Chapter 4 To study intermolecular recombination of 11 kb element in *E. coli*

4.1 Introduction

Recombination occurs in all organisms, allowing for shuffling, deleting and rearranging genes. It mainly involves breaking and rejoining of the DNA molecule(s) and is divided into homologous recombination and site-specific recombination. Homologous recombination occurs between any homologous regions and is an important phenomenon for genetic exchange, reassortment of genes, repair of broken DNA, etc. Site-specific recombination (SSR) occurs between specific sites of 5-50 base pairs in size (Chen & Rice, 2003).

Non-homologous recombination which is catalyzed by specific proteins can be further divided into two categories, SSR and transposition. While SSR is recombination between two defined sequences, transposition involves recombination between specific sequence and non-specific DNA sites. SSR is responsible for the integration of viral genome, switching on or off of the genes, inversion of DNA segments and genetic variations (Azaro, 2002; Grindley, 2002;). SSR can also be intermolecular or intramolecular. Intermolecular recombination can occur with a variety of types of DNA templates: linear DNA templates acquired via transduction, transformation, conjugation may combine with linear chromosomes and plasmids; circular dsDNA chromosomes with plasmids; and between two circular dsDNA templates. Intermolecular recombination results in the integration of DNA segment whereas intramolecular recombination results in the deletion of DNA or inversion of DNA when the target sites are present in direct or inverted orientations, respectively (Figure 1) (Grindley, NDF, 2006).

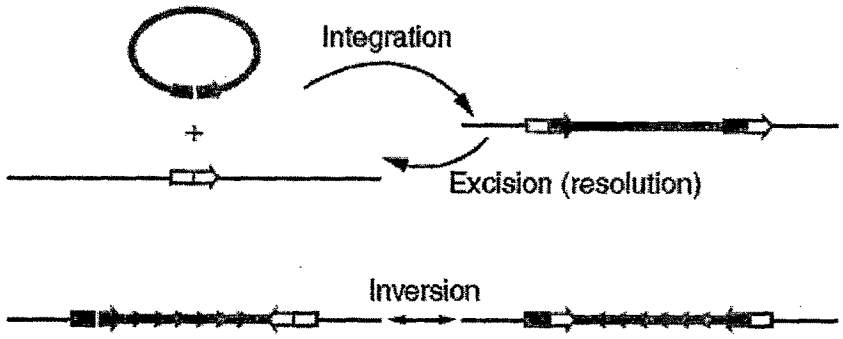


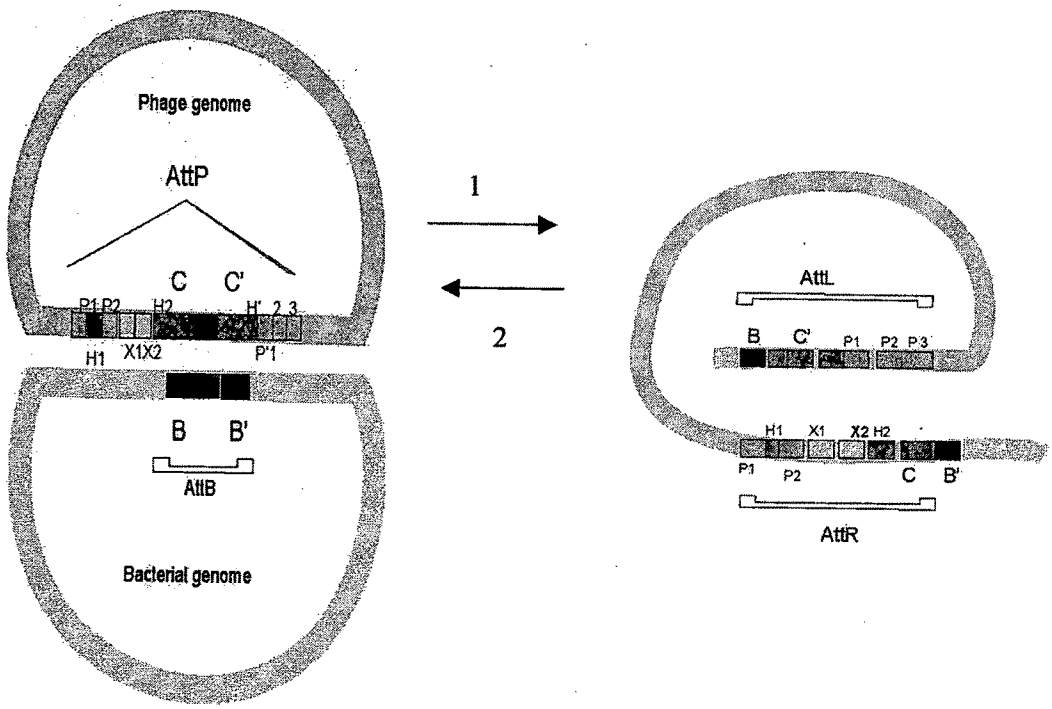
Figure 4.1: possible outcomes of site-specific recombination

Integration of λ phage genome in to the bacterial host chromosome during the lysogenic cycle is well established case of intermolecular SSR. This process is mediated by the λ integrase involves *att*P site on the phage genome and *att*B site on the bacterial genome. Besides the recognition sequences, additional sequences are necessary for integration which are the binding sites for architectural proteins like IHF. Excision of the phage genome from the bacterial chromosome, an intramolecular SSR event, occurs by looping of DNA which is mediated by specific Xis protein along with Int and IHF proteins. Currently, about 300 SSR are characterized in viruses, bacteria, fungal and mammals.

XisA mediates a site specific recombination event in the late stage of heterocyst maturation to excise the *nifD* element leading to the formation of *nifHDK* operon (Lammers et al., 1986). The excised 11kb element remains in the form of circle in the heterocyst cell but it does not undergo replication. Entire 11 kb *nif D* element has been cloned in the pBR322 vector to generate pMX25 plasmid, which has been used extensively to study the rearrangement in heterologous host *E. coli.* In *E. coli,* the fate of the excised 11kb element is not known. Additionally, it has been postulated that *nifD* element and other DNA elements of *Anabaena* PCC 7120, *viz fdxN* and *hupL* elements, could have been derived from cyanophages (Prabhavati & Apte, 1994). This is supported by the fact that these elements are not present in some heterocystous cyanobacteria.

Hence, the present study has been done to investigate intermolecular recombination of 11 kb element by introducing plasmids containing 11bp target site in *E. coli*.

Figure 4.2 Intermolecular and intramolecular recombination events during integration of λ genome into bacterial host genome



1. Intermolecular recombination of λ phage into bacterial genome
2. Intramolecular recombination in Bacterial genome leading to release of phage genome

4.2 Material and methods:

4.2.1

For routine growth and maintenance of plasmids, *E. coli* cells were grown in the LB medium with appropriate concentration of antibiotic. For monitoring the recombination events, M9 minimal medium was used. Transformation was done by Hanahan method.

4.2.2 Bacterial strains and plasmids used in the study

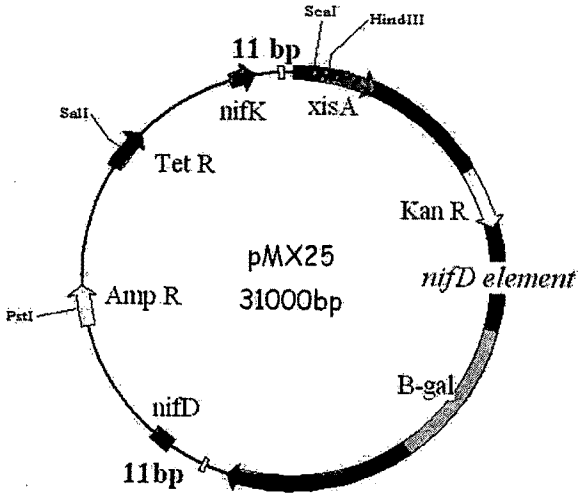
Table 4.1

E. coli / Plasmids Strains	Genotype / Features	Source/Reference
JM101	*FΔtraD36lacI^{q}Δ (lacZ)M15 proA$^+$B$^+$/supE thi Δ (lac-proAB)*	Yanisch-Perron, 1985
Plasmids		
pMX25	Amp^R, Tet^R, Km^R LacZ$^+$and contains two 11bp target site	Lammers *et al.*, 1986
pKK#1	Trim^R and contains one 11 bp target site	Karunakaran, 2000
pMC 71A	Chlm^R	(Buchanan-Wallaston *et al.*, 1981)

4.2.3 Plasmids:

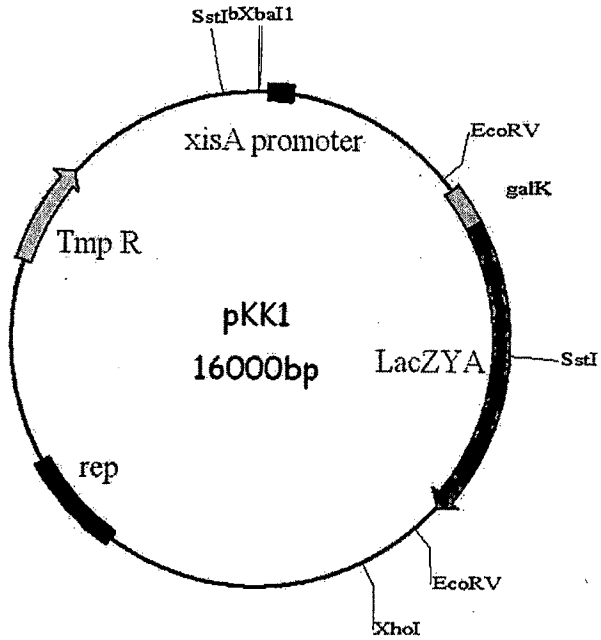
4.2.3.1 pMX25

An 17kb EcoRI DNA fragment of *Anabaena* PCC 7120 containing the entire 11kb *nifD* element flanked by 11bp target site 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.

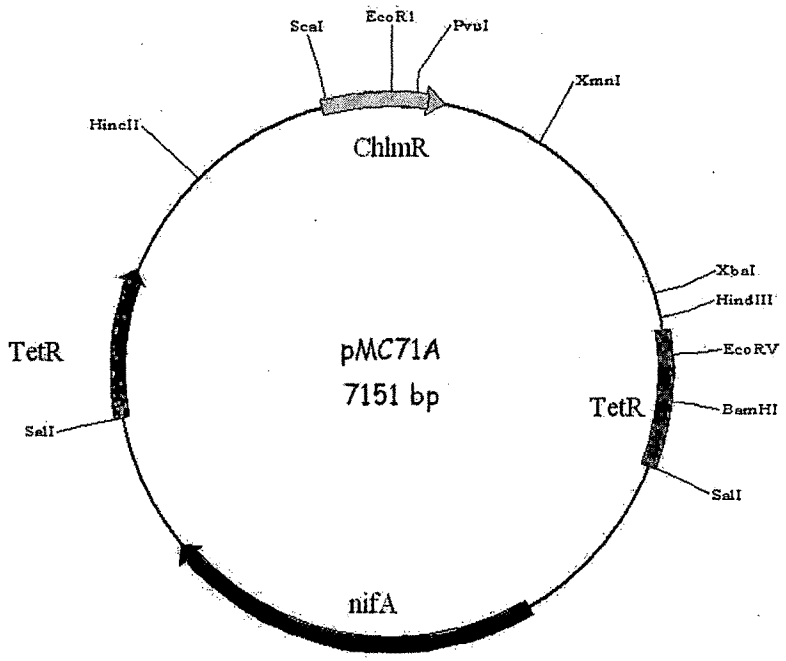


4.2.3.2 pKK1

1kb HindIII fragment from pAN207.65 plasmid, carrying the *xisA* gene promoter has been cloned in the unique HindIII site of pMU575 plasmid in the upstream region of the *lacZYA* operon. It contains one copy of the 11bp target site. It has trimethoprim resistance. Size of pKK1 is 16 kb.



4.2.3.3 pMC 71A



pMC71A is a derivative of the plasmid pACYC184. The *nifA* gene from of *Klebsiella pneumoniae* was inserted into SalI site within the Tetracycline resistance gene of vector. The *nifA* gene is expressed constitutively under tetracycline resistance gene promoter. The vector has chloramphenicol resistance gene. The size of pMC71A is 7.1 kb.

4.2.4 Methodology:

4.3.1 Intermolecular recombination at 11 bp target site

pMX 25 plasmid containing two 11 bp target site with entire *nifD* element, was used for intermolecular recombination experiments with single target site containing pKK1 plasmid. The possible outcomes of the intramolecular or intermolecular recombination of these plasmids will be:

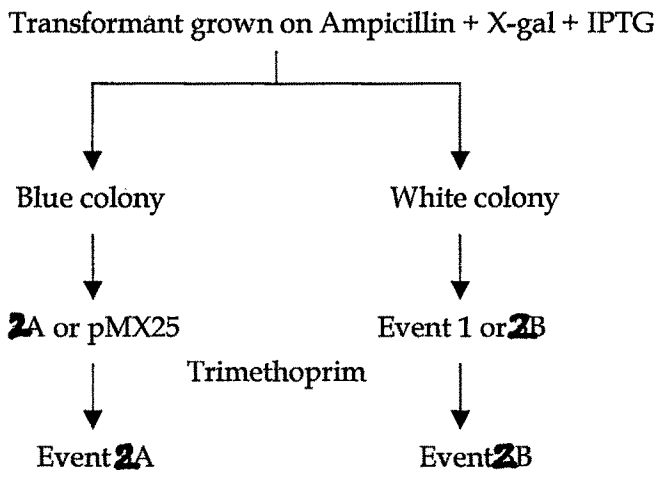
(i) **Intramolecular recombination:** The formation of pMXΔ25 plasmid lacking *nifD* element resulting in the loss of kanamycin resistance and *lacZ* gene, thereby forms white colony on X-gal containing plate.

(ii) **Intermolecular recombination:** Three intermolecular recombination events are possible involving pMX25, pMXΔ25 pKK1 and circular *nifD* element. If *nifD* element combines with the pKK1 plasmid, it will result in the transposition of the *nifD* element.

4.2.5 : **Strategy employed for selection of intramolecular or intermolecular recombination**

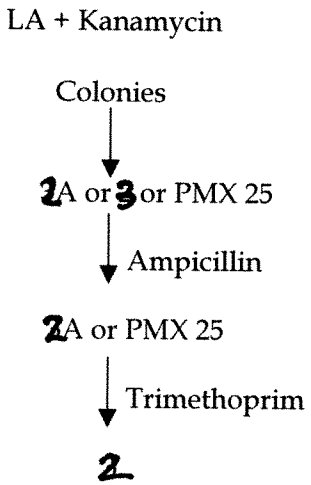
E. coli JM101 containing all three plasmids will be grown in M9 minimal medium for 24 hours, plasmids will be monitored for intramolecular and intermolecular recombination events. Antibiotic resistance and the β-galactosidase activity will be used for determining these events as shown in the figure (3). The screening procedure for monitoring the recombination events is shown in strategy. In the ampicillin resistance selection, blue colonies will be because of intact pMX25 plasmid or intermolecular recombination of pMX25 plasmid with pKK1, which can be differentiated by selecting on trimethoprim plate. Intact pMX25 plasmid does not show resistance to trimethoprim, while the recombined molecule will have trimethoprim resistance. In similar manner, kanamycin and trimethoprim resistance was used for the selection.

Selection procedure 1:



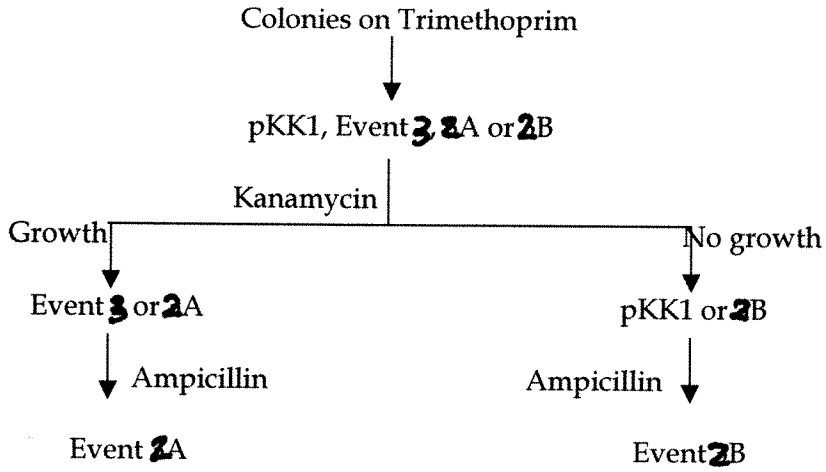
Negative controls used:
LA+ Chloramphenicol+ Trimethoprim

Selection procedure 2:



Negative control:
LA+ Chloramphenicol+ Trimethoprim

Selection strategy 3:



4.2.6 Methodology:

E. coli JM101 cells were transformed with the all three plasmid sequentially and the selection was done by antibiotic resistance followed by restriction digestion pattern analysis of plasmids. *E. coli* JM101 containing all three plasmids was grown for 24 hours in the presence of respective antibiotics in M9 minimal medium. Cells were harvested and plasmids were isolated using modified alkali lysis method. *E. coli* JM101 competent cells were transformed with the plasmids and grown on different antibiotics and X-gal containing plates.

4.3 Results

In the first selection strategy, where selection was done on ampicillin-X-gal plate, higher percentage of colonies were white than blue colonies. Blue colonies as well as white colonies upon streaking on trimethoprim plate, could not grow (table 2). In the second selection strategy, Kanamycin was used, all colonies were screened for ampicillin resistance where higher number of the colonies could not grow on the ampicilin. Those grown on the ampicillin were further selected on Trimethoprim, where no colony showed growth (table 3).

Table 4.2 Strategy 1 results:

No. of colonies on Ampicllin plate			
9251 ± 680			
No. of Blue colonies		No. of white colonies	
4210± 247		5032± 917	
No. of colonies grown Trim	No. of colonies not grown on Trim	No. of colonies grown Trim	No. of colonies not grown on Trim
0		0	

Table 4.3: Result of strategy 2:

No. of colonies on kanamycin plate			
7213±787			
No. of colonies grown on ampicillin		No. of colonies not grown on ampicillin	
3422 ± 264		3791 ± 545	
No. of colonies grown Trim	No. of colonies not grown on Trim	No. of colonies grown Trim	No. of colonies not grown on Trim
0		0	

Strategy 4.4 result obtained using strategy 3

No. of colonies on Trimethoprim plate			
1720 ± 273			
No. of colonies grown on kanamycin		No. of colonies not grown on kanamycin	
0		1720	
No. of colonies grown on Ampicillin	No. of colonies not grown on Ampicillin	No. of colonies grown on Ampicillin	No. of colonies not grown on Ampicillin
0	0	0	1720

4.4 Discussion

Present study has been carried out to monitor the fate excised 11 kb element in *E. coli* after growth for 24 hours in M9 minimal medium, where it *xisA* gene activity was highest (Karunakaran, 2000). After the plasmid isolation, upon retransformation on Ampicillin plate, both blue and white colonies were obtained. Blue colonies suggest that the plasmid pMX25 could be intact or pKK#1 might have co-transformed, which was rule out by negative control (Trim). Almost 45% of the colonies which remained blue and ampicilin resistance, was because of intact plasmid pMX25, which is not going on any genetic shuffling, further conformed by selecting on kanamycin. So the XisA is not produced in enough amount to carry out the recombination or it could be that all the copies of the plasmid is not getting excised, so even few copies of the intact plasmid in the colony, contributes to blue colour.

Remaining ~55% colonies on ampicillin was white, indicating the excision of 11kb element from the pMX25 plasmid. However, none of the white colony showed trimethoprim resistance, indicating that the excised 11kb element have not recombined with the pKK#1, containing one copy of the 11bp target site.

Selection using kanamycin also produced similar kind of pattern, where almost ~50% colonies retained the ampicillin resistance while rest, lost it and did not show any other resistance feature indicating no other event other than excision. Colonies on the trimethoprim were considerable less than on ampicillin and kanamycin. The single copy number of pKK#1 could be the reason for less number of colonies as compared to pMX25 (medium copy 15-20). The colonies on trimethoprim did not show any additional characters, due to intermolecular recombination or transposition.

Intermolecular recombination has been observed in *E. coli,* where two DNA molecules have been introduced from outside and shown to undergo intermolecular recombination in *vivo* (Degryse E, 1996). Such phenomenon has also been studied in other prokaryotes like Bacillus subtilis, where chromosome has been shown to participate (Biswas *et al.,* 1992). In case of V(D)J recombination, plasmids containing the cross-over sites has been introduced from outside and shown to undergo intermolecular recombinstion (Tevelev & Schatz, 2000). However in our study, we could not detect any intermolecular recombination or transoposition event. We could monitor only excision event. Earlier studies has shown that the presence of *nifA* in M9 medium after 24 hours gives around 60-70% of rearrangement. Here after isolating the plasmids and retransforming it, it gave rearrangement up to 55% percent which was comparable with earlier results, indirectly matching the earlier data. Almost half of the cells are not showing any genetic shuffling. Expression of *xisA* gene could be the limiting factor, as the protein has not been detected, even after 60-70% rearrangement. XisA could be protease sensitive or it could be produced in very less amount. Another reason could be plasmid copy number. pMX25 plasmid is a medium copy number plasmid (pBR322 based), so it could be possible that instead of all copies only few copies undergoing some kind of genetic change, even the excision, but since the remaining intact plasmid gives the phenotype, it is not possible to detect such events. To overcome this, one can use a single copy plasmid containing the entire 11kb element and monitor the recombination event in presence of *nifA*.

4.4 Summary

pMX25 plasmid did not show any intermolecular recombination or transposition event. It showed excision frequency at around 50-55%, comparable with earlier results.

Table 4.5 Possible fate of 11 kb element and their characteristics

Event	*lacZ*	Kan^r	Cm^r	Tm^r	Tet^r	Amp^r
Plasmid pMX25	+++	+++	---	---	+++	+++
Deletion 1	---	---	---	---	+++	+++
Trans-Position 2A	+++	+++	---	+++	---	---
Trans-Position 2 B	---	---	---	+++	+++	+++
Inter molecular Recombination 3	+++	+++	---	+++	+++	+++