CHAPTER III

Role of NifA of *Klebsiella pneumoniae* on the excision of *nifD* element of *Anabaena* PCC 7120.

3.1 : INTRODUCTION

Excision of *nifD* element of *Anabaena* PCC 7120 is dependent on the XisA protein encoded within the element and the excision does not occur if this protein is absent (Golden *et al.*, 1988; Brusca *et al.*, 1990). The results reported in Chapter II also suggest that increase in the excision of *nifD* element may be due to increased XisA activity. Thus it appears that excision of *nifD* element could be increased by increasing the amount of XisA protein. Attempts to overexpress the XisA protein in *E. coli* by very strong promoters like P*tac* have not been successful (Brusca *et al.*, 1990). Additionally, *E. coli* overexpressing *xisA* gene could not grow very well, suggesting toxic effects of XisA. In order to increase the excision of *nifD* element it may be desirable to increase the expression of *xisA* gene only under specific conditions. Analysis of putative promoter region of *xisA* gene could help in achieving these objectives.

The promoter region of the *xisA* was shown in the **Figure 3.1**. Putative promoter region of *xisA* (-100bp to -170bp) upstream of the second ATG contains two divergent overlapping potential promoters P1 and P2 (Lammers *et al.*, 1986). The P1 is stronger than P2 and can cause transcription away from the open reading frame. As a consequence, it can create a strong interference in *xisA* transcription. *xisA* gene is expressed only in heterocysts but not in vegetative cells. Two DNA binding proteins BifA and factor 2 were isolated from the vegetative cells which could bind to the putative promoter regions of *xisA* gene. It was postulated that these two binding proteins repress the expression of *xisA* gene in the vegetative cells. A negative regulatory (NRE) located between -65bp to -192bp strongly controls the *xisA* expression (Brusca *et al.*, 1990). At least two DNA-binding proteins BifA (now it is called NtcA) and factor2, associated with the NRE (between -152bp to -223bp) and regulates the expression (Ramasubramanian *et al.*, 1994).

FIG 3.1 PROMOTER REGION OF*xisA* GENE OF *Anabaena* sp. Strain PCC 7120

BifA/NtcA protein of *Anabaena* PCC 7120 binds to the consensus binding motif TGT (N_9 or N_{10}) ACA. NtcA protein is similar to NifA and CRP binding motifs (Botsford and Harman, 1992; Morett and Buck, 1989; Morett *et al.*, 1991) but NtcA does not show amino acid similarities to NifA (Gussin *et al.*, 1986; Wei *et al.*, 1993). BifA/NtcA mutant of *Anabaena* PCC 7120 showed pleiotropic effects and the protein is necessary for the expression of many genes involved in the nitrate metabolism. DNA binding property of NtcA of *Anabaena* PCC 7120 *in vitro* depends upon the status of sulfhydral groups of cystine residues and the status of cystine residues may be important for transcriptional activator and repressor activity. NtcA regulated genes have -15 sequences of the σ70 but do not have -35 consensus sequence.

NifA belongs to a large family of Enhancer-binding proteins (EBPs) that activate transcription from distant sites and uses the sigma^{54} (σ^{54}) containing holoenzyme form of the RNA polymerase ($E\sigma^{54}$) (Kustu *et al.*,1989; Morett and Segovia, 1993; Shingler,1996). σ^{54} is an evolutionarily unique class of initiation factor that binds to the -12/-24 promoters (TGGCACN $_5$TTGCN) in the absence of core RNA polymerase (Buck and Cannon, 1992; Lonneto *et al.*, 1992; Cannon *et al*, 1997). $E\sigma^{54}$ is unable to carryout transcription unless an EBP, such as NifA is present (Popham *et al*, 1989; Merrick, 1993; Lee *et al.*, 1994). In the current model for the transcriptional activation, an oligomeric form of EBP, bound far from the promoter of Upstream Activator Sequences (UAS) contacts the $E\sigma^{54}$ closed promoter complex, so that it is converted to an open, transcriptionally active form, in a process shown to require ATP hydrolysis (Weiss *et al.*, 1991; Berger *et al.*, 1995).

Interestingly, the BifA/NtcA binding sites and NifA binding sites are identical and the putative promoter region of *xisA* gene contains three binding sites for NtcA protein. Additionally, essential sequences for the σ54 *i.e.* $GGN_{10}GC$ are also present in the promoter regions of *xisA* gene. Thus, xisA gene expression could be modulated in *E. coli* by NifA protein of *K. pneumoniae*.

In this chapter, the effect of *nifA* gene of *K. pneumoniae* expressed in *E. coli* from promoter of Tet ^{R} on excision of *nifD* element of *Anabaena* PCC 7120 was tested.

3.2 : MATERIALS AND METHODS

3.2.1: Strains

Strains	Genotype	Reference
GN#1	*JM101 (pMX25 & pMC71A)*	This study
PK#1	*DH5α (pMX25 & pMC71A)*	This study
NK#1	*JM101rpoN::208 Tn10*	This study
ET8045	*rbs lacZ::IS1gyrA hutC$_k$ rpoN::208 Tn10*	Merrick (1983)
NK#2	*NK#1 (pMX25 & pMC71A)*	This study
NK#6	*NK#1 pMX25*	This study

3.2.2 : pMC71A: (Buchanan-Wallaston *et al.*, 1981)

pMC71A is a derivative of the plasmid pACYC184. The *nifA* gene from of *Klebsiella pneumoniae* was inserted into Sal I site within the Tetracycline resistance gene of vector. The *nifA* is expressed constitutively under the tetracycline resistance gene promoter. The vector has chloramphenicol resistance gene. The size of pMC71A is 7kb **(Fig 3.2).**

3.2..3: Monitoring excision of *nifD* element containing pMX25 and pMC71A plasmids in *E. coli.*

A single colony of *E. coli* containing pMX25 and pMC71A was grown overnight in 15ml Luria Broth with kanamycin, ampicillin and chloramphenicol. Dilutions were made in saline and 10^{-4} plated on Luria agar containing ampicillin and chloramphenicol X-Gal . The 0h plating was done to determine the number of white colonies present in the starting culture. Kanamycin was added to ensure that the rearranged colonies were eliminated.

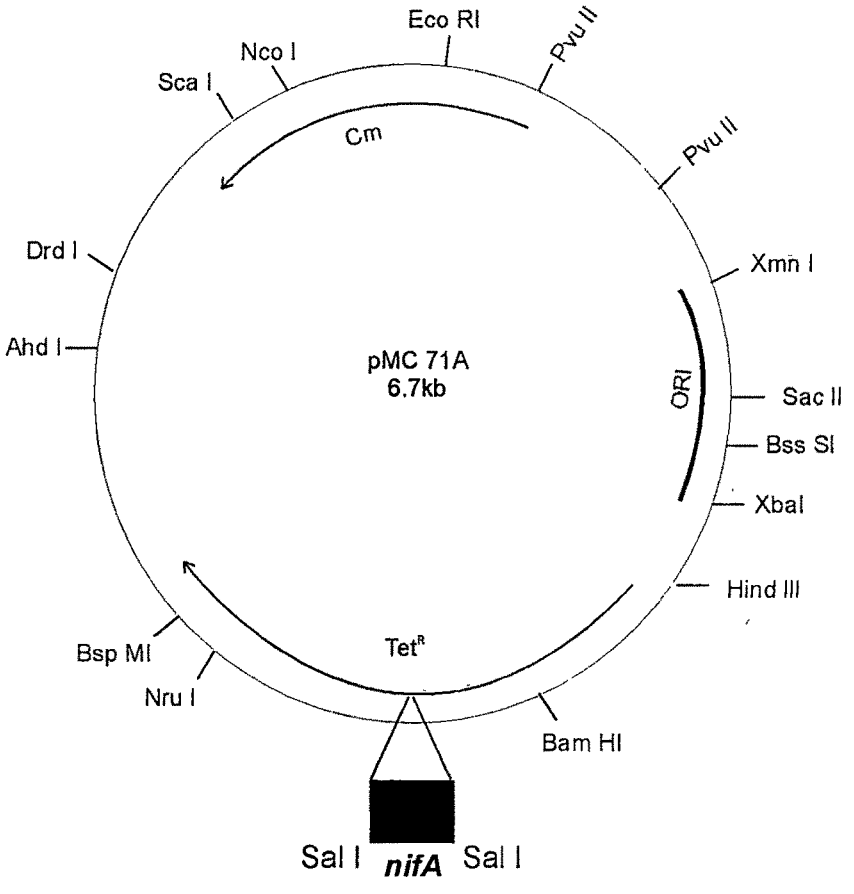


Fig. 3.2 : Restriction map of pMC71A plasmid

1.0 ml culture was used for further experiments. This culture was spun at 5000 rpm at 4° C for 5min. Then the pellet was washed twice with either Luria broth or M9 minimal medium

From the washed culture 1% (150µl) of the inoculum was reinoculated in Luria broth /M9 minimal medium containing ampicillin and chloramphenicol. The cultures were allowed to grow for 24 h at 37°C. After 24 h, the dilutions were made as above and plated on X-Gal ampicillin and chloramphenicol plates. The plates were kept at 37°C and the total excision was calculated using this formula

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

3.2.4: Construction of *ntrA* mutant of *E. coli* JM101.

The construction of the *ntrA* mutant of *E. coli* JM101 was carried out by P1 transduction (as described in 2.2.10.1). To check for the *ntrA* mutants, the transductants were restreaked on M9 minimal medium supplemented with or lacking arginine. The transductants unable to grow without arginine were scored as *ntrA* mutants.

3.2.5: Rearrangement of *nifD* element in GN#1, PK#1, NK#2 and NK#6.

The rearrangement was monitored using blue and white colony assay as described in (2.2.9). M9 minimal medium was supplemented with 200µg/ml glutamine for growing cells of *E .coli* NK#2 and NK#6. For maintaining pMC71A in the strains GN#1, PK#1 and NK#2 chloramphenicol was added in the medium.

3.6: Time dependent excision of *E. coli* GN#1 on Luria broth and M9 minimal medium.

A single colony of *E. coli* GN#1 was inoculated into 15ml Luria Broth with kanamycin, carbenicillin and chloramphenicol. The culture was allowed to grow overnight at 37°C. Serial dilutions of the culture were made in saline (10^{-4}) and 0.05-0.1ml from the dilution were plated on Luria agar plates containing X-Gal carbenicillin and chloramphenicol plate for estimation of number of blues and whites in the starting culture. The cells were pelleted and washed twice with Luria broth or M9 medium containing 40mM NH_4Cl. The washed cells were reinoculated into 15ml Luria broth or M9 medium containing NH_4Cl. At different growth time points the culture was diluted appropriately and plated on X-Gal carbenicillin/chloramphenicol plate. The excision frequency was determined as described earlier. The OD was taken at 620nm in spectrophotometer. 1ml culture was withdrawn at each time point and diluted for plating and rest used for OD reading.

3.3: RESULTS

3.3.1: Role of NifA protein in the excision of pMX25.

In order to determine the role of NifA protein, the excision of pMX25 was monitored in the absence and presence of the plasmid pMC71A. The pMC71A contains the *nifA* gene of *K. pneumoniae*. The *nifA* gene is under the control of tetracycline resistance gene promoter and is constitutively expressed. The restriction pattern of pMX25 and plasmids from *E. coli* strains GN#1 (JM101 pMX25 and pMC71A) and PK#1(DH5α pMX25 and pMC71A) is shown in the **Fig 3.3.**

The excision was monitored in the *E. coli* PK#1 and GN#1 **(Fig .3.4)** in Luria broth and M9 minimal medium containing 40mM NH_4Cl as nitrogen source. The results are given in the **Table 3.1.** In PK#1 *(recA⁻)* strain, the excision frequency in the presence of pMC71A is 5 fold higher in Luria broth and 25% higher in M9 minimal

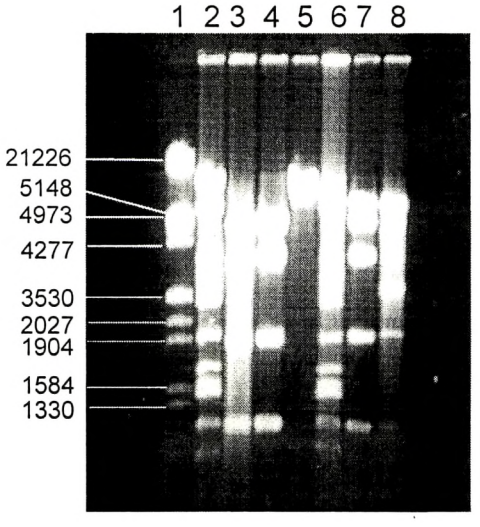
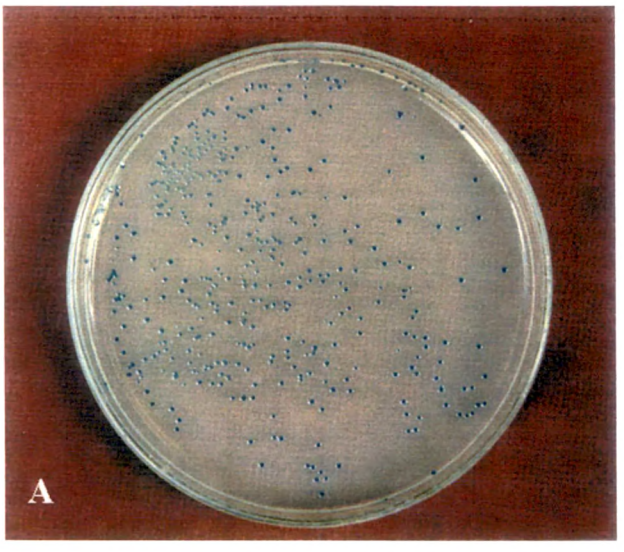


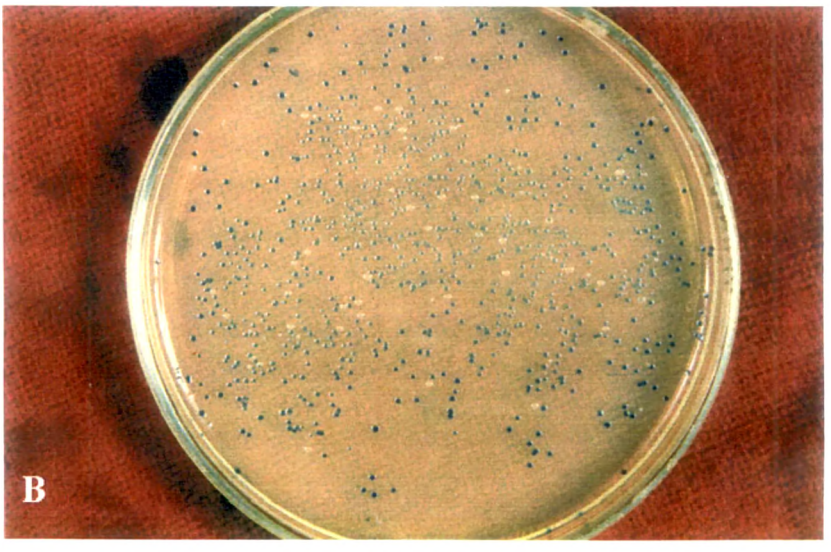
Fig. 3.3 Eco RI digestion pattern of plasmid DNA from blue and white colonies of JM 101 containing pMX25, pMXΔ25 and GN#1

Lanes : 1.Lambda DNA digested with HindIII/Eco RI;
Eco RI digestion of 2. Blue colony (JM 101 pMX25),
3 & 4. White colony (JM 101 pMXΔ25), 5.pMC71A,
Eco RI digestion of 6. GN#1 blue colony,7. GN#1 white colony and 8.PK#1 white colony.

Fig 3.4 : Excision of the *nifD* element of *Anabaena* sp. strain PCC 7120 in *E. coli* strains GN#1 and PK#1 in luria broth and M9 minimal medium

A. GN#1 0h
B: GN#1 24h luria broth
C: GN#1 24h M9 minimal medium
D: PK#1 24h M9 minimal medium


A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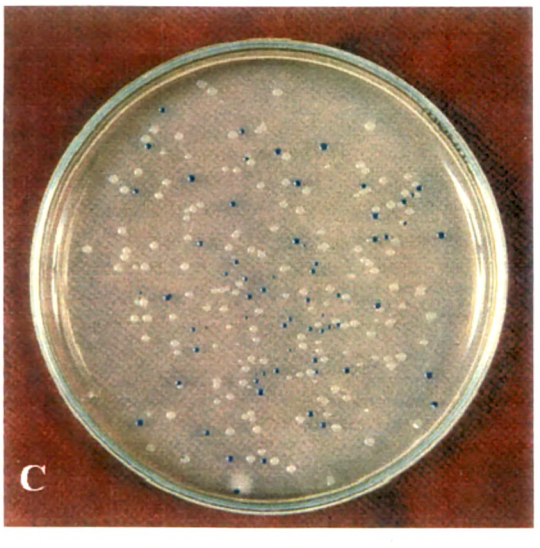
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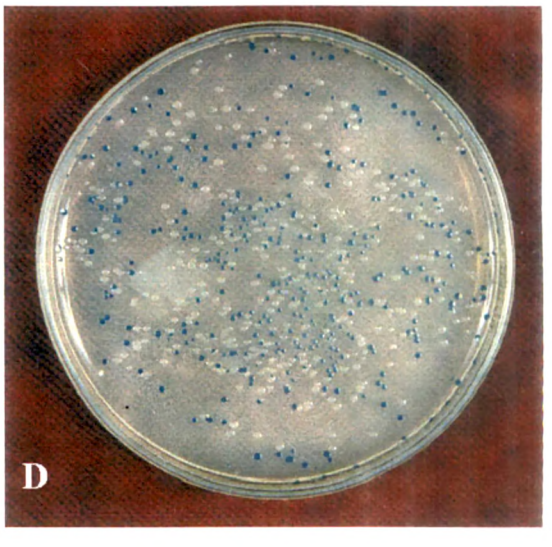
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medium grown cells when compared to cells lacking the pMC71A (DH5α pMX25 (*recA*$^-$). But in the case of GN#1 (*recA*$^-$) the excision in Luria broth was 2 fold increase in the presence of pMC71A but in M9 minimal medium grown cells the increase was 7 fold (70%) as compared to that of (JM101 pMX25) **(Table 3.1).**

Table 3.1 : Effect of *nifA* of *Klebsiella pneumoniae* on the excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* PK#1 and GN#1.

Media / Time (h)	**Number of Blue colonies**	**Number of White colonies**	**Total**	**Percent Excision Mean ±S.D**
	PK#1			
0 h	4860	22(8)*	4874	0.28±0.34
Luria broth/24h	2928	144	3072	4.68±1.86
M9 minimal medium/24h	1279	1507	2786	62.51±9.75
	GN#1			
0 h	5018	21(7)*	5032	0.27±0.27
Luria broth /24h	1078	57	1135	5.02±1.58
M9 minimal medium /24h	794	2392	3186	75.07±8.99

* Number of Kanamycin resistant colonies after restreaking into plates.
Results are of three independent experiments.

Plasmids extracted from the blue and white colonies from PK#1 and GN#1 were subjected to EcoRI digestion **(Fig 3.5).** The gel pattern in all cases, was similar to that reported by Lammers *et al.* (1986). These results indicated that the NifA protein greatly enhances the excision of pMX25 both under Luria broth as well as M9 minimal medium conditions. However, in the M9 minimal medium the excision frequency enhancement was significantly more than that in cells grown in Luria broth.

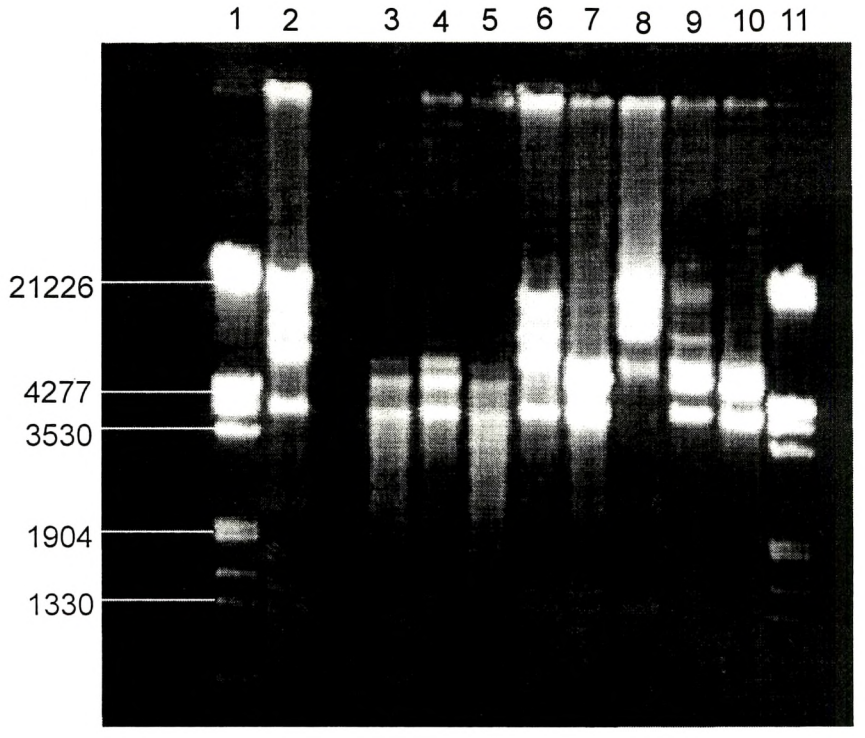


Fig. 3.5 : Eco RI digestion pattern of plasmid DNA from blue and white colonies PK#1 and GN#1 strains after growth in Luria broth and M9 minimal medium for 24h.

Lanes : 1. Lambda DNA digested with Hind III/Eco RI, Plasmid DNA from *E.coli* GN#1

2. blue colony in Luria broth, 3. white colony in Luria broth,
4. white colony in M9 minimal medium, 5. White colony in M9 minimal medium, Plasmid DNA from *E.coli* PK#1 6. blue colony in Luria broth,
7. white colony in Luria broth, 8. blue colony in M9 minimal medium,
9 & 10. white colony in M9 minimal medium and
11. Lambda DNA digested with Hind III/Eco RI

3.3.2: Role of *ntrA* of *E. coli* on the excision of pMX25.

Since NifA increases the transcription of genes, which are expressed by σ^{54} (encoded by *ntrA* gene), the *rpoN208::Tn10* allele was transduced into JM101 by P1 transduction using the donor strain ET8045. The *rpoN* phenotype was checked as described in 3.2.4. A *ntrA* mutant designated NK#1 was constructed by P1 transduction and used for further work. The pMX25 transformed into NK#1 (NK#6) was grown in either Luria broth or M9 minimal medium. The results are shown in the **Table3.2.** It was found that the excision frequency was less compared to that of JM101 containing pMX25. Additionally, there was no significant increase in the excision in the M9 minimal medium grown cells.

Table 3.2 : Excision of the *nifD* element of *Anabaena* PCC 7120 in *E. coli* (NK#6) σ^{54} mutant.

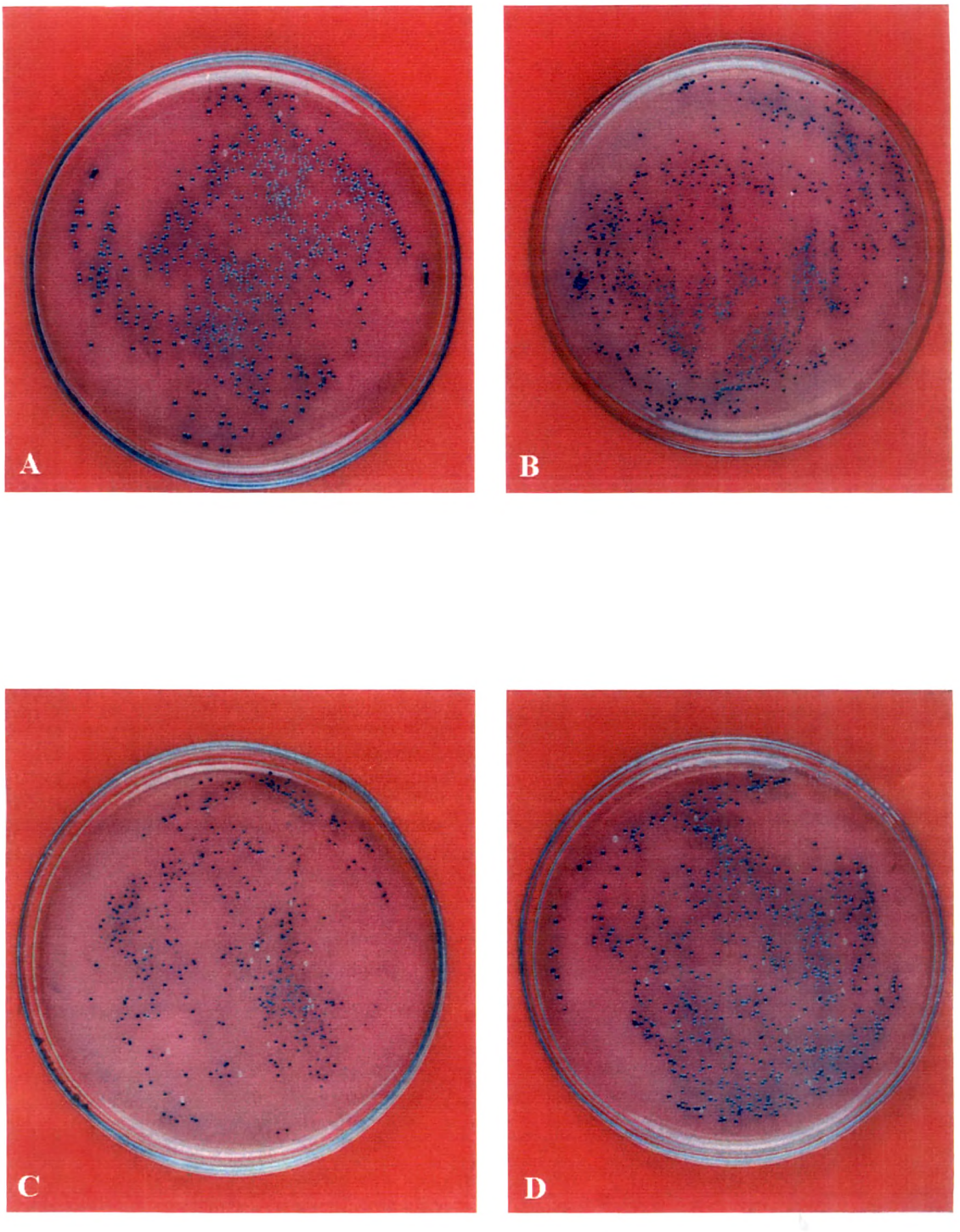
Media / Time (h)	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D
0	7217	14(6)*	7229	0.19±0.17
Luria broth /24h	3240	43	3283	1.30±0.62
M9 minimal medium/24h	5741	101	5843	1.74±0.51

* Number of Kanamycin resistant colonies after restreaking into plates.
Results are of three independent experiments.

In order to find the role of NifA on excision frequency the plasmid pMC71A was incorporated into NK#6, the resulting in strain NK#2. Excision was monitored in cells grown in Luria broth and M9 minimal medium conditions. The results are presented in the **Figure 3.6** and **Table 3.3.** The excision frequency was same in both the strains NK#6 and NK#2 (which contains the *nifA* gene) demonstrating that the *ntrA* of *E. coli* plays an important role in the excision of the *nifD* element in *E. coli.*

Fig 3 6 Excision of the *nifD* element of *Anabaena* sp. strain PCC 7120 in *E. coli* strains NK#6 and NK#2 in luria broth and M9 minimal medium

A. NK#2 after growth on M9 minimal medium for 24h
B: NK#6 0h
C: NK#6 after growth on luria broth for 24h
D NK#6 after growth on M9 minimal medium for 24h


A
B
C
D

The plasmids were extracted from the blue and white colonies of both NK#6 and NK#2 grown either in Luria broth and M9 minimal medium and digested with EcoRI and HindIII. The gel pattern is shown in **Fig 3.7**, which is similar to the earlier pattern.

Table 3.3 : Excision of the *nifD* element of *Anabaena* PCC 7120 in *E. coli* (NK#2)σ^{54} mutant containing *nifA* gene of *K. pneumoniae*.

Media / Time (h)	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D
0	6274	33(9)*	6298	0.23±0.24
Luria broth /24h	4832	73	4905	1.39±0.62
M9 minimal medium/24h	4388	78	4466	1.74±0.77

* Number of Kanamycin resistant colonies after restreaking into plates.
Results are of three independent experiments.

3.3.3: Growth dependent excision of *E. coli* GN#1 in Luria broth and M9 minimal medium.

The increase in the excision of *nifD* element by NifA protein was monitored after 24h. In order to find out the excision frequency at different time points, during the growth phase, the *E. coli* GN#1 was grown in Luria broth. The results presented in the **Table 3.4**, indicated that at cells at 0h, only 0.4% excision frequency which increased with growth and reached to about 5.8% at 12h by this time the growth had already reached its maximum level. The excision did not increase much (6.6%) even at 24h.

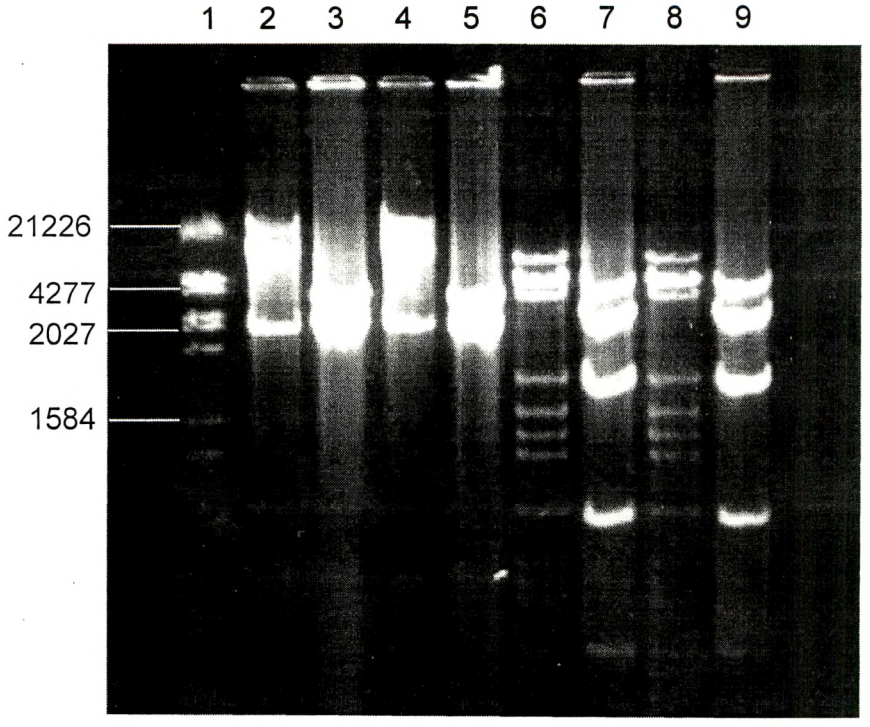


Fig. 3.7 : Eco RI and HindIII digestion pattern of plasmid DNA from blue and white colonies obtained from *E. coli* strains NK#6 and NK#2 after growth in Luria broth and M9 minimal medium for 24h.

Lanes : 1. Lambda DNA digested with HindIII/Eco RI;
Eco RI digestion pattern of pMX25 unrearranged (blue) and rearranged (white) from NK#6 : 2. blue colony in Luria broth,
3. white colony in Luria broth, 4. blue colony in M9 minimal medium,
5. white colony in M9 minimal medium,
HindIII digestion pattern of pMX25 unrearranged (blue) and rearranged (white) from NK#2 : 6. blue colony in Luria broth,
7. white colony in Luria broth, 8. blue colony in M9 minimal medium and
9. white colony in M9 minimal medium.

Table 3.4 : Growth dependent excision of the *nifD* element of *Anabaena* PCC 7120 in *E. coli* containing *nifA* gene of *K. pneumoniae* in Luria broth.

Time (h)	OD $_{620nm}$	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D
0	0.70±0.14	1501	14(8)*	1507	0.40±0.17
3	0.10±0.02	896	7	903	0.77±0.50
6	0.49±0.01	913	21	934	2.24±0.25
9	0.72±0.01	996	39	1035	3.76±0.92
12	0.73±0.02	1425	89	1514	5.87±0.60
24	0.63±0.03	1949	128	2077	6.61±0.43

* Number of kanamycin resistant colonies after restreaking on to plate.
Results are of three independent experiments.

Growth phase dependent excision experiments in *E. coli* GN#1 were repeated in M9 minimal medium containing NH_4Cl. The results were presented in the **Table 3.5**. The excision exhibited similar pattern to that in Luria broth. Growth was slower in M9 minimal medium and reached to O.D. of 0.7 and 0.9 by about 24h and 48 h, respectively. The excision had reached the maximum value of 75% at 24h. These results demonstrated that the excision was correlated with the growth.

Table 3.5 : Growth dependent excision of the *nifD* element of *Anabaena* PCC 7120 in *E. coli* containing *nifA* geneof *K. pneumoniae* in M9 minimal medium.

Time (h)	OD $_{620nm}$	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D.
0	0.68±0.11	1693	13(7)*	1699	0.35±0.29
6	0.06±0.01	754	32	786	4.07±0.73
12	0.09±0.02	2380	376	2756	13.64±1.89
18	0.10±0.02	582	303	885	34.23±4.17
24	0.72±0.06	569	2203	2772	77.38±3.28
48	0.93±0.02	1178	3790	4758	76.44±5.14

* Number of kanamycin resistant colonies after restreaking into plates.
Results are of three independent experiments.

3.3.4: Monitoring excision independent of the growth phase.

The excision in M9 minimal medium in GN#1 was very high and correlated with the growth. In order to determine whether growth in M9 minimal medium was necessary for the excision, the GN#1 was grown in Luria broth and cells in the logarithmic phase were transferred to M9 minimal medium. The excision was monitored at different time points. The results are presented in the Table 3.6. The starting O.D. was 0.4 and increased to a maximum growth of 1.0 O.D. by 16 h. Excision at 3 h was about 4 % and increased to the maximum value of 18% by 16 h. The results indicated that although there was an increase in the excision frequency, it is much less compared to that obtained in cells grown in M9 minimal medium.

Table 3.6 : Growth independent Excision of the *nifD* element of A*nabaena* PCC 7120 in *E. coli* containing *nifA* gene of *K. pneumoniae* in M9 minimal medium.

Medium	Time (h)	OD $_{620nm}$	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D
Luria broth	0	0.39±0.04	1071	7(4)*	1074	0.37±0.25
M9 minimal medium	4	0.39±0.04	732	29	761	3.81±0.90
	8	0.55±0.17	571	29	600	4.80±2.84
	12	0.82±0.19	335	54	389	13.9±1.96
	16	1.06±0.11	1103	252	1355	18.6±2.81
	24	1.13±0.11	1224	269	1463	18.0±4.85

Luria broth grown cells were transferred to M9 minimal medium.
* Number of kanamycin resistant colonies after restreaking into plates.
Results are of three independent experiments.

3.4: DISCUSSION

NifA of *K. pneumoniae* increased the excision of *nifD* element of *Anabaena* PCC 7120 to very high levels *i.e.* 70% in M9 minimal medium. This is very interesting since all the efforts aimed at increasing the excision never reached values greater than 1% not only with the *nifD* element but also of 55kb *fdxN* element in *Anabaena* PCC 7120 (Carrasco *et al.*,1994). It is very surprising that the increase in excision was very high in M9 minimal medium but not reached these values in Luria broth even though NifA is constitutively expressed from the promoter of tetracycline resistance gene and therefore expected to produce the *nifA* gene product to more or less same levels under both grown conditions. These results compared to the RecA-dependent excision results, in that the RecA- dependent excision frequency was higher under M9 minimal medium growth conditions than that in Luria broth grown cells. In the Discussion 2.4, it was suggested that some proteins may be involved in the increasing the *xisA* expression. The results presented in this chapter support the suggestion that additional proteins could involved in the excision of *nifD* element, which acts by increasing the *xisA* expression. It is possible that these proteins may be facilitating the synapse formation of the two target direct repeats, which are separated by 11 kb. It is known that many site-specific recombinations involve accessory proteins which support the formation of synapses (Sadowski, 1993).

ntrA mutant does not show increase in the excision of *nifD* element of *Anabaena* PCC 7120 in the presence of NifA protein even in M9 minimal medium. This suggests that NifA binds to the sites present in the putative promoter regions of *xisA* and activates the transcription *via* σ^{54}, similar to NifA function in *K. pneumoniae*.

Growth dependent excision of *nifD* element showed that excision was correlated with the phase of growth. The time at which maximum excision is achieved will be very useful in monitoring the expression of *xisA* gene and may also help in the detection of XisA protein. This is very important since so far all efforts in detecting XisA protein have not been successful. Detection and purification of XisA is very important in

understanding the mechanism of excision of *nifD* element. Unfortunately, Luria broth grown *E. coli* did not show high excision. If they were successful then they would have simplified the XisA detection.

Although NifA protein of *K. pneumoniae* increases the excision of *nifD* element in *E. coli* upto 70% under minimal medium conditions such an increase has not been observed in the vegetative cells of *Anabaena* PCC 7120 which express NifA-like NtcA protein. It has been postulated that NtcA of *Anabaena* PCC 7120 can act as an activator as well as repressor depending upon oxidation or reduction status in a gene specific manner (Jiang *et al.*, 1997). The repression of *xisA* gene in vegetative cells of *Anabaena* PCC 7120 can be mediated by NtcA and factor 2 as suggested by Chastain *et al.* (1990) and Ramasubramanian *et al.* (1994). The results presented here show that in addition to XisA some other accessory proteins are necessary for the excision of *nifD* element in *E. coli.* Similar proteins may be required for the excision of the *nifD* element in *Anabaena* PCC 7120. Monitoring the effect of NtcA protein on the excision of *nifD* element in *E. coli* and monitoring the expression of *xisA* gene in *Anabaena* PCC 7120 in vegetative cells and heterocysts will help clarify these issues.