± 0.09	$1.8 \times 10^6 \pm 0.30$	$1.0 \times 10^4 \pm 0.20$	0.6±0.01
36	0.41± 0.06	$6.5 \times 10^6 \pm 1.2$	$3.4 \times 10^4 \pm 0.80$	0.5±0.40
48	0.42± 0.06	$5.4 \times 10^6 \pm 1.4$	$2.0 \times 10^4 \pm 0.50$	0.4±0.26
		Anaerobic 20mM KNO_3		
12	0.16± 0.07	$4.8 \times 10^6 \pm 0.40$	$1.5 \times 10^5 \pm 0.30$	3.1±0.12
24	0.42± 0.11	$3.3 \times 10^6 \pm 0.60$	$1.2 \times 10^5 \pm 0.30$	3.6±0.38
36	0.42± 0.01	$6.6 \times 10^7 \pm 0.70$	$6.2 \times 10^5 \pm 0.30$	1.3±0.30
48	0.42± 0.01	$7.1 \times 10^7 \pm 0.80$	$4.3 \times 10^5 \pm 0.40$	0.6±0.21

E. coli cells containing pAn 207.65 were grown overnight in M9 minimal medium containing ampicillin. They were transferred to M9 minimal medium containing different N sources and grown under Aerobic and Anaerobic conditions without ampicillin. The number of cells was determined from the number of colonies on Luria agar plates.

Results are of three independent experiments

Table 4.7 : Growth dependent antibiotic susceptibility of *E. coli* JM101 containing pAn 256 plasmid in M9 minimal medium.

Time (h)	Condition	Medium *	Without Ampicillin	With Ampicillin	% Viability on Ampicillin Plate
0		NH_4Cl	3.3×10^7 ±0.21	3.2×10^7 ±0.24	96±5
24	Aerobic	NH_4Cl	4.3×10^7 ±0.27	4.6×10^7 ±0.16	106±9
48		NH_4Cl	2.5×10^6 ±0.26	2.6×10^6 ±0.12	104±11
24	Anaerobic	KNO_3	3.2×10^7 ±0.15	3.3×10^7 ±0.11	103±7
48		KNO_3	2.4×10^7 ±0.13	2.8×10^7 ±0.12	116±6

* M9 minimal medium with 40mM NH_4Cl or 20mM KNO_3
Results are of three independent experiments.

4.3.7: Effect of IPTG on the endonuclease activity of excisase A in *E. coli.*

The *xisA* gene is in same orientation as P_{lacZ} promoter in pAn207.65 plasmid. In order to determine whether *xisA* could be expressed using P_{lacZ} promoter, endonuclease activity was monitored in the presence and absence of IPTG in the *E .coli* JM101 containing pAn207.65. Results were shown in the **Table 4.8.** In these experiments, the culture was grown over night on M9 minimal medium instead of Luria broth. No significant difference was found in the viability of cells grown in the presence and absence of IPTG.

Table 4.8: Growth dependent antibiotic susceptibility of *E. coli* JM101 containing pAn207.65 plasmid in M9 minimal medium with and without IPTG.

Time (h)	O.D. 620nm	Condition*	Without Ampicillin	With Ampicillin	% Viability on Ampicillin Plate
0	0.13	NH_4Cl	3.4×10^7 ±0.35	4.2×10^6 ±0.35	12.4±0.14
12	0.02	-IPTG	1.9×10^7 ±0.42	1.5×10^6±0.30	8±0.56
	0.05	+IPTG	1.6×10^7 ±0.40	4.5×10^5 ±0.42	3±0.55
24	0.20	-IPTG	5.2×10^7 ±0.36	5.0×10^5 ±0.38	1±0.5
	0.30	+IPTG	4.5×10^7 ±0.43	2.1×10^5 ±0.25	0.5±0.1

* M9 minimal medium with 40mM NH_4Cl or 20mM KNO_3.
Results are of three independent experiments.

4.3.8: Endonuclease activity of *E. coli* OK#1.

The endonuclease activity of excisase was also studied with *E . coli* strains OK#1(*recA*$^+$) and OK#2 (*recA*$^-$) each containing the plasmids pMC71A and pAn207.65. It was observed that *E. coli* OK#1 grown over night in Luria broth with ampicillin and chloramphenicol showed 0.22% viability on plates containing ampicillin as compared to the plates without antibiotic (**Table 4.9**). *E. coli* OK#2 grown over night in Luria broth containing ampicillin and chloramphenicol did not show any viable cells upon plating on Luria agar containing ampicillin (Data not shown). The plasmids were extracted from over night grown cultures of OK#1 and OK#2 and were digested with EcoRI. pAn207.65 plasmid was found in *E. coli* OK#1 but not detected in *E. coli* OK#2 **(Fig. 4.5).**

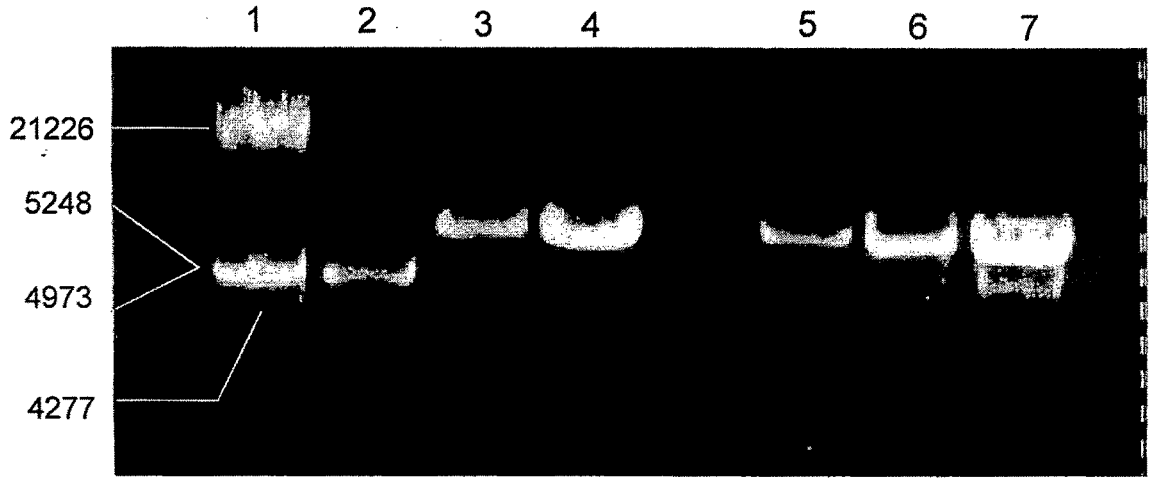


Fig 4. 5 : Eco RI digestion pattern of pAn207.65 & pMC71A plasmid DNA from different *E.coli* strains grown over night in Luria broth .

Lanes : **1**. Lambda DNA digested with HindIII /Eco RI; Plasmid DNA digested with Eco RI,**2**. DH5∝ (pAn207.65) , **3**. DH5α(pMC71A), **4**. OK#2, **5**. JM 101 (pMC71A) and **6 & 7**. OK#1

Table 4.9: Endonuclease activity of *E. coli* OK#1 grown in Luria broth.

Time (h)	OD620 nm	Without antibiotic	+Chloram-phenicol	+Ampicillin	+Chloram-phenicol and Ampicillin	%Viability on Ampicillin
0	0.73±0.08	$5.33x10^8$± 0.92	$6.73x10^8$± 2 19	$1.16x10^6$± 0.40	$0.8x10^6$± 0.52	0.22±0.09

Results are of three independent experiments.

4.4 : DISCUSSION

E. coli GN#1(*recA*$^+$) and PK#1 (*recA*$^-$) containing pMX25 and pMC71A plasmids had low viability only in the medium containing ampicillin but not with chloramphenicol. Detailed experiments with *E. coli* GN#1 showed the loss of viability increased with growth phase and was also more in M9 minimal medium as against that in Luria broth. However, the loss in viability was found on only ampicillin but not on chloramphenicol, which suggests that viability, loss could be due loss of pMX25 by abortive excision events. Such a view was proposed by Lammers *et al.* (1986) to explain the presence of ampicillin sensitive white sectors in the blue colonies containing pMX25.

It was surprising that ampicillin sensitive colonies survived in the growth medium even upto 24h in the presence of *ampicillin.* Many more ampicillin sensitive *E. coli* may have been killed during growth. However, when similar experiments were performed with *E. coli* GN#1 in the absence of both the antibiotics, the viability of *E. coli* on ampicillin plates was found to be similar to that grown in the presence of both antibiotics. These results could be accounted if the ampicillin got inactivated, in the medium during early stages of growth, due to the secretion of β-lactamase by the ampicillin resistant *E. coli.*

In addition to the loss of viability, these experiments have also showed that the excision frequency when grown in the absence of chloramphenicol and ampicillin was only 25% **(Table 4.4C)** whereas when the cells were grown in the presence of ampicillin and chloramphenicol was about 70% (Results from Chapter III). This may be accounted if the accessory proteins are limiting the excision and the cleaved *nifD* element may be acted upon by the exonucleases.

Earlier experiments with overexpression of *xisA* in *E. coli* suggested that excisase A had toxic effects (Brusca *et al.*, 1990). In order to determine whether the toxicity of excisase A is related to its excision activity, the loss of pAn207.65, which contains single target site, was monitored under different conditions. In all conditions about 90% loss of viability was found in the cells grown in the presence of ampicillin. Furthermore, the nature of nitrogen source, anaerobic growth conditions or the presence of IPTG did not further effect the viability.

The pAn256 plasmid that contains only 11 base pair target site but no excisase A had no effect on viability. Interestingly, *E. coli* OK#1 and OK#2 strains showed specific loss of ampicillin resistance but not chloramphenicol resistance marker. The viability was only 0.22% in the case of OK#1 when grown over night in Luria broth. The loss of viability was very high in OK#2 that it was difficult to estimate the number of viable cells. OK#2 is recA$^-$ isogenic strain of OK#1 which suggests that this precipitous loss of viability of *recA*$^-$ could be due largely to the instability of the strain to repair the dsDNA breaks, gaps, created by *xisA* action on chromosomal DNA.

These results indicate that excisase A cleaves the 11 base pair target sites and the toxic effects on *E. coli* may also occur by specific endonuclease activity on the genomic DNA. However, direct demonstration of endonuclease activity need to be demonstrated. So far, studies on the mechanism of excisase A have been hindered by

the absence of purified protein. Direct demonstration of endonuclease activity with excisase A on plasmids containing the target sites have been found to be very difficult, that the protein was not even detected. Studies on *xisA* gene expression may help in detection and purification of excisase A.