Chapter 6: To determine the regulation of XisA promoter using GFP reporter gene

6.1 Introduction

Anabaena PCC 7120 undergoes *xisA*-mediated excision of *nifD* element in the last stages of the heterocyst differentiation. Putative promoter region of *xisA* (-100bp to -170bp) upstream of the second ATG contains two divergent overlapping potential promoters P1 and P2 (Figure 6.1) (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. A negative regulatory element (NRE) located between -65bp to -192bp controls the *xisA* expression (Brusca *et al.*, 1990). NtcA and factor 2 have been shown to be associated with the NRE (between -152bp to -223bp) and regulate the expression of *xisA* (Ramasubramanian *et al.*, 1994).

NtcA protein of *Anabaena* PCC 7120 binds to the consensus binding motif $TGTN_{9/10}ACA$. *Anabaena* PCC 7120 *ntcA* mutant showed pleiotropic effects with defective nitrate metabolism, heterocyst differentiation and nitrogen fixation (Frias *et al.*; Wei *et al.*, 1994)). DNA binding property of NtcA of *Anabaena* PCC 7120 *in vitro* depends upon the status of sulfhydral groups of cystine residues but site directed mutagenesis of the cystine residues had retained normal functions (Jiang *et al.*, 1997). NtcA regulated genes have -15 sequences of the σ70 but do not have -35 consensus sequences. NtcA expression increases immediately after the nitrogen step down and it activates many other genes involved in the alternative nitrogen source metabolism and heterocyst differentiation (Golden, 1998, 2003). Some NtcA regulated genes get activated at early stage of heterocyst differentiation while others in the middle and others

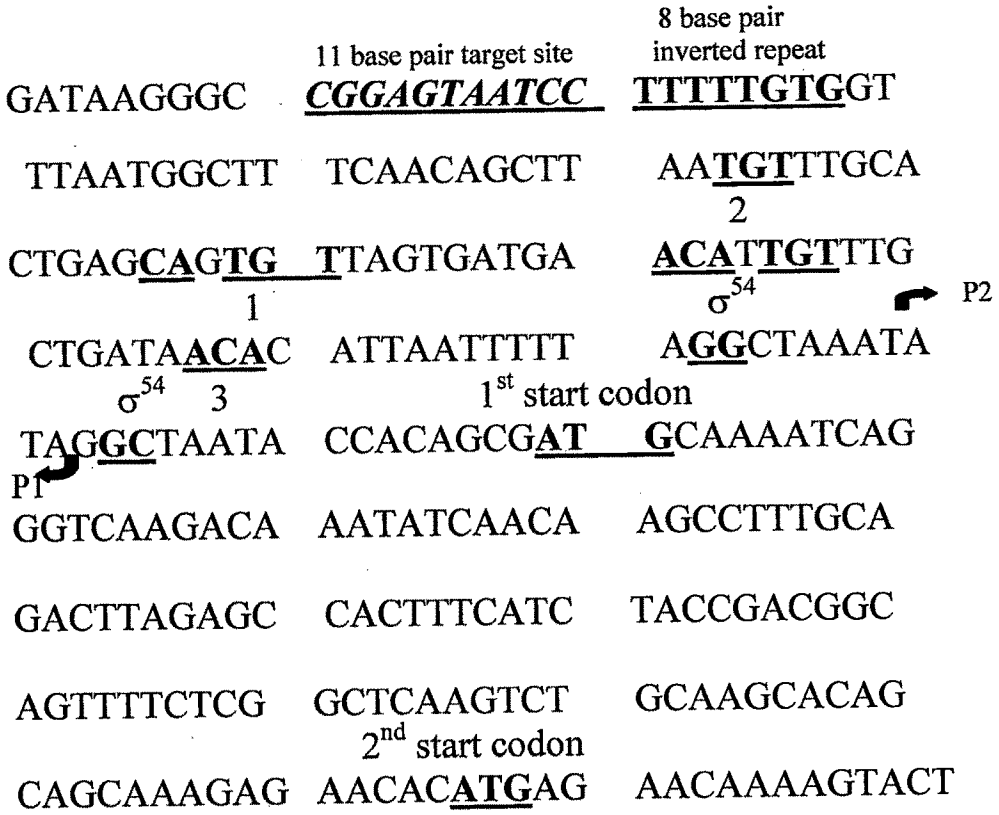
like *xisA* gets activated only at the last stages of the heterocyst differentiation, even though NtcA binding sequences in their promoter region are identical.

Binding of NtcA has been shown to depend on two factors. Signal for the absence of the nitrogen source is 2-ketoglutarate, which starts accumulating after the nitrogen step down (Luque *et al.*, 2004; Olmedo-verd *et al.*, 2005). 2-OG directly interacts with the NtcA and induces conformational change in the NtcA protein. Hence, it has been suggested that binding of the NtcA to the promoter regions depends on the levels of 2-ketoglutarate.

Another factor affecting the NtcA activity is redox potential (Jiang *et al.*, 1997). Binding of NtcA has been shown to be stronger in anaerobic condition. In the heterocyst development, anaerobic condition is gradually increases with modification in the cell wall composition, absence of oxygen generating PS II system, cytochrome oxidase and uptake hydrogenase.

Details of the mechanism of expression of genes associated with DNA rearrangements and nitrogen fixation has not been well established. Expression of *nifH* occurs in the late stages of heterocyst differentiation even in the absence of DNA rearrangements (Golden & Weist, 1988). Many studies have been carried out on *xisA* gene associated with excision of *nifD* element. *xisA* has been cloned since 1986, but not been purified so far, no attempts has been made to study its expression and regulation. In this chapter, attempts have been made to study the *xisA* expression during physiological conditions using GFP reporter assay in *E. coli* and its transformation in *Anabaena* PCC 7120.

Figure 6.1 Putative promoter region of *xisA*



1-3 - NtcA binding sites

6.2 Material and methods:

All the Restriction enzymes were purchased from New England Biolab, (USA) and Roche. DNA extraction Kit and plasmid preparation kit was obtained from the Qiagen limited (USA). All the media and chemicals were purchased from Sigma chemicals, USA.

Table 6.1 Bacterial strains and plasmids used in the study

Strains	Genotype/specific characteristics	Reference
JM101	*F'traD36lacI^{q}Δ(lacZ)M15 proA$^+$B$^+$/supE thi Δ(lac-proAB)*	Yanisch-Perron *et al.* 1985
JM101recA$^-$	*F'traD36lacI^{q}Δ(lacZ)M15 proA$^+$B$^+$/supE thi Δ(lac-proAB)recA*	Karuankaran, 2000
HB101	*thi*-1, *hsd*S20 (r_B^-, m_B^-), *sup*E 44, *recA* 13, *ara*-14 *leu*B6, *pro* A2, *lacY1*, *rpsL*209 (str^r), *xyl*-5, *mtl*-1	Boyer & Roulland-Dussoix, 1969
DH5α	*F'endA1hsdR17(r_K^- m_K^+)supE44 thi-1recA1gyrA96(nal^r) relA1(lacZYA-argF)U169deoR (∅80dlacΔ (lacZ)M15*	Hanahan, 1983
Anabaena PCC 7120	Wild type	Haselkorn
Plasmids		
pBlueScriptSK	Amp^R	Morris *et al.*, 1986
pAM1956	Kan^R/Neo^R, contains *gfp* gene	Apte SK
pAN207.65	Amp^R	Lammers *et al.*, 1986
pRL443	Km^s derivative of RP4,Tet^R , Amp^R	Elhai *et al.*, 1997
pRL623	MobColK, M.AvaI, M.Eco47II, M.EcoT22I, Chl^R	Elhai *et al.*, 1997
pAM999	Contains *ntcA* gene, Amp^R	Ramasubramnium *et al.*, 1994.
pXis::*gfp*	Contains pXis upstream of *gfp*	This study

pMC71A	Contains *nifA* from *K. pneumoniae*, Chl^R	Buchanan-Wallaston *et al*, 1989

6.2.2 Media used:

For routine growth and transformation, *E. coli* was grown at 37°C in LB media at 200 RPM. The medium was supplemented with 100μg/ml ampicillin, 50μg/ml of kanamycin, 30 μg/ml of chloramphenicol and 30μg/ml of tetracycline for maintenance of plasmids.

6.2.2.1 Preparation of BG11 media:

BG11 medium was prepared by using following components:

For 1000ml

Component	Working solution	stock solution
Na_2EDTA	1 ml	0.1g/100ml
Citric Acid	1 ml	0.6g/100ml
K_2HPO_4	1 ml	3.048g/ 100ml
$Na_2CO_3.H_2O$	1 ml	2.0g/ 100ml
Hoogland's reagent	1 ml	1000X stock
Components added separately after autoclaving		
$MgSO_4.7H_2O$	1 ml	7.5g/ 100ml
$CaCl_2.2H_2O$	1 ml	3.6g/ 100ml
$Fe(NH_4)_3$).citrate	1 ml	0.6g/ 100ml

For BG 11^+ medium $NaNO_3$ is added 1.5 gm/1000 ml, which is not added for $BG11^-$ media. For preparation of BG11 agar media, BG11 2X is prepared and mixed with 3% equal volume of agar to make it BG11 agar medium.

Buffer composition: (used in electroporation) :

10mM HEPES

100mM LiCl

50mM $CaCl_2$

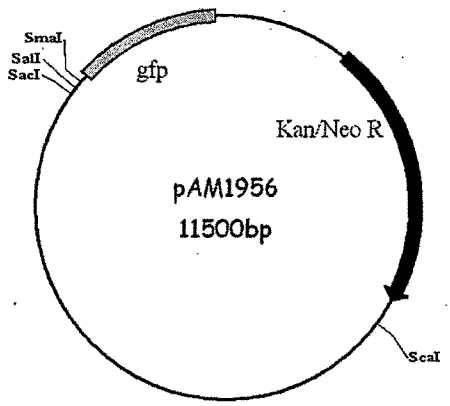
6.2.3:

Anabaena PCC 7120 cultures were maintained by sub culturing regularly on BG11 media. Before sub culturing, old cultures were spun at 6000 rpm/3 mins at room temperature, supernatant was discarded and cells were resuspended in 20 ml of BG11 media. This washing step was repeated thrice and cells were finally resuspended in 400ml of BG11 media and kept on shaker with illumination overnight without shaking. Next day onwards kept on shaking at 115 rpm till further sub culturing.

6.2.4 Plasmid used for the Triparental conjugation:

6.2.4.1 Helper Plasmid: pRL623 has been used as helper plasmid. It contains Chloramphenicol resistance and methylase gene Eco47II M of *Anabaena* PCC 7120, which methylates the Cargo plasmid at GGNCC sequence which is sequence for AvaI, AvaII and AvaIII and prevents its degradation in *Anabaena.*

6.2.4.2 pAM1956 (Cargo plasmid): It is a shuttle vector of *E. coli* and *Anabaena.* It contains Kanamycin/Neomycin resistance gene. It has GFP reporter gene and contains MCS upstream of it. The Size of the vector is 11.5 kb.



6.2.4.3 Conjugal plasmid: pRL443, a variant of pRL4 is used as a conjugal plasmid as it contains *tra* gene for transfer, *mob* gene which codes DNA nicking

protein that specifically recognizes the *bom* site, *OriT* site and mob site. This plasmid helps in carrying the cargo plasmid to the host cells.

6.2.5 Cloning of *xisA* promoter:

pAN207.65 vector was digested with HindIII to release 980 bp fragment containing *xisA* promoter. This fragment was purified from the gel using the Qiagen gel extraction kit as given in the manual. pBSSK plasmid was linearized with HindIII and purified using gel extraction kit. This vector was ligated with *xisA* promoter region and the ligation mixture was transformed in *E. coli* JM101 competent cells. Transformants were selected by Blue-white selection and confirmation of cloning and direction of the cloning was done by restriction digestion with ScaI, SalI and ScaI/Sal I enzymes. The pXis-SK was digested with SacI-SalI enzyme to release the promoter. pAM1956 was linearized by SacI-SalI digestion and ligated with *xisA* promoter to generate pXis::*gfp*. Confirmation of the clones was done by restriction digestion using ScaI, SalI and SacI enzymes.

Figure 6.2 Strategy used for cloning of PxisA

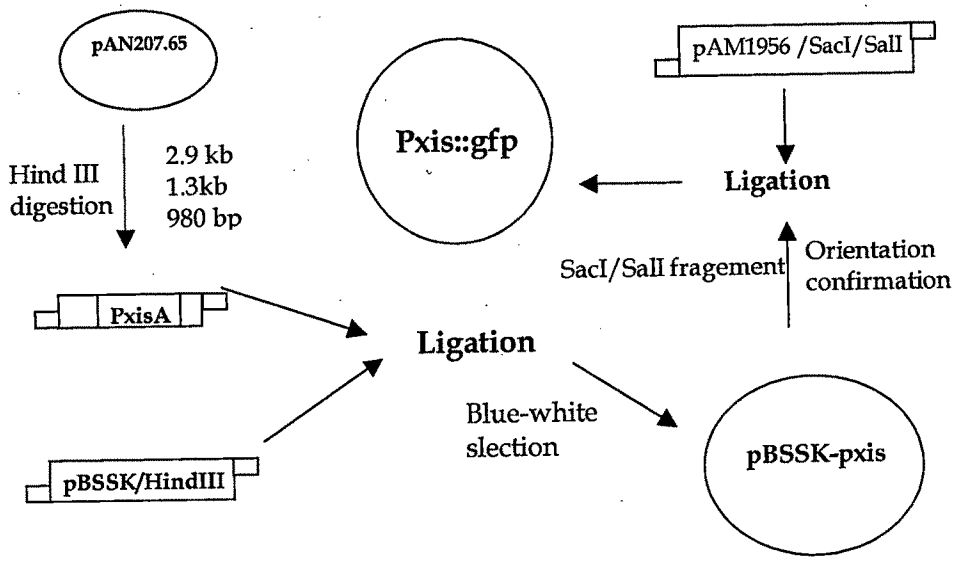
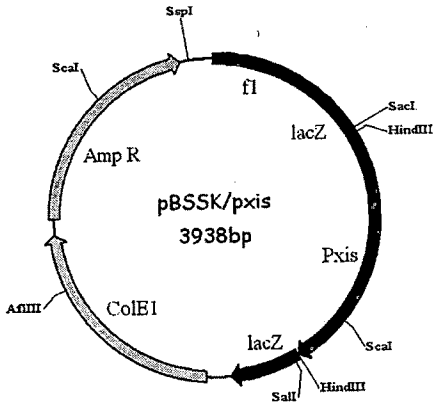
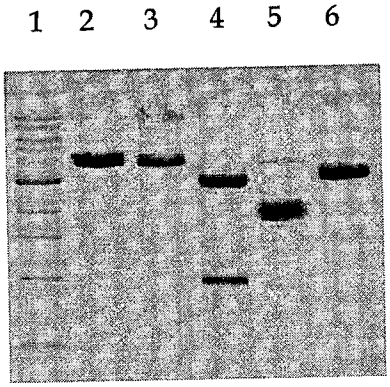


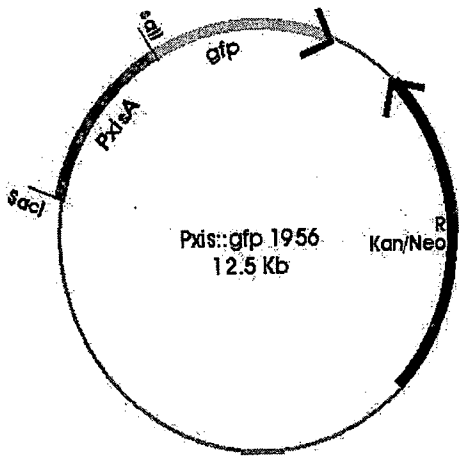
Figure 6.3: Plasmid map and restriction digestion pattern of pBBSk/PXis

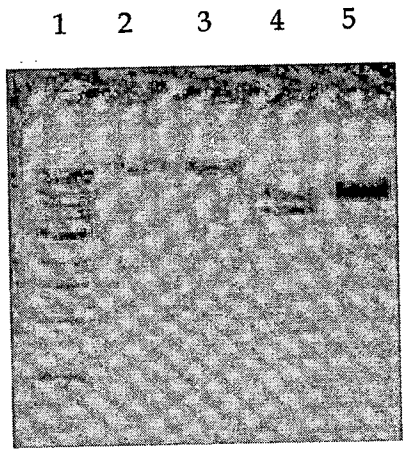




1. 1 kb ladder
P*xisA*-SK digested with
2. SacI 3. Sal I
4. SacI/SalI 5. ScaI
6. pBS-Sk/Sac I digest

Figure 6.4 Plasmid map and restriction digestion pattern of Pxis::*gfp* plasmid





1. 1 Kb ladder
2. PXis::gfp /SacI digest
3. PXis::gfp /Sal I digest
4. PXis::gfp /SacI/Sal I/ ScaI digest
5. PXis::gfp /ScaI digest

6.2.6 Transformation of PXisA::*gfp* in *Anabaena* PCC 7120 by triparental conjugation:

Transformation of PXisA:: *gfp* in *Anabaena* PCC 7120 was tried by three methods

1. Electroporation
2. Natural transformation
3. Tri parental conjugation

6.2.6.1. Electroporation and natural transformation

For electroporation *Anabaena* PCC 7120 was grown for almost 42 h to reach the logarithmic phase which was determined by measuring the chlorophyll a content at every 6 h using spectrophotometric method (ref). After Chl a content was 3μg/ml, 50μg chl a equivalent ~17 ml of *Anabaena* cells were taken for electroporation. Cells were spun at 6000rpm for 3 minutes at room temperature, resuspended in 1.3 ml of buffer and incubated for 1 h at room temperature with intermittent mixing. Conjugal plasmid PXis::*gfp*, pRL443 and pRL 623 were incorporated in *E. coli* HB101 by step wise transformations. Plasmids were isolated from *E. coli* HB101 triple transformant and 1μg of DNA was used for incorporation in to *Anabaena*.

For natural transformation, after addition of 1μg DNA, *Anabaena* cells were incubated for 1 h at room temperature after which 1.3 ml of 2X BG11- medium was added and kept in microtitre plate for 1 day with low light intensity (recovery phase).

For electroporation conditions used:

Gap between electrode - 2mm

Voltage – 12KV

Electric field – 6KV/cm

Time – 5 ms

Buffer - 10mM HEPES, 100mM LiCl, 50mM $CaCl_2$

After electroporation, cells were immediately transferred to microtitre plate and 2X BG11- media was added as in case of natural transformation and rest protocol remained same.

After one day, 1.3 ml from 2.6 ml culture, was spun, resuspended in 200µl of BG11 medium, spread on autoclaved 0.22µ Whatman filter paper on $BG11^+$ agar plate containing neomycin (25µg/ml) and plates were kept on low light intensity for 1 day. While remaining 1.3 ml of cells were directly inoculated in 2.7ml BG11+ media containing neomycin (7.5µg/ml) and kept at room temperature for a week in presence of light after which it was plated on $BG11^+$ agar plate containing neomycin (25µg/ml). Filter papers containing the cells were transferred on every second day to fresh $BG11^+$ agar plate containing neomycin (25µg/ml).

6.2.6.2 Tri parental conjugation:

Biparental conjugation was done by mixing overnight grown *E. coli* HB101 strains containing the pRL443 (amp^R, tet^R) and pRL623 (chl^R), incubated overnight at 37°C. Next day, conjugates were selected on the ampicillin, tetracycline and chloramphenicol antibiotics. Double transformants were confirmed by plasmid isolation followed by restriction digestion pattern analysis.

E. coli HB101 (pRL443/ pRL623) strain was then conjugated with the *E. coli* HB101 (PXis::*gfp*) by bi-parental conjugation, as described earlier, to generate the *E. coli* HB101 (pRL443/ pRL623/ PXis::*gfp*) by selection on ampicillin, tetracycline, kanamycin and chloramphenicol. Presence of all three plasmids was confirmed by restriction digestion analysis.

Anabaena PCC 7120 cells were grown on BG11 media containing nitrogen source till log phase. *E. coli* cells were grown overnight in Luria broth with all required antibiotics. *Anabaena* cells were washed thrice with BG11 media and finally resuspended in 1 ml. Overnight grown *E. coli* cells were washed thrice with Luria broth and finally resuspended in 1ml luria broth. 1ml of *Anabaena* and 1ml of *E. coli* cells were mixed in a glass tube and kept on the illuminatory shaker at 37 °C for 3 h after which cultures were streaked on autoclaved 0.22µ

Whatman filter paper on $BG11^+$ agar plate and incubated with low light intensity for a day. Next day filter paper were transferred to $BG11^+$ agar plate with neomycin (25μg/ml) and incubated in presence of high light intensity. After every 2 days, filters were transferred to fresh $BG11^+$ agar plate containing neomycin (25μg/ml).

After a few sub culturing, *Anabaena* cells from electroporation and natural transformation, in the liquid media as well as on the plates, were dying while control cells grew normally. However, after a few more sub culturing, green tiny colonies started appearing on the filter paper among the dead cells, which were further used for sub culturing.

6.2.6.3 PCR of neomycin gene to confirm the transformation:

To confirm *Anabaena* PCC 7120 transformants, PCR of neomycin gene was done using cells as template with following conditions. Initial denaturation at 94°C for 4 minutes followed by 4 cycles of (94°C for 1 minute, 45°C for 1 minute, 72°C for 1 minute), 30 cycles of (94°C for 30 s, 48°C for 30 s, 72°C for 45s) and final extension at 72°C for 10 minutes. Further confirmation of transformation was done by checking the fluorescence of the *Anabaena* cells.

6.3 Results

After cloning the promoter of XisA upstream of *gfp*, cells were checked for the fluorescence which showed *E. coli* cells showing GFP. In order to study the regulation of XisA promoter in *E. coli*, following cultures were used to find out the effect of physiological conditions and presence of NifA and NtcA.

Table 6.2 Cultures used for analysis:

1. JM101 pAM 1956	2. JM101 Psba::*gfp*
3. DH5α PxisA::*gfp*	4. JM101 PxisA::*gfp*
5. JM101recA$^-$ PxisA::*gfp*	6. JM101 PxisA::*gfp* + pMC71A
7. JM101 PxisA::*gfp* recA-+ pMC71A	8. JM101 PxisA::*gfp* + pAM999 (*ntcA*)

All cultures were grown on Luria broth as well as on M9 minimal medium, samples were withdrawn at log and stationary phase and fluorescence was measured at 480/520 nm. Results are given in the figure 6.5-6.24 and summary is given in the table 6.2-6.5.

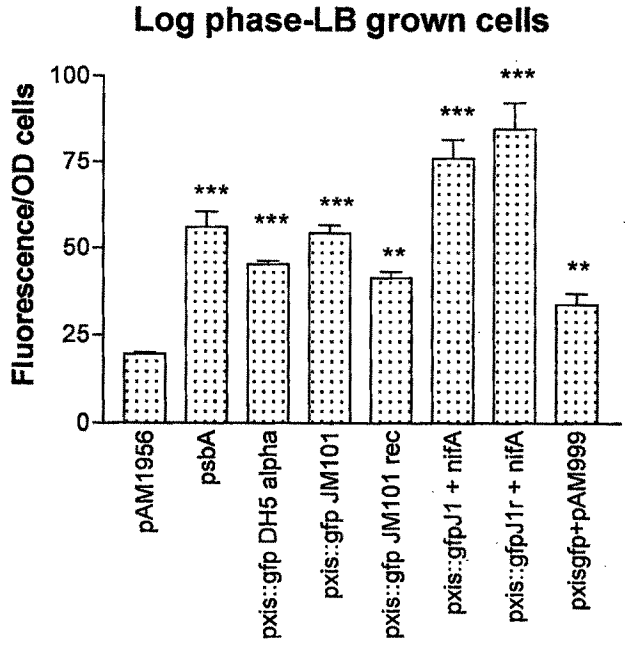


Figure 6.5 Fluorescence shown by various cultures at log phase on LB medium

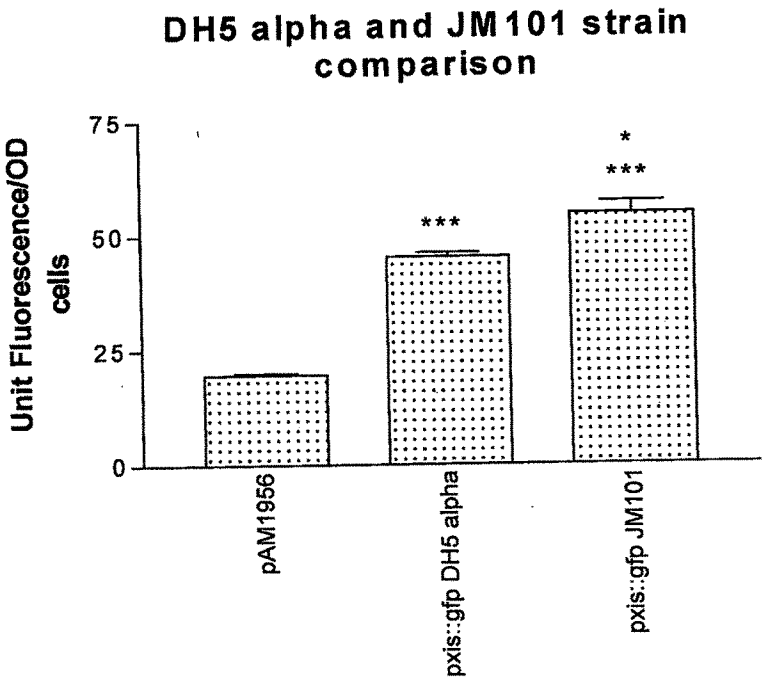


Figure 6.6 Comparative analysis of Log phase, LB grown DH5α and JM101 strain containing Pxis::gfp

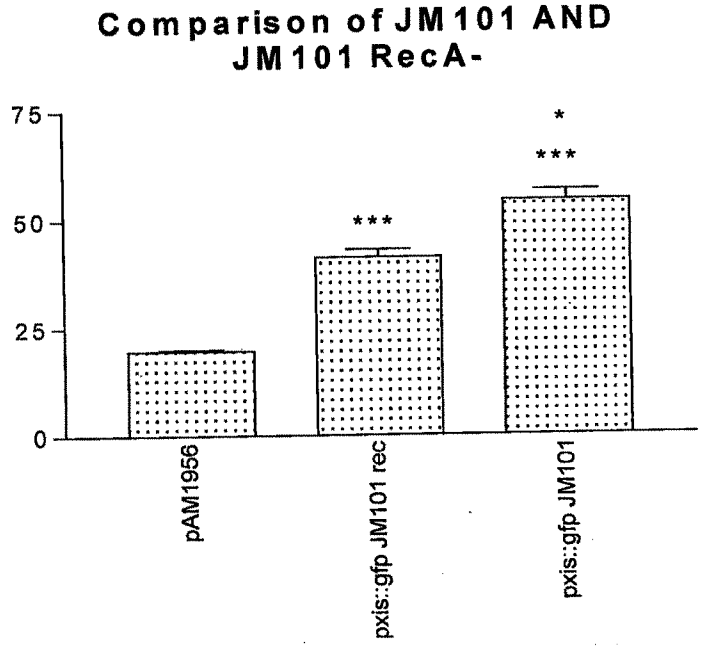


Figure 6.7 Comparative analysis of Log phase, LB grown JM101 and JM101 recA$^-$ strain containing Pxis::*gfp*

(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)

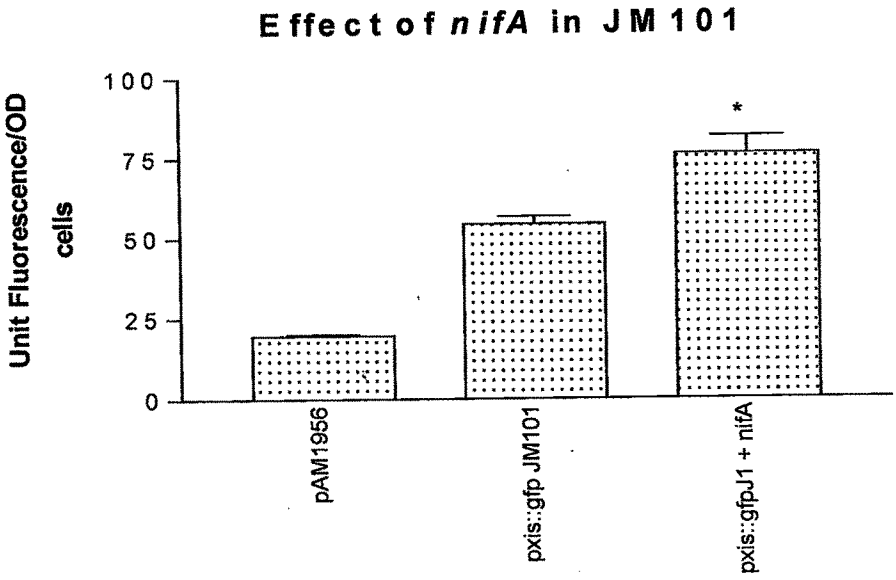


Figure 6.8 Comparative analysis of Log phase, LB grown JM101strain containing Pxis::*gfp* with and without NifA strain

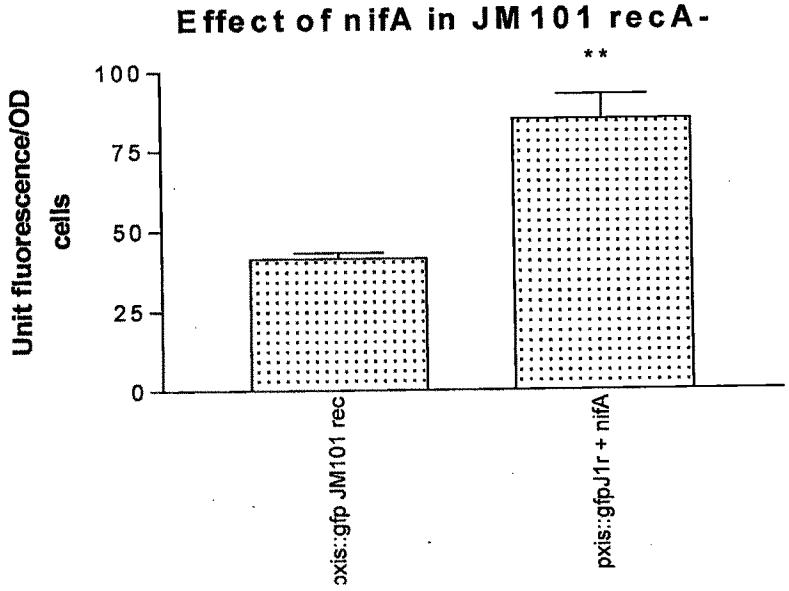


Figure 6.9 Comparative analysis of Log phase, LB grown JM101strain RecA- containing Pxis::*gfp* with and without NifA strain

(* - P< 0.05, ** - P < 0.01 *** - P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)

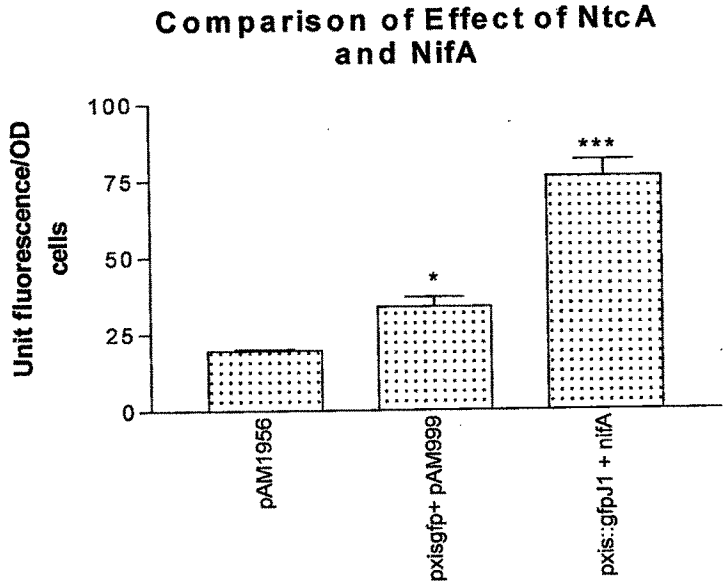


Figure 6.10 Comparative analysis of Log phase, LB grown JM101strain containing Pxis::*gfp* with NifA and NtcA

(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)

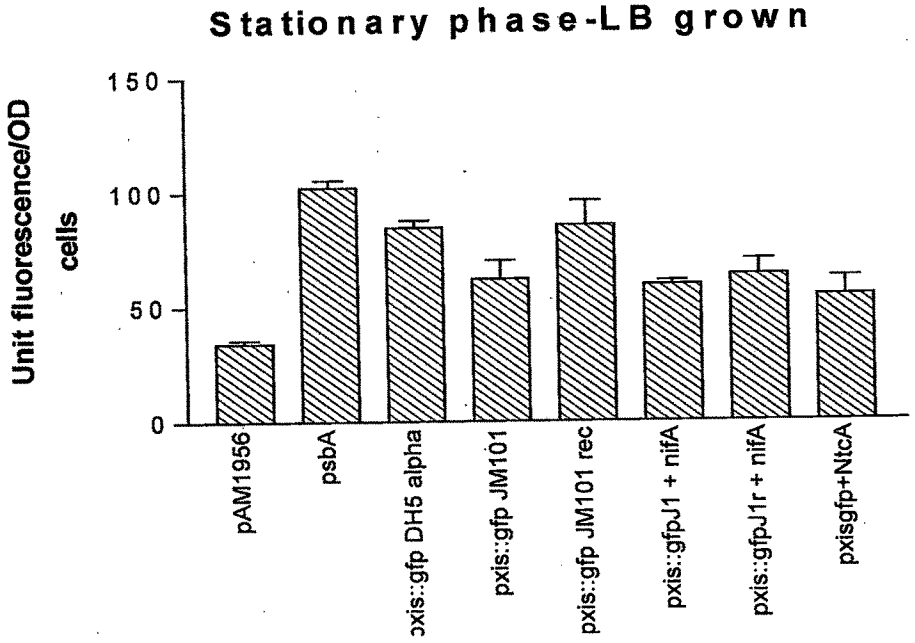


Figure 6.11 Fluorescence shown by various cultures at stationary phase on LB medium

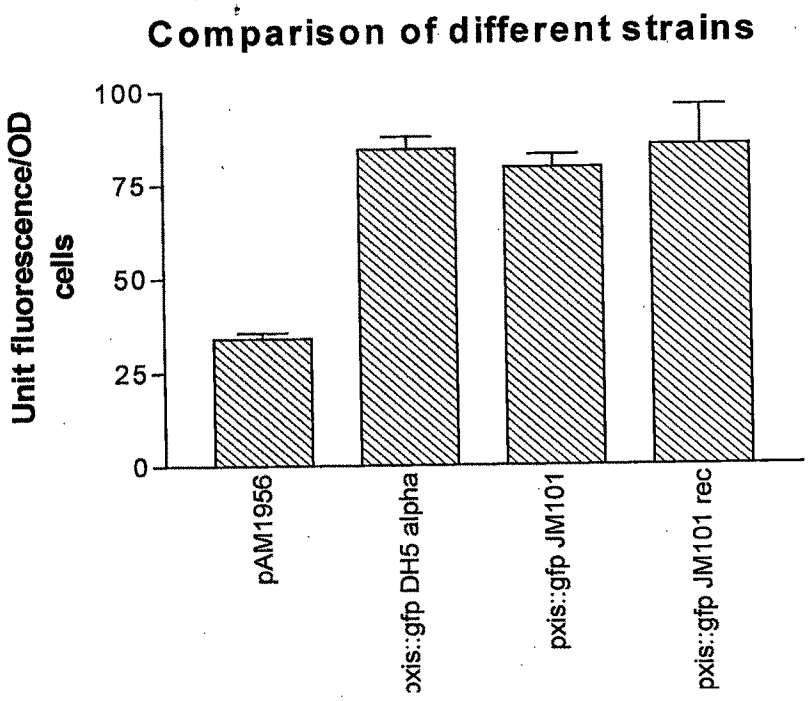


Figure 6.12 Comparative analysis of stationary phase, LB grown DH5α, JM101 and JM101 recA$^-$ containing Pxis::*gfp*

(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)

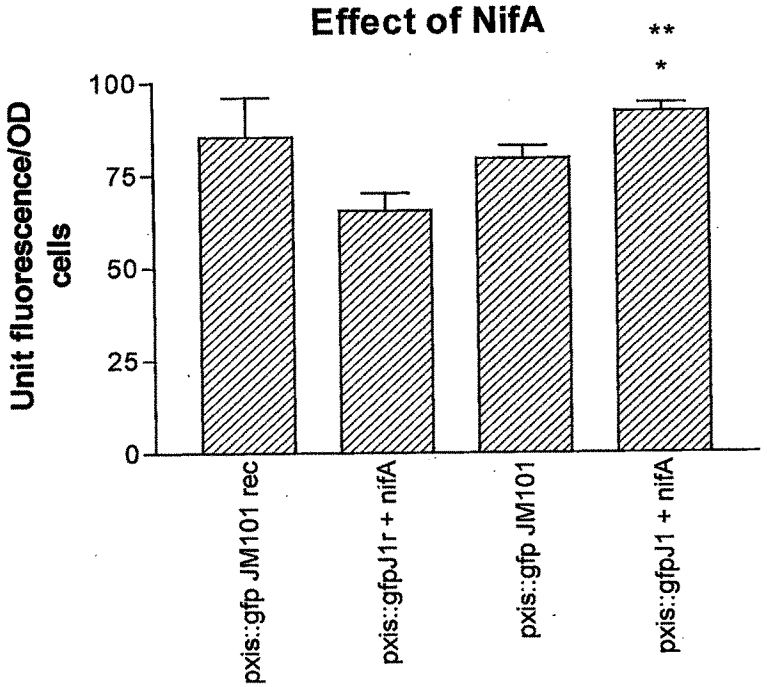


Figure 6.13 Comparative analysis of Stationary, LB grown JM101strain containing Pxis::*gfp* with and without NifA strain

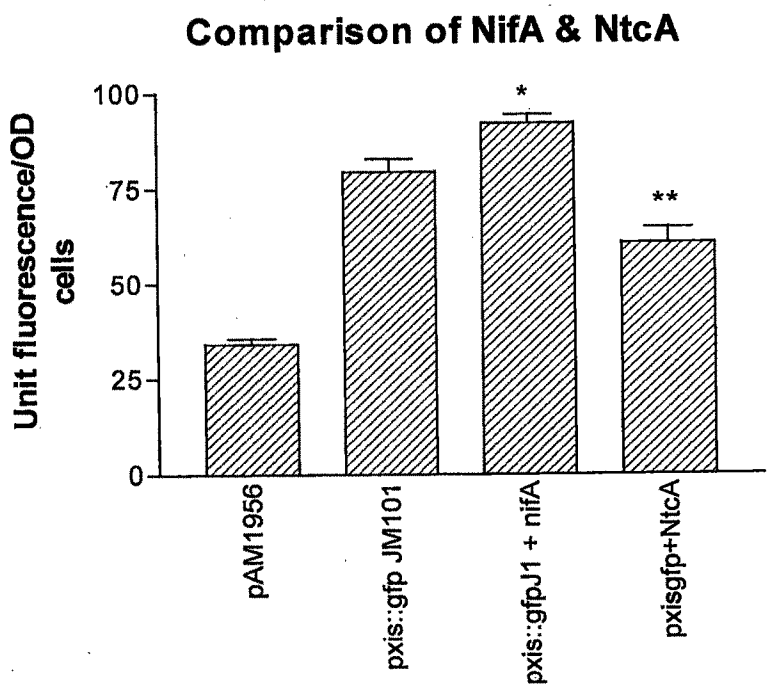


Figure 6.14 Comparative analysis of Stationary, LB grown JM101strain containing Pxis::*gfp* with NifA and NtcA
(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)

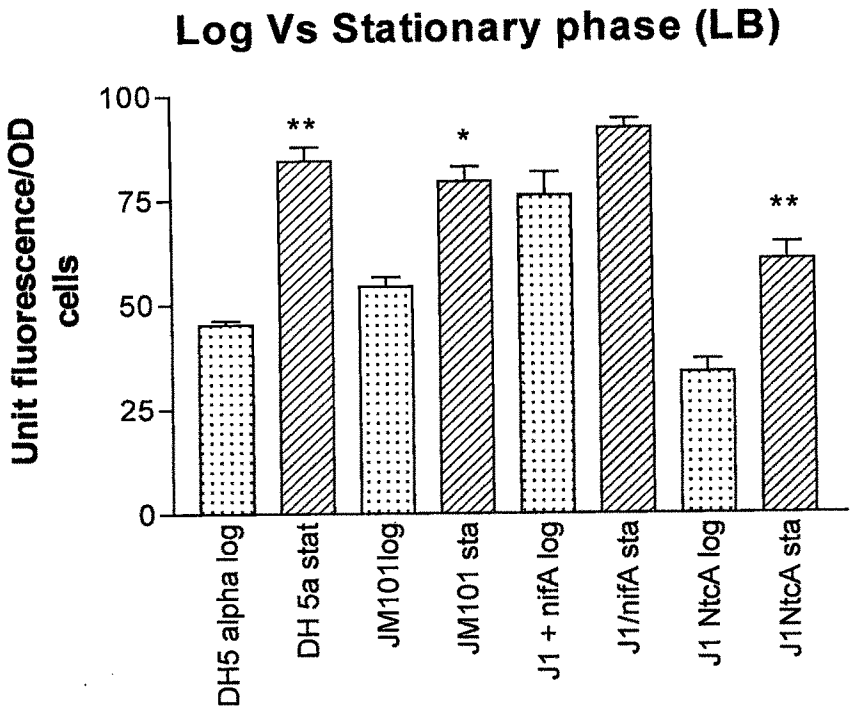


Figure 6.15 Comparative analysis of various cultures at log phase and stationary phase on LB medium.

(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)

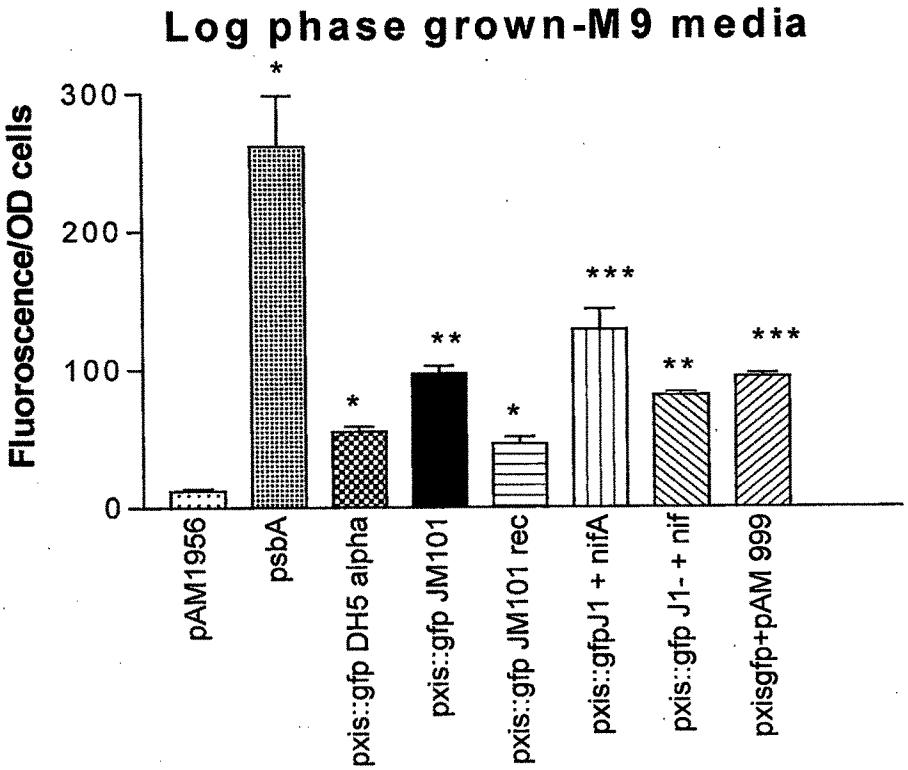


Figure 6.16 Comparative analysis of various cultures grown up to log phase on M9 minimal medium

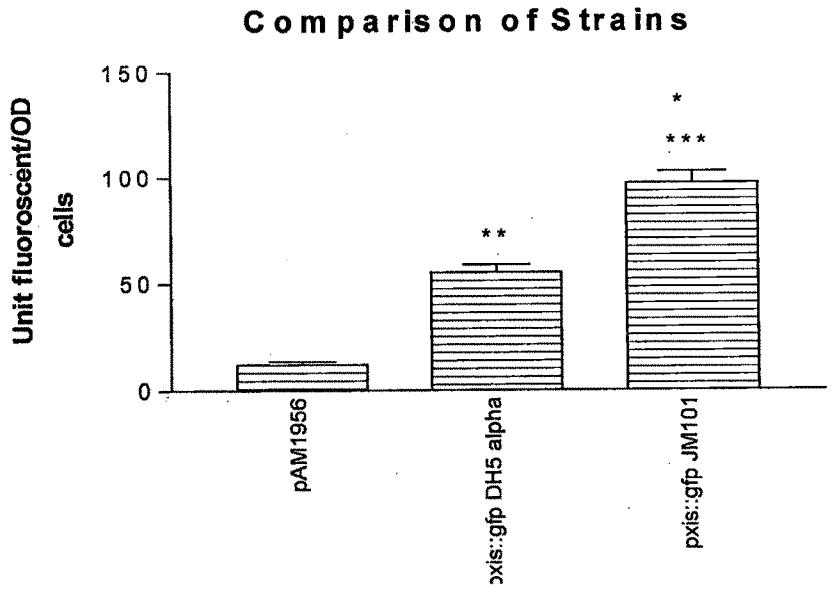


Figure 6.17 Comparison of DH5α Vs JM101 strain containing pXis::*gfp* (* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)

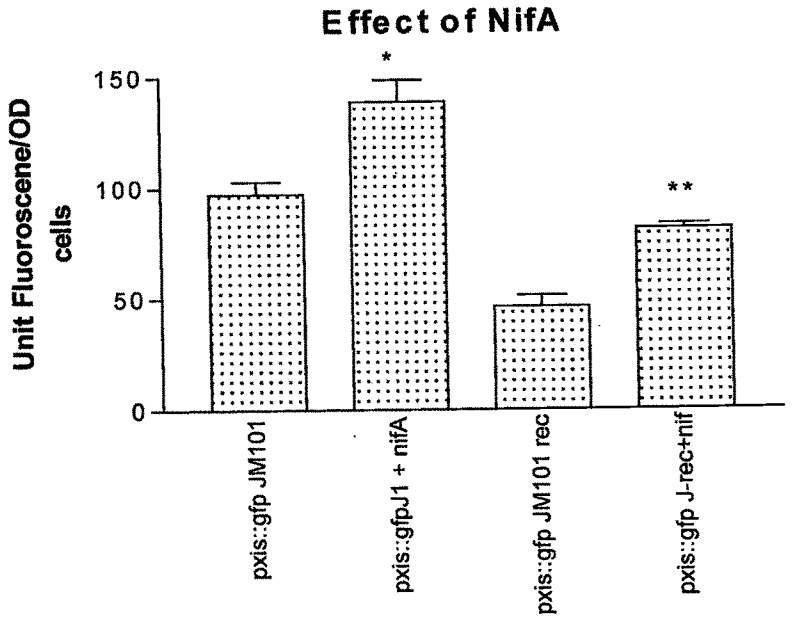


Figure 6.18 Comparison of JM101 and JM101 recA- strain containing pXis::*gfp* with and without NifA.

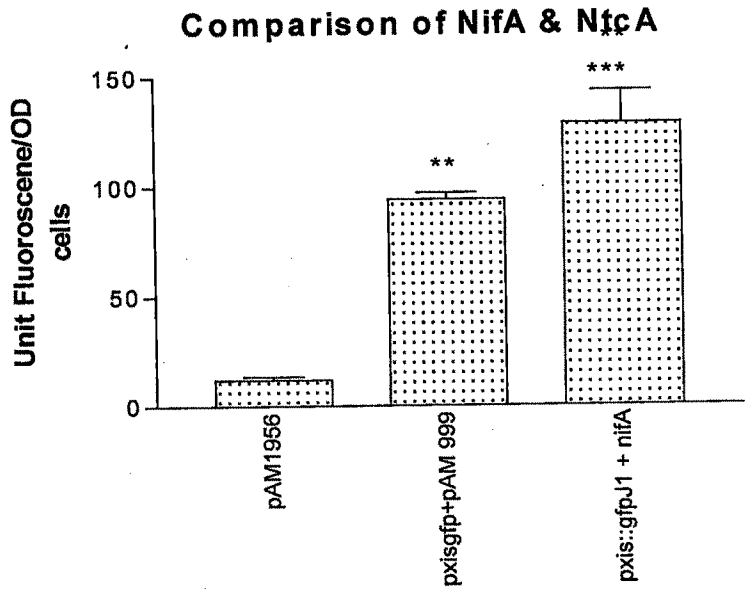
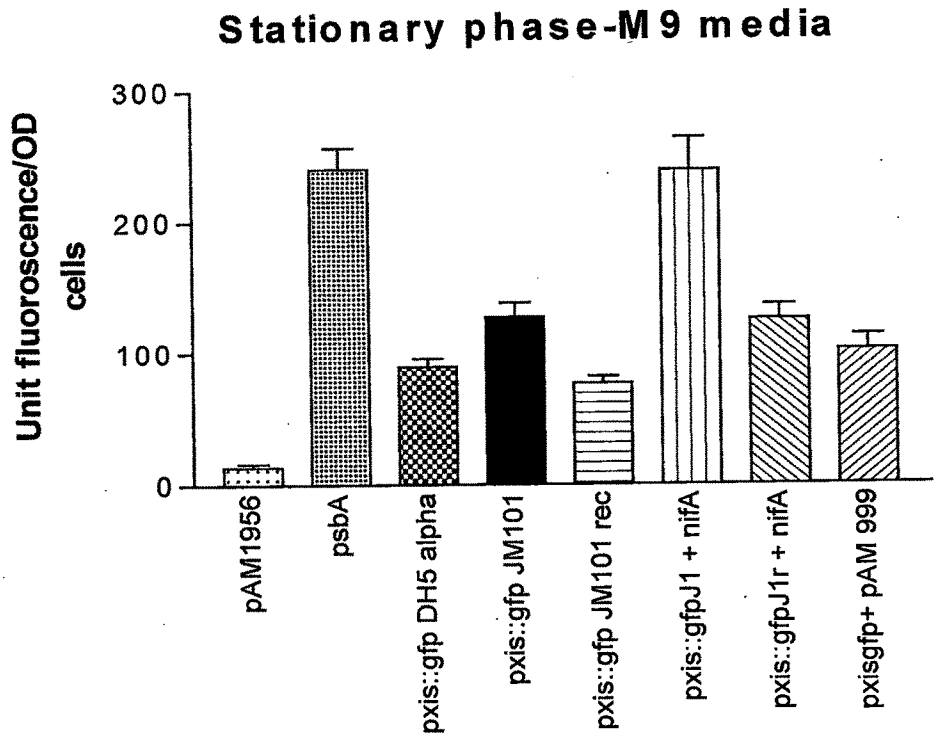


Figure 6.19 Comparison of JM101 strain containing pXis::*gfp* with NtcA and NifA.

(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 independent experiments)



Unit 6.20 fluorescence of various stationary phase grown cultures in M9 minimal medium

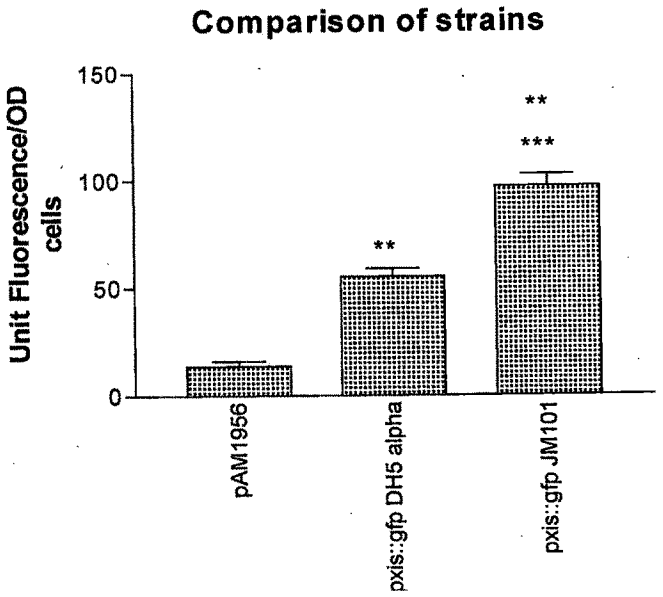


Figure 6.21 Comparison of DH5α and JM101 strain grown up to stationary phase in M9 minimal medium
(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4 expts)

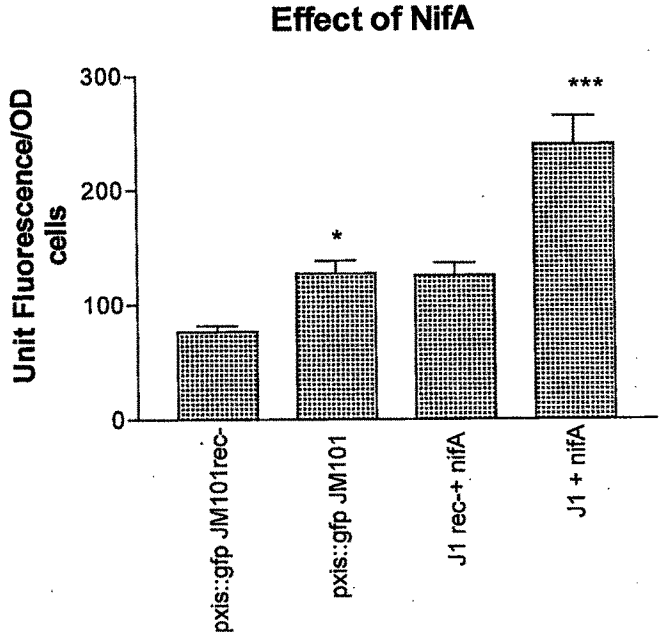
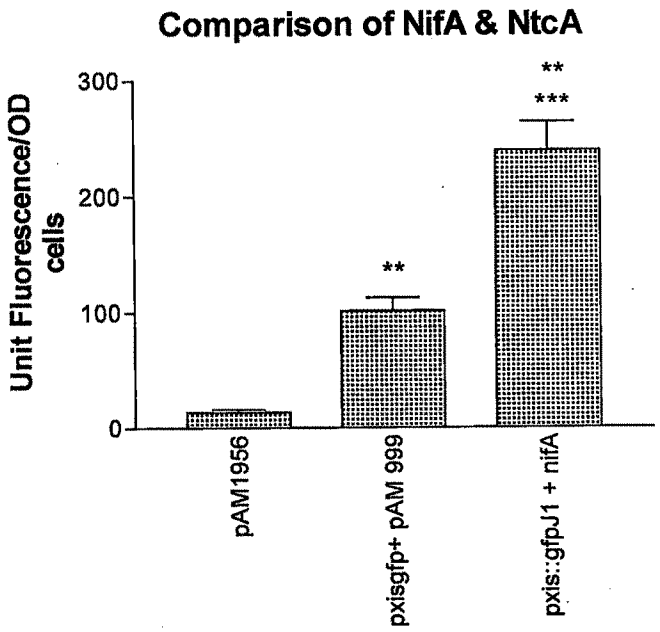


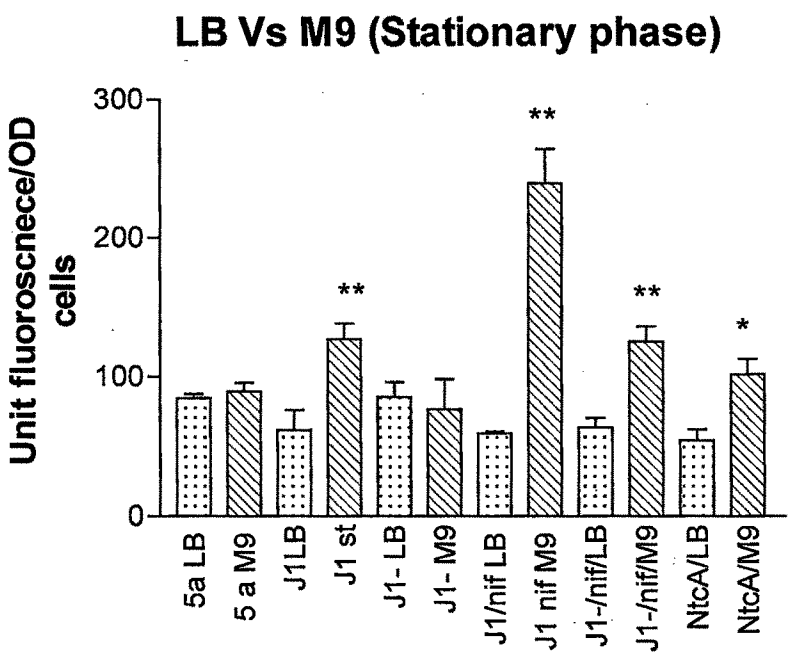
Figure 6.22 Comparative analysis of JM101 strains with and without NifA, at stationary phase on M9 minimal medium



6.23 Comparative analysis of JM101 strain with NifA and NtcA

(* - P< 0.05, ** - P < 0.01 ***- P < 0.001, values expressed as Mean ± S.E.M of 4

independent experiments)



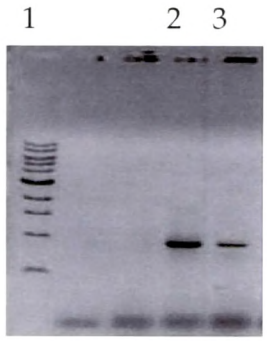
6.24 Comparison of GFP Unit of Stationary phase grown cultures in Luria broth and M9 minimal medium.

Table 6.3 Summary of the XisA promoter studies using GFP as reporter under different conditions

Culture	LB-Log phase	M9 log phase	LB-stationary phase	M9 stationary phase
JM101 pAM1956	20 ± 1	34 ± 2	12 ± 2	14 ± 2.5
JM101 Ppsba::*gfp*	56 ± 5	102 ± 4	262 ± 36	240 ± 16
DH5 α Pxis::*gfp*	45 ± 2	84 ± 4	55 ± 4	89 ± 6
JM101 Pxis::*gfp*	54 ± 3	79 ± 4	97 ± 6	127 ± 11
JM101recA$^-$ Pxis::*gfp*	41 ± 3	85 ± 11	46 ± 5	77 ± 5
JM101 Pxis::*gfp* + pMC71A	76 ± 6	92 ± 4	129 ± 15	239 ± 25
JM101 recA$^-$ Pxis::*gfp* + pMC71A	84 ± 8	65 ± 3	81 ± 2	125 ± 11
JM101 Pxis::*gfp* + pAM999	33 ± 3	61 ± 5	94 ± 3	102 ± 11

6.3.2 Transformation of Pxis::*gfp* in *Anabaena* PCC 7120

Out of all methods employed, natural transformants and tri-parental conjugation gave few isolated colonies on the filter paper after around 3-4 weeks. These colonies were then subcultured on different plates. Cells were taken from the colonies and used to amplify the neomycin resistance gene for the confirmation of successful transformation and fluorescence was monitored from the filaments. Results are given in the figures 6.25.



1. 1 kb ladder
2 & 3 PCR amplification of *nptII* gene

6.25 Colony PCR of *nptII* from *Anabaena* PCC 7120 transformants

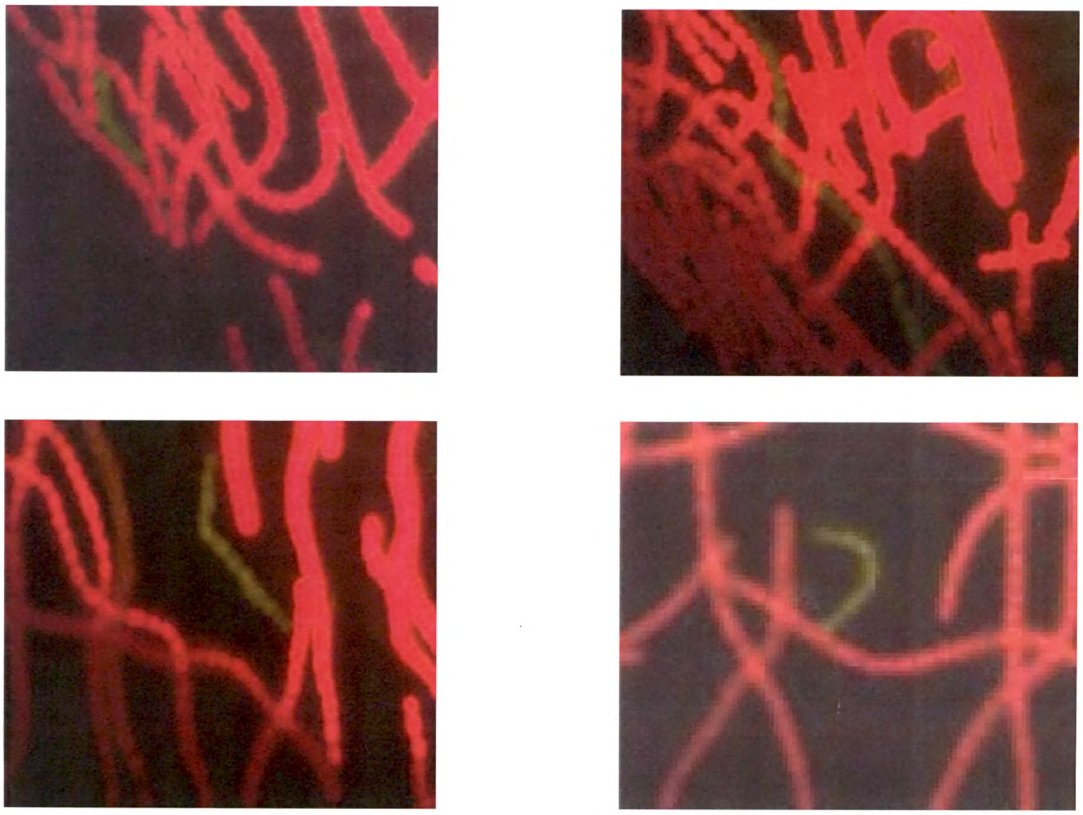


Figure 6.26 *Anabaena* PCC 7120 filaments showing GFP after transformation Pxis::*gfp*

6.4 Discussion

The present study was centered on the regulation of *xisA* expression using GFP reporter gene. Promoter of *xisA* was cloned upstream of *gfp* and fluorescence was monitored under different physiological conditions in *E. coli.*

Under all conditions, fluorescence shown by culture were significantly higher than the control i.e. *gfp* without promoter. However, the extent of increase was different. DH5α and JM101 *recA*$^-$ strain showed around 1.5 fold increase while the JM101 strain (recA$^+$) showed ~2.5 fold increase in the fluorescence. This suggests that RecA increases the expression of *xisA*.

Nature of the medium and growth status also influenced the fluorescence of GFP. Log phase grown cells in Luria broth showed increase in fluorescence similar to that of stationary phase grown cells, scenario was different in M9 grown cells. M9 grown cells showed higher expression of *gfp* as compared to LB grown cells and stationary phase grown cells on M9 showing higher expression than log phase grown cells.

Presence of the transcriptional activators NifA and NtcA increased the *gfp* expression, higher in case of M9 stationary phase as compared to M9 Log phase and LB grown cells. Presence of NifA showed ~10 fold increase in the *gfp* expression in M9 stationary phase. Presence of NtcA though, did not show much increases (~4 fold) in stationary phase as compared to log phase (~2 fold), while such increase was not found in LB medium.

Present data shows similar pattern as reported earlier but do not match the rearrangement frequency obtained under such conditions (Karunakaran, 2000). While the increase in *xisA* expression is several folds higher, the rearrangement frequency increases from 10 % to 70%. This suggests that XisA alone is not sufficient for the excision of *nifD* element but requires accessory

factors. This view has been supported by earlier results that just 11bp target site is not sufficient for the recombination, but additional sequences are required, other than nearby 8 bp repeat, to which such accessory factors bind (Chapter 1). The accessory factor(s) seem to be media/growth phase dependent. Role of such factors could be structural or involved in the protein-protein interaction to stabilize the complex or may be helping in DNA looping. NifA of *K. pneumoniae* has DNA bending activity. Thus, NifA may be binding at the 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. Since the NifA/NtcA binding sites are similar, it could be possible that NtcA may also be involved in the DNA bending.

Pxis::*gfp* transcription fusion has been successfully transformed in *Anabaena* PCC 7120, which can be used to study the expression of *xisA* under different physiological conditions.