



Summary

Introduction

Anabaena PCC 7120, filamentous heterocystous cyanobacterium, has distinct and developmentally regulated nitrogen fixation. In this organism, there are three genetic rearrangements occur in *nifD*, *fdxN* and *hupL* genes and fragments involved are *nifD* element, *fdxN* element and *hupL* element and size are of 11,278 bp, 59,428 bp and 9,419 bp, respectively. These elements should get excised for the expression of these genes. *xisA* encodes a site specific recombinase which is involved in the *nifD* element excision from chromosome under nitrogen limitation condition.

nifD element rearrangement, reported in *E. coli* and suggested the requirement for XisA and some *E. coli* proteins, the latter providing the same function as in *Anabaena* PCC7120. An *in vitro* rearrangement system may be needed to examine this possibility. Earlier results in our laboratory demonstrated that excision of *nifD* element could be enhanced under different conditions involving XisA protein, this conditions are: (i) Minimal medium in stationary phase, and (ii) presence of NifA of *Klebsiella pneumoniae* which is dependent on NtrA, σ factor. Details of the mechanism of excision and requirement of any accessory proteins are still not known.

NtcA is a DNA binding protein whose predicted amino acid sequence shows similarity to the family of prokaryotic regulatory proteins represented by the cyclic AMP receptor protein (CRP). An *ntcA* mutant of *Anabaena* sp. strain PCC 7120 requires ammonium for growth and is defective for heterocyst formation, indicating that NtcA is required for the utilization of nitrate and dinitrogen. NtcA binds at three sites in the upstream region of *xisA* gene. NtcA of *Anabaena* PCC7120 has similar and overlapping DNA binding sites to that of NifA of

Klebsiella pneumoniae. Monitoring the effect of NtcA in the excision of *nifD* element could provide an insight in to the rearrangement phenomena occurring in *Anabaena* PCC 7120.

Excisase A has carboxyl terminal domain similar to the **Int** domain of integrase family proteins. Earlier studies had showed that Excisase A to possess specific endonuclease activity in addition to recombinase activity. The relationship between the structural domains of the XisA to these two activities are not known. Thus, it is necessary to determine the role of N-terminal and C- terminal region of XisA in the excision of *nifD* element.

Excisase A protein has not been detected so far, which hindered the understanding of the mechanism of excision. *Eschericia coli* strain DH5 α containing variants of *xisA* gene with deletions at the N-terminal when expressed under the regulation of *tac* promoter on a multicopy plasmid, showed poor growth. The reason for the toxicity to *E. coli* was explained by the accumulation of high level of XisA protein. It was supported by the observation that *E. coli* XL1-Blue strain containing these plasmids grew normally, presumably because the strain contain low level of XisA protein due to the presence of *lacI^f* gene that overproduces the Lac repressor that allows only limited transcription from the *tac* promoter on multicopy plasmids.

Hence the objectives of the present study are –

1. Effect of Nitrogen sources on the excision of *nifD* element of *Anabaena* PCC7120
2. Role of *Anabaena* PCC 7120 NtcA on the excision of *nifD* element in *E. coli* .
3. Role of N terminal and C terminal domains of *xisA*.
4. Detection and characterization of XisA protein.

1. Effect of nitrogen source on the excision of *nifD* element of *Anabaena* strain PCC 7120 in *Escherichia coli*.

Effect of the presence of amino acids on the excision of *nifD* element in *E. coli* DH5 α (*recA*⁻) and JM101 (*recA*⁺) strains was monitored in the minimal medium containing casein acid hydrolysate. *E. coli* grown on M9 minimal with 40mM NH₄Cl demonstrated around 6 fold increase in excision as compared to that on Luria broth. In M9 minimal containing 1% casein and 100mM glucose which has C/N ratio similar to Luria broth, the excision frequency was around 10 fold and 2 fold higher as compared to that in Luria broth and M9 with 40mM NH₄Cl, respectively. Presence of NifA of *Klebsiella pneumoniae* also increased the excision frequency by 15 fold and 5 fold higher under similar conditions. Using PxisA::lazZ, promoter activity of *xisA* was found to be almost half in casein (1%) medium with 100mM glucose as compared to that on Luria Broth. Activity of Excisase A was also high in stationary phase as compared to the logarithmic phase in all conditions. These results are in agreement with the excision studies in *E. coli* strains grown on Luria broth and minimal medium which showed that the excision of *nifD* element is not only dependent on XisA protein but requires additional host proteins.

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

NtcA protein regulates the transcription of many genes involved in the nitrogen metabolism in cyanobacteria. Earlier we have reported the increase in the excision of *nifD* element of *Anabaena* PCC 7120 in minimal medium to about 50% and 70% in *E. coli* DH5 α

and JM101 strains, respectively, in the presence of *nifA* gene of *Klebsiella pneumoniae*. Present studies of the excision of *nifD* element in *E. coli* in minimal medium showed 3 fold increases in the presence of NtcA but not in Luria broth. NtcA influence on *xisA* gene expression was monitored in *E. coli* using *PxisA*- β -galactosidase transcriptional fusion. β -galactosidase activity was higher in the M9 minimal medium than in LB medium. Thus, NtcA could enhance the excision of *nifD* element by activating the expression of *xisA* gene. These studies demonstrate that NtcA and NifA proteins act in a similar manner to increase the excision of *nifD* element and *xisA* gene expression in *E. coli* when grown in M9 Minimal medium. These studies also suggest that NtcA could participate in excision in the heterocysts of *Anabaena* PCC 7120 by increasing the expression of *xisA* gene. Identification and characterization of accessory (host) proteins other than NtcA, could also help in understanding the mechanism of excision of *nifD* element in the heterocysts.

3. Role of N terminal and C terminal domains of *xisA*.

Site-specific recombinase XisA belongs to Integrase family. Nucleotide sequences showed presence of the *Int* domain towards C terminal region of the *xisA* gene. To characterize its functional domains, N-terminal and C-terminal domains of *xisA* gene were amplified with specific primers. N-terminal domain of the *xisA* was cloned in pACYC184 and pMC71AGm plasmids. C terminal of the *xisA* was cloned first in the pTTQ18 plasmid downstream to the Ptac in MCS and the C terminal of *xisA* along with Ptac was amplified with the specific primers. The amplicon was then cloned in pMC71AGm plasmid. These

plasmids are being used to monitor rearrangement and endonuclease activities using the *xisA* defective pMX32 substrate plasmid.

4. Detection and characterization of XisA protein.

Earlier when *xisA* was expressed in *E. coli* under strong promoter like Tac, it was found to exhibit toxicity. If the toxicity was due to the endonuclease activity could be circumvented by expression of the *xisA* under strong promoter which is targeted to the periplasm. Hence, MBP::XisA was constructed to target the fusion protein to the periplasm. The fusion protein was detected at very low concentration in the periplasm when *E. coli* BL21 (DE3) strain was induced with 1mM IPTG at 37 °C. Even after decreasing the temperature to 25°C, the protein concentration was not increased.

5. Cloning, expression and partial purification of *Anabaena* PCC 7120 XisA protein

Toxic proteins were expressed using tight regulatory control of expression in pVN6 plasmid. *xisA* gene was cloned under T7 Promoter in pVN6 plasmid. IPTG induction showed the presence of XisA protein on SDS-PAGE. Further purification of XisA protein is being carried out along with determining *in vitro* activity, which will be carried out using another substrate plasmid containing two 11bp target sites. XisA protein was partially purified using MonoQ column which suggests XisA protein is able to bind to positive charged molecule, while it showed poor binding to the negatively charged matrix in column chromatography. *In vitro* activity found absent with partially purified XisA protein.