CHAPTER I

INTRODUCTION

1.1 General introduction

Cyanobacteria (formerly called as Blue green algae) are a group of photosynthetic bacteria. Many of them are able to fix atmospheric dinitrogen to ammonia. Cyanobacteria originated during Mesozoic era 3 X 10^9 years ago (Brock, 1973) and are supposed to have contributed significantly to oxygenation of primitive earth atmosphere (Schopf, 1975). Chloroplasts of higher plants are believed to have evolved through endosymbiosis of cyanobacteria and plant cells (Walsby, 1986). Cyanobacteria are the only diazotrophs, which can fix nitrogen as well as can perform oxygenic photosynthesis. Cyanobacteria are the only source of nitrogen biofertilizer derived from solar energy and they are the major nitrogen fixers in the oceans of this planet (Capone and Carpenter, 1982; Fogg *et al.*, 1973). In tropical rice cultivation, cyanobacteria either as free-living or symbiotic association with water fern *Azolla* increase the grain yield by 15-20% (Venkatraman, 1972).

Cyanobacteria exhibit different morphological features ranging from unicellular forms to filamentous (Rippka *et al.*, 1979). The unicellular and filamentous nonheterocystous forms fix nitrogen in the dark phase of the carbon cycle whereas nitrogen fixation in some of the filamentous forms, such as *Anabaena* and *Nostoc,* occurs in specialized cells called heterocysts, which provide the anaerobic environment for nitrogen fixation.

The filamentous cyanobacterium *Anabaena* sp. protect extremely oxygen-sensitive nitrogenase by spatially separating nitrogen-fixation and oxygenic photosynthesis in two different cell types, the oxygen evolving vegetative cells and the nitrogen-fixing heterocyst. Upon deprivation of combined nitrogen, about 5-10% of the vegetative cells differentiate into heterocysts, resulting in a semi-regular spacing of these specialized cells along the trichome. Upon differentiation, morphological as well as biochemical changes in the developing heterocysts leads to the establishment of a

microaerobic environment tolerated by nitrogenase (Haselkorn, 1978; Wolk, 1982; Apte, 1992; Fay, 1992; Adams and Duggan, 1999). The heterocysts maintain a very low internal partial oxygen pressure (pO_2) by many different mechanisms (Wolk *et al.*, 1994). For instance, oxygen producing PSII is inactivated in heterocysts but is present in other vegetative cells. Heterocysts are enveloped by a layer of glycolipids and they provide a substantial barrier to the entry of oxygen and this layer is in turn covered by a layer of polysaccharides that protects it from physical damage. In addition, oxygen is reduced to water by respiration. Nitrogen metabolism of cyanobacteria, especially nitrogen fixation, has been significant since its regulation is coordinated with photosynthesis.

1.2 Assimilation of ammonium, nitrate and nitrite in cyanobacteria.

The most abundant forms of nitrogen in both aquatic and terrestrial habitats are the inorganic species, dinitrogen (N_2) and to a lesser extent nitrate and ammonium. Some of the proteins and genes involved in the nitrogen assimilation in bacteria are given in **Table 1.1.**

Table 1.1 : Proteins and genes involved in Nitrogen metabolism in bacteria.

Proteins/Genes	Nature of the Protein product
GlnA	Glutamine synthetase
GlnB	P_{II}
NirA	Nitrite reductase
NarB	Nitrate reductase
nrt ABCD	Nitrite/nitrate membrane transporter
NtcB	Nitrite dependent (nitrate assimilation)
NRT	Nitrate transport system
NtcA	Positive/Negative regulator for ammonium Assimilation

Synechococcus PCC 7942 does not possess glutamate dehydrogenase (Lightfoot *et al.*, 1988). Hence, glutamine synthetase (GS) / glutamine oxoglutarate aminotransferase (GOGAT) enzymes are necessary for ammonia assimilation (Boussiba and Gibson, 1991). GS catalyses the ATP dependent synthesis of glutamine from glutamate and ammonium ions. The cyanobacterial enzyme was purified from both non-nitrogen fixing and nitrogen fixing species (Sampaio *et al.*, 1979; Orr *et al.*, 198; Florencio and Ramos, 1985; Blanco *et al.*, 1989; Merida *et al.*, 1990;). It closely resembles the *E. coli* GS and has 12 subunits of approximately 50 kDa which are arranged in two superimposed hexagonal rings.

Generally all cyanobacteria can utilize nitrate and nitrite as sole sources of nitrogen for growth (Guerrero and Lara, 1987). Nitrate assimilation involves nitrate uptake by an active transport system (NRT) and a sequential intracellular reduction to nitrite and ammonium by nitrate reductase (NR, a molybdenum containing enzyme) and nitrite reductase (NiR), respectively (Flores *et al.*, 1983). An active transport system sensitive to ammonium appears to drive nitrate and nitrite uptake. Additionally, a passive diffusion of nitrate insensitive to ammonium contributes to nitrate uptake (Flores *et al.*, 1983; 1987; Martin-Nieto *et al.*, 1989; Lara *et al.*, 1987; Tischner and Schmidt, 1984). In *Synechococcus* PCC 6301, NR and NiR enzymes were purified and their sizes are 75kDa and 50kDa, respectively. Bisen and Shanthy (1991) purified the NR from the *Anabaena doliolum.*

1.2.1: Biochemical regulation of nitrate and nitrite metabolism.

Ammonium-mediated repression of GS has been observed in many cyanobacterial species. However, no evidence exists for a covalent modification of the enzyme by adenylation as found in enteric bacteria, even in the case of *Synechocystis* PCC 6803, a species reported to display a marked inhibition of GS activity by ammonium and to exhibit a response to nitrogen starvation similar to that of enteric bacteria (Fischer *et al.*, 1981; Merida *et al.*, 1990; 1991a; 1991b). In *Synechocystis* PCC 6803, the GS

shows ADP-ribosylation but similar modification is not found in *Anabaena* PCC 7120 (Silman *et al.*, 1995). However, *glnA* of *Anabaena* PCC 7120 is functional in *Synechocystis* PCC6803 and is subject to ammonium inactivation. Fischer *et al.* (1981) showed that the *Anabaena* GS could not get adenylated in *E. coli*. In contrast, inactivation occurs when ammonium is added to cells maintained for 11h under nitrogen fixing condition wherein differentiation of heterocyst occurs. This suggests that the regulatory mechanisms of GS activity might differ between nitrogen fixing and non-nitrogen fixing strains and might depend on the nitrogen sources provided before transfer to the medium lacking a combined nitrogen source (Orr and Haselkorn, 1982). Comparison of the genetic and biochemical regulation of nitrogen metabolism in enterobacteria and cyanobacteria are given in **Table 1.2.**

In nonheterocystous nitrogen fixing strains, the uptake and reduction of nitrate are suppressed or at least decreased when ammonium is present (Guerrero and Lara, 1987; Bisen and Shanthy, 1991). In unicellular non-heterocystous nitrogen fixing strains, the regulation of the activity of nitrate reducing systems, NR and NiR, proceeds mainly through a repression by ammonium (Flores *et al.*, 1983; Herrero *et al.*, 1981; 1984; 1985; Herrero and Guerrero, 1986). In nitrogen fixing filamentous strains, the regulation is exerted through a combined process involving both induction by NO_3 and NO_2 and repression by ammonium itself.

The absence of adenylation of GS in cyanobacteria has been interpreted for many years as an indicator of the lack of bacterial-like nitrogen regulatory system. In enteric bacteria, the P_{II} protein gets uridylated in response to low intracellular glutamine and high α-ketoglutarate (αKG) and regulates the activity of GS via adenylation /deadenylation of the protein and its synthesis via the modulation of transcription initiation of *glnA* by NtrBC system. NtrB acts as a "modulator" or "sensor" and NtrC acts as a "response regulator" or transcriptional activator (Stock *et al.*, 1989). Harrison *et al.* (1990) purified the P_{II} protein from *Synechocystis* PCC 6301 and the corresponding gene *glnB* from *Synechococcus* PCC 7942. It encodes

for a 12.4kDa protein which shows 62% sequence identity with *glnB* gene product of other bacteria. DNA/DNA hybridization experiments indicate that an equivalent of the *glnB* gene is present in all cyanobacteria including *Anabaena* PCC 7120 and *Calothrix* PCC 7601 (Tsinoremas *et al.*, 1991).

Forchhammer and Tandeau de Marsac (1994) identified the *glnB* gene product (P_{II}) from the non-nitrogen fixing unicellular *Synechococcus* sp strain PCC 7942. P_{II} protein was found to be modified by the formation of a phosphomonoester bond at a serine residue instead of uridylylated as is characteristic in enteric bacteria. Forchhammer and Tandeau de Marsac (1995a) identified the P_{II} protein as trimeric protein and each subunit can be phosphorylated. *In vitro* assay for P_{II} phosphorylation showed that it is catalyzed by a protein kinase, which specifically utilizes ATP as a phosphoryl donor and transfers γPO_4 of ATP to Ser49 of P_{II}. Phosphorylation results in the formation of three isoforms of P_{II} with increasing negative charge. The recognition site of P_{II} kinase, out of the three seryl residues one which is recognized, is two amino acids away on the N-terminal to the tyrosine-51 that is uridylylated in other bacteria. The sequence surrounding the target seryl residue is RYRGSEY, which resembles the minimal recognition motif of eukaryotic c-AMP dependent protein kinases *i.e.* RXS (Kemp and Pearson, 1990). Comparison of this sequence stretch in P_{II} with the corresponding proteobacterial P_{II} protein shows two differences. First, the phosphorylated seryl 49 residue replaces an alanyl residue , which is present in all proteobacteria. Second, Arg 45 in *Synechococcus* sp strain PCC 7942 P_{II} substitutes for Leu in homologous sequence. In analog Arg 45 may contribute to the recognition site of the P_{II} kinase.

The P_{II} kinase activity was shown to be stimulated by αKG (Forchhammer and Tandeau de Marsac, 1995b). The concentration of αKG required to stimulate P_{II} kinase is in the physiological range found in *Synechococcus* PCC 7942 cells grown in the presence of nitrate (Marques *et al.*, 1992). Unlike the P_{II} uridylyl

transferase and uridylyl removing enzyme characterized in proteobacteria, the activity of P_{II} kinase form cyanobacteria did not respond to glutamine. The kinase activity was lower in ammonium-grown cells than in nitrate-grown cells which suggests that the kinase is regulated by the nitrogen status of the cell.

The P_{II} protein in *Synechococcus* PCC 7942 signals the cellular state of nitrogen assimilation relative to carbon dioxide fixation by phosphorylation at a seryl residue. The modification of P_{II} is independent of a specific light regimen in unicellular *Synechococcus* PCC 7942 (Forchhammer and Tandeau de Marsac, 1995a). However, incubation in the dark shifted its status towards the unmodified form when nitrate was present. Conversely, P_{II} remained modified in the dark when nitrate was absent. It was suggested that PSII favours P_{II} phosphorylation whereas dark favours its dephosphorylation. The dependence of nitrate utilization upon the availability of CO_2 is well documented in cyanobacteria and green algae (Eisele and Ullrich, 1977; Magnum *et al.*, 1973).

Similar to enterobacteria, *glnB* mutant of *Synechococcus* PCC 7942 (MP2) could utilize nitrate, glutamine and ammonium as nitrogen sources. In the presence of ammonium, the MP2 mutant could grow in the medium buffered to a pH of less than 8.0; above this pH cells ceased to grow and displayed degradation of chlorophyll-a. MP2 mutant also excretes nitrite into medium when grown in the presence of nitrate (Forchhmmaer and Tandeau de Marsac, 1995a). Levels of NR and NiR were decreased in ammonium adapted cells in this mutant as compared to that in the wild-type. This suggests that the synthesis of NR and NiR are subject to ammonium mediated repression even in the absence of P_{II} protein. Lee *et al.* (1999) identified two promoters in the *glnB* operon : one is constitutively expressed and other is NtcA dependent promoter, which gets expressed only under nitrogen starved conditions (Gracia -Dominquez and Florencio, 1997). The phosphorylation of P_{II} in the wild type is inversely correlated to nitrogen availability and directly correlated to higher CO_2 levels.

Table 1.2 : Comparison of the regulation of nitrogen metabolism in Enteric and cyanobacteria.

	Enteric bacteria	Cyanobacteria
Biochemical regulation		
Intracellular component for sensing modification of GS	α-KG/Gln adenylation/deadenylation	α-KG ADP-ribosylation[1]
Nature of P_{II} modification	uridylation/deuridylation	serine phosphorylation[2]
Enzymes of P_{II} modification	uridyl tansferase	serine kinase
Site of modification	Tyr51	Ser49
Genetic regulation		
Sigma factor	σ^{54}	σ^{70}
Sensor kinase	Ntr B	Not known
Response regulator	Ntr C active Dimer- Tetramer	Ntc A (active as reduced disulfide bond)[3]
	Phosphorylated dimer	Ntc B (nitrite specific)
Nitrogen fixation		
Sigma factor	σ^{54}	σ^{N} (not known)
Stimulus sensor	Nif L	--- (not known)
Response regulator	Nif A	--- (not known)

1 Found in *Synechocysitis* but not in *Anabaena* PCC 7120.
2 In *Synechococcus* PCC7942.
3 In *Anabaena* PCC 7120.

1.2.2 Molecular biology of ammonium, nitrate and nitrite metabolism in cyanobacteria :

The *glnA* gene has been sequenced from the nitrogen fixing species *Anabaena* PCC 7120 (Tumer *et al.*, 1983). In ammonium grown cells the *glnA* is transcribed from two *E. coli* like promoters P2 and P4 located between -155 and -273 (Tumer *et al.*, 1983; Schneider *et al.*, 1987). The major mRNA transcription starts from *nif* type promoter (P1) located at -93. This promoter is not recognized *in vitro* by the purified *Anabaena* RNA polymerase, suggesting that it requires a specific σ factor or some other effectors to be functional. BifA/NtcA protein has been identified in *Synechococcus* PCC7942 and *Anabaena* PCC 7120, which is a transcriptional regulator of nitrogen metabolism in cyanobacteria (Vega-Palas *et al.*, 1992; Wei *et*

al., 1994). *Anabaena glnA* gene has been shown to be expressed in *E. coli* at a level similar to that of activated *E. coli glnA* gene without requirement of the *ntrC* gene product and independent of the ammonia concentration (Tumer *et al.*, 1983).

Crespo *et al.*, (1998) identified GS type III (encoded by *glnN)* from the cyanobacterium *Pseudoanabaena* PCC 6903. This cyanobacterium lacks typical prokaryotic GS-I encoded by *glnA*. The expression of *glnN* is nitrogen dependent. NtcA protein from *Synechocystis* PCC 6803 (which is expressed in *E. coli*) was found to bind to the *Pseudoanabaena* PCC 6903 *glnN* gene. Although *glnN* gene of *Synechocystis* PCC 6803 has four out of six binding sites for NtcA, it was not possible to demonstrate *in vitro* binding of NtcA in this region (Reyes *et al.*, 1997). In *Pseudoanabaena* PCC 6903 the *glnN* gene promoter contains five out of six matching NtcA consensus sequences (Crespo *et al.*, 1998). Under nitrate growing conditions, the *Pseudoanabaena* PCC 6903 *glnN* promoter is stronger than that of *Synechocystis* PCC 6803 *glnN* gene.

In the unicellular cyanobacterium *Synechococcus* PCC 7942, the genes encoding for NRT (*nrtABCD)* (Omata, 1991; Omata *et al.*, 1989; Kuhkemeier *et al.*, 1984 a & b), NR (*narB*) (Kikuchi *et al.*, 1996) and NiR (*nirA)* (Luque *et al.*, 1993; Suzuki *et al.*, 1995) form an operon, *nirA-nrtABCD-narB* (*nirA* operon). Transcription of *nirA* operon is induced by removal of ammonium from the medium. Upstream to the *nirA* operon two genes (*nirA* and *ntcB)* involved in the maximum nitrate assimilation and transcribed divergently from the *nirA* operon and this operon was also repressible by ammonium (Maeda *et al* ., 1998).

Cai and Wolk, (1997b) identified several loci that are activated upon deprivation of nitrogen in *Anabaena* PCC 7120. They identified three genes that respond most rapidly and are closely linked and situated within *nirA* and *nrtC* and between *nrtD* and *narB* genes whose products are responsible for uptake and reduction of nitrite and nitrate. *narB* from *Anabaena* PCC 7120 shows extensive sequence similarity to

the NR from a diversity of prokaryotes whereas *nirA* shows extensive sequence similarity to the NiR of other cyanobacteria and higher plants (Cai and Wolk, 1997 b; Frias *et al.*, 1997; Maeda *et al.*, 1998). Nitrate and its ,catabolites, nitrite are actually transported into cells by a membrane transporter complex that is encoded by *nrt ABCD* (Luque *et al.*, 1992; 1994., Omata *et al.*, 1989; 1993). A strain bearing *narB* to *luxAB* was used for monitoring gene expression. The initial activation of *nrtC*, *nrtD* and *narB* moderates, perhaps in response to mobilization of internal nitrogenous reserves within 2.5h..

1.2.3: Genetic regulation of nitrogen metabolism in cyanobacteria.

A transcriptional regulator NtcA has been identified as responsible for the induction of expression of *nirA* operon and the *glnA* gene in the absence of ammonium (Luque *et al.*, 1994). NtcA is a DNA-binding protein whose predicted amino acid sequence shows similarity to those of the family of prokaryotic regulatory proteins represented by the cyclic AMP receptor protein CRP (Vega-Palas *et al.*, 1992; Wei *et al.*, 1994). The predicted amino acid sequence of the *Anabaena* PCC 7120 NtcA/BifA shows 77% identity to that of *Synechococcus* PCC 7942. In the latter, NtcA is required for the full expression of genes subject to ammonium repression (Vega-Palas *et al.*, 1990).

NtcA of *Synechococcus* PCC 7942 binds to DNA at $GTAN_8TAC$ sequence whereas BifA/NtcA of *Anabaena* 7120 binds to $TGTN_{10}ACA$. In the promoter region of *ntcA* gene there is a binding site GTN_{10} ACA at the upstream region. DNase protection assay showed that regions protected by NtcA from *Anabaena* PCC 7120 covered the whole recognition sites in the target sites indicates that NtcA is a dimer. *ntc*A gene from the *Anabaena* PCC 7120 was cloned in *E. coli* and formed a DNA protein complex similar to that of *Anabaena* (Wei *et al.*, 1994). Control extracts from *E. coli* harboring only vector could not form the complex suggesting

that NtcA binds to upstream region of its own gene. The partially purified NtcA protein binds and forms a complex at the -171 to -21 region in the promoter.

NtcA is repressor of *gorA*, encoding for glutathione reductase, in *Anabaena* PCC 7120 under nitrogen fixing conditions. Low levels of *gorA* and *rbcLS*, which codes for ribulose-1,5-bis-phosphate carboxylase/oxygenase, in heterocysts represents that they are negatively regulated by NtcA. NtcA functions as a repressor of *rbsLS* by blocking initiation of transcription (Ramasubramanian *et al.*, 1996). Like other members of CRP family, NtcA acts as an activator and a repressor of the target genes depending upon the location of the target sequence in relation to the promoter (Callado-Vides *et al.*, 1991). Most of the CRP members form dimers when they interact with DNA, each monomer binding to single half site of being dyad symmetry with the other half site.

Jiang *et al.* (1997) using electrophoretic mobility assays showed that the NtcA protein from *Anabaena* PCC 7120 interacts with the promoter sequence of *gorA* gene thereby providing a novel example of NtcA acting as repressor found only previously for *rbsLS* gene. NtcA of *Anabaena* PCC 7120 contains two cystine residues at Cys157 and Cys164, which are located close to the N-terminus of the helix-turn-helix DNA binding motif. The first Cys157 residue is conserved in all cyanobacteria including non-heterocystous forms such as *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942. The second cystine residue exists uniquely in the NtcA of heterocystous cyanobacteria (Jiang *et al.*, 1997). NtcA is regulated *in vitro* a redox dependent mechanism involving cystine residues of the NtcA protein. This suggests that response not only to nitrogen status but also to the cellular redox status, a function that might be particularly significant during heterocyst differentiation. The upstream non-coding region of *Anabaena* PCC 7120 *gorA* gene contains 3 putative *E. coli* sigma 70 promoter (-10 to -35 hexamer sequence) and one NtcA binding site that overlaps the middle promoter. The middle promoter was used only during the growth on ammonium but not on other sources such as nitrate or dinitrogen. These

results suggests that NtcA binding to its target sequence might prevent RNA polymerase from binding to middle promoter under growth on nitrate or dinitrogen.

In *E. coli* and *Salmonella typhimurium* OxyR responds to oxidative stress and one of the six cystine residues of the OxyR is critical in redox sensing (Stroz *et al.*, 1990). In contrast to NtcA, the oxidized form of OxyR functions as an activator. In *E. coli*, Fnr (another CRP family member), which allows the use of electron acceptor other than oxygen under anaerobiosis requires, the cystine clusters for anaerobic response (Merville and Gunsalus, 1993,). NtcA of *Anabaena* PCC 7120 requires free thiol residues for binding to DNA or activation of transcription similar to eukaryotic enhancer that regulates the AP-1 (Abate *et al.*, 1990) and USF (Pognonec *et al.*, 1992)

Ramasubramanian *et al.* (1994) showed that multiple transcripts of NtcA are produced under nitrogen limited conditions. The *ntcA* gene expression was differentially regulated (Ramasubramanian *et al.*, 1996). The peak of *ntcA* expression occurs at 12h, there was a drop at 18h, followed by a gradual increase in expression. The NtcA mutant of *Anabaena* does not form heterocysts indicating that it has an important role in heterocyst development. Through protein-DNA interaction NtcA finely regulates the expression of *nirA* and *glnA* during growth on nitrate as well as N-starvation (Luque *et al.*, 1994; Cohen-Kupiec *et al.*, 1995) and also shown that NtcA autoregulates its own gene expression (Luque *et al.*, 1994).

P_{II} protein does not directly regulate the NtcA activity in response to the nitrogen status or that its function is redundant for this purpose. If phosphorylated P_{II} protein is required for the activation of NtcA the phenotype, then the phenotype of the P_{II} deficient mutant would be similar to that of NtcA deficient mutant which constitutively express *narB*, *nirA* and *glnA* genes at low level irrespective of the nitrogen source (Vega-Palas *et al.*, 1990). Since P_{II} protein is not acting via

NtcA, there may be some other factor involved in the expression of *glnA In contrast to enterobacteria, genes of nitrate metabolism in cyanobacteria are dependent on constitutive general sigma factor. nir* genes of *Synechococcus* 7942 does not appear to have –35 consensus sequence instead NtcA binding sites are located in that region.

1.3 : Nitrogen-fixation in non-heterocystous and heterocystous cyanobacteria.

The cyanobacterial "conventional" nitrogenase complex, which converts dinitrogen to ammonia, is highly homologous to its counterparts in other nitrogen-fixing bacteria (Haselkorn, 1986, 1989; Apte, 1993). All cyanobacterial nitrogenases studied so far, consists of two oxygen sensitive proteins: the iron-molybdenum protein (FeMo protein) and iron protein (Fe protein) which reduce nitrogen only when together. The heterotetrameric FeMo protein is encoded by the *nifD* and *nifK* genes of α-subunit of 55kDa and β-subunit of 55-60kDa, respectively. The homodimeric Fe protein encoded by *nifH* gene synthesize the polypeptide of 30-36kDa. In this complex the electron flow, which is coupled to ATP hydrolysis, goes from ferredoxin to the Fe protein and finally to the MoFe protein (Van Baalan, 1987; Guerrero and Lara, 1987). Like in other nitrogen-fixing bacteria the cyanobacterial nitrogenase complex is irreversibly inactivated by oxygen. Kintemich *et al* (1988) and Thiel *et al.*(1995) showed the existence of two "alternative" nitrogenases in *Anabaena variabilis* ATCC 29413. A vanadium dependent nitrogenase and Fe nitrogenase probably contain only Fe-S clusters as prosthetic group. The MoFe and Fe proteins from *Anabaena* and *Plectonema* complement of MoFe and Fe proteins of each other and with other diazotrophs (Hallenbeck *et al.*, 1979; Smith *et al.*, 1987).

Cyanobacterial nitrogenase catalyses the reduction of N_2, proton (to H_2), acetylene (C_2H_2) and cyanide and is sensitive to CO. It is regulated at the level of both enzyme activity and at the gene expression (Haselkorn,1986; Wolk, 1982; Fay, 1992;

Stewart, 1980; Stewart and Rowell, 1986). Dinitrogen is not considered to be an inducer, but nitrate, ammonium, urea as well as amino acids like glutamine and glutamate have shown to suppress the nitrogen fixation.

1.3.1: Oxygen dependent regulation of nitrogen fixation.

Presence of oxygen or its active species physically degrades nitrogenase polypeptides by simulating certain proteases. In unicellular and filamentous non-heterocystous cyanobacteria, the nitrogenase complex is evenly distributed in the cytoplasm and is therefore not separated from photosynthetic apparatus (Smoker *et al.*, 1989; Stal and Bergman, 1990). In such strains the "temporal" separation between photosynthesis in the presence of light and nitrogen fixation in the dark contribute to create an appropriate microanaerobic environment to protect the nitrogenase complex from inactivation by oxygen which is produced in photosynthesis (Fay, 1992). In case of unicellular strains, *Gloeothece* and *Synechococcus,* temporal separation between both biological processes does not seem obligatory (Wolk, 1982) and the nitrogen fixation is most likely regulated by the availability of intercellular glycogen (Gallon and Chaplin, 1988). In *Oscillatoria* sp. culture grown photoheterotrophically under alternative light/dark cycle fix nitrogen in both light and darkness, but at higher rates in darkness indicating that respiration might provide reductant and ATP for nitrogen fixation (Gallon *et al.*, 1991).

1.3.2 Heterocyst differentiation :

Spatial separation photosynthesis and nitrogen fixation is found in heterocyst forms. In filamentous cyanobacteria grown under aerobic conditions, the expression of nitrogenase complex is confined to the heterocysts whose formation specifically occurs in response to a lack of utilizable source of fixed nitrogen (Haselkorn, 1978; Stewart, 1982). Nitrogen fixation is thus linked to a developmental process which

includes a series of sequential structural and physiological modifications which are necessary to create an anaerobic environment conducive for nitrogen-fixation (Haselkorn, 1978; Wolk, 1982; Bothe, 1982; Van Baalen, 1987; Adams, 1992; Tandeau de Marsac,1993; Adams and Duggan,1999). Generally not more than 5-10% of the vegetative cells within any filament differentiate into heterocyst. Mature heterocyst is slightly larger than the vegetative cells and is surrounded by a thick cell wall which contains extra three additional layers external to the vegetative cell type envelope and provide a physical barrier to diffusion of oxygen into the heterocyst.

Several structural and biochemical modifications accompany heterocyst differentiation to facilitate the nitrogen fixation (Haselkorn, 1978). Large structures like carboxysomes, polyphosphate bodies and thylakoids are destroyed to create the space for nitrogenase and its accessories (Stewart, 1977). During heterocyst development, a thick envelope is synthesized and the envelope consists of an external polysaccharide layer and an inner laminated layer of glycolipids that function to limit gas permeation. For nutrients to move between the heterocyst and the adjacent vegetative cells, a channel or pore is formed that contain a neck region of reduced diameter at the end of which the plasma membrane of the heterocyst and vegetative cells are narrowly separated. A structure called microplasmadesmata appear to join the two plasma membranes. The inner thylakoid membranes are rearranged within heterocyst, with the formation of honeycomb of membranes, rich in oxidative enzymes, near the poles of the developing heterocysts.

Photosystem II is destroyed and thus oxygen is not evolved in the heterocyst (Thomas, 1970; Van Gorkom and Dorze, 1971; Tel-Or and Stewart, 1975). Uptake hydrogenase and superoxide dismutase are produced to remove traces of oxygen from the site of nitrogenase activity. Under illumination, ATP is mainly supplied via cyclic photophosphorylation within heterocyst, while in darkness it is provided via

oxidative phosphorylation. High levels of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) supports nitrogenase activity by providing the source of reductant as well as ATP (Apte *et al.*, 1978; Lockau *et al.*, 1978), while GS activity increases to facilitate assimilation of fixed ammonia (Dharmawardene *et al.*, 1973; Wolk *et al.*, 1976). When interconnections between the vegetative and heterocyst cells get established, the former provide organic carbon compounds to the latter. The heterocyst provides the glutamine to neighboring vegetative cells (Bothe and Neuer, 1988).

Factors such as pH (Fernandez-Valiente and Leganos, 1989), light /dark shifts and availability of external CO_2 (Reich and Boger, 1989) also have regulatory effect on nitrogen fixation in heterocysts. In *Anabaena* ATCC 33047 Ca^{2+} has been reported to be involved in the protection of nitrogenase complex from inactivation by oxygen (Rodriquez *et al.*, 1990).

1.3.3 : Model for heterocyst pattern formation.

Heterocysts are present in semi-regular pattern of every tenth cell in the filament. While the filaments of *Anabaena* when subjected to nitrogen deprivation, certain cells may become nitrogen deficient before others. If nitrogen deprivation merely allow expression of a pre-existing pattern, and is not causally involved in the localization of heterocyst formation, there may be no spatial relationship between the cells that differentiate and the cells that first become nitrogen deficient. Two models are proposed for the pattern formation in cyanobacteria (Wolk, 1991). According to the first model, known as the *altruistic model,* the cells which first become nitrogen deficient inhibit nearby cells from differentiating, for example by releasing products of proteolysis, and themselves proceed to differentiate. Second *selfish model,* postulates that cells that first become nitrogen deficient scavenge nitrogen from their neighbors until a cell, scavenged from both sides becomes highly nitrogen deficient,

and in response differentiates. In this case differentiation commences distant from, rather than at the site of initial nitrogen deficiency.

1.3.4 : Molecular biology of heterocyst differentiation.

During heterocyst development, about 600-1000 genes in the genome of *Anabaena variabilis* are exclusively transcribed (Lynn *et al.*, 1986). Several regulatory and structural genes that are required for heterocyst differentiation in *Anabaena* PCC 7120 have been identified and cloned (Buikema and Haselkorn, 1993; Wolk, 1996). Nitrogen control gene, *ntcA*, is required for an early response to nitrogen deprivation, for induction of nitrate assimilatory genes as well as for later responses in heterocyst differentiation (Frias *et al.*, 1994; Wei *et al.*, 1994). Two σ factors *sigB* and *sigC* augment vegetative sigma factor *sigA* in response to nitrogen deprivation of *Anabaena* PCC 7120. However, mutations in *sigB* or *sigC* did not impair heterocyst differentiation nor nitrogen fixation (Brahamsha and Haselkorn, 1992).

Pattern formation may depend on one or more genes that express spatially constitutively *i.e.* in all vegetative cells grown on fixed nitrogen (Apte and Naresh Kumar, 1996). Elhai and Wolk (1990) carried out high efficient measurement of luminescence at a single cell level. They showed that *in vivo* assessment of transcription from single genes at the single cell level is a critical tool to study the development in *Anabaena* and that the transcription events are developmentally regulated.

In *Anabaena* PCC 7120 genes that are induced upon removal of fixed nitrogen are divided into two groups *Group H* necessary for heterocyst formation and those *Group V* (vegetative growth) that are not required for heterocyst differentiation. In order to identify the genes whose promoters respond rapidly to N deprivation, a derivative of transposon Tn5 was used to generate random transcriptional fusion of promoter less bacterial luciferase genes *luxAB* into the genome of *Anabaena* PCC

7120 (Wolk *et al.*,1991). Mutants TLN10, TLN12, TLN21 all of which are transcriptionally activated within 30 minutes of removal of ammonium from the milieu have the insertions at different positions at *nirA-nrt ABCD-narB* gene cluster, that is required for assimilation of nitrate (Cai and Wolk, 1997a). Mutants TLN2 and TLN6 are activated within an hour, and these mutants are capable of heterocyst differentiation and nitrogen fixation.

Khudyakov and Wolk (1996) identified a locus in *Anabaena* PCC 7120 called *hanA*, which encodes for HU protein. The mutant of *hanA*, AB22, exhibits pleotrophic effect, includes slow growth rate, altered pigmentation, cellular fragility, resistance to the phage A-4(L) and unable to differentiate heterocysts. The expression of *hanA* of *Anabaena* PCC 7120 is regulated by NtcA. Upstream of the *hanA* gene are the nucleotide sequences located at 241-256 that perfect match the NtcA binding site. The HU protein was shown to participate directly or indirectly in the regulation of the expression of *hetR*, a transcriptional regulator necessary for heterocyst differentiation. It was suggested that the Het$^-$ phenotype of the mutant AB22, results in the impaired expression of *hetR*.

Using nitrosoguanidine mutagenesis, Ramaswamy *et al.* (1996) isolated a mutant AMC260 of *Anabaena* PCC 7120 that is unable to reduce nitrate and forms 6-8% heterocyst in the presence of nitrate. The corresponding chromosomal fragment 1.8kb was cloned and a 1.2kb ORF called *moeA* was identified. The deduced amino acid sequence of the predicted MoeA polypeptide showed 37% homology to the *E. coli* MoeA which is required for the biosynthesis of molybdopterin cofactor required for various molybdoenzymes including NR (Johnson and Rajagopalan,1987; Nohno *et al.*, 1988). Similar mutants which are defective in NR activity were isolated from *Synechococcus* PCC 7002 (Stevens and Smith, 1994) and from *Nostoc muscorum* (Bagchi and Singh, 1984).

Zhang *et al* (1998) cloned a gene *prpA* from *Anabaena* PCC 7120, encoding a protein similar to eukaryotic type of phosphoprotein phosphatase PP1, PP2A and PP2B. An eukaryotic type protein *pknE* was found 301bp downstream to the *prpA*. Both the proteins were overexpressed in *E. coli*. Immunodetection and RNA/DNA hybridization experiments suggests that these two genes are unlikely to be coexpressed despite of close linkage. PrpA is expressed constitutively under different nitrogen conditions while *pknE* is regulated during the process of heterocyst development. *pknE* strongly expressed in the presence of nitrate with its expression decreasing during the first few hours of nitrate deprivation and then increasing again. Inactivation of *prpA* or *pknE* affects similar biological process (heterocyst structure formation and nitrogen fixation activity) strongly suggesting that these two genes are involved in regulation of similar targets in *Anabaena*. Mutants of *prpA* or *pknE* produce heterocysts with aberrant structures , which may account for their poor performance in nitrogen fixation and ultimately their inability to sustain growth in the absence of combined nitrogen.

1.3.4.1 HetR

Buikema and Haselkorn (1991) identified a mutant *hetR, Anabeana* 216, defective in heterocyst differentiation in *Anabaena* PCC 7120. The *hetR* gene encodes a 299 amino acid protein. Over-expression of *hetR* in wild-type and *Anabaena* 216 increases the frequency of heterocysts under N deprivation conditions and in media containing fixed nitrogen. Under nitrogen deficient conditions, it leads to supernumerary heterocyst wherein an array of two or more adjacent heterocysts develop.

The *hetR* gene is abundantly transcribed as two mRNA species of 1.4 kb and 1.9kb which remain transcribed at low levels under nitrogen replete conditions, although it is not required for vegetative cell growth (Buikema and Haselkorn, (1991). *hetR* is an autoregulatory gene that is induced within 2 h after nitrogen step down (Black

and Wolk, 1994; Buikema and Haselkorn, 1991). Upon 6 to 18h. of N starvation, the level of *hetR* RNA increases by 3-5 fold and decreases slightly after 18 h. *hetR* mRNA seems to be transcribed from multiple promoters (Buikema and Haselkorn,1991). The transcripts of the *hetR* gene were found to be present mostly in the heterocysts or proheterocysts. The *hetR* gene was overexpressed and purified in *E. coli.* (Zhou *et al,* 1998). The HetR was unstable and degraded easily in solution. Phenylmethane sulfonyl fluoride (PMSF), a serine protease inhibitor, prevented the degradation and could modify HetR. Dansyl fluoride (DnsF) and other serine protease inhibitors also covalently modify HetR. The pI of the native hetR under nitrogen deprivation conditions is much more acidic than that of rHetR (Zhou *et al,* 1998). One possibility that *hetR in vivo* is auto digestive until it is modified after cells receive the signal of nitrogen deprivation. The activity of HetR is dependent upon the calcium concentration and it was suggested that HetR may be calcium dependent protease. During early phases of heterocyst differentiation, intracellular proteolysis activity was increased several times in *Anabaena* (Wood and Haselkorn ,1979).

1.3.4.2 : PatS.

Yoon and Golden (1998) identified the *patS* gene which encodes a 17 amino acid peptide which is involved in the spacing pattern of heterocysts presumably by preventing the differentiation of the cells between two heterocysts. It was also shown that heterocyst differentiation can be suppressed completely by the external addition of the pentapeptide RGSGR corresponding to the 5aa at the carboxy terminal of PatS protein. Over-expression of the 54bp gene *patS* blocked heterocyst differentiation in *Anabaena* PCC 7120. A null mutant of *patS* showed an increased frequency of heterocysts and abnormal pattern. *patS* appears to control heterocyst pattern formation through intercellular signaling mechanism. Interestingly, *patS* mutant strain AMC451 formed heterocyst on nitrate containing medium. This mutant shows multiple contiguous heterocysts with interspersed short stretches of vegetative cells.

Long chains of upto 10 contiguous heterocysts were occasionally formed. Heterocyst formation in AMC451 was inhibited by exogenously added RGSGR, indicating rescue of the mutant phenotype by the pentapeptide. However, the pattern was still abnormal. Using GFP-*patS* the expression was localized in developing proheterocysts and induction of *patS* occurs after 12h of nitrogen step down.

PatS thus seems to play a key role in heterocyst pattern formation by inhibiting the formation of adjacent heterocyst and by maintaining a minimum of vegetative cell between heterocyst (Haselkorn , 1998). It is proposed that the first cells to differentiate increase the production of *patS* to inhibit neighboring cells from forming heterocysts. The *patS* signal is likely to be processed carboxy terminal pentapeptide that is confined to the periplasm of the gram negative cyanobacteria. A processed *patS* peptide originating from differentiating proheterocysts may diffuse along the filaments contiguous periplasmic space and it is taken up by the neighboring cells creating a gradient of inhibitory signal. The intracellular target of the *patS* signal is not known.

1.3.4.3 : Other Group H genes.

Liang *et al.* (1992) identified the *patA* gene that encodes a predicted protein of 379 amino acids whose C-terminal domain exhibits a very high homology with *cheY* which is involved in the regulation of chemotaxis as well as other response regulators of two-component regulatory system in several eubacteria (Stock *et al.*, 1991; Bourret *et al.*, 1991). *patA* is expressed as a species of 1.4kb mRNA and the expression is abundantly increased approximately the same timing as the expression of *hetR*. Like *hetR*, its transcription is at a low level in the presence of fixed nitrogen. Mutation in *patA* gene leads to the filaments with mostly terminal heterocysts instead of the normal spacing pattern (Liang *et al.*, 1992). Interestingly, the *patA* mutation suppresses the multi heterocyst phenotype produced by extra copies of *hetR* gene. This suggests that *patA* and *hetR* are members of same

environmental sensing regulatory circuit in *Anabaena.* Based on the homology of *patA* to *cheY* family regulator proteins, it has been proposed that the *patA* is transcriptional activator controlled by phosphorylation of an aspartate residue (Liang *et al.*, 1992).

Another Group H gene, *hetC* has an open reading frame of 1044 amino acids and shows homology to HylB family of bacterial protein exporters. It is induced within 4 h after nitrogen step down, hours after the induction of *hetR.* Transcription of *hetC* requires both HetR and a functional HetC protein, implying that *hetC* like, *hetR* is autoregulatory. In the *hetC* promoter region, -620 to –605, there is a binding site for NtcA protein which acts as *cis*-element and stimulates the expression of *hetC. HetR* strongly represses the expression of *hetC* in vegetative cells.

The *patB* gene encodes for a protein with helix-turn-helix motif and containing ferredoxin (Liang *et al.*, 1993). Mutation in the *patB* leads to greatly delayed heterocyst formation but eventually the pattern shows extra heterocysts. Mutation in *hetN* gene results in the absence of heterocysts. The *hglB* (formerly *hetM)* gene is located upstream of *hetN.* HglB protein has two functional domains; the amino terminus is similar to an acyl carrier protein and the central portion has similar to keto reductases. An insertion mutation in the *hglB* gene results in inability to produce heterocyst glycolipids (Black and Wolk, 1994; Bauer *et al.*, 1997).

Two heterocyst-specific genes, *devA* and *hetP,* were found between 5' from initiation codons and 3' untranslated regions of HetC (Khudyakov and Wolk, 1997). Mutation in *hetP* also blocks heterocyst differentiation and strains containing extra copies of *hetP* forms multiple contiguous heterocyst in media lacking combined nitrogen (Fernadez-Pinas *et al.*, 1994).

Maldener *et al.* (1994) showed that the pleotropic phenotype of mutant M7 was caused by transposition of Tn5-1063 into an ORF named *devA*. Expression studies using *luxAB* as a reporter, it was shown that *devA* expression increases by approximately eight fold in whole filaments after 14h of nitrogen step down. The deduced amino acid sequence of *devA* showed striking similarity to the ATP binding subunit of ABC (ATP binding cassette) transporters. Fideler *et al.* (1998) identified three cistrons of the *devABC* operon encoding the complete transporter. The *devB* and *devC* cistrons encode proteins with similarities to membrane fusion proteins. Mutation in each of these genes results in an identical phenotype; the heterocyst specific glycolipids forming the laminated layer of the envelope are absent.

In *Anabaena* PCC 7120, synthesis of the heterocyst polysaccharide envelope is dependent on the gene *hepA*. (Zhu *et al.*, 1998). The transcriptional start site of *hepA* is located 104 bp 5' of its translational initiation codon. A 785 bp ORF denoted *hepC* was found further upstream. Inactivation of *hepC* led to constitutive expression of *hepA* and prevented the synthesis of heterocyst envelope polysaccharide. However, the glycolipid layer of the heterocyst envelope was synthesized. A *hepK*, mutation blocked both in the synthesis of heterocyst envelope polysaccharide and in the induction of *hepA*. The predicted protein of *hepK* resembles the sensory protein-histidine kinase of two component regulatory system. Analysis of regions between *hepC* and *hepA* indicated that -574 to -440 and -340 to -169 bp region is required to induce *hepA* upon nitrogen deprivation. The inactivation of *hepC and hepK* blocks the expression of *hepA*. Induction of *hepA* in response to nitrogen deprivation requires an intact *hepK* gene. A protein that receives a phosphate group from HepK may regulate directly or indirectly the *hepA* promoter. The heterocyst specific glycolipid synthesis, which is critical for nitrogen fixation (Buikema and Haselkorn, 1993; Wolk, 1996), is not affected by the inactivation of either *prpA* or *pknE*.

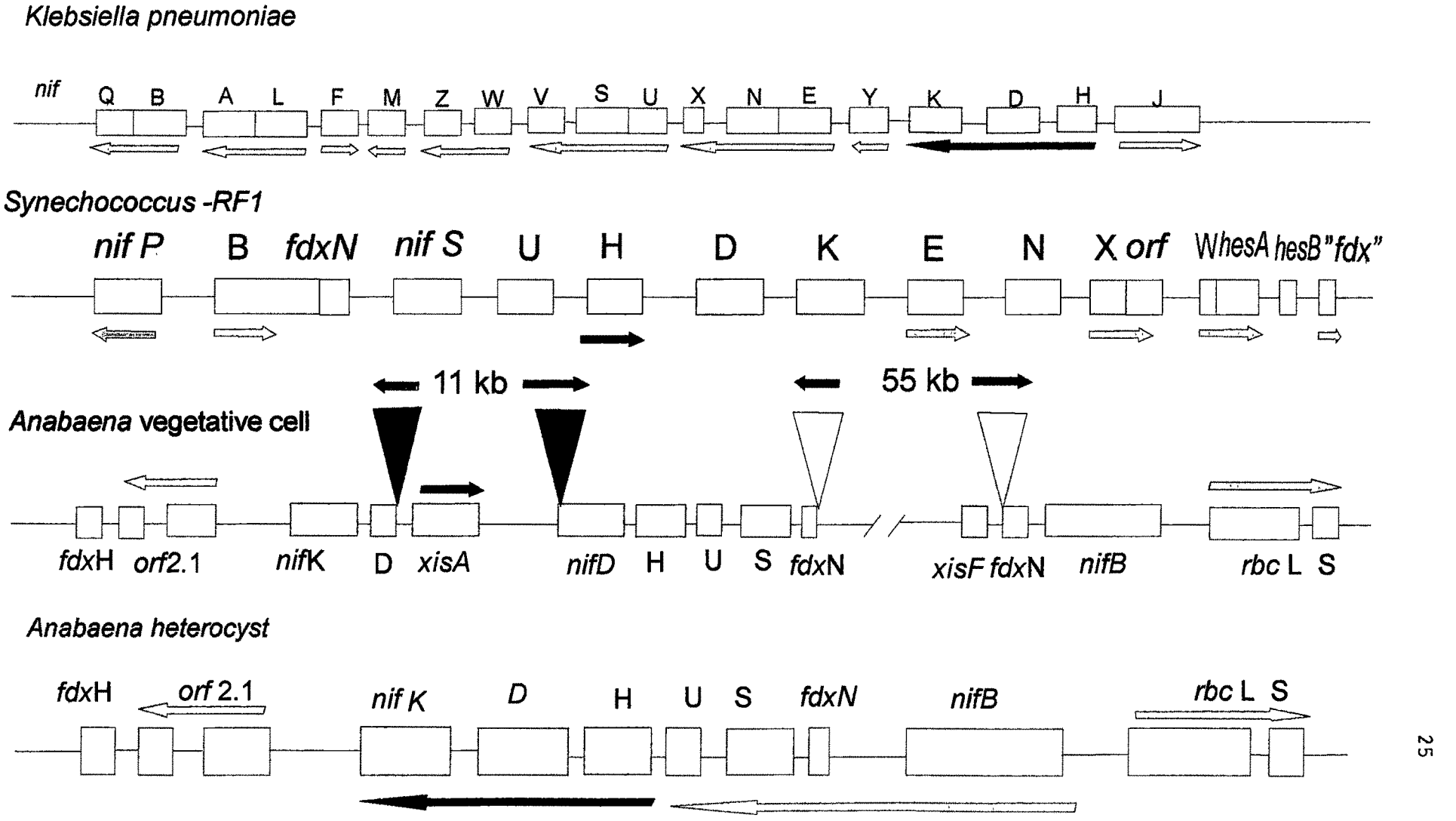
1.3.5: Organization of nitrogen fixation genes in cyanobacteria.

The organization of nitrogen fixation (*nif)* genes in cyanobacteria differs significantly from that found in other diazotrophs (**Fig. 1.1**). In the facultatively anaerobic enterobacterium *Klebsiella pneumoniae* 20 *nif* genes clustered in one region of the chromosome which are involved in the process of nitrogen reduction by molybdenum-containing nitrogenase enzyme complex (Arnold *et al.*, 1988). The molybdenum-containing nitrogenase is composed of a Fe protein, a dimer of identical subunits (NifH) containing [4Fe-4S] cluster, and MoFe protein a heterodimer of two NifD and two NifK subunits and includes two iron molybdenum cofactors [FeMoco] and two P clusters (Smith and Eady, 1992; Kim and Rees, 1994).

In the unicellular cyanobacterium, *Synechococcus* strain RF-1, the nitrogen fixing activity in diurnal/dark entrained culture was found to exhibit a circadian rhythm (Huang and Grobbelaar, 1995). In this organism the organization of *nif* gene cluster was recently elucidated (Huang *et al.*, 1999). It was found that sixteen *nif and* "*nif*-associated" genes (only expressed under conditions of nitrogen fixation) were present in this cluster which is in a continuous arrangement spanning approximately 18kb with seven transcriptional units. The structural genes for nitrogenase *nifH, nifD* and *nifK* are arranged in *nifHDK* operon similar to that of other diazotrophs. The *nif* and *nif* associated genes located downstream from *nifK* in *Synechococcus* RF1 were found to be arranged *nifE-nifN-nifX-orf-nifW-hesA-hesB-'fdx'.* The organization is nearly the same as *Anabaena* except that the *orf-3* located between *nifK* and *nifE* was not found *Anabaena.* The operon containing *nifP* located upstream of *nifB.* (**Fig. 1.1**).

In the well studied heterocystous cyanobacterium *Anabaena* PCC 7120, the major *nif* gene cluster consists of several open reading frames (ORFs). Two DNA interrupting elements and four operons are dispersed in over 80kb of DNA (Mazur *et al.*, 1980; Rice *et al.*, 1982; Golden *et al.*, 1985; Apte and Prabhavathi, 1994). At least

Fig.1.1 ORGANIZATION OF *nif GENES in Klebsiella pneumoniae* & Cyanobacteria



+ 11 kb circle & 55 kb circle

14 genes associated with nitrogen fixation have been identified in *Anabaena* PCC 7120. These include *nifHDK* operon (Mazur *et al.*, 1980; Rice *et al.*, 1982) the *nifB, fdxN, nifS, nifU* operon (Mulligan and Haselkorn., 1989) a *fdxH* gene which encodes for a heterocyst specific ferredoxin (Bohme and Haselkorn, 1988) and another operon containing *orf1*.

Using PCR strategy, Stricker *et al.* (1997) identified *nifVZT* genes form *Anabaena* PCC 7120. The *nifVZT* were not located close proximity to the main *nif* gene cluster in *Anabaena* PCC 7120 and therefore *nifVZT* forms a second *nif* gene cluster in this strain. Overlaps between *nifV* and *nifZ* and between *nifZ* and *nifT* genes were found and the finding of 1.8kb transcript corresponding to these genes indicated that *nifVZT* might form one transcriptional unit. The *Anabaena* PCC 7120 *nifV* gene could complement *nifV* mutant of *Rhodobacter captulatus* R2291. Transcripts of *nifV* were induced in a nitrogen depleted culture and also by iron-depletion independently of the nitrogen status. *nifV* encodes for homocitrate synthase. Hoover *et al.* (1988; 1989) have shown homocitrate to be an integral part of FeMo co. NifZ appears to be involved in the formation or accumulation of active MoFe protein but it is not essential for nitrogen fixation in *Klebsiella pneumoniae* (Paul and Merrick, 1989). *nifT* expression in *K. pneumoniae* also has a limited effect on nitrogen fixation (Simon *et al.*, 1996). Therefore there was no clear role of NifZ/NifT is known. It has been speculated that *nifZ/nifT* may also be involved in the biosynthesis of FeMo cofactor of nitrogenase (Stricker *et al.*, 1997).

1.3.6 : DNA Genomic Rearrangements in Anabaena.

Three genes involved in nitrogen fixation in *Anabaena* PCC 7120 are interrupted by large DNA elements. An 11kb DNA interrupting element present in *nifD* gene (Golden *et al.*, 1985; Golden, 1988), a 55kb DNA element in the *fdxN* gene (Golden *et al.*, 1988), and a 10.5kb DNA element in the *hupL* gene (Carrasco *et al.*, 1995)

(**Table 1.3**). The *nifHDK* and *nifBfdxN nifSU)* operons are located next to each other while the *hupL* operon is located >700kb away on the *Anabaena* chromosome (Kuritz *et al.*, 199?). The interrupting elements are excised out precisely during heterocyst differentiation by means of site-specific recombination events, resulting in gene rearrangement and formation of functional operons. The excised circles persist in the heterocysts with no known function (Haselkorn, 1989; 1992 ; Apte and Prabhavathi, 1994). The predicted ORF of the *nifD* element encodes a polypeptide for 480 amino acids. After excision an new ORF is created by the fusion of the *nifD* ORF to *nifK* 5' flanking sequence. This new ORF codes for 497aa, of which C-terminal 43 residues replaced 26 C-residues of vegetative cell ORF. The new ORF shows similarity to *Rhizobium* NGR 234 of *Parasponia* α subunit of nitrogenase (Golden *et al.*, 1985).

Three different excisases, the XisA, XisF and XisC, encoded by three independent genes namely *xisA* (Lammers *et al.*, 1986), *xisF* (Carrasco *et al.*, 1994) and *xisC* (Carrasco *et al.*, 1995) are involved in three excision events. Interestingly, each of these genes resides near the left border of the same DNA element, which it excises during development *i.e.* 11kb (**Fig. 1.2**), 55kb and 10.5kb elements harbor *xisA, xisF* and *xisC,* respectively. The 11kb element is flanked by a 11bp direct repeat (CGGAGTAATCC) and the 55kb by a 5bp direct repeat (TATTC) at each end (Golden *et al.*, 1987, 1988,1991; Mulligan *et al.*, 1988; Mulligan and Haselkorn, 1989) while the 10.5kb has a 16bp direct repeat (CACAGCAGTTATATGG) at the left and right borders (Carrasco *et al.*, 1995). Each excisase carries out a site-specific recombination event involving the respective direct repeats and results in the excision of the DNA between the direct repeats (Haselkorn, 1992). Mutation in the *xisA* and *xisF* genes result in the Nif phenotype and lack of diazotrophic growth (Weist and Golden, 1988; Carrasco *et al.*, 1994). Mutations in the *xisC* have not been reported as yet and are expected to bring about decrease in the efficiency of nitrogen fixation (Carrasco *et al.*, 1995).

Fig. 1.2 EXCISION OF *nif*D ELEMENT OF *ANABAENA* sp. strain PCC7120

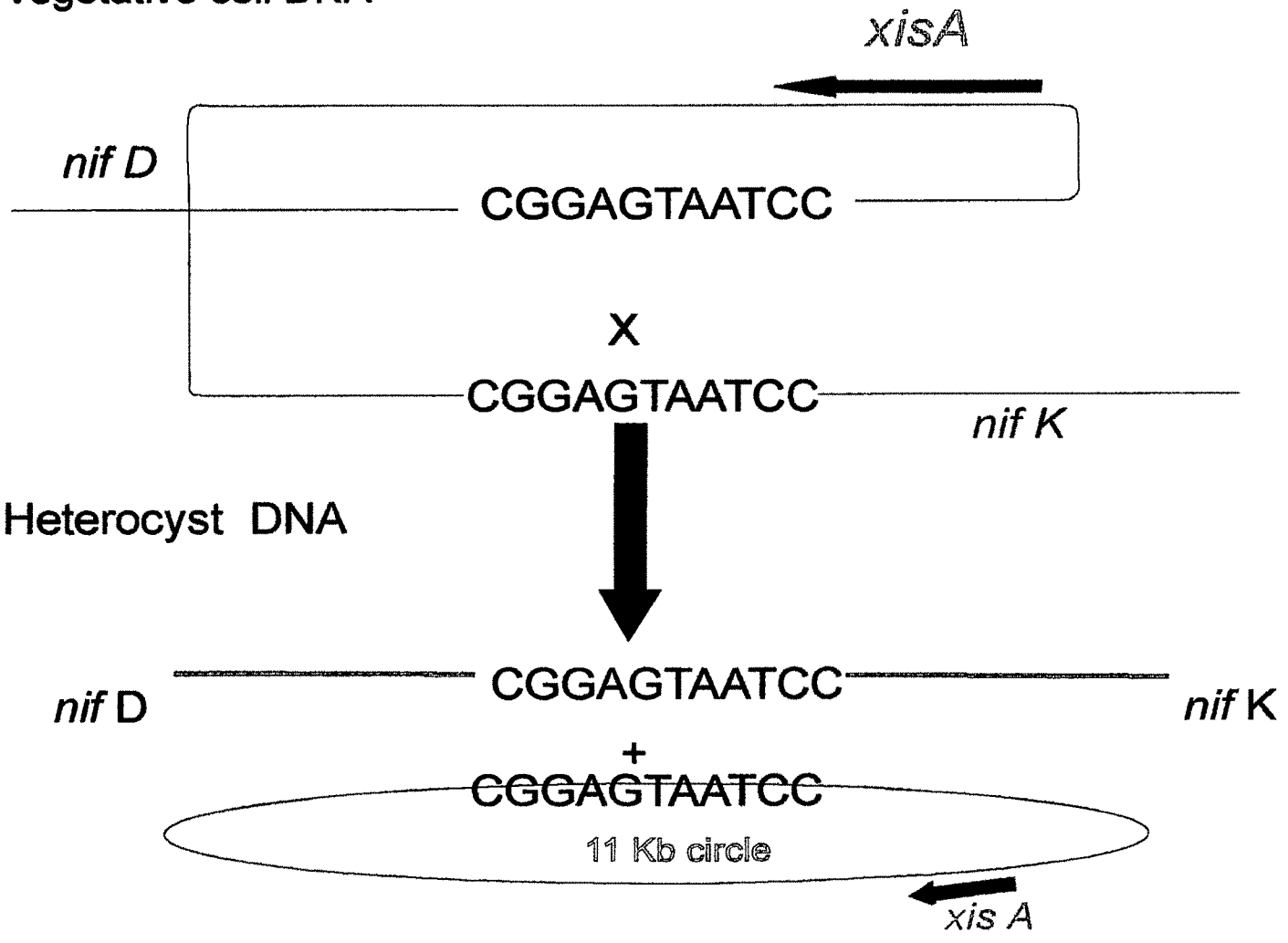


Table 1.3 : DNA Rearrangements in *Anabaena* PCC 7120.

NAME OF THE ELEMENT	DISRUPTED OPERON	SIZE (Kb)	SEQUENCE OF DIRECT REPEAT	EXCISASE	SIMILARITY WITH OTHER PROTEINS
NifD	*nifHDK*	11	GGATTACTCCG	XisA	Integrase family
fdxN	*nifB-fdxN-nifS-nifU*	55	TATTC	XisF	SpoIV CA of *B. subtilis*
HupL	*hupL*	16	CACAGCAGTTATAT	XisC	Integrase family

Neither transcript nor protein products of the genes encoding the excisases are detected so far in *Anabaena*. The exact transcription start sites of these genes are not known and the functional promoter is not yet mapped (Brusca *et al.*, 1990; Carrasco *et al.*, 1994). The only way to measure the activity has been to screen for the respective gene rearrangements. Based on these studies, the expression of this genes occurs late during heterocyst development (Haselkorn, 1992; Carrasco *et al.*, 1994). All the three excisases are developmentally regulated *i.e.* only expressed in the heterocysts.

The deletion of the 55 kb element results in the formation of the *fdxN* ORF and presumably allows for the expression of the downstream genes in the *nifB-fdxN-nifS-nifU* operon by the *nifB* promoter (Mulligan *et al.*, 1988). Excision of *fdxN* occurs independently form the 11kb *nifD* element excision (Carrasco *et al.*, 1994;; Golden and Wiest, 1988; Golden *et al.*, 1987). The presence of the 11 and 55kb elements in *Nostoc* Mac, *Anabaena cylindrica* and *Anabaena* M131are also reported (Carrasco and Golden, 1995). The Marine cyanobacterium *Anabaena* CA lacks both the elements (Carrasco and Golden, 1995).

The predicted HupL polypeptide is homologous to the large subunit of [NiFe] uptake hydrogenase, *hupL* is expressed in a manner similar to other nitrogen-fixation

genes; *hupL* message was detected only during late stages of heterocyst differentiation. An ORF, named *xisC* was identified near one end of the *hupL* DNA element is presumed to encode element's site-specific recombinase.

A 1.5kb ORF of XisC was identified 115bp inside the right border of the *hupL* element. Comparison with Genbank sequences identified the homology with a single protein XisA. XisA protein sequence that was reported earlier (Lammers *et al*, 1986) has been corrected (Nunes-Duby *et al.*, 1998). Although XisC is 25% larger than the XisA, the amino acid sequence can be aligned along its full length with XisC. The sequence is 61% similar and 43% identical and they appear to belong to Intergrase family (Nunes-Duby *et al.*, 1998). The transcription of *xisA/xisC* genes has been difficult to study because of their low levels of expression and transcription start site has not been identified so far for both genes. However, two *Anabaena* PCC 7120 DNA binding proteins NtcA and factor2 has been shown to bind the sequence upstream of *xisA* gene. The NtcA binding sites are not present in the upstream to *xisC*. *Anabaena* PCC 7120 uptake hydrogenase is required to improve the efficiency of nitrogen-fixation. *Anabaena* PCC 7120 *xisF* recombinase, which is required for excision of *fdxN* element, belongs to the resolvase family and is homologous to *Bacillus subtilis* SpoIVCA site-specific recombinase. The *hupL* may differ from the *nifD* and *fdxN* rearrangement in that failure to produce hydrogenase would not be expected to block nitrogen fixation in heterocyst nor growth on nitrogen free medium but could only decrease the efficiency of nitrogen fixation (Carrasco *et al.*, 1995).

1.4 : Excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli.*

Proper rearrangement of the *nifD* element of *Anabaena* PCC 7120 has been demonstrated in *E. coli* with plasmids containing the entire *nifD* element (Lammers *et al.*,1986). Insertional inactivation of the *xisA* gene of these plasmids abolishes

the rearrangement and this suggested that *xisA* is necessary for the excision (Golden and Weist, 1988). 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* PCC 7120 open reading frame larger than 180bp. pAM461 does not rearrange in the absence of a complementary plasmid expressing the *xisA* gene. Thus, XisA is sufficient for excision in *E. coli*. Toxic effects of XisA hindered the utility of *E. coli* to understand the mechanism of excision of *nifD* element including the nature of XisA protein (Details are given in Chapter 4).

1.5 : Evolution of the *nifD* element.

Brusca *et al.* (1990) constructed a mutant *Anabaena* PCC 7120, lacking the 11 kb element. This mutant grows normally as wild type strain and the ratio of rearranged and unrearranged chromosomes in *Anabaena* PCC 7120 remains constant whether it is expressing *xisA* gene product at low or higher levels. This result may be due to integration of the excised the *nifD* element in a reverse process. However, there is no evidence for the replication of the excised circle in vegetative cells. If the excision is reversible, heterocysts may contain accessory factors that prevents the integration of the excised element, since the element is completely excised in heterocysts (Haselkorn, 1992).

Moderately thermophilic heterocystous cyanobacterium *Fischerella ATCC* 27929 (PCC 7115) has a contiguous *nifD* organization (Saville *et al.*, 1987) and the dominant symbiont (*Anabaena azollae)* in the water Fern *Azolla caroliniana* also lacks the *nifD* element (Meeks *et al*, 1988). In contrast, cyanobacteria cultured from *Azolla caroliniana* contains the *nifD* element (Meeks *et al*, 1988). *Anabaena torulosa* contains sequences similar to that of *Anabaena* PCC 7120 (Apte and Thomas, 1987). *Anabaena variabilis* contains the *nifD* element but does not contain

fact that nitrogen fixation in heterocystous cyanobacteria does not require the presence of *nifD* element, 55kb element located in the *fdxN* is not found in many heterocystous cyanobacteria (Haselkorn, 1989). It has been suggested that these elements may have evolved from a cyanophage.

1.6 Objectives of the present work :

Proper excision of *nifD* element in *E. coli*, albeit at a lesser efficiency, allows to study the details of the excision. Present study was directed towards understanding the following aspects of the excision of *nifD* element of *Anabaena* PCC 7120 using the *E. coli* model system. Is the excision of *nifD* element in *E. coli* is mediated by any accessory factors ? Is the toxicity of XisA related to excision activity, which involves site specific endonuclease digestion and ligation ? Would it be possible to increase the excision frequency by increasing the expression of *xisA* gene in *E. coli* ? Could the expression of *xisA* promoter be monitored in *E. coli* ?

Accordingly the work presented here deals with the following –

(i) In order to understand the role of accessory factors, excision of the *nifD* element of *Anabaena* PCC 7120 was monitored in different *E. coli* strains under various media and culture conditions.

(ii) Role of NifA protein of *Klebsiella pneumoniae* in the excision of the *nifD* element of *Anabaena* PCC 7120 was monitored in *E. coli*.

(iii) To study the role of *ntrA* of *E. coli* on the excision of the *nifD* element of *Anabaena* PCC 7120 was monitored in *ntrA* mutant of *E. coli*.

(iv) In order to understand the toxic effects of XisA, stability of plasmids containing direct repeats of the XisA protein was tested in *E. coli*.

(v) The expression of the *xisA* promoter was studied using a transcriptional expression vector.