CHAPTER II

Excision of the *nifD* element of *Anabaena* PCC 7120 in *E. coli* strains grown under different culture conditions.

2.1 INTRODUCTION :

E. coli containing entire *nifD* element of *Anabaena* PCC 7120 excises the *nifD* element DNA at a excision frequency of about 0.3% (Lammers *et al.*,1986). Sequencing of the rearranged plasmid DNA showed that nature of the excision event is identical to that of *Anabaena* PCC 7120. Golden and Weist (1988) constructed the *xisA* mutant using site-directed inactivation of the *xisA* gene in *Anabaena* PCC7120 chromosome. Although the *xisA* mutant produces heterocysts and correctly excise the 55kb *fdxN* element, they fail to excise the 11kb element and are unable to fix nitrogen. This indicates that *xisA* is required for the *nifD* rearrangement in *Anabaena* heterocysts and that the DNA rearrangement is necessary for proper expression of the nitrogen fixing genes. Brusca *et al.* (1990) constructed a substrate plasmid pAM461 that contains the left and right borders of *nifD* element but does not contain an intact *Anabaena* PCC7120 open reading frame larger than 180bp. pAM461 does not rearrange in the absence of a complementary plasmid expressing the *xisA* gene. This result suggests that in *E. coli* the *nifD* element is able to properly rearrange in the absence of any other *Anabaena* gene products.

Excision of *nifD* element belongs to site-specific recombinations. Since, many site-specific recombinations involve accessory proteins, proper excision of the *nifD* element in *E. coli* might also involves accessory proteins of *E. coli* in the excision event (Lammers *et al.*, 1986). Excision of the *nifD* element in *E. coli* was considered not dependent on RecA since *E. coli* MT8820TR contains a *recA* mutation. Additionally, these experiments did not show much variation in the excision frequency when the *E. coli* was given heat shock or grown under different nature of nitrogen sources. This result is surprising as the excision in *Anabaena* PCC 7120 occurs in the heterocysts, which are formed only under nitrogen starvation conditions.

Brusca *et al.* (1990) overexpressed the *xisA* gene in *Anabaena* PCC 7120 vegetative cells and showed that the excises in the vegetative cells. This suggests that in *E. coli*

the excision/deletion events are infrequent and apparently not tied to N-metabolism or anaerobic growth. Interestingly, Razquin *et al.* (1994) showed that under iron stress conditions the excision of the 11kb *nifD* element occurred in *Anabaena* PCC 7120 even in the presence ammonium. Thus, it appears that the frequency of excision of the *nifD* element in *E. coli* may be influenced by certain accessory proteins. In order to determine such components, the excision of *nifD* element was monitored in *E. coli* strains, which were grown in different media and culture conditions.

2.2 : MATERIALS AND METHODS

2.2.1 : 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
**-Required concentration of NH_4 Cl was added from 1M stock solution.
Unless otherwise indicated here M9 minimal medium refers to M9 minimal medium containing 40mM NH_4Cl.

Antibiotic stock solutions (1000 X for rich medium)

Ampicillin/Carbenicillin : 100mg/ml in H_2O.
Kanamycin : 50mg/ml in H_2O
Tetracycline : 25mg/ml in 70% Ethanol
Chloramphenicol : 40mg/ml in Ethanol
Trimethoprim : 60mg/ml (in Dimethyl formamide)

For minimal medium, the antibiotic concentrations were used at half the concentration that is used for the rich medium.

X-Gal : 20mg/ml (in Dimethyl formamide)
IPTG : 100 mM

2.2.2: Strains

Strains	Genotype	Reference
JM101	*F'traD36lacI^{q}Δ(lacZ)M15 proA$^+$B$^+$/supE thi Δ(lac-proAB)*	Yanisch-Perron. *et al. (1985)*
DH5α	*F'endA1hsdR17(r_k^- m_k^+)supE44 thi-1 recA1gyrA96(nal^r) relA1(lacZYA-argF)U169deoR (∅80dlacΔ (lacZ)M15*	Hanahan (1983)
GJ216	*recA srl::Tn10*	Gowrishankar (1986)
RK#1	JM101 *recA$^-$ srl::Tn10*	This study
RK#2	JM101 recA$^+$ *srl::Tn10*	This study

2.2.3 : Plasmids

2.2.3.1 : pMX25 (Lammers *et al.* 1986).

An 17kb EcoRI DNA fragment of *Anabaena* PCC 7120 containing the entire 11kb *nifD* element along with the complete *nifK* gene and some region of *nifD* gene was subcloned into unique EcoRI site of the vector pBR322. The mini-mu-transposon carrying the kanamycin gene and β-gal (*lacZ*)gene was inserted inside the 11kb *nifD* element (outside the *xisA* ORF). The pBR322 plasmid component provides resistance to tetracycline and ampicillin. The size of the pMX25 is 31kb (**Fig 2.1**).

2.2.3.2 : pMXΔ25: (Lammers *et al.* 1986).

Upon rearrangement the 11kb *nifD* element was excised out from pMX25 along with the kanamycin resistance and β-gal (*lacZ*)genes. The rearranged plasmid contains the *Anabaena* region containing *nifK* and the *nifD* genes in the pBR322 plasmid. This rearranged plasmid size is 10kb and designated pMXΔ25 (**Fig 2.2**).

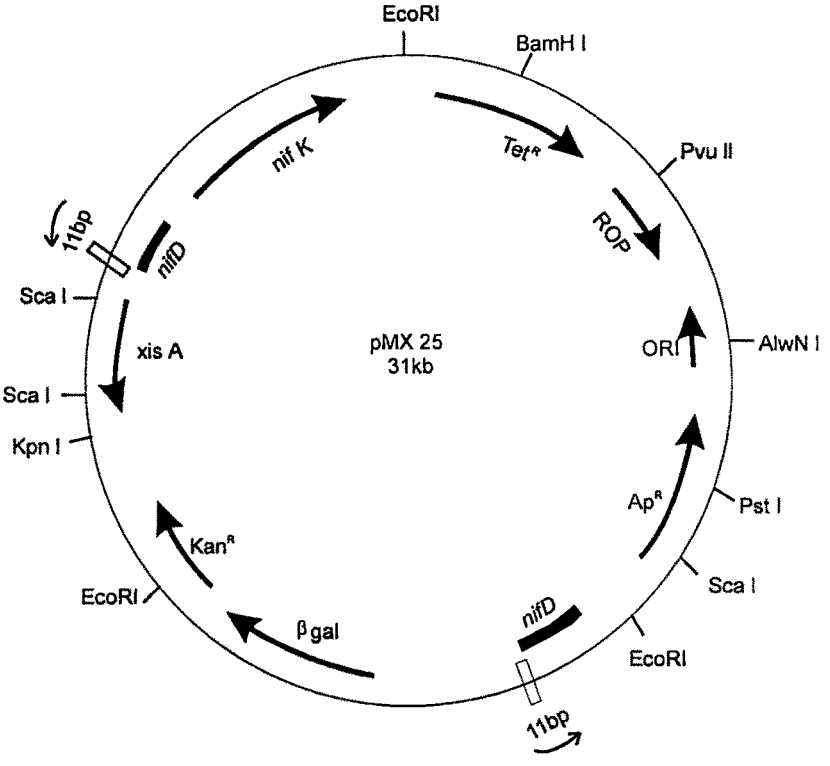


Fig. 2.1 : Restriction map of pMX25 plasmid

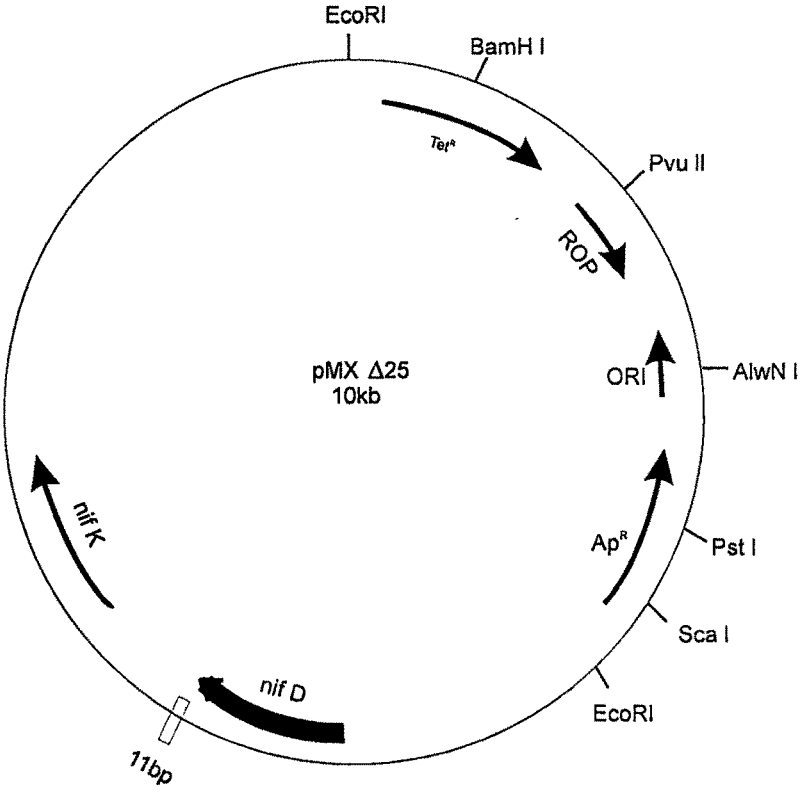


Fig. 2.2 : Restriction map of pMXΔ25 plasmid

2.2.4 : Isolation of plasmid DNA.

The plasmid DNA isolation was done by the alkali lysis method as described by Brinboim and Doly (1979).

2.2.5: Transformation of plasmid DNA.

The method of transformation was carried out using $CaCl_2$ method as described by Cohen *et al.* (1972).

2.2.6 : Restriction enzyme digestion analysis.

0.5-1.0μg DNA sample was used for each restriction enzyme digestion. 2-4U of the restriction enzymes were used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 20μl. The digestion was allowed to go for overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were photographed.

2.2.7: Ligation (Sambrook *et al.*, 1989).

50-100ng of DNA was used in each ligation reaction. Vector to insert ratio was maintained at either 1:1 or 1:2. The ligation reaction was usually done in 15μl volume containing ligation buffer and 0.5-1.0U of T_4 DNA ligase. The ligation reaction was carried out at 16°C for 12-16h.

2.2.8: Agarose gel electrophoresis.

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose gel in 90mM Tris-Borate buffer at 5v/cm for 2-4h. The gel was stained in 1μg/ml ethidium bromide for 30min at room temperature and the bands were visualized by fluorescence under the UV-light.

2.2.9: Monitoring excision of *nifD* element by monitoring rearrangement of pMX25 plasmid in *E. coli.*

Rearrangement assay of Lammers *et al.* (1986) was used with modifications.

A single colony of *E. coli* containing pMX25 plasmid overnight was grown in 15ml Luria broth with kanamycin, and ampicillin. 0.1ml cells from 10^{-4} dilution (diluted in saline) were plated on Luria agar containing ampicillin X-Gal. The 0h plating was done to ensure that there are no whites present in the starting culture. Kanamycin was added to ensure that the rearranged colonies are eliminated.

From this overnight grown culture 1.0 ml was spun at 5000 rpm at 4° C for 5min. The pellet was washed twice with either Luria broth or minimal medium with ammonium chloride and suspended in same volume of Luria broth or minimal medium with ammonium chloride.

1% of (150μl) of the cells was reinoculated either in 15ml of Luria broth or minimal medium containing only ampicillin. The cultures were allowed to grow for 24 h at 37°C. After 24 h of growth, again the dilutions were made and plated on Luria agar containing X-gal and ampicillin. The excision was calculated using this formula.

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

After counting the number of colonies whites and blues were picked and restreaked on Luria agar containing kanamycin and Luria agar containing ampicillin. The white colonies grew only on ampicillin,containing plates but not with kanamycin but blue colonies grew on both plates. Plasmids from white and blue colonies were digested with restriction enzymes to confirm the pattern's resemblance to the rearranged and unrearranged DNA respectively.

2.2.10: Construction of *recA* mutant of *E. coli* JM101.

2.2.10.1: P1 Transduction (Gowrishankar, 1985).

2.2.10.1.a: Growth of P1 lysate on GJ216 (*recA⁻srl::Tn10)*.

Grow *E. coli* GJ216 in 1ml Luria broth+5mM $CaCl_2$ to saturation. To 0.3ml of the culture. 50-75µl P1 lysate was added and left at room temperature for 20min. Soft agar containing 125µl of infection mixture was overlayed on Luria agar supplemented with $CaCl_2$ (5mM) and glucose (0.2%) plate. After 8-10h of incubation at 37°C, 2ml of Luria broth-containing $CaCl_2$ was added to the plate, followed by addition of 1-2 drops of chloroform and continuation of incubation for next 30min. The supernatant was decanted into a sterile tube, 2-3 drops of chloroform was added, mixed vigorously by vortexing, and was spun down at 3000 rpm for 10min. The supernatant was transferred to a clean tube.

2.2.10.1.b: Transduction of JM101 using P1 grown on GJ216.

Grew the *E. coli* JM101 in 3ml of Luria + $CaCl_2$ broth. To 1ml cells, 500µl P1 lysate was added and allowed infection to occur for 15 min. The cells were collected after centrifugation and washed twice with Luria broth-citrate (25mM). Cells were suspended in 5ml of Luria broth-citrate and they were kept for 45 min at 37°C for the expression of antibiotic resistance gene to occur. The transduction mixture was spread on Luria agar citrate plate containing tetracycline. Control cells were treated similarly but without the addition of P1 lysate.

2.2.10.2: Scoring the *recA* phenotype.

Transductant colonies from the selection plate were purified on Luria agar Tet-citrate plate. The *recA* phenotype was checked as follows: The colonies from the selection plate were purified on Luria agar citrate-tetracycline plate. The freshly prepared inoculum was exposed to UV (240nm at 30cm) for 20 seconds and were incubated in the dark at 37°C in order to prevent photoreactivation. The transductants that grew after UV exposure was scored as $recA^+$, those that did not grow under these conditions were scored as $recA^-$ transductant. One transductant from each class RK#2 and RK#1 respectively was used for further studies.

2.2.11: Growth characteristics of *E. coli* containing pMX25 and pMXΔ25 plasmids.

A single colony of *E. coli* was inoculated in Luria broth and grown overnight. 1ml of the cells were pelleted and washed twice with Luria broth/M9 minimal medium and resuspended in same volume of fresh Luria broth/M9 minimal medium. Same number of cells from each culture were reinoculated into Luria broth/M9 with antibiotics. OD_{600} was measured at different time points and plotted.

2.2.12: Competition experiment of blue Vs white *E. coli* colonies on minimal medium.

E. coli JM101 containing rearranged pMXΔ25 (white) and unrearranged (blue) pMX25 plasmids were grown overnight in Luria broth containing ampicillin for the former and ampicillin and kanamycin in the latter case. 1ml of the cells were spun down and washed twice with M9 minimal medium and suspended in 1ml of M9 minimal medium. Dilutions were made in saline (10^{-4}) and plated on Luria agar containing ampicillin for white colony and Luria agar containing kanamycin for blue colony. The numbers of CFU from these two plates were calculated. The blue and white colonies were mixed in the proportion of 99% blue and 1% whites,

allowed them to grow for 24 h at 37°C. After 24 h the culture was diluted and plated on X-Gal plates. The percent blue and whites was calculated as described earlier.

2.2.13: Effect of Fe^{3+} on the rearrangement of *nifD* element in *E. coli.*

To study the effect of Fe^{3+} on rearrangement of *nifD* element, the cells were grown in the minimal medium supplemented with 0.9μM Ferric ammonium citrate (Fe limiting condition) and 30μM Ferric ammonium citrate (Fe excess condition). The rearrangement assay was same as described earlier.

2.3 : RESULTS

2.3.1 : Rearrangement of pMX25 in *E. coli.*

The excision of the *nifD* element of *Anabaena* PCC 7120 in *E. coli* was studied using the plasmid pMX25. *E. coli* colonies with pMX25 plasmid exhibit resistance to ampicillin and kanamycin and form blue colonies on the indicator X-gal plate. The rearranged colony after excision retains ampicillin resistance gene but looses the kanamycin resistance and lacZ genes and therefore gives rise to white colonies on the indicator plate. Lammers *et al.* (1986) studied the rearrangement of the *nifD* element using pMX25 in *recA*⁻ strain of *E. coli* MT8820TR (*recA* mutant) and reported that the excision occurred at the frequency of 0.3% after it was grown overnight in Luria broth. In our experiments with *E. coli* DH5α containing pMX25, the excision frequency was 0.45% for cells grown overnight on Luria broth **(Table2.1)**. The excision was found to be same when the *E. coli* was grown under anaerobic conditions or after giving heat shock at 45°C for 5min., followed by overnight growth in the presence of ampicillin or growth under nitrogen limitation (Lammers *et al.*, 1986).

Table 2.1 : Excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* DH5α and JM101 containing pMX25 grown in Luria broth.

Media/Strain	Number of Blue colonies	Number of White colonies	Total number of colonies	Percent excision Mean ± S.D.
Luria broth/ DH5α	3757	12	3469	0.45 ± 0.26
Luria broth /JM101	2084	15	2099	0.7 ± 0.02

Results are of three independent experiments.

The excision frequency was slightly higher in cells grown for 24h when compared to that in freshly grown overnight Luria broth **(Table 2.2)**. The excision frequency in DH5α was increased to 1.11% in Luria broth but no further increase in the excision frequency was observed when the *E. coli* was grown for longer periods (Data not provided). Interestingly, the excision frequency was found to increase from 0.45% to 2% in the presence of M9 minimal medium **(Table 2.2)**.

Table 2.2 : Effect of growth medium on the excision of *nifD*element of *Anabaena* PCC 7120 in *E. coli* DH5α andJM101 containing pMX25.

Media/Time (h)	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D.
	JM101 pMX25			
0 h	2402	14(9)*	2407	0.20±0.15
Luria broth /24 h	4219	84	4303	1.95±0.32
M9 minimal medium/24h	5399	661	6060	10.90±7.48
	DH5α pMX25			
0 h	2972	0	2972	0.00±0.00
Luria broth /24 h	4343	49	4392	1.11±0.62
M9 minimal medium/24h	2177	44	2221	1.98±0.59

* Number of Kanamycin resistant colonies after restreaking in to plate.
Results are of three independent experiments.

2.3.2 Role of RecA on the excision of pMX25 in *E. coli*.

The excision was monitored in *E. coli* strain JM101 which was found to be 0.7% when grown overnight in Luria broth **(Table 2.1)**. Since the excision frequency was more in JM101as against DH5α, the excision was also monitored in cells grown in M9 minimal medium in which the *E. coli* N-metabolism is expected to be different under these conditions. The results are presented in the **Fig 2.3** and **Table2.2**. The excision was 5 fold higher in JM101 than that in DH5α grown in M9 minimal medium. But the increase was not found to be significant when these strains were grown in Luria broth. The restriction pattern of the plasmids from blue and white colonies from JM101 and DH5α is identical to that reported by Lammers *et al.* (1986) **(Fig 2.4 and 2.5).**

Table 2.3 : Effect of growth media on the excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* RK#1 and RK#2 containing pMX25.

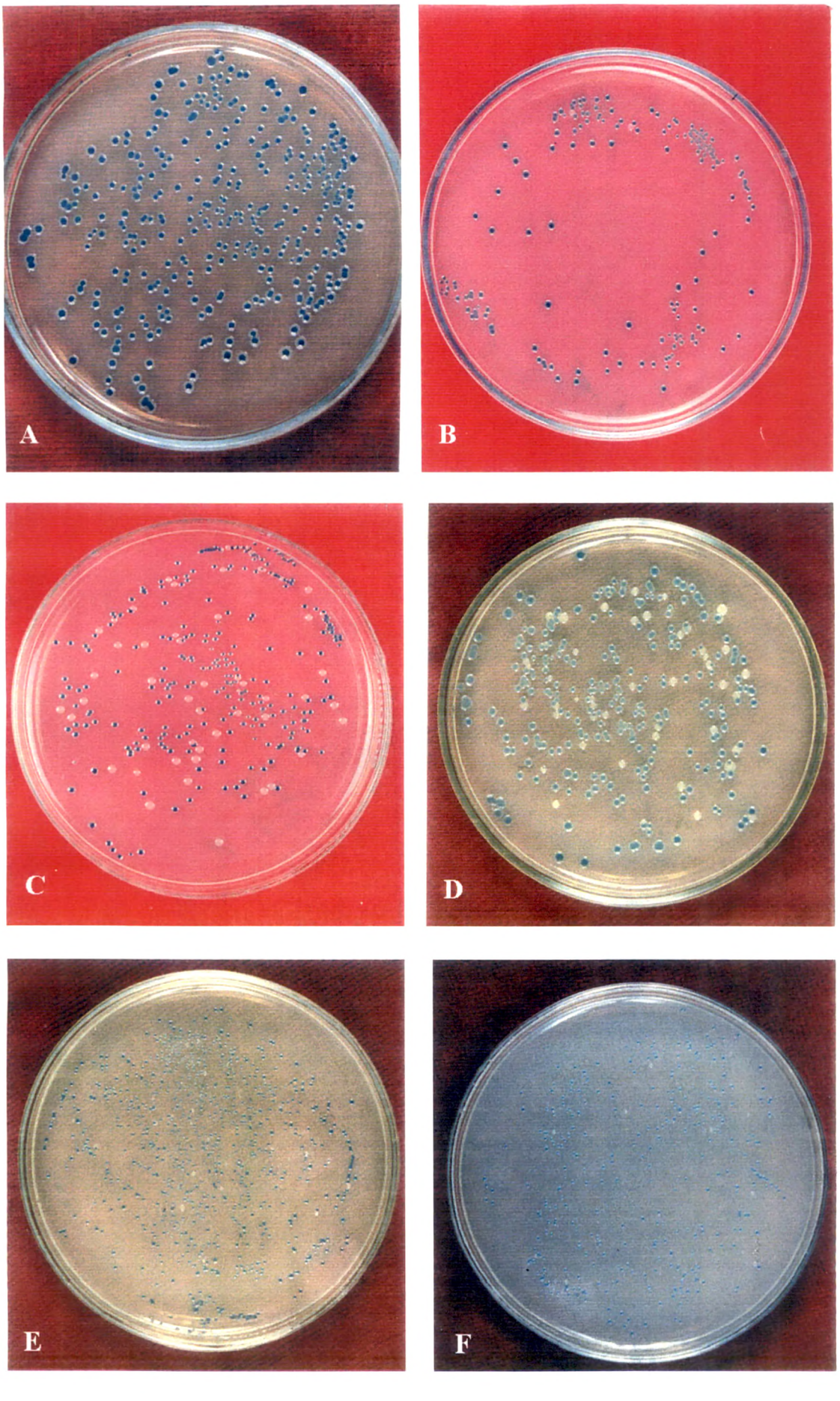
Media/Time (h)	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D.
		RK#1		
0 h	3052	11(6)*	3057	0.16±0.15
Luria broth /24 h	4534	26	4560	0.57±0.45
M9 minimal medium/24h	2331	40	2371	1.68±0.98
		RK#2		
0 h	2182	20(16)*	2186	0.18±0.15
Luria broth /24 h	1203	50	1253	3.99±1.66
M9 minimal medium/24h	1286	186	1472	12.63±7.10

* Number of Kanamycin resistant colonies after restreaking in to plate.
Results are of three independent experiments.

In order to find out the role of *recA* on the excision in *E. coli*, the *recA* mutant of JM101 was constructed using P1 transduction. Isogenic JM101 RK#1 (*recA*$^-$) and RK#2 (*recA*$^{+}$) strains were used for monitoring the rearrangement of pMX25 after

Fig 2.3 : Excision of the *nifD* element of *Anabaena* sp. strain PCC 7120 in different *E. coli* strains containing pMX25

A. JM101 (pMX25) 0h
B JM101 (pMX25) 24h luria broth
C: JM101 (pMX25) 24h M9 minimal medium
D: RK#1 24h M9 minimal medium
E: DH5α (pMX25) 24h M9 minimal medium
F: RK#2 24h M9 minimal medium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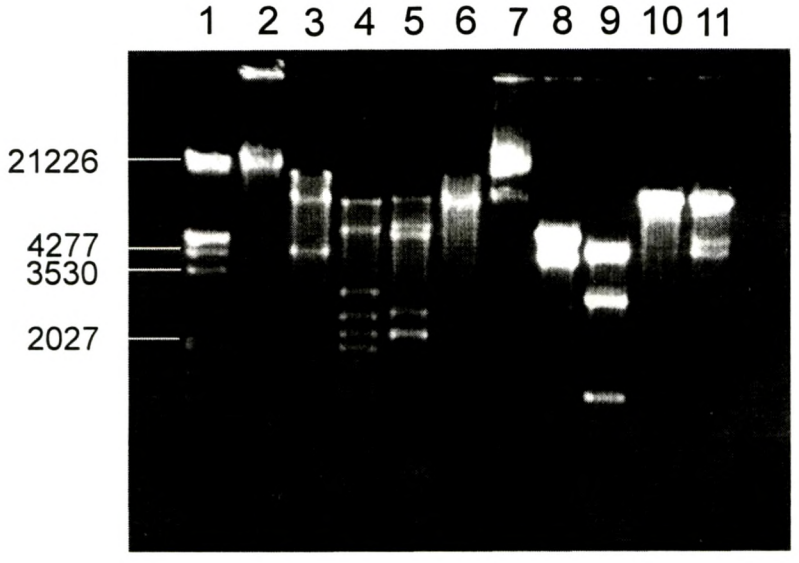

Fig. 2.4 : Restriction endonuclease digestion pattern of pMX25 and pMX△ 25 plasmids.

Lanes: 1. Lambda DNA digested with HindIII /Eco RI; 2. pMX25 uncut plasmid DNA, pMX25 digested with, 3. Eco RI, 4. HindIII, 5. PvuII, 6. BamHI, 7. pMX 25 uncut plasmid DNA, pMX 25 digested with 8. Eco RI, 9. HindIII, 10. BamHI and 11. Pvul.I

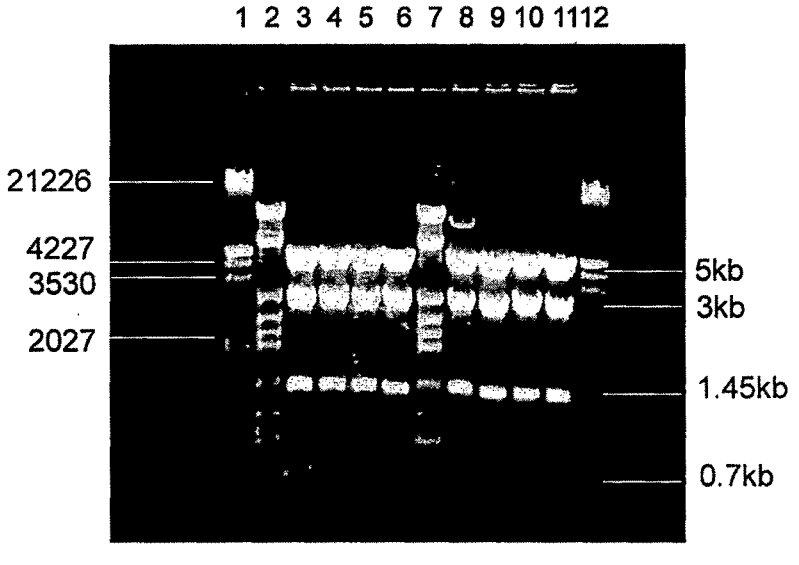

Fig. 2.5 : HindIII digestion pattern of plasmids from blue and white colonies of *E. coli* JM101 and DH5α containing pMX25 after growth in Luria broth and M9 minimal medium for 24h.

Lanes: 1. Lambda DNA digested with HindIII/Eco RI, HindIII digestion of pMX 25 plasmid unrearranged (blue)and rearranged (white) DNA from DH5α, 2. blue colony in Luria broth, 3 & 4. white colony in Luria broth, 5 & 6. white colony in M9 minimal medium, HindIII digestion of pMX 25 plasmid unrearranged (blue)and rearranged (white) DNA from JM 101, 7. blue colony in Luria broth, 8 & 9. white colony in Luria broth, 10 & 11. white colony in M9 minimal medium and 12. Lambda DNA digested with HindIII/Eco RI,

their growth in either Luria broth or M9 minimal medium. Results indicated that in the *recA* mutant of JM101 (RK#1) the frequency of the excision was same as in DH5α and the isogenic JM101 *recA*$^+$ strain (RK#2) the frequency is same as the parent. The plasmid restriction pattern was similar to the parental strains (Fig 2.6). This result clearly indicated that the difference in excision frequency that was observed between DH5α and JM101 could be ascribed to *recA* mutation present in DH5α, excision in isogenic pair of JM101 differing in *recA* genotype compared to the two strains (DH5α and JM101) initially used in the study.

Table 2.4 : Effect of Fe^{3+} and N source on the excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* DH5α containing pMX25.

Media	Time (h)	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ±S.D
Luria broth	0	1295	0	1295	0.00±0.00
-Fe/-N	24	1090	18	1108	1.62±0.56
+Fe/-N	24	859	15	874	1.71±0.43
-Fe/+N	24	850	20	870	2.29±0.57
+Fe/+N	24	700	23	723	3.20±0.72
-Fe/-N	48	784	16	800	2.00±0.34
+Fe/-N	48	870	14	884	1.58±0.26
-Fe/+N	48	944	26	970	2.70±0.57
+Fe/+N	48	903	22	925	2.37±0.45

Grown on M9 minimal medium. +N = 40mM NH_4Cl; -Fe = 0.9 μM; +Fe = 30μM.
Results are of three independent experiments.

Table 2.5 : Competition of blue and white colonies on M9 minimal medium (Initial 99 % Blue & 1% White colonies inoculated on to medium).

Media/ Time (h)	Number of Blue colonies	Number of White colonies	Total	Percent Excision Mean ± S. D.
M9 minimal medium /24h	2889	452	3341	13.52±2.25

Results are of three independent experiments.

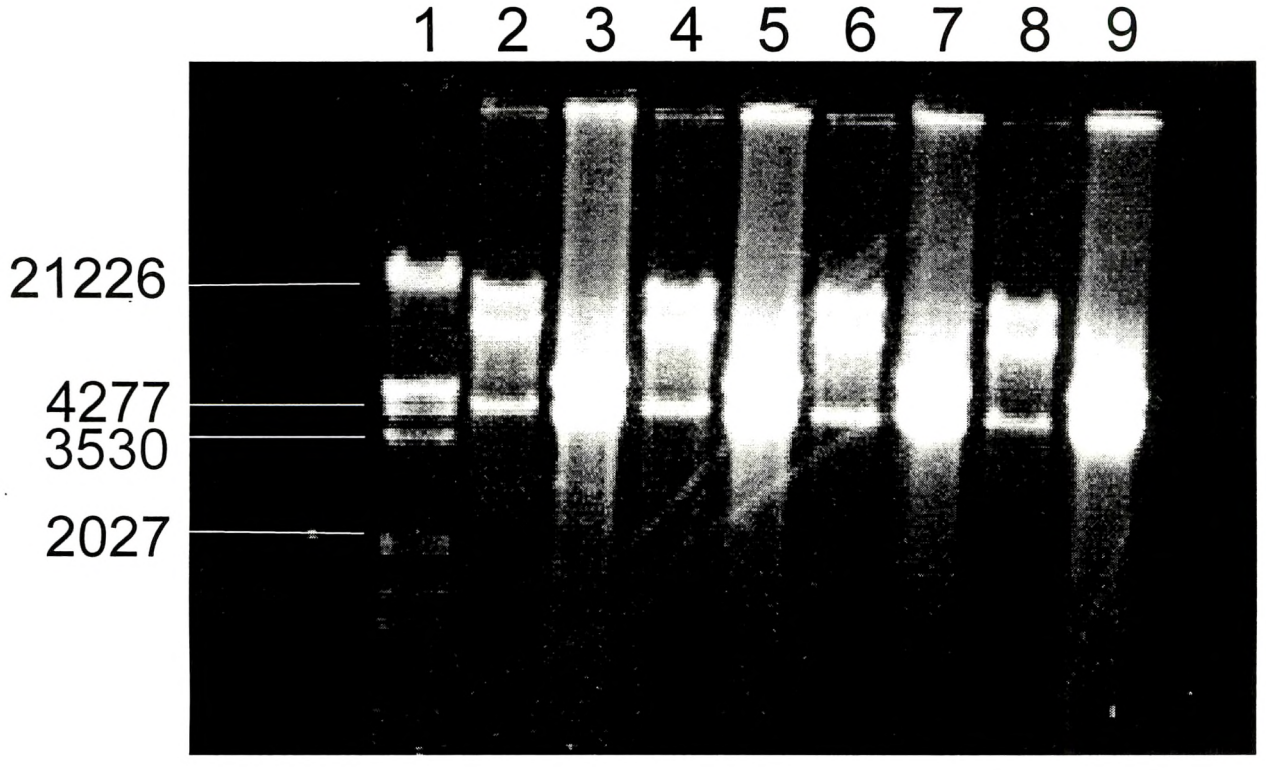

Fig.2. 6 : Eco RI digestion pattern of plasmids from blue and white colonies of *E. coli* RK#1 and RK#2 containing pMX25 after growth in Luria broth and M9 minimal medium for 24h.

Lanes: 1.Lambda DNA digested with HindIII/Eco RI; HindIII digestion of pMX 25 plasmid unrearranged (blue)and rearranged (white) DNA from RK#1, 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 of pMX 25 plasmid unrearranged (blue)and rearranged (white) DNA from RK#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.

Though, the effect of recA mutation on the excision was not substantial, the 5 fold increase was consistent and highly reproducible.

2.3.3 : Appearance of anomalous ' white' colonies of *E. coli.*

E. coli strains were grown on Luria broth containing ampicillin and kanamycin and were transferred to Luria broth or M9 minimal medium for monitoring excision. In order to test that phenotype of the starting *E. coli* strains (at 0h), the strains were plated on X-Gal/ plates. Surprisingly, some white colonies were observed , amongst them many whites were kanamycin resistant. The 'white' colonies turned blue only upon restreaking on Luria agar containing X-gal kanamycin and ampicillin. Furthermore, the DNA isolated from the 'white' colonies, produced only blue transformants, and the digestion pattern of plasmid DNA from 'white' colony was same that of DNA from blue colony indicating unrearranged structure.**(Fig 2.7).** The reason for the appearance of these white colonies is not clear. Thus, these white colonies were not included in counting the rearranged colonies.

2.3.4 : Effect of iron status on the excision of *nifD* element.

Razquin *et al.* (1994) showed that under iron stress conditions the excision of the 11kb *nifD* element in *Anabaena* PCC 7120 occurred even in the presence of ammonium. In order to find out whether the iron stress condition in *E. coli* could increase the frequency of the excision, the rearrangement was monitored in *E. coli* DH5α in iron deficient and sufficient conditions. The results were shown in the Table 2.5. Under -N/-Fe condition the excision frequency was 1.62% but there is slight increase of excision under +N/+Fe (3.20%). The results indicated that the excision frequency was not significantly affected under Fe stress conditions and that indicated the excision in *E. coli* and *Anabaena* PCC 7120 do not follow completely

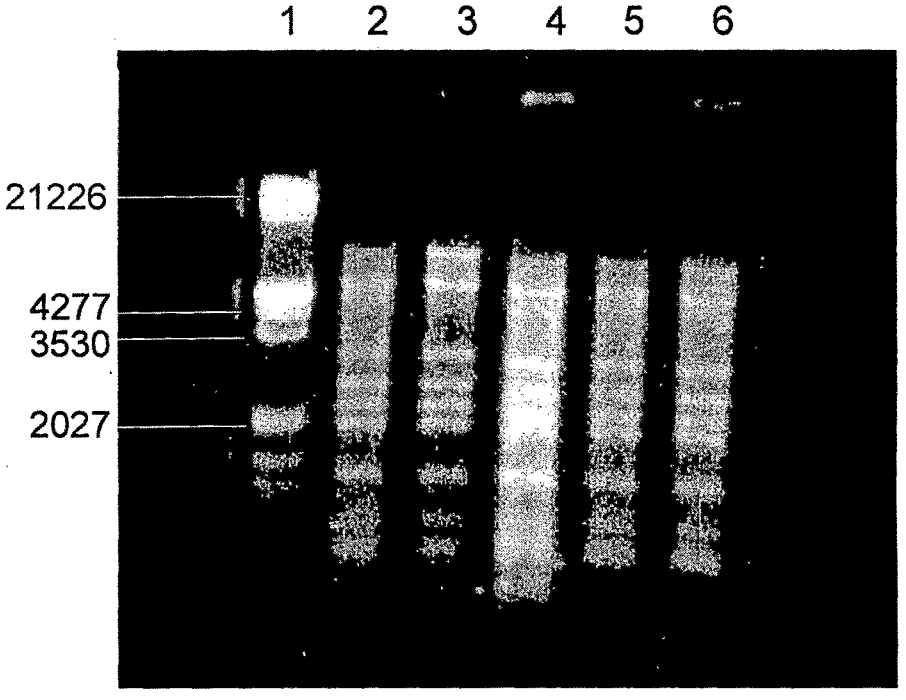

Fig.2.7 : Hind III digestion pattern plasmid DNA from white colonies appeared from different *E.coli* strain at 0 h.

LANES: 1. Lambda DNA digested with Hind III/Eco RI, 2. JM 101, 3. RK#1 4. RK#2, 5. NK#6, 6. JM 101.

identical pathway. The accessory proteins required for the repression as well as activation of the excision in *Anabaena* PCC 7120 may not be present in *E. coli.*

2.3.5 : Growth characteristics of Blue and White colonies on Luria broth and M9 minimal medium :

Plasmid sizes of unrearranged and rearranged are 31kb and 10 kb, respectively. *E. coli* containing these unrearranged (Blue colony) and rearranged (White colony) plasmids may show different growth rates. In order to find out the growth characteristics of blue and white colonies in Luria broth and minimal medium, the growth pattern was determined. The growth pattern in Luria broth for white and blue colonies was similar with the generation time being of 72 and 78 min, respectively **(Fig 2.8).** However, in the minimal medium the generation time of white colony (66 min) was about half of that of blue colony (114min) **(Fig 2.9).** This indicated that the white colonies grew faster than blue colonies in minimal medium but not in Luria broth. Hence, the increase in number of white colonies appearing after growth in minimal medium for 24 h is a result of both the rearrangement in the plasmid in the white colonies and faster growth of the rearranged colonies over the blue colonies.

2.3.6: Competition of blue and white colonies in minimal medium.

Estimation of the contribution of growth advantage of white against blues for accurate estimation of excision frequency, with starting inoculum that consisted of 99% Blue and 1% white cells were determined after 24h of growth in minimal medium. The results are presented in the **Table 2.5.** It was seen that after 24h growth in minimal medium the white colonies were 13% after 24h of growth.

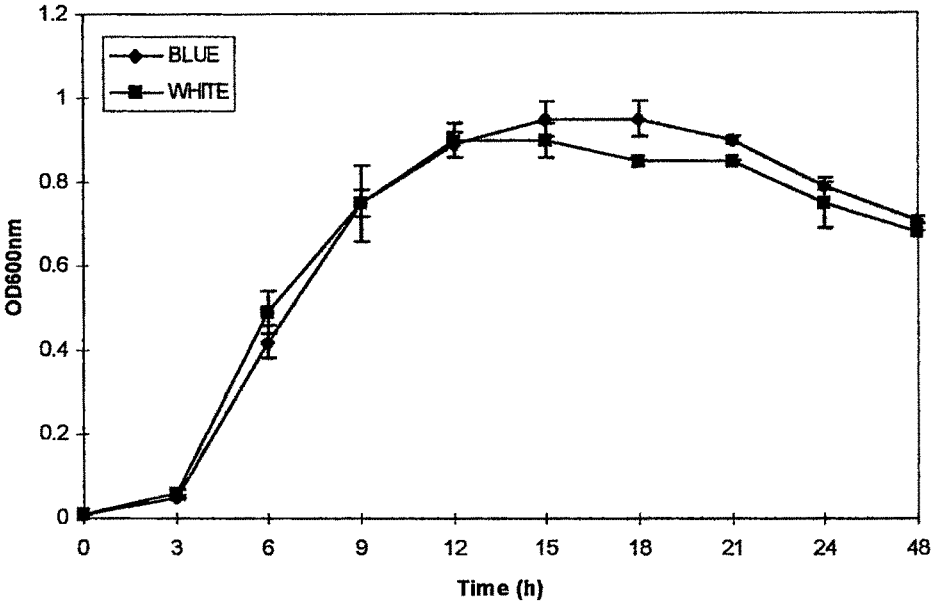

Fig 2.8 : Growth curve of *E. coli* (JM101) containing unrearranged pMX25 plasmid (blue colony) and rearranged pMXΔ25 plasmid (white colony) in Luria broth

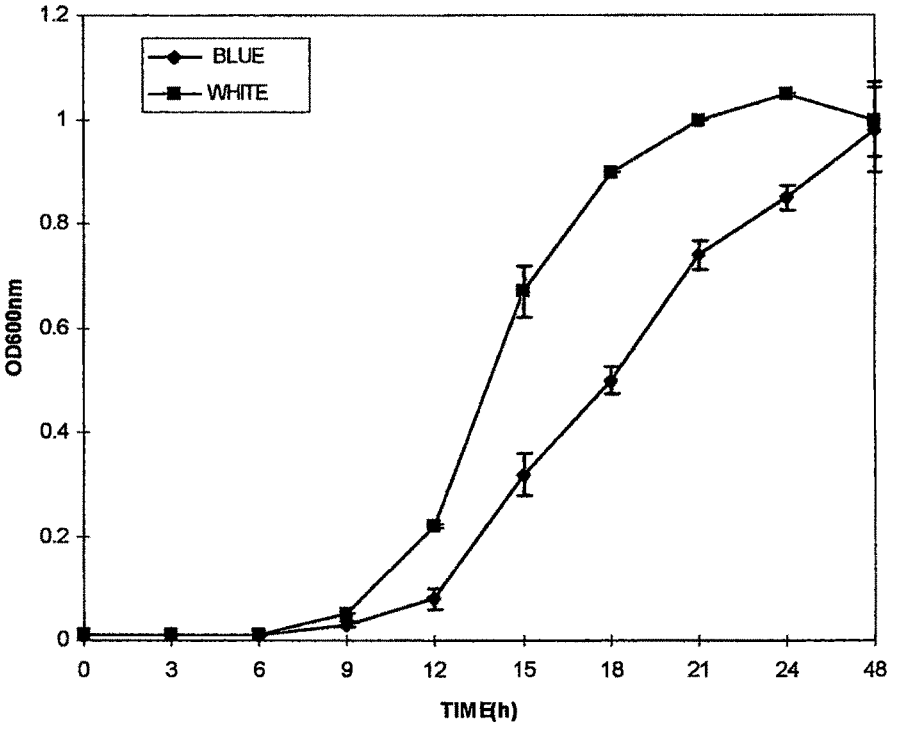

Fig 2.9 : Growth curve of *E. coli* (JM101) containing unrearranged pMX25 plasmid (blue colony) and rearranged pMXΔ25 plasmid (white colony) in M9 minimal medium

2.4 : DISCUSSION

Excision of *nifD* element of *Anabaena* PCC 7120 occurs in all heterocysts whereas it occurs in *E. coli* less frequently. *E. coli* DH5α showed an increase in the excision frequency from 0.3% after over night growth to about 1% after 24 h growth. Additionally, excision increased to about 2% in the minimal medium. These increases cannot be completely accounted by the faster growth of *E. coli* containing rearranged plasmids.

Earlier Lammers *et al.* (1986) concluded that *recA* gene is not involved in the excision as the excision experiments were carried out in MT8820TR which is *recA* mutant. However, excision in *E. coli* JM101 is about 5-6 fold higher than in DH5α when grown in minimal medium. Similar results were obtained with isogenic $recA^+$ and $recA^-$ strains of JM101 grown in minimal medium. The restriction endonuclease digestion pattern of the rearranged plasmids from all these strains were similar to that reported by Lammers *et al.* (1986). Thus it appears that RecA enhancement of excision is mediated through excisase A. Since significant increase in the excision is found in minimal medium, accessory proteins may be present at higher levels in minimal medium. Sato *et al.* (1996) showed that excision of 48 kb occurs *skin* element of *Bacillus subtilis* could occur in the absence of CisA recombinase encoded by *spoIVCA* at low level. The CisA independent excision was suggested to be *recA*-dependent since *skin*-less mutants could not be obtained from *recA* mutant. Interestingly, the frequency of generating *skin*-less strains from a *spoIVCA* null mutant was much lower that that from the *spoIVCA* point mutation (Sato *et al.*, 1996) suggesting that RecA is mediating its role via CisA protein.

Iron starvation does not seem to influence the excision of *nifD* element in *E. coli* but brings about the excision in *Anabaena* PCC 7120 even in the presence of ammonium. This is expected if the excision is repressed in *Anabeana* PCC 7120 and the derepression occurs under iron starvation conditions. An iron containing transcriptional repressor may be converted into inactive form by sensing the oxygen

status (Apte & Naresh Kumar, 1996). Such repression may not be present in *E. coli* as excision of *nifD* element occurs even in the Luria broth whereas it normally occurs in *Anabaena* PCC 7120 under nitrogen starvation conditions in the heterocysts.