# CHAPTER V

## Expression of *xisA* promoter of *Anabaena* PCC 7120 in *Escherichia coli.*

## 5.1: Introduction

*Anabaena* PCC 7120 expresses genes involved in heterocyst differentiation and nitrogen fixation upon nitrogen starvation. Elhai and Wolk (1990) used firefly luciferase to study the gene expression in the vegetative cells and heterocysts of *Anabaena* PCC 7120. The $P_{nifHDK}$ was fused into *luxAB*, the expression of the promoter fusion occurs only in the heterocysts whereas the construct $P_{glnA}$ -*luxAB* expresses in both cell types *i.e.*, vegetative cells and heterocysts, and $P_{rbcLS}$ -*luxAB* express only in the vegetative cells. Induction of the $P_{nifHDK}$ -*lacZ* occurs after 13-16h after removal of combined nitrogen. There are no reports of the expression of *xisA* or $P_{xisA}$ - reporter gene fusion to study the expression of *xisA*. The regulation of *xisA* during heterocyst development is not understood and the attempts to map its promoter were not successful (Golden and Weist, 1988).

In the previous chapters, it has been shown that NifA protein of *K. pneumoniae* causes dramatic increase in the excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* cells grown in M9 minimal medium and this increase was not found in *E. coli ntrA* (σ54) mutant. Thus, it appears that NifA protein increases the expression of *xisA* gene, which is mediated by σ54. In order to determine the role of *xisA* gene on the excision of *nifD* element, β-galactosidase structural gene was fused to the putative $P_{xisA}$ promoter and the expression was monitored under different growth conditions.

## 5.2: MATERIALS AND METHODS

### 5.2.1: Strains

| Strains | Genotype | Reference |
|---|---|---|
| GN#2 | *JM101 (pKK#1 & pMC71A)* | This study |
| GN#3 | *JM101 (pMU575 &pMC71A)* | This study |
| GN#4 | *JM101 (pMU575&pAn207.65)* | This study |
| GN#5 | *JM101 (pKK#1 & pAn207.65)* | This study |
| RK#1 | *JM101 recA⁻ srl:: Tn10* | This study |
| RK#3 | *RK#1 (pKK#1 & pMC71A)* | This study |
| RK#4 | *RK#1 (pMU575& pMC71A)* | This study |
| RK#5 | *RK#1 (pMU575 & pAn207.65)* | This study |
| RK#6 | *RK#1 (pKK#1 & pAn207.65)* | This study |
| NK#1 | *JM101 rpoN::208 Tn10* | This study |
| NK#3 | *NK#1 (pKK#1&pMC71A)* | This study |
| NK#4 | *NK#1 (pMU575&pAn207.65)* | This study |
| NK#5 | *NK#1 (pKK#1&pAn207.65)* | This study |
| NK#7 | *NK#1 (pMU575&pMC71A)* | This study |

### 5.2.2: Plasmids

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

Same as given in chapter 4. Section 4.2.1

**5.2.2.2: pMU575** (Yang and Pittard, 1987).

It is a transcriptional fusion vector carrying trimethoprim resistance gene. The ribosome binding site and the first 55 codons of *galK* are fused to the eighth codon of *lacZ*. Stop codons are present in all the three reading frames upstream of ribosome binding site, which prevents translational interference from the cloned DNA. The λtL- terminator present upstream of the polylinker prevents transcriptional interference from other promoters.

### 5.2.2.3: Construction of pKK#1 plasmid.

The plasmid pAn207.65 carries the *xisA* gene promoter on a 1kb HindIII fragment **(Fig 5.1)**. The fragment was purified from the gel and ligated to HindIII digested pMU575 plasmid. The recombinant plasmid pKK#1 **(Fig 5.2)** contains the *xisA* promoter at the multiple cloning site fused to *lacZ* gene. *E. coli* transformed with pKK#1 show blue colonies on X -Gal plates. The presence of the *xisA* promoter is confirmed by the presence of 1kb fragment upon digesting the pKK#1 with restriction endonuclease HindIII **(Fig 5.3)**. The resultant plasmid has Trimethoprim resistance.

### 5.2.2.4: Orientation of the HindIII fragment in pKK#1 plasmid.

The orientation of the HindIII insert in pKK#1 was determined by digestion of the plasmid with restriction enzyme ScaI **(Fig. 5.4A)**, and BamHI plus ScaI **(Fig 5.4 B)**. The fact that 1kb size DNA fragment was obtained in the double digest indicates that the promoter is oriented in the same direction as for the direction of transcription of β-galactosidase gene.

### 5.2.3: β-Galactosidase Assay (Miller, 1992).

Composition of Z Buffer:/liter

| | |
|---|---|
| 60mM $Na_2HPO_4.2H_20$ | : 16.1g |
| 40mM $NaH_2PO_4.H_20$ | : 5.5g |
| 10mM KCl | : 0.75g |
| 1mM $MgSO_4.7H_2O$ | : 0.246g |
| 50mM β-Mercaptoethanol | : 2.7ml |

Adjust the pH to 7.0. Store in refrigerator.

Ortho-nitro phenyl-β-galactoside (4mg/ml) in Z-Buffer.

1M $Na_2CO_3$.

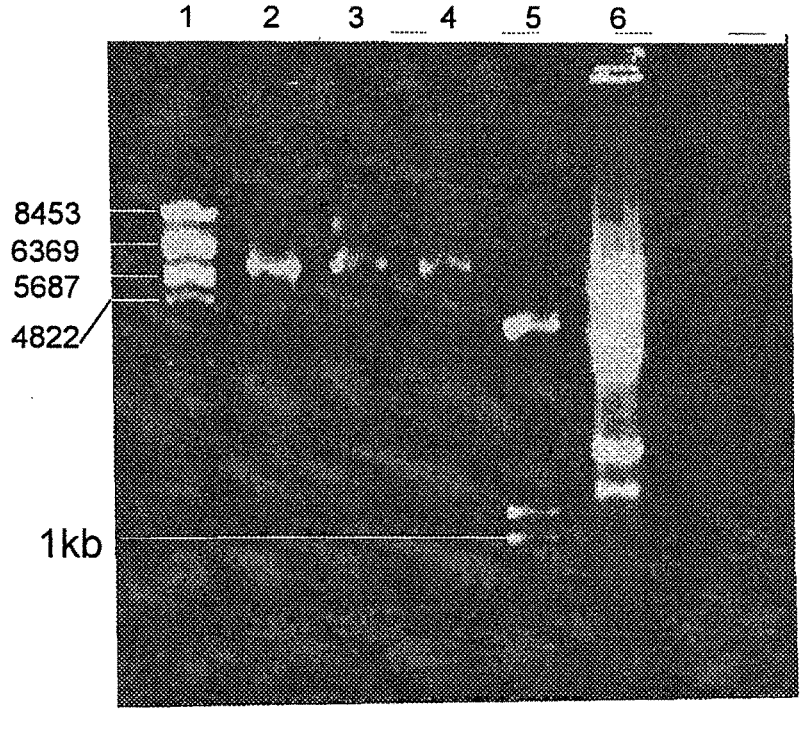
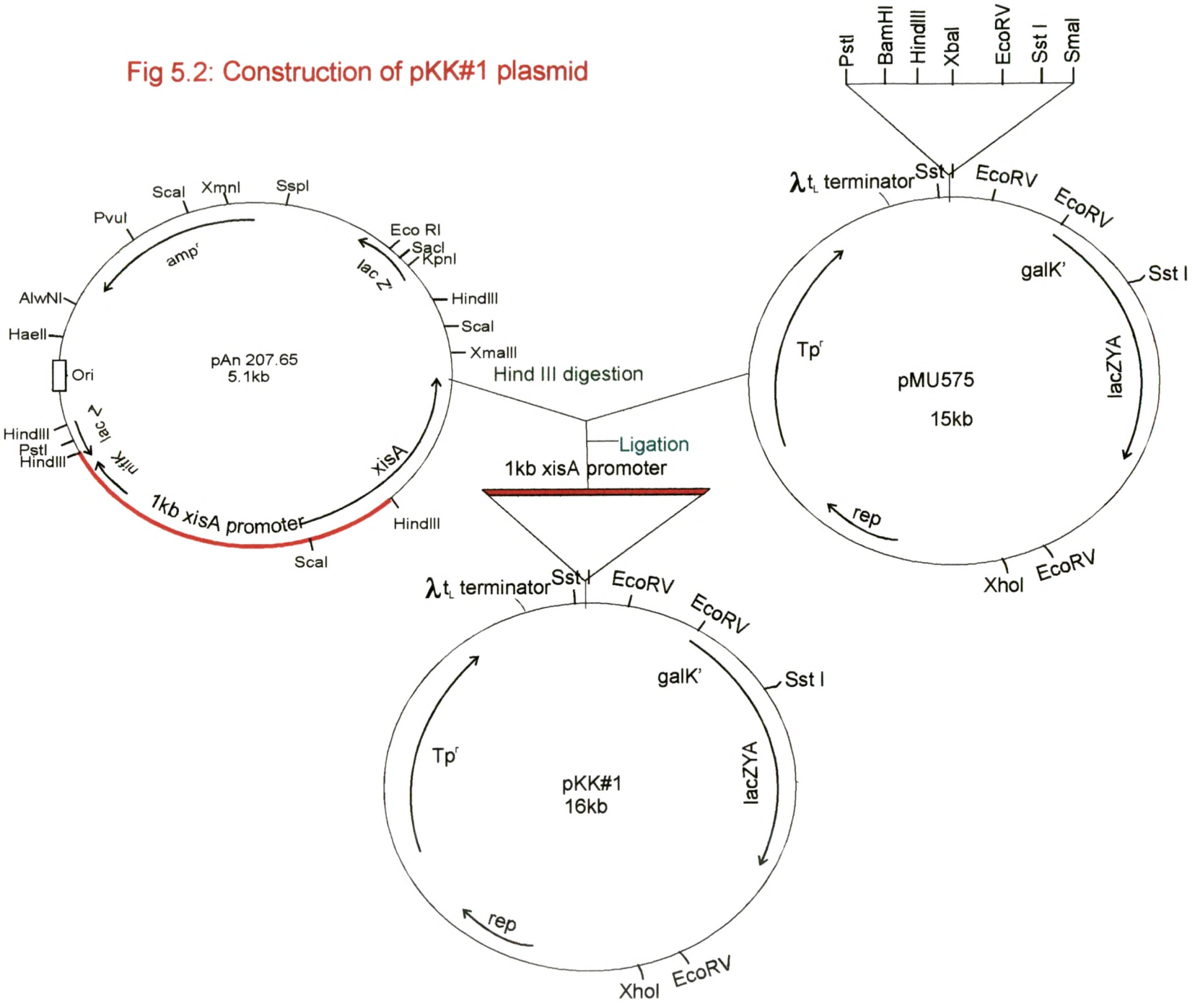



Fig. 5.1 : Restriction endonuclease digestion pattern of pAn 207.65

Lanes : 1. Lambda DNA digested BstEII, pAn 207.65 plasmid DNA digested, 2. KpnI, 3. pAn207.65 uncut plasmid DNA, pAn 207.65 plasmid DNA digested 4. Eco RI, 5. Hind III and 6. ScaI

Fig 5.2: Construction of pKK#1 plasmid



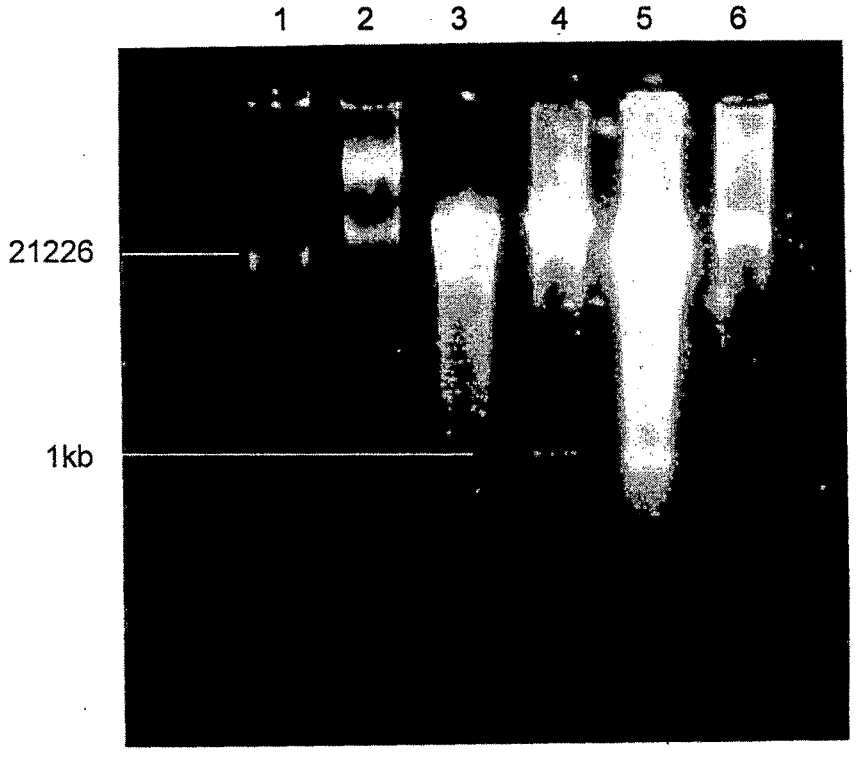



Fig. 5.3 : HindIII digestion pattern of pMU575 and pKK#1 plasmids

Lanes :1. Lambda DNA digested with HindIII/Eco RI; 2. pMU575 uncut plasmid DNA, HindIII digestion pattern of plasmid DNA of 3. pMU575 , 4. pKK#1, 5.GN#2 ( pKK#1 and pMC71A) and 6. PMU575.

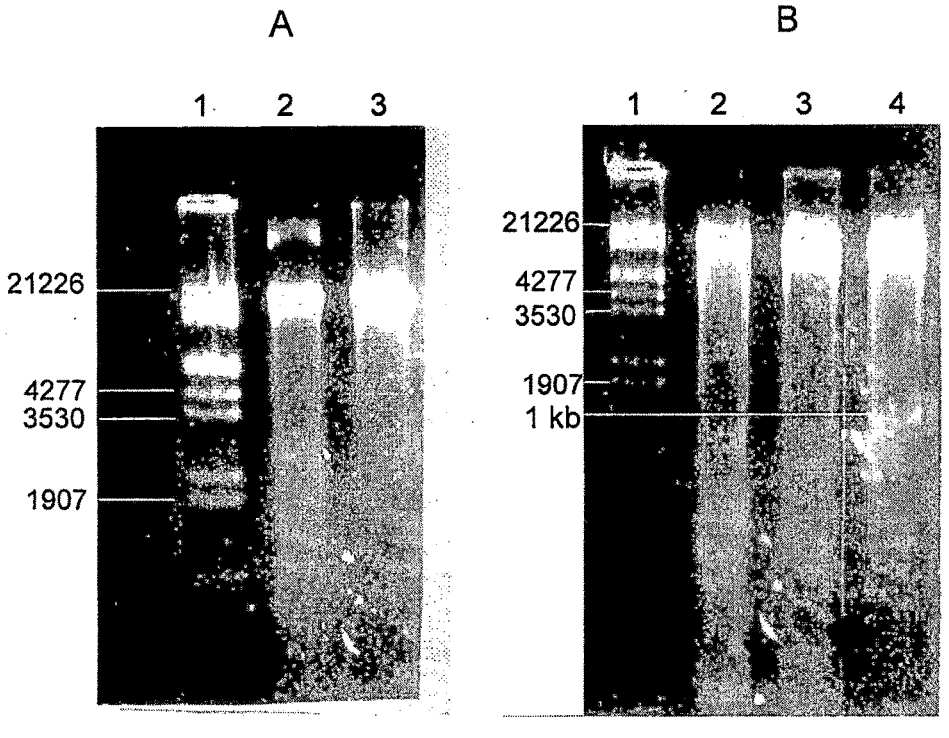



Fig. 5.4 Restriction endonuclease digestion pattern of pMU575 & pKK#1

A : Lane 1. Lambda DNA digested with HindIII/Eco RI; 2. pMU575 digested with Sca I, 3. pKK#1 digested with Sca I

B : Lane 1.Lambda DNA digested with HindIII/Eco RI; 2. pMU575 digested with Bam HI, 3.pKK#1 digested with Bam HI, 4. pKK#1 digested with Bam HI/Sca I

*E. coli* strains were grown overnight in Luria broth/M9 minimal medium with appropriate antibiotics at 37°C until the cultures reach a density of 2-5X$10^8$ cells/ml (an $OD_{600}$ of 0.28-0.70). The cultures were kept on ice for 20min. 0.05- 0.2 ml volume of the cultures was added to the assay medium (Z-Buffer). The cells were ruptured by adding 2 drops of chloroform and 1 drop of 0.1% SDS to each assay mixture. The tubes were vortexed for 10sec.

The reaction was started by adding 0.2ml of ONPG, the contents were mixed for few seconds. After sufficient yellow colour has developed, the time of reaction was recorded and the reaction was stopped by adding 0.5ml of 1M $Na_2CO_3$ solution. For each tube the optical density was measured at 420nm as well as 550nm.

For *E. coli*, the scattering = 1.75 X $OD_{550nm}$

The enzyme units were calculated as follows:

$$\text{Units of } \beta\text{-galactosidase} = \frac{1000 \text{ X } (OD_{420} - 1.75\ OD_{550})}{t \text{ X } v \text{ X } OD_{600}}$$

t- Time of the reaction in minutes.

v- volume of the culture used in ml in the assay.

These units are proportional to the increase in the o-nitrophenol per minute per bacterium.

For assaying β-galactosidase activity in stationary phase, the cultures were grown for 24h ($OD_{600}$ of 1.6-2.0), then dilution were made in Z-Buffer (1:10), 0.1-0.2 ml volumes used for assay.

## 5.3: RESULTS

### 5.3.1 : Construction of $P_{xisA}$-β galactosidase fusion plasmid.

Transformation of DH5α to trimethoprim resistance with the ligation mixture containing linearized pMU575 and 1kb HindIII insert of pAn207.65 yielded 50% dark blue, 16% light blue and 34% whites. The 1kb promoter region of *xisA* gene was subcloned into the pMU575 which gave rise to the plasmid pKK#1 (**Fig 5.2**). The presence of the insert in the right orientation in the plasmid confers blue phenotype to DH5α transformants in X-Gal plate. To confirm the presence of the insert, plasmid DNA from 15 bark blue colonies was digested with HindIII. 1kb insert was found to be released by all (Data not shown). Out of this one clone was used for further studies. The transformants-containing vector alone did not form any blue color on X-Gal plates. The selected clone was digested with HindIII and the results were presented in the **Fig 5.3**. The plasmid was transformed in to *E. coli* strains JM101, RK#1 and NK#1. pMU575 was also transformed in these strains and this served as negative control. To study the effect of NifA on $P_{xisA}$-lacZ expression the plasmid pMC71A was transformed into the each of these strains containing either pMU575 or pKK#1.

### 5.3.2: Expression of *xisA* in *E. coli.*

Since the rearrangement was higher in stationary phase cell than in the exponentially growing cells, the β- galactosidase activity was monitored of $P_{xisA}$-lacZ was also monitored in cells in different growth phases. The activity was measured in the logarithmic phase at O.D. of 0.28-0.7 and also in stationary phase cells as described in 5.2.3. But in these experiments different time points for logarithmic phase and stationary phase cells were used because the excision was high in the stationary phase.

The β-galactosidase assay of different *E. coli* strains under Luria broth and M9 minimal medium growth conditions were presented in the **Fig 5.5** and **Fig. 5.6**, respectively. The control *E. coli* strain containing the pMU575 plasmid showed minimal activity (4-12U) in all conditions even in the presence of pMC71A plasmid. All strains showed higher activity in the stationary phase, which was about 3 fold higher than in the logarithmic phase in Luria broth and the activity was highest, *i.e.* 1600 units in RecA strain. Although increase in β-galactosidase activity was found in the stationary phase in cells grown in minimal medium, the increase was 1.5-2 fold in the presence of NifA. There was no significant change in the activities in Luria broth and minimal medium grown cells during their logarithmic phase of growth in all strains. *E. coli recA*$^-$ and *ntrA*$^-$ mutant strains had not exhibited any significant difference in the activities in Luria broth whereas the mutants RK#3 *(recA*$^-$*)* and NK#3 ( *ntrA*$^-$ ) had less than 50% activity of GN#2 (WT) strain. This suggests that RecA and σ54 significant increase the expression of *xisA* gene in the stationary phase in M9 minimal medium.

### 5.3.3: Autoregulation of *xisA* expression.

In order to study autoregulation of XisA, the plasmid pAn207.65 was transformed in to the strains containing pKK#1 plasmid. The restriction digestion of the plasmids obtained from the transformants is given in the **Fig 5.7**. The β-galactosidase activity was monitored in the logarithmic phase as well as in the stationary phase. **Fig 5.8** shows the log phase activity cells grown in Luria broth and in minimal medium. No significant effect on expression of $P_{xisA}$-lacZ was seen by the presence of *xisA* gene (plasmid pAn207.65) in cells grown in Luria broth but the activity doubled in minimal medium grown cells of JM101 and in *ntrA*$^-$ mutant strains but not in *recA*$^-$ strain. Thus autoactivation of *xisA* gene is significant in the logarithmic phase in M9 minimal medium. However, in the stationary phase there was significant increase in

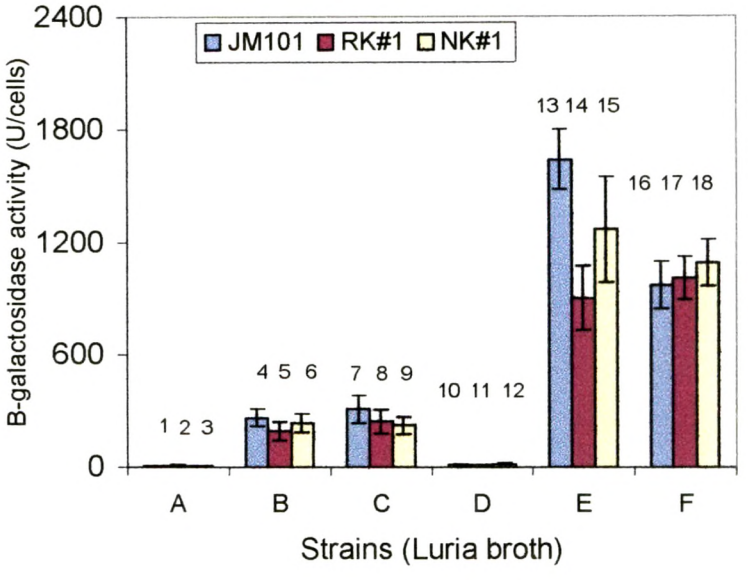



Fig. 5.5 : β- galactosidase activity in logarithmic and stationary phase cells of different *E. coli* strains grown in Luria broth.

A,B,C- logarithmic phase
D,E,F- stationary phase

1 - JM101 (pMU575)
2 - RK#1 (pMU575)
3 - NK#1 (pMU575)
4,13- JM101 pKK#1
5,14- RK#1 pKK#1
6,15- NK#1 pKK#1
7,16 - GN#2
8,17 - RK#3
9,18- NK#3
10- GN#3
11- RK#4
12- NK#7

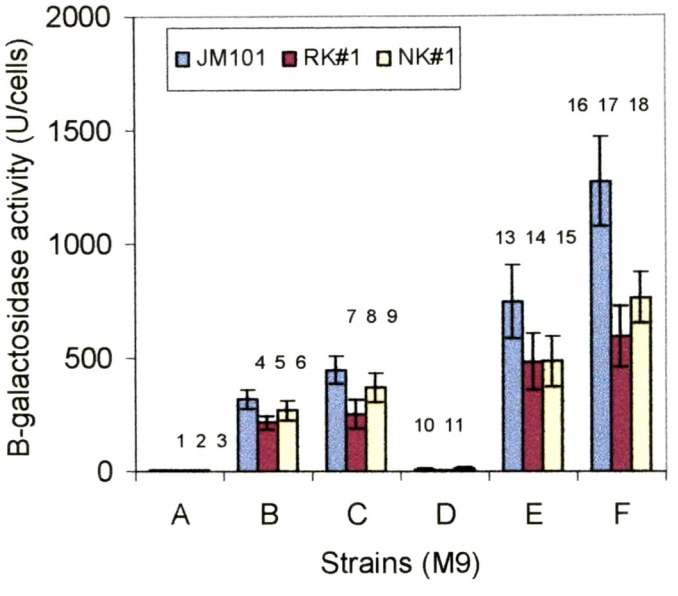



Fig. 5.6 : β- galactosidase activity in logarithmic and stationary phase cells of different *E. coli* strains grown in M9 minimal medium.

A,B,C- logarithmic phase
D,E,F- stationary phase

1 - JM101 (pMU575)
2 - RK#1 (pMU575)
3 -NK#1 (pMU575)
4,13 - JM101 pKK#1
5,14 - RK#1 pKK#1
6,15 - NK#1 pKK#1
7,16 - GN#2
8,17 - RK#3
9,18 - NK#3
10 - GN#3
11 - RK#4
12 - NK#7

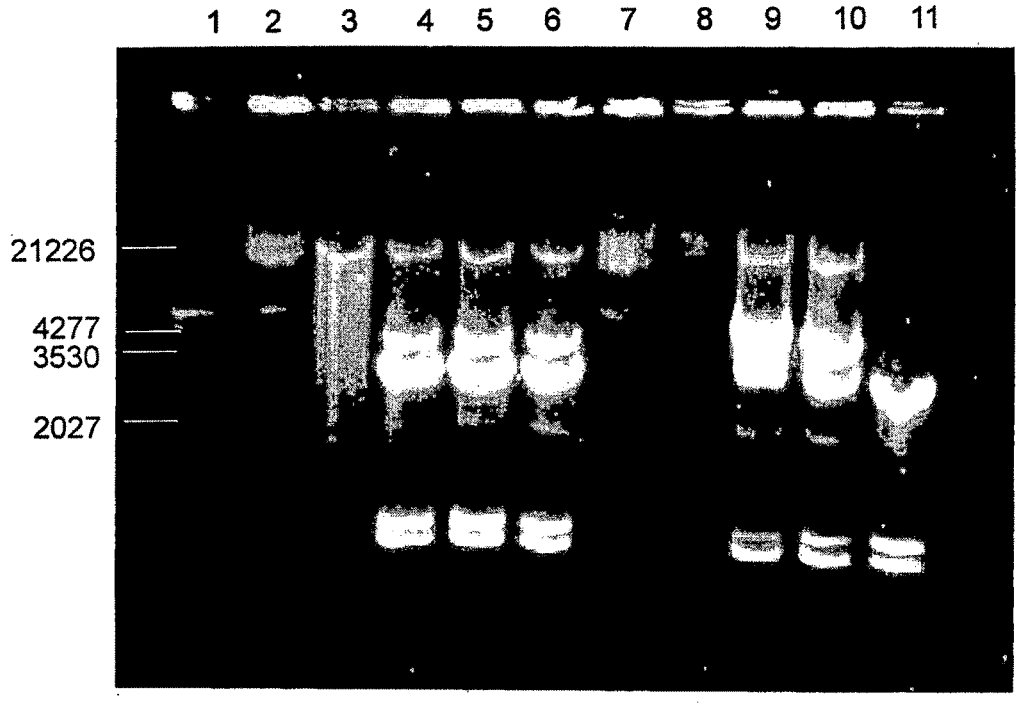



Fig. 5. 7 : HindIII digestion pattern of plasmid DNA from different *E. coli* strains.

Lanes : 1. Lambda DNA digested with HindII/IEco RI;
2. pKK#1 uncut plasmid DNA, 3. pKK#1 digested with HindIII,
HindIII digestion pattern of plasmid DNA from *E.coli :* 4. GN#5(WT),
5. RK#6 *(recA)*, 6. NK#5 (*ntrA*), 7. GN#5 uncut plasmid DNA
8. GN#3 uncut plasmid DNA, HindIII digestion pattern of plasmid DNA from *E.coli :* 9. GN#4(WT), 10. RK#5 *(recA)*, 11. NK#4 (*ntrA*).

the activity as compared to the logarithmic phase in Luria broth but not in the M9 minimal medium. *recA* mutants had no significant increase (**Fig. 5.9**). Thus the role of excisase in autoactivation is variable but appear to require RecA protein.

## 5.4 : DISCUSSION

*Anabaena* PCC 7120 *xisA* gene expression was monitored in *E. coli* under different growth conditions of growth, by using the plasmid construct containing $P_{xisA}$-lacZ gene fusion. Interestingly, *xisA* expression was found in significant levels under all conditions. x*isA* expression was increased 2-3 fold in cells grown in Luria broth in the stationary phase compared to that in logarithmic phase. But the excision of *nifD* element was not high in Luria broth grown cells even in stationary phase. The *xisA* expression in M9 minimal medium grown cells was similar to that in Luria broth. Thus, the excision of the *nifD* element requires some other factor(s) a conclusion inferred also from lack of excision in *E. coli ntrA* mutant.

*xisA* expression is less by about 1.5 fold in *E. coli recA* and *ntrA* mutants compared to their isogenic wild type strains. Thus, it appears that RecA and σ54 proteins increase the expression of *xisA* gene but excisase A not sufficient for excision of *nifD* element even in the presence of *K. pneumoniae* NifA protein. Another surprising result was that, XisA also autoactivates *xisA* gene expression.

Although *Anabaena* PCC 7120 does not have σ54, RecA dependent expression of *xisA* may be functional in this organism. NtcA/BifA has been shown to be transcriptional activator of many genes in cyanobacteria and it was expected to repress *xisA* gene expression (Ramasubramanian *et al.*, 1994). Such interpretation was necessary to account for the lack of excision in the vegetative cells. Results presented here suggest that excisaseA alone may not be sufficient for the excision of *nifD*

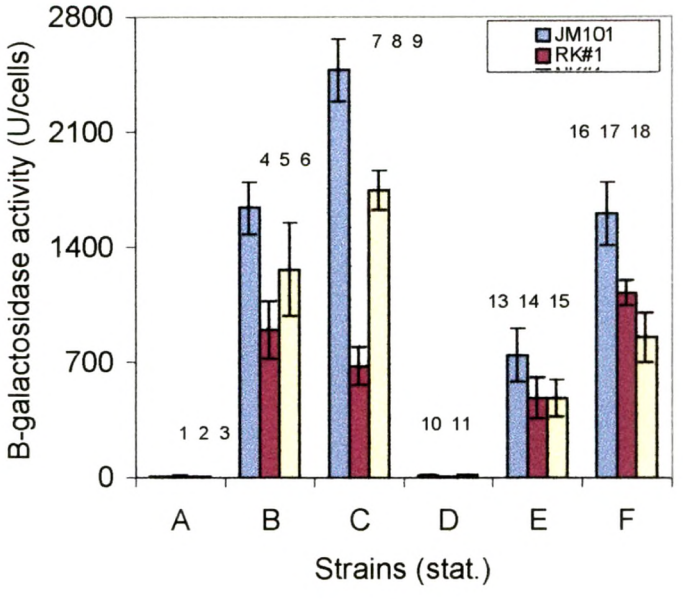



Fig. 5.9 : β- galactosidase activity in stationary phase cells of different *E. coli* strains grown in Luria broth and M9 minimal medium.

A,B,C- logarithmic phase Luria broth
D,E,F- logarithmic phase M9 minimal medium

1. JM101 (pMU575)
2. RK#1 (pMU575)
3. NK#1 (pMU575)
4,13- JM101 pKK#1
5,14- RK#1 pKK#1
6,15- NK#1 pKK#1
7,16- GN#5
8,17- RK#6
9,18- NK#5
10- GN#4
11- RK#5
12- NK#4

element in *Anabaena* PCC 7120 and may also require some other factors similar to the σ54 dependent proteins postulated for the excision in *E. coli*. Hence, NtcA/BifA may not be a repressor of *xisA* gene in vegetative cells and excisaseA may also get expressed in vegetative cells. However, the results of NifA of *K. pneumoniae* may not be extrapolated to the functions of NtcA of *Anabaena* PCC 7120.

Since *xisA* expression was found high even in cells grown in Luria broth in the stationary phase, the role of *K. pneumoniae* NifA protein in bringing about *nifD* element excision does not appear to be mediated by increasing σ54 dependent expression of *xisA* gene. Our analysis of DNA sequences near the 11 base pair target site which is in the major fraction of the *nifD* structural gene within the *nifD* element of *Anabaena* PCC 7120 showed the presence of additional binding sites for NtcA which are identical to *K. pneumoniae* NifA binding sites. NifA of *K. pneumoniae* has been found to have DNA bending activity. Thus, NifA may be binding to these sites which are present on both the ends of *nifD* element and bring the 11 base pair target sites to close proximity by DNA bending. It not known whether NtcA of *Anabaena* PCC 7120 also has DNA bending function. It will be interesting to know whether NtcA of *Anabaena* PCC 7120 mimics the effects of *K. pneumoniae* NifA protein in *E. coli*.