Chapter 4

Role of *Anabaena* PCC 7120 NtcA on the excision of *nifD* element in *E. coli*

4.1 Introduction

The *ntcA* gene encodes a Nitrogen control transcriptional regulator in cyanobacteria. In the N2-fixing, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, *ntcA* is an autoregulatory gene that is transcribed from a complex promoter region that includes a constitutive promoter (P(2)) and promoters that are induced upon N step-down (P(1) and P(3)) (Olmedo-Verd *et al.*, 2006). *ntcA* not only regulates the expression of genes involved in nitrogen and carbon metabolism but also coordinates iron acquisition and nitrogen metabolism by activating the expression of pkn41 and pkn42 (Cheng *et al.*,2006). NrrA protein, a nitrogen-responsive response regulator of OmpR family encoding gene *nrrA* is depended on the NtcA (Ehira and Ohmori, 2006). A characteristic fetures of NtcA activated promoter was found in upstream of the gene *all4312* encoding protein All4312 which has domains similar to two components response regulators (Muro-Pastor *et al.*, 2006). Overexpression of *ntcA* in a *hetR* background promoted expression of devBCA in response to ammonium withdrawal and excision of the 11-kb *nif D* element even in the presence of combined nitrogen. Some NtcA-dependent heterocyst-related genes can be expressed independently of HetR. Heterocysts were produced in response to nitrogen step-down, the NtcA overproducing strain could not grow diazotrophically (Olmedo-Verd *et al.*, 2005).

DNA binding property of NtcA *in vitro* depends upon the redox status in both *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 but independent of cysteine residues in *Anabaena* PCC 7120 (Jiang *et al.*, 1997; Alfonso *et al.*, 2001; Wisen *et al.*, 2004). High 2-oxoglutarate levels enhance NtcA binding in *Synechococcus* PCC 7942 (Vazquez-Bermudez *et al.*, 2002b; Tanigawa *et al.*, 2002). Although NtcA activates the expression of genes

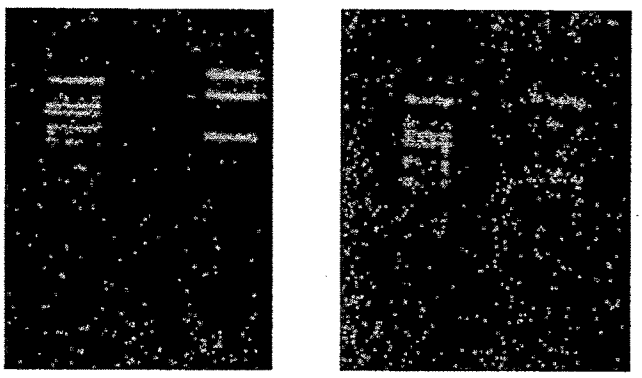
associated with nitrate/nitrite metabolism and heterocyst differentiation (Flores & Herrero, 2005), its role in the expression of *xisA* gene is not very clear. Overexpression of NtcA in *Anabaena* PCC 7120 results in normal heterocysts formation and DNA rearrangement but defective in nitrogen fixation (Olmedo-Verd *et al.*, 2005).

In heterocystous cyanobacteria Anabaena PCC 7120, the transcription factor NtcA is required for activation and repression of extensive number of genes involved in nitrogen metabolism, heterocyst differentiation and nitrogen fixation (Muro-Pastor *et al.*, 2001b). An *ntcA* mutant of *Anabaena* PCC 7120 cannot grow on nitrate and is devoid of heterocysts (Frias *et al.*, 1994, Wei *et al*, 1994). NtcA binds to TGT(N_9or$_{10}$)ACA which is identical to the binding sites of *nifA,* a transcriptional activator of nitrogen fixing (*nif*) genes in many diazotrophs. NtcA interacts with *xisA*, *glnA*, *rbcL* and *nifH* promoter fragments *in vitro* (Ramasubramanian *et al.*, 1994). Excisase A, encoded by *xisA* gene, is a site-specific recombinase involved in the excision of an 11,278 bp DNA element from *nifD* gene during heterocyst development (Golden *et al.*, 1985; Chastain *et al.*, 1990). NtcA binds to three sites in the upstream region of *xisA* gene. Earlier Lammers *et al.*, (1986) demonstrated the rearrangement of *nifD* element in *E. coli* occurring at low frequency (~ 0.3%). Our studies showed the increase in the rearrangement frequency up to 50% and 70% in *E. coli* DH5α and JM101 strains, respectively, in minimal medium in the presence of *nifA* gene of *Klebsiella pneumoniae* (Karunakaran, 2000). We report here that *ntcA* increases the *nifD* rearrangements in minimal medium but not in Luria broth. P*xisA-lacZ* fusions showed the increase in the expression of *xisA* in the presence of *ntcA* gene in Luria broth and minimal medium.

4.2 Materials and Methods

Please refer chapter titled Material and Methods

4.3 Results and Discussion



(a) (b)

Fig. 1 Restriction digestion pattern of pMX25 (a) and pMX32 (b) plasmids by EcoR1.

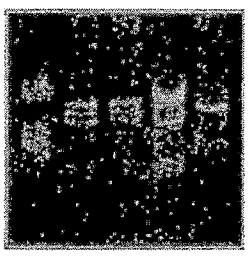


Fig. 2a Restriction digestion pattern of *E. coli* JM101 containing pMX25 and pAM1323 plasmids by EcoR1. Lane 1 Molecular weight marker λ DNA cut with EcoR1, Lane 2 pMX25 + pAM1323 cut with EcoR1, Lane 3 pMX25 + pAM1323 cut with EcoR1, Lane 4 pAM1323 cut with EcoR1.

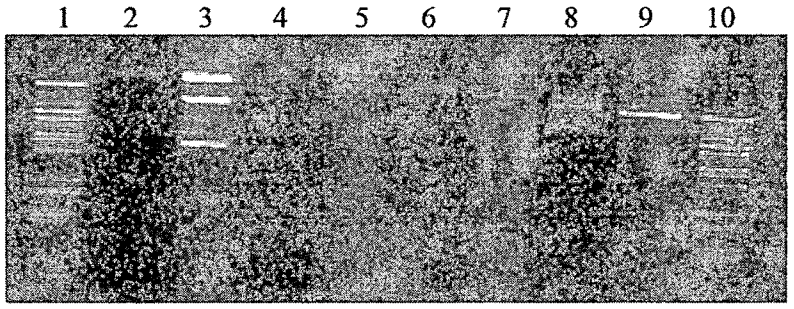


Fig. 2b Restriction digestion pattern of *E. coli* JM101 containing pMX25 and pAM1323 plasmids. Lane 1- λBst EII Marker; Lane2- pMX25, undigested; Lane3- pMX25, EcoRI digested; Lane4- pMX25, pAM1323, undigested, Sample-1; Lane5- pMX25, pAM1323, EcoRI digested, Sample-1; Lane6- pMX25, pAM1323, undigested, Sample-2; Lane7- pMX25, pAM1323, EcoRI digested, Sample-2; Lane8- pAM1323, undigested; Lane9- pAM1323, EcoRI digested; Lane10- λBst EII Marker.

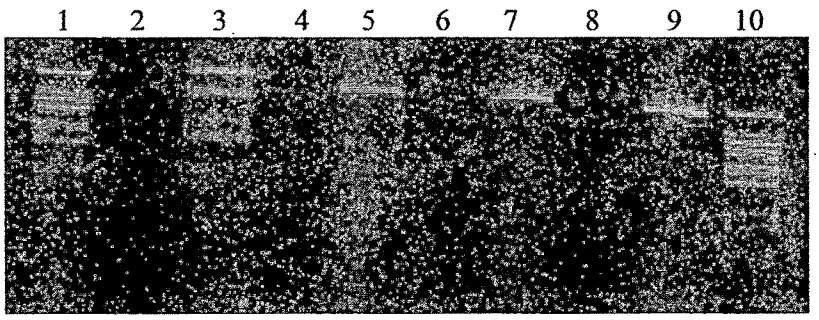
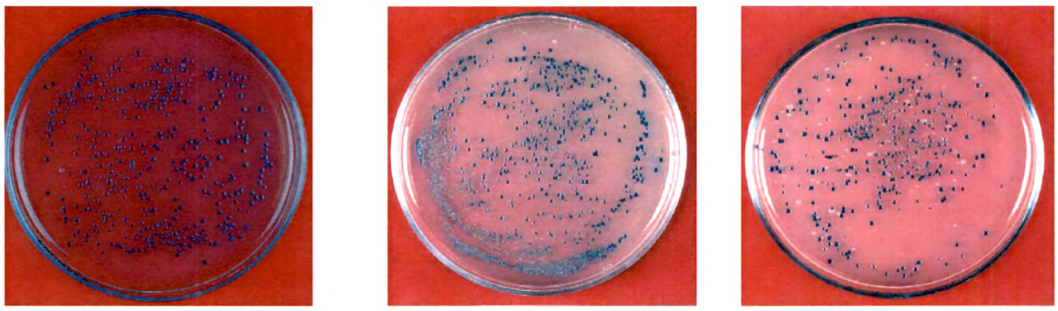


Fig. 2c Restriction digestion pattern of *E. coli* JM101 containing pMX32 and pAM1323 plasmids. Lane 1- λBst EII Marker; Lane2- pMX32, undigested; Lane3- pMX32, EcoRI digested; Lane4- pMX32, pAM1323,undigested, Sample-1; Lane5- pMX32, pAM1323, EcoRI digested, Sample-1; Lane6- pMX32, pAM1323, undigested, Sample-6; Lane7- pMX32, pAM1323, EcoRI digested, Sample-6; Lane8- pAM1323, undigested; Lane9- pAM1323, EcoRI digested; Lane10- λBst EII Marker.



a **0 h** **b** **24 h LB** **c** **24 h M9**

Fig. 3 Excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* JM101 containing pMX25 and pAM1323 plasmids.

Table 1 Excision of *nifD* Element of *Anabaena* Strain PCC7120 in *E. coli* JM101 in the presence of *ntcA* gene in Luria broth and Minimal medium during stationary phase.

Condition/Time	No. Of Blue Colonies	No. Of White Colonies	Frequency of Rearrangement
0 h	400	5	1.0±0.01
24 h (LB)	201	2	0.98±0.49
24 h M9 minimal media	1107	435	29.75±6.17

Fig. 4 Restriction digestion pattern of pKK1 and pAM1323 plasmids by EcoR1.

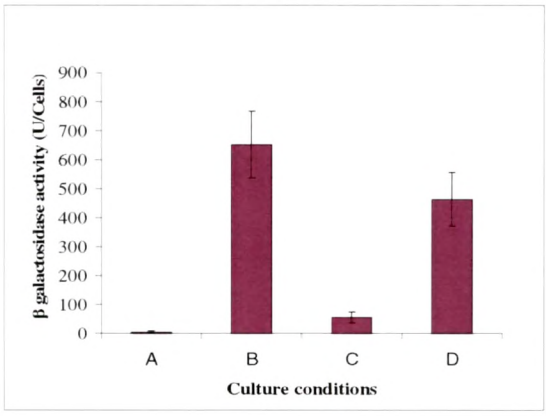
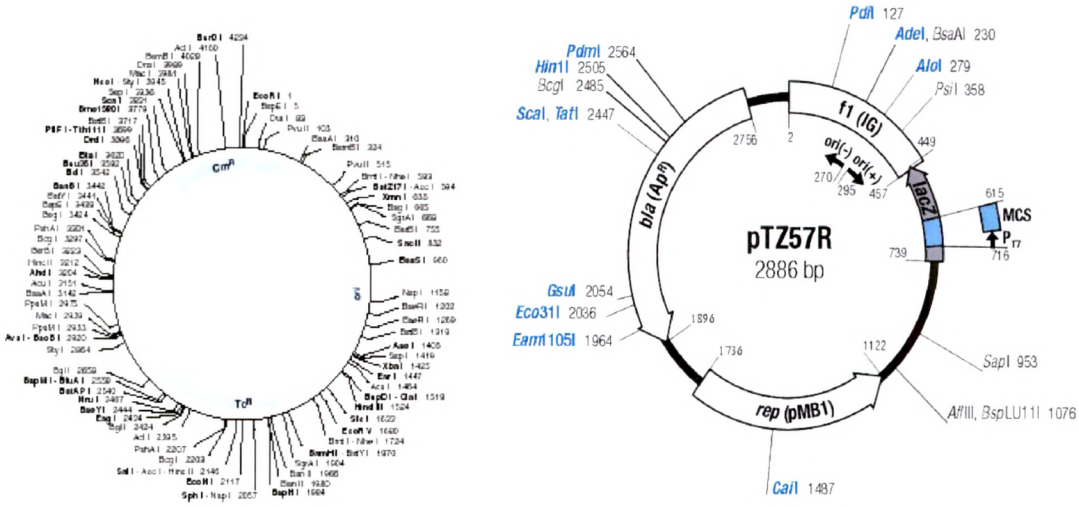


Fig. 5 β-galactosidase activity of P*xisA*-l*acZ* reporter fusion in *E. coli* JM101 containing *ntcA* gene in the stationary phase of Luria broth and M9 minimal medium. A. JM101 with pMU575 and pAM1323 plasmids in LB during stationary phase, B. JM101 with pKK1 and pAM1323 plasmids in LB during stationary phase, C. JM101 with pMU575 and pAM1323 plasmids in M9 minimal medium during stationary phase, D. JM101 with pKK1 and pAM1323 plasmids in M9 minimal medium during stationary phase.

Fig. 6 Isolation of genomic DNA of *Anabaena* sp. strain PCC 7120.

Fig. 7 Amplification of *ntcA* gene of *Anabaena* sp. Strain PCC 7120.



(A) (B)

Fig. 8 Map of plasmid (A) pACYC184 and (B) pTZ57R.

4.3.1 Effect of NtcA on the excision of *nifD* element

In order to determine the role of NtcA protein, the excision of pMX25 and pMX32 were monitored in the presence of the plasmid pAM1323 in *E. coli* JM101. Restriction digestion pattern of plasmid pMX25 and pMX32 by EcoR1 (**Fig. 1(a), Fig 1(b) and Fig. 2a, 2b and 2c**). The pAM1323 contains the *ntcA* gene of *Anabaena* sp. strain PCC 7120. Results of the rearrangement of substrate plasmid is shown in Luria agar containing Ampicillin and Spectinomycin (**Fig. 3**) In presence of NtcA rearrangement of *E. coli* JM101 harboring pMX25 was monitored. Excision frequency of pMX25 was found around 1% in Luria broth after 24 h, which is otherwise equal to the rearrangement frequency of pMX25 alone where XisA plays an important role in the rearrangement (**Table 1**). In M9 minimal medium rearrangement frequency was increase up to 30% which is almost 30 fold higher in compare to the M9 minimal medium where NtcA is absent. In both, Luria broth and M9 minimal medium growth of *coli* was very poor. Here, NtcA of *Anabaena* sp strain PCC 7120 shows different effect based on the different media.

Nif A showed excision frequency of *nifD* element at 70% in M9 minimal medium where nitrogen source is NH_4Cl. Though binding site is same for NifA and NtcA but its effect is different on *xisA* gene. In case of Luria broth both are deprived as accessory protein to increase in rearrangement frequency along with RecA of host. NifA has better effect on the rearrangement in Luria broth in compare to NtcA since NifA could increase rearrangement form 1% to 5% at the same time NtcA has no effect on the excision frequency in Luria broth. Both NifA and NtcA are weak in Luria broth to increase the rearrangement of *nifD* element in rich media where nitrogen source is not defined. In M9 minimal medium where nitrogen source is defined as NH_4Cl, rearrangement frequency is increasing drastically from 13 % to above 70% in case of NifA where it is expressed under strong promoter like *Ptet*, whereas in case of NtcA excision frequency was increased form 13% to 30%. In both the case in stationary phase growth excision frequency is increasing which suggest that rearrangement is also driven by some other factor other than these two. Earlier reported that RecA also plays a role but promoter activity of *xisA* gene was remain almost same in the

stationary phase of growth in Luria broth as well as in M9 minimal medium. NtcA, a protein of CRP member can act as a positive and negative regulator. In our case of *xisA* gene NtcA is able to bind at three site and able to form a complex at three position (Chastain *et al.,* 1990), which suggest that different complex have different activity, here in case of Luria broth the complex which is forming may be increase the XisA expression compare to M9 minimal medium but at the same time also because of high level of it may be covering the target site of the *xisA* so there is only 1% rearrangement. There are three binding site present in upstream of *xisA* gene. NtcA binds to coding and noncoding region of *xisA* gene; size is 143 bp which include 63bp from coding and 66bp form noncoding region of *xisA* (Chastain *et al.,* 1990). This binding site is 30 bp away forms the recombination site of the 11 kb nifD element. NtcA binding activity is present in cells grown under nitrogen replete and nitrogen depletes conditions (Ramasubramanian *et al.,* 1994). NtcA like other CRP is an archetypal DNA-bending protein (Gaston *et al.,* 1992). Bending of DNA is necessary for the expression some time, here also the same role NtcA is providing to the *xisA* gene expression. In case of M9 minimal medium it may be very easy to do this activity since may be three dimensional conformation of *nifD* element is important in the excision of it. This is otherwise also easy in minimal medium in compare to Luria broth.

Effect of NtcA binding on the expression of the XisA

It is known that NtcA shows its effect by binding in the upstream of the *xisA* gene. To understand its role of NtcA in the excision of *nifD* element of *Anabaena* sp strain PCC 7120 in *E. coli* promoter activity of *xisA* gene was monitored by β-galactosidase assay using plasmid pKK1. pKK1 plasmid has *Pxis::lacZ* transcriptional fusion where *lacZ* structural gene is expressed under promoter of *xisA* gene. In presence of plasmid pKK1 and control plasmid pMU575, β-galactosidase was measured in *E. coli* strain JM101. Restriction digestion pattern of the plasmid pAM1323 and pKK1 by EcoRI is shown in **Fig. 4**. 650 U of β-gal was found in the E. coli strain JM101 grown on Luria broth in stationary phase, while in stationary phase of M9 minimal medium it was reported only 460 U. So β-gal activity of *PxisA* found low in M9 minimal medium which is around 200 U less in comparison (**Fig. 5**).

Promoter activity of *xisA* gene is indirect measure of the excision frequency, in many cases where activity is high then also rearrangement is low suggests that only amount of XisA protein is not important in the excision of *nifD* element but other accessory proteins other then NtcA and RecA are also involved in the rearrangement. Promoter activity *xisA* gene is almost three fold less in Luria broth and half in M9 minimal medium during stationary phase of growth in *E. coli* strain JM101 harboring both plasmid pKK1 and pAM1323 in compare to *E. coli* strain JM101 harboring only pKK1 alone, which suggests activity of the *xisA* gene is not directly proportional to the excision frequency. Promote activity of *Pxis* in presence of plasmid pAM1323 during stationary phase is comparable to the logarithmic phase growth of the *E. coli* in presence of pKK1 alone. In the log phase of growth the rearrangement frequency is very low in JM101 harboring substrate plasmid pMX25.

4.3.3 Cloning of *ntcA* in pACYC184

To clone PCR product directly in the plasmid vector is very difficult. So Plasmid pTZ57R, TA cloning vector form MBI Fermentas was used and then through subcloning form plasmid pTZ57R. It was cloned in the plasmid pACYC184 at BamHI and SalI Restriction site. But unfortunately no colony gave recombinant plasmid.