Chapter 1

Introduction

1.1 General Introduction

Cyanobacteria are phylum of bacteria that obtain their energy from photosynthesis (Brock, 1973). They are often referred as blue-green algae, even though it is now known that they are not algae. Nonetheless, description is still sometimes used to reflect their appearance and ecological role. Fossil traces of cyanobacteria are claimed to have been found from around 3.8 billion years ago, but recent evidence has sparked controversy over this assertion. Cyanobacteria played a dominant role in fixing carbon dioxide into sugars. Cyanobacteria are now one of the largest and most important groups of bacteria on earth. They are supposed to have contributed significantly to oxygenation of primitive earth environment (Schopf, 1975). Cyanobacteria are the only diazotrophs which perform oxygenic photosynthesis and 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 are a morphologically diverse but phylogenetically cohesive group of gram-negative eubacteria (Haselkorn, 1978; Rippka *et al.*, 1979; Wolk, 1982; Apte, 1992; Fay, 1992; Adams and Duggan, 1999). All cyanoacteria are photoautotrophic, using ATP and NADPH generated in the light reactions for autotrophic CO_2 assimilation by enzymes of the Calvin-Benson, or reductive pentose phosphate, cycle. They synthesize glycogen as the storage material in light which is subsequently catabolised to provide mainteneance energy during the dark periods of natural day-night cycles. Cyanobacteria include unicellular, colonial and filamentous forms (Rippka *et al.*, 1979). Some filamentous cyanophytes form differentiated cells, called *heterocysts*, which are specialized for nitrogen fixation, and resting or spore cells called *akinetes*. Each individual cell typically has a thick, gelatinous cell wall, which stains gram-negative. The cyanophytes lack flagella, but may move about by gliding

along surfaces. Most are found in fresh water, while others are marine, occur in damp soil, or even temporarily moistened rocks in deserts. Some live in the fur of sloths, providing a form of camouflage.

Photosynthesis in cyanobacteria generally use water as an electron donor and produce oxygen as a by-product. In most forms the photosynthetic machinery is embedded into folds of the cell membrane, called thylakoids. The large amounts of oxygen in the atmosphere are considered to have been first created by the activities of ancient cyanobacteria. Due to their abilities to fix nitrogen in aerobic conditions they are often found as symbionts with a number of other groups of organisms as fungi (lichens), corals, pteridophytes (Azolla), angiosperms (Gunnera) etc.

Cyanobacteria are the only group of organisms that are able to reduce nitrogen and carbon in aerobic conditions, a fact that may be responsible for their evolutionary and ecological success. The water-oxidizing photosynthesis is accomplished by coupling the activity of photosystem (PS) II and I. They are also able to use in anaerobic conditions only PS I—cyclic photophosphorylation—with electron donors other than water (hydrogen sulfide, thiosulphate, or even molecular hydrogen) just like purple photosynthetic bacteria. Furthermore, they share an archaebacterial property - the ability to reduce elemental sulfur by anaerobic respiration in the dark. Perhaps the most intriguing thing about these organisms is that their photosynthetic electron transport shares the same compartment as the components of respiratory electron transport. Actually, their plasma membrane contains only components of the respiratory chain, while the thylakoid membrane hosts both respiratory and photosynthetic electron transport.

Phycobilisomes which are attached to thylakoid memebrane, act as light harvesting antennae for photosystem II. The phycobilins present in phycobilosomes are responsible for the blue-green pigmentation of most cyanobacteria. Variations to this theme is mainly due to carotenoids and phycoerythrins which give the cells the red-brownish coloration. The color of light influences the composition of phycobilisomes, in turn to cynobacteria. In green light, the cells accumulate more phycoerythrin, whereas in red light they produce more phycocyanin. Thus the bacteria appear green in red light and red in green light.

Cyanobacteria colour is a process, which is known as complementary chromatic adaptation and its a way for the cells maximize the use of available light for photosynthesis.

A few genera, however, lack phycobilins and have chlorophyll *b* as well as chlorophyll *a*, giving them a bright green colour. These were originally grouped together as the prochlorophytes or chloroxybacteria, but appear to have developed in several different lines of cyanobacteria.

Chloroplasts found in eukaryotes (algae and higher plants) most likely represent reduced endosymbiotic cyanobacteria (Walsby, 1986). This endosymbiotic theory is supported by various structural and genetic similarities. Primary chloroplasts are found among the green plants, where they contain chlorophyll *b*, and among the red algae and glaucophytes, where they contain phycobilins. It now appears that these chloroplasts probably had a single origin. Other algae likely took their chloroplasts from these forms by secondary endosymbiosis or ingestion.

The cyanobacteria were traditionally classified by morphology into five sections, referred to by the numerals I-V. The first three - Chroococcales, Pleurocapsales, and Oscillatoriales - are not supported by phylogenetic studies. However, the latter two - Nostocales and Stigonematales - are monophyletic and make up the heterocystous cyanobacteria.

Most taxa included in the phylum or division Cyanobacteria have not been validly published under the Bacteriological Code. Except:

- The classes Chroobacteria, Hormogoneae and Gloeobacteria
- The orders Chroococcales, Gloeobacterales, Nostocales, Oscillatoriales, Pleurocapsales and Stigonematales
- The families Prochloraceae and Prochlorotrichaceae
- The genera Halospirulina, Planktothricoides, Prochlorococcus, Prochloron, Prochlorothrix.

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 was the first photosynthetic organism whose genome was completely sequenced (in 1996, by the Kazusa Research Institute, Japan). It continues to be an important model organism. (Retrieved from "http://en.wikipedia.org/wiki/Cyanobacteria")

1.2 Cyanobacteria are important in the nitrogen cycle

Nitrogen being an important constituent of living system, its role in environment is maintained by nitrogen cycle. The conversion of atmospheric dinitrogen into ammonia by symbiotic, associative and free-living bacteria - is of tremendous importance to the environment and to the agriculture throughout the world. Loss of nitrogen in the process of denitrification was replenishes by the nitrogen fixation. Availability of fixed nitrogen is frequently the limiting factor for crop productivity; making demands on global agriculture to provide food security as the world's population increase in the coming years. Human interference in the nitrogen cycle has prompted concerns regarding the increased emissions of nitrogen oxides, soil acidification and water eutrophication. The fixed nitrogen that is provided by biological nitrogen fixation is less prone to leaching and volatilization as it is utilized *in situ* and therefore the biological process contributes an important and sustainable input into agriculture (Capone, 2001). The ability to fix nitrogen is found in most bacterial phylogenetic groups, including green sulphur bacteria, Fimbribacteria, actinomycetes, cyanobacteria and all subdivisions of the Proteobacteria. Methanogen in group of Archea can only fix nitrogen. The ability to fix nitrogen is compatible with a wide range of physiologies includes aerobic (*Azotobacter*), facultatively anaerobic (*Klebsiella*), anaerobic heterotroph (*Clostridium*), anoxygenic (*Rhodobacter*) or oxygenic (*Anabaena*) phototrophs and chemolithotrophs (*Leptospirillum ferrooxidans*).

Cyanobacteria are very important organisms for the health and growth of many plants. In the cultivation of rice, the floating fern *Azolla* is actively distributed among the rice paddies. The fern houses colonies of the cyanobacterium *Anabaena* in its leaves, where it fixes nitrogen. The ferns then provide an inexpensive natural fertilizer and nitrogen source for the rice plants when they die at the end of the season. Cyanobacteria also form symbiotic relationships with many fungi, forming complex symbiotic "organisms" known as lichens .

1.3 Nitrogen assimilation and nitrogen control in cyanobacteria

Many cyanobacteria can assimilate atmospheric nitrogen and have the capability to assimilate some amino acids, particularly arginine and glutamine (Flores and Herrero, 1994). The assimilation of most of these compounds provides intracellular ammonium, which is a preferred nitrogen source. Thus, in the presence of ammonium, the genes encoding permeases and enzymes for the assimilation of nitrogen sources alternative to ammonium are repressed, a process known as 'nitrogen control'.

1.3.1 Mechanism of assimilation of combined nitrogen

Incorporation of nitrogen-containing compounds, which are frequently found at low concentrations (e.g. below 1μM) in the environment, takes place through permease that are located in the cytoplasmic membrane. Multicomponent ATP binding cassette (ABC)-type transporters have been shown to be involved in the uptake of nitrate, nitrite (Omata *et al.,* 1993; Luque *et al.,* 1994) and urea (Valladares *et al.,* 2002) in a number of cyanobacteria. ABC-type permeases are also required for the transport of arginine and glutamine (Quintero *et al.,* 2001). These permeases use ATP to drive an active, concentrative transport of their substrates. On the other hand, a secondary transporter of the major facilitator superfamily has been identified as the nitrate-nitrite transporter in some marine cyanobacteria (Sakamoto *et al.,* 1999). The transport of ammonium is also mediated by secondary permeases, in this case of the Amt family (Montensionos *et al.,* 1998; Vazques-Bermudez *et al.,* 2002). Amt permeases were probed with (^{14}C) methylammonium in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 revealed a membrane potential-driven transport (Vazques-Bermudez *et al.,* 2002).

Intracellular nitrate is sequentially reduced to nitrite and ammonium by nitrate reductase and nitrite reductase, which are the products of the *narB* and *nir* genes respectively (Luque *et al.,* 1993; Rubio *et al.,* 1996). Cyanobacterial nitrate reductase is homologous with Mo-containing bacterial oxidoreductases but is unique in that it uses ferredoxin as an electron donor, forming tight 1:1 complexes (Hirasava *et al.,* 2004). The Mo cofactor is of the Mo-*bis*-molybdopterin guanine dinucleotide type (Rubio *et al.,* 1998; 1999; 2002) and the

enzyme also contains a [4Fe-4S] cluster (Jepson *et al.,* 2004). In this enzyme system, electrons flow from reduced ferredoxin to the iron–sulphur cluster and then to the Mo cofactor, where nitrate is reduced to nitrite. Cyanobacterial nitrite reductase is homologous with ferredoxin-dependent higher-plant nitrite reductase and contains a [4Fe-4S] cluster and sirohaem as prosthetic groups (Luque *et al.,* 1993; Knaff, 1996). Electrons from reduced ferredoxin are transferred to the iron–sulphur cluster and then to sirohaem, where nitrite is reduced to ammonium. The *narB* and *nir* genes are clustered together with the nitrate/nitrite permease-encoding genes in numerous cyanobacteria forming an operon with the structure *nir*-permease genes-*narB*. High conservation of this gene arrangement, in which the expression level is higher for the upstream than for the downstream genes in the operon (Fryas *et al.,* 1997), suggests that it ensures the production of a balanced amount of the different proteins of the pathway.

Regarding organic sources of nitrogen used by cyanobacteria, urea is degraded to ammonium and CO_2 by bacterial Ni^{2+}-dependent urease (Valladares *et al.,* 2002), whereas arginine is catabolized by an unusual pathway that combines the urea cycle and the arginase pathway rendering ammonium and glutamate as final products (Quinterno *et al.,* 2000).

Nitrogen source used for growth, intracellular ammonium is incorporated into carbon skeletons through the glutamine synthetase–glutamate synthase pathway (reviewed in Flores and Herrero, 1994). In cyanobacteria, lack 2-oxoglutarate dehydrogenase, the main metabolic role of 2-oxoglutarate is in the incorporation of nitrogen (Vasquez-Bermudez *et al.,* 2000) and 2-oxoglutarate is an indicator of the C to N ratio of the cells (Muro-Pastor *et al.,* 2001).

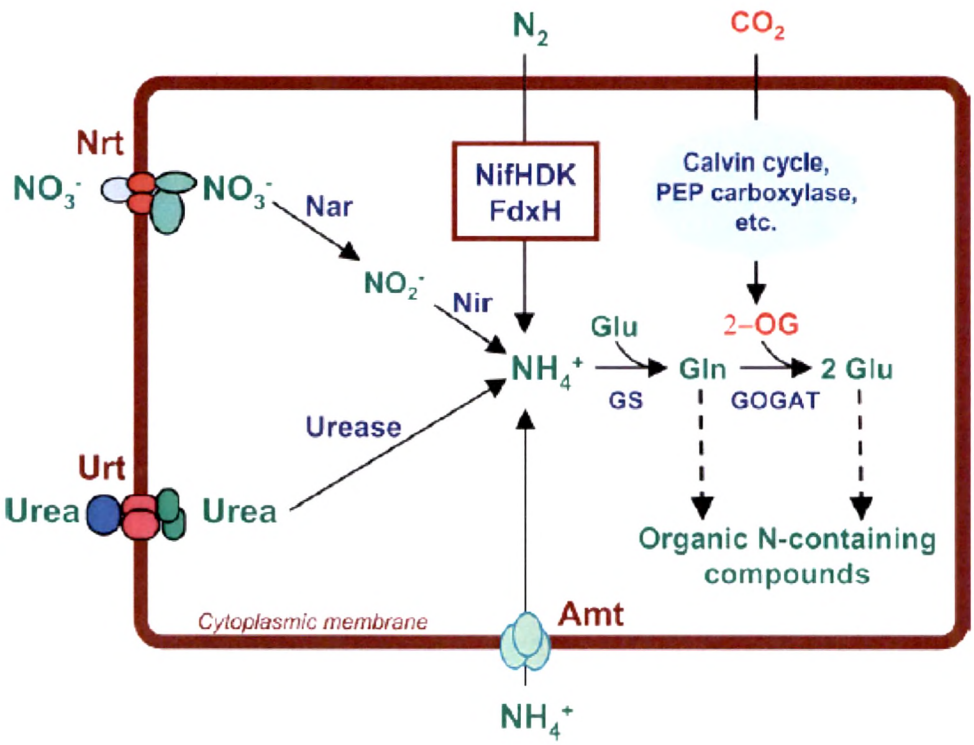

Fig. 1 Main nitrogen assimilation pathways in cyanobacteria (Flores and Herrero, 2005). Nrt, ABC-type nitrate/nitrite transporter; Urt, ABC-type urea transporter; Amt, ammonium permease; Nar, nitrate reductase; Nir, nitrite reductase; NifHDK, nitrogenase complex; FdxH, heterocyst-specific ferredoxin; PEP carboxylase, phosphoenolpyruvate carboxylase; 2-OG, 2-oxoglutarate; GS, glutamine synthetase; GOGAT, glutamate synthase.

Figure 1 presents a scheme of the main nitrogen assimilation pathways that can be found in cyanobacteria. The scheme highlights the production of intracellular ammonium during the assimilation of different nitrogen sources and the role of 2-oxoglutarate as the C-skeleton for the incorporation of nitrogen into organic material. However, not all these pathways are present at the same time in a cyanobacterial cell, their expression being strictly regulated by the nitrogen source and also by the availability of carbon.

1.3.2 Nitrogen control

PII

Advances in the sequencing of genomes discovered that PII proteins are represented in all three domains of life found in almost all free-living bacteria, archaea, eukaryotic algae and plants. It becomes evident that these proteins play central roles in microbial nitrogen control (Forchammer, 2004). PII family can be classified into three closely related subgroups, the products of genes *glnB*, *glnK* and *nifI* (Arcondeguy *et al.,* 2001). Effect of light qualities on phosphorylation of protein revealed a 12.5 kDA protein in unicellular cyanobacterium *Synechococcus elongates* strain PCC6301. Its N terminus has high homology to the N-terminus of the GlnB form *E. coli* (Haririson *et al.,*1990). Later on *glnB* homologue was detected in the related cyanobacteria *S. elongates* strain PCC 7942 (Tsinoremas *et al.,* 1991). Occurrence of *glnB* homologues was screened by southern blot hybridization in other cyanobacterial strains. In all cases of cyanobacteria, a hybridization signal corresponding to one homologue was detected (Tsinoremas *et al.,* 1991). GlnB were subsequently cloned form the unicellular strain *Synechocystis* sp. PCC 6803 (Garcia-Dominguez and Florencio, 1997) and from the filamentous heterocystous strains *N. punctiforme* (Hanson *et al.,* 1998) and *Anabeana* sp. PCC 7120 (Gonzalez *et al.,* 2000).

In cyanobacteria, a transcriptional regulator NtcA functions as a global nitrogen controller while PII senses the C/N balance of the cells by a mechanism different form that of enterobacteria, and acts on the regulation of processes not previously recognized as being influenced by PII in any other organism (Fig. 2). These include the Nrt-dependent transport of nitrate and nitrite, the NtcA mediated transcription activation under nitrogen stress and ArgB, a N-acetylglutamate kinase, the enzyme phosphorylate N-acetylglutamic acid, an important role in biosynthesis arginine (Heinrich *et al.,* 2004). ArgB regulation by PII demonstrate that the pathway for the biosynthesis of arginine, an excellent nitrogen donor molecule in cyanobacteria and regulated by the C/N equilibrium of the cell. Regulation of an amino acid biosynthetic pathway by C/N equilibrium is a remarkable discovery for understanding the global co-ordination of cyanobacterial metabolism. The cyanobacterial signal transduction PII protein (*glnB* gene product), binds ATP and 2-oxoglutarate and is phosphorylated when the cells experience a high C to N ratio, it having been suggested that it

operates as a 2-oxoglutarate sensor (reviewed in Forchhammer, 2004). The PII protein is required for the activation of NtcA dependent genes specifically under conditions of nitrogen stress (Paz-Yepes *et al.*, 2003; Aldehni *et al.*, 2003), and it appears to promote gene expression more efficiently in its phosphorylated form (Paz-Yepes *et al.*, 2003). Therefore 2-oxoglutarate may affect NtcA activity directly and indirectly through PII. *In vivo* positive effects of 2-oxoglutarate on the expression of nitrogen-regulated genes have been observed in an engineered *Synechococcus* strain bearing the *E. coli* 2-oxoglutarate permease (Vazquez-Bermudez *et al.*, 2003).

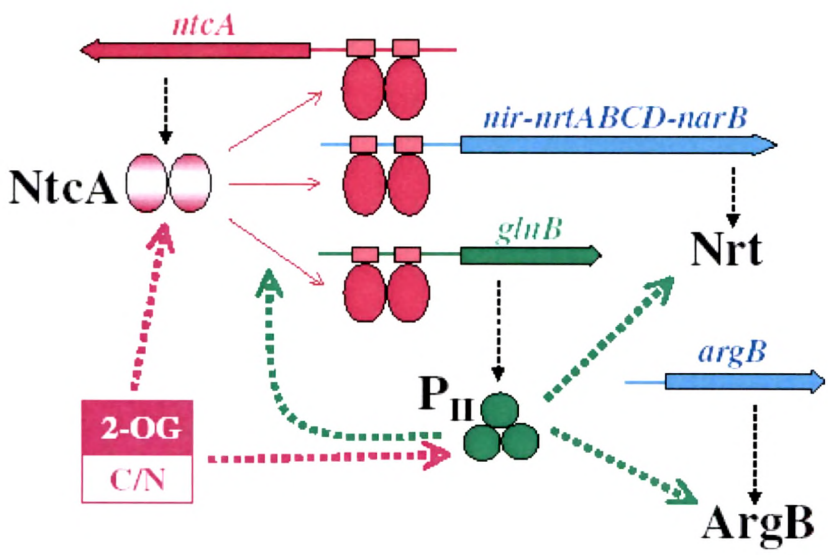

Fig. 2 Nitrogen regulation of cyanobacteria: Interconnections between the regulatory proteins NtcA and PII (Herrero, 2004).

NtcA

In presence of limiting concentration of ammonium with an adequate supply of carbon, cyanobacterial cells sense a high C to N ratio that determines expression of genes encoding permeases and enzymes required for an efficient assimilation of ammonium or for the assimilation of alternative nitrogen sources. This activation of gene expression requires NtcA (Vega-Palas *et al.,* 1990), a transcriptional regulator that belongs to the family of bacterial transcription factors whose best-known representative is catabolite activator protein (CAP) of *E. coli* (Vega-Palas *et al.,* 1992). NtcA is transcription factor found in various species of cyanobacteria (Frias *et al.,* 1993; Wei *et al.,* 1993; Lindell *et al.,* 1998) Modeling of NtcA based on the CAP structure and preliminary X-ray structural data confirms that NtcA bears a DNA-binding helix–turn–helix motif in its C-terminal domain (Wisen *et al.,* 2004). NtcA binds to specific sites in the promoters of the regulated genes activating their transcription (Luque *et al.,* 1994). The DNA-binding site of NtcA shows the sequence signature $GTAN_8TAC$ that is usually found 22 nt upstream from a promoter -10 box (Luque *et al.,* 1994). This promoter structure for NtcA-activated genes, which conforms to the structure of bacterial class II promoters, has been found in most cyanobacterial nitrogen-regulated genes investigated to date (reviewed in Herrero *et al.,* 2001).

The *ntcA* gene is autoregulatory and the NtcA protein appears to be subjected to regulation being activated when the C to N ratio of the cell is high (Luque *et al.,*2004). Although the precise mechanism of modulation of NtcA is still not known, two elements that influence NtcA activity have recently been identified: 2-oxoglutarate and the PII protein. Binding of NtcA to some N-regulated promoters is stimulated by 2-oxoglutarate (Tanigawa *et al.,* 2002; Vazquez-Bermudez *et al.,* 2002), suggesting that this metabolite can represent an allosteric effector for NtcA.

Some NtcA-dependent promoters appear to require additional regulators or transcription factors for proper operation. This is the case of the *Anabaena nir* operon promoter that requires binding of NtcB (a LysR-family transcription factor) in addition to binding of NtcA (Frias *et al.,* 2000). Use of this promoter is also dependent on CnaT, a

putative glycosyl transferase that influences transcription through a mechanism that is yet to be worked out (Frias *et al.,* 2003).

In addition to the regulation of gene expression, some elements of the nitrogen assimilation system are subjected to post-translational regulation in cyanobacteria. The nitrate/nitrite permease is a target of regulation, being inhibited when the cells sense a high N to C ratio. The non-phosphorylated form of the PII protein appears to effect specific inhibition of the permease when the cells are incubated under conditions that determine low 2-oxoglutarate levels (Lee *et al.,* 1998; 2000). Additionally, in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, glutamine synthetase is subjected to ammonium-promoted inactivation by two small protein factors whose expression is repressed by NtcA when the cells are incubated in the absence of ammonium (Garcýa-Domýnguez *et al.,* 1999; 2000). Moreover, NtcA also participates in the activation of expression of genes whose products are required during the differentiation process or are active in the mature heterocyst. In the mature heterocyst, the *glnA* gene encoding glutamine synthetase, *petH* encoding ferredoxin:$NADP^+$ reductase and the *nifHDK* operon encoding the nitrogenase complex are also expressed from NtcA-activated promoters (Valladares *et al.,* 1999). It has been recently shown that genes for the metabolism of cyanophycin, a nitrogen reserve made of aspartate and arginine that accumulates conspicuously in the heterocysts, are expressed from multiple promoters also including NtcA-dependent promoters (Picossi *et al.,* 2004).

Some of the heterocyst-related NtcA-dependent promoters do not present the structure of the standard, class II NtcA type promoter, but rather show the structure of bacterial class I promoters with the NtcA-binding site being located ~90 nt upstream from the transcription start point. NtcA effects nitrogen control not only for triggering the developmental process but also for its progression and in the N_2-fixing heterocyst, and does so through the use of different types of NtcA-dependent promoters. NtcA appears to be a global nitrogen regulator that plays an equivalent role in cyanobacteria to that played by NtrC in many other prokaryotes.

The *ntcA* gene has been identified in 11 other cyanobacterial species, and homologous genes have been sequenced from *Synechocystis* strain PCC 6803 and *Anabaena* strain PCC 7120 (Frias *et al.*, 1993). In *Anabaena* spp., NctA was originally identified as factor VF1, later termed BifA, which binds to sites upstream of the *xisA* gene (Chastain *et al.*, 1990; Wei *et al.*, 1993).

In *Anabaena* spp., NtcA-binding sites are present in the *glnA* and *nir* upstream regions and an *ntcA* mutant fails to induce nitrate and nitrite reductase and to express the major *glnA* transcript (RNAI) which is induced under conditions of nitrogen limitation (Frias *et al.*, 1994; Tumer *et al.*,1983). An *ntcA* mutant also fails to induce nitrogenase, but although NtcA (BifA) has been reported to bind weakly *in vitro* to the upstream region of the *nifH* gene, this region does not contain sequences characteristic of NtcA-regulated promoters. Expression of nitrogenase in *Anabaena* spp. requires heterocyst development, which is in turn dependent on expression of the *hetR* gene (Buikema and Haselkorn, 1991). Induction of *hetR* and induction of heterocyst development are both absent in an *ntcA* mutant (Frias *et al.*, 1994) but *hetR* promoters are also not characteristic of NtcA-dependent promoters, suggesting that a gene earlier in the developmental process than *hetR* may be the site of NtcA action. NtcA, like some other transcriptional regulators of the Crp family, may also be able to act as a repressor, e.g., at the promoter of the *rbcLS* operon (Ramasubramanian *et al.*,1994). RbcL encodes a subunit of ribulose-1,5-bisphosphate carboxylase which is involved in carbon fixation and therefore expressed only in vegetative cells. To date, no sensor protein that interacts with NtcA has been identified, and although the involvement of a PII protein was postulated (Vega-Palas *et al.*, 1992), the phenotype of a *glnB* mutant in *Synechococcus* strain PCC 7942 is quite distinct from that of an *ntcA* mutant (Forchhammer and Tandeau de Marsac, 1995). If PII were absolutely required for the activation of NtcA, the phenotypes should be similar, indicating that at least for the regulation of NtcA activity, PII is dispensable. Thus, there may exists several branched signaling pathways originating from the primary nitrogen sensor to control nitrogen assimilation at different levels in cyanobacteria, and the primary sensor has still to be identified.

Two further nitrogen-regulated genes, *nirB* and *ntcB*, have recently been found in *Synechococcus* strain PCC 7942, in which they appear to constitute an operon (*nirB ntcB*)

transcribed divergently from *nirA* (Suzuki *et al.,* 1995). Transcription of *nirB ntcB* is elevated under conditions of nitrogen limitation and is NtcA dependent. The deduced protein sequence of NirB shows no similarities to known proteins, but a *nirB* mutant decreases nitrite reductase activity and excretes nitrite into the medium, suggesting that it is in some way required for maximum nitrite reductase activity. The deduced protein sequence of NtcB shows it to be a member of the LysR family of transcriptional activators, and the growth rate of an *ntcB* mutant is reduced during growth on nitrate or nitrite but not on ammonium. However, the precise role of NtcB in *Synechococcus* nitrogen metabolism is unclear.

1.4 Nitrogen fixation and heterocyst formation

A developmental pattern of single heterocyst separated by approximately ten vegetative cells is established to form a multicellular organism composed of two interdependent cell types. Heterocyst differentiation must require global changes in gene expression (Wolk *et al.,* 1994; Wolk, 2000). During formation of heterocyst from a vegetative cell, major structural and biochemical changes occurs that affect nitrogen fixation (Bohme, 1998). Upon nitrogen deprivation phycobili proteins are broken down. At the same time, around the outer membrane of the heterocyst a double layered envelope is formed, which decreases the diffusion of oxygen. Vegetative cells are connected through a pore, equipped with microplasmodesmata. Heterocyst imports carbohydrates, this act as reductant and energy sources for nitrogen fixation, and in turn exports glutamine. Changes in the thylakoid structure of heterocyst are associated with degradation of photosystem II that lacks oxygen evolving activity, and rubisco (the main enzyme complex responsible for CO_2 fixation). Therefore, reductant is almost exclusively channeled to the reduction of nitrogen to ammonia, which in turn reacts with glutamate derived form the imported carbohydrates. Both oxygen and nitrogen diffuse into the cells, but increased respiratory activity in membranes near to the polar ends of heterocyst depletes the oxygen concentration. Hydrogen produced by nitrogenase reaction feeds into an uptake hydrogenase system, which is induced upon heterocyst formation. This reacts with oxygen to produce water, contributing to the ATP pool required for biosynthetic reactions such as nitrogen fixation (Wolk *et al.,* 1994) Fig. 3.

Heterocysts have a unique structure and physiology (Fay, 1992); they obtain photosynthate, probably sucrose (Curatti *et al.,* 2002), form nearby vegetative cells and, in return, supply those cells with fixed nitrogen as amino acids. Metabolites and signals must be exchanged between heterocyst and vegetative cells along the filament to support growth and regulate the developmental pattern of *Anabaena* PCC 7120. The machinery for transport of these molecules is not well understood, but it has been proposed that the continuous periplasm might function as a means along the filament. Interestingly, heterocyst pattern and the physiology of the cyanobacterial partner are notably altered in symbioses with plants (Meeks and Elhai, 2002) Fig. 4.

The regulation of heterocyst development involves a response to the external signal of nitrogen deprivation, uncharacterized internal cues related to physiology and possible the cell cycle, and intercellular communication between cells along filaments (Wolk *et al.,* 1994; Meeks and Elhai, 2002). During heterocyst development, global changes in gene expression must occur, this regulation is not well understood and there is a conspicuous lack of identified transcription factors (Wolk *et al.,* 1994; Meeks and Elhai, 2002). However, the expression pattern of several genes has been studied and a few have been placed into an ordered sequence (Wolk *et al.,* 1994; Cai and Wolk, 1997 and Wolk, 2000). The activity of *Anabaena* promoters appears to be confined either to vegetative cells or to heterocyst, with genes active in both cell types possessing multiple promoters (Tumer *et al.,* 1983; Elhai and Wolk, 1990; Black *et al.,* 1993; Ramasubrmanian *et al.,* 1996). The regulation of nitrogen-fixation (*nif*) genes in *Anabaena* PCC7120 is tightly coupled to the development of heterocyst (Elhai and Wolk, 1990). However, a clear consensus *nif* promoter has not been identified.

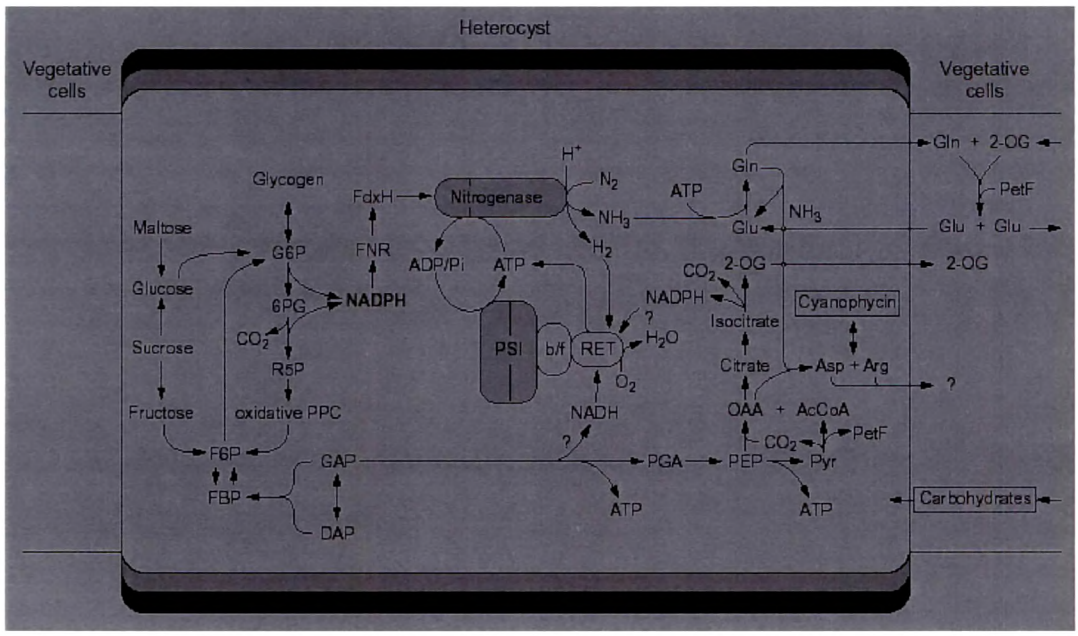

Fig. 3 Heterocyst metabolism and nitrogen fixation. AcCoA, acetyl coenzyme A; Arg, arginine; Asp, aspartate; b/f, cytochrome b6f complex; F6P, fructose 6-phosphate; PetF, vegetative cell type ferredoxin; Glu, glutamate; Gln, glutamine; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; 6PG, 6-phosphogluconate; PGA, 3-phosphoglycerate; Pi, inorganic phosphate; R5P, ribose 5-phosphate. (Trends in Plant Science. **3:**346-351.1998).

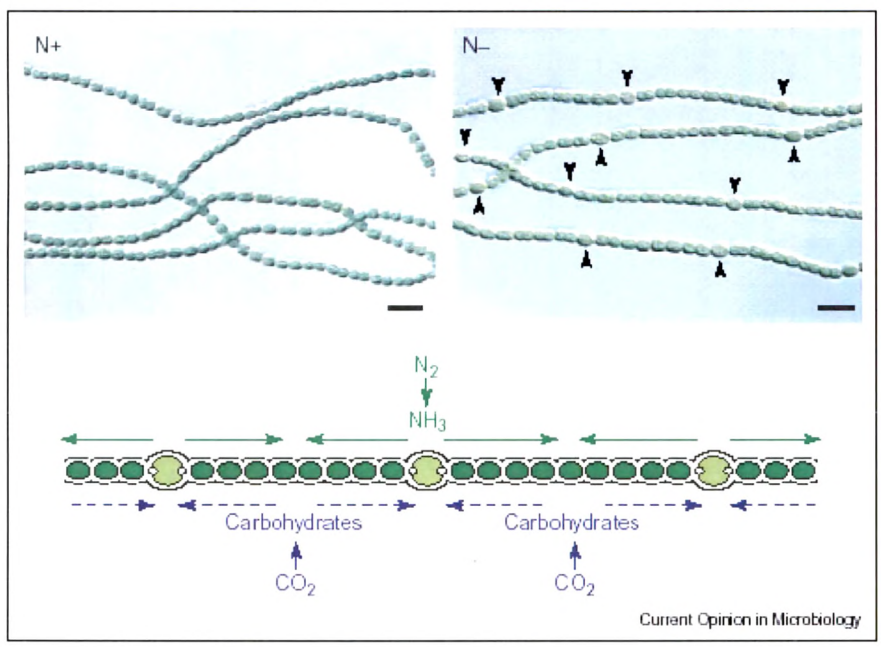

Fig. 4 The filamentous heterocystous cyanobacterium *Anabaena* PCC 7120 grown with (N+) or without (N-) combined nitrogen (Golden and Yoon, 2003). Heterocysts are indicated by arrowheads.

1.5 Role of NtcA in heterocyst formation

The *Anabaena* PCC 7120 *ntcA* gene is required for the full activation of genes that are involved in nitrogen metabolism, as well as the regulation of several other genes, and is required for the early stages of heterocyst development (Herrero *et al.*, 2001). NtcA has been shown to mediate nitrogen control in response to 2-oxogluarate levels. A combination of genetic and physiological studies in *Synechococcus* PCC 6803 indicates that nitrogen status is perceived via 2-oxoglutarate levels (Muro-Pastor *et al.*, 2001). It has then shown that binding of NtcA to *ntcA* and *glnA* promoters of *Synechococcus* PCC 7942 is enhanced by 2-oxoglutarate in vitro, and more importantly, that in vitro transcriptional activation of these promoters by NtcA requires 2-oxoglutarate (Tanigawa *et al.*, 2002). The increased binding affinity for NtcA on the *Synechococcus* PCC 7942 *glnA* promoter in the presence of 2-oxoglutarate was independently determined (Vazquez-Bermudez *et al.*, 2002).

The number of genes that have been confirmed as regulated by NtcA continues to grow. The *Anabaena* PCC 7120 *devBCA* operon encodes an ABC-type exporter and is essential for heterocyst maturation. NtcA binds to the *devBCA* promoter and *ntcA* is required for the transcription of the operon (Fiedler *et al.*, 2001). The *Anabaena* PCC 7120 *urtABCED* genes encode an ABC-type permease that is required for urea uptake. Expression of the operon is induced under nitrogen-limiting conditions and requires NtcA for full induction (Valladares *et al.*, 2002). NtcA is strictly required for heterocyst development (Frias *et al.*, 1994; Wei *et al*, 1994), apparently linking differentiation to nitrogen deficiency, and is needed for the induction of the key heterocyst development regulator HetR (Frias *et al.*, 1994; Muro-Pastor *et al.*, 2002). In heterocyst expression of ferric uptake regulator (Fur) proteins are principally responsible for maintaining iron homeostasis in prokaryotes. Limiting amount of iron reduces photosynthetic rates and cell growth in cyanobacteria. Levels of furA mRNA and FurA protein increases significantly in response to nitrogen deprivation. NtcA regulate the expression of the *furA* gene .

1.6 Role of HetR protein in regulation of heterocyst development

HetR is key regulator of heterocyst development. *Anabaena* PCC7120 *hetR* mutants fail to produce heterocyst and extra copies of *hetR* on a plasmid cause a multiple contiguous heterocyst (Mch) phenotype. Expression of the *hetR* gene from the copper regulated *petE* promoter leads to heterocyst differentiation under repressing conditions (Buikema and Haselkorn, 2001). The use of a *hetR-luxAB* fusion has shown that *hetR* is expressed in a spatial pattern and is positively autoregulated (Black *et al.,* 1993); upregulation of *hetR* requires *ntcA* (Muro-Pastor et al., 2002) and is inhibited by *hetN* overexpression (Callahan and Buikema, 2001; Li *et al.*,2002). There is a mutual dependence for the increased expression of *hetR* and *ntcA* after nitrogen step-down (Muro-Pastor *et al.,* 2002). HetR protein is an unusual serine-type protease that might show posttranslational modification during development (Zhou *et al.,* 1998a; Zhou *et al.,* 1998b). The *hetR* gene has been isolated and sequenced from different heterocystous and filamentous nonheterocystous cyanobacteria, and was shown to be required for heterocyst development in *A. variabilis* (Schiefer *et al.,* 2002); however, expression of *A. variabilis* Nif2 nitrogenase in vegetative cells under anaerobic conditions did not require *hetR*.

The non-heterocystous nitrogen-fixing cyanobacterium *Trichodesmium* fixes nitrogen in specialized cells called diazocytes. *Trichodesmium* contains several genes that are involved in heterocyst development, including *hetR*, and it has been suggested that *hetR* is required for diazocyte development because this gene shows a diurnal expression pattern that might correspond to diurnal patterns of *nifH* expression and diazocyte differentiation (El-Shehawy *et al.,* 2003).

1.7 Role of *hetF* gene

The *N. punctiforme hetF* gene is required for heterocyst formation and for the normal localized expression of *hetR* (Wong and Meeks, 2001). Similar to the effect seen with *hetR*, extra copies of *hetF* produced a Mch phenotype, but only after nitrogen step-down. The predicted HetF protein shows no significant similarity to other proteins and the *hetF* gene was present in only heterocystous cyanobacteria; it was, therefore, proposed that HetF

somehow restricts *hetR* expression and the accumulation of HetR protein to differentiating heterocysts. HetF absence does not affects the NtcA depentedent expression of *hetR* transcription but it affects transcription dependent on HetR and accumulation of it.

1.8 HetC is required during early development

The gene *hetC* encodes a protein that is similar to bacterial ABC protein exporters and is involved in early regulation of heterocyst differentiation (Khudyakov and Wolk, 1997). HetC mutants cannot form heterocysts but do produce a pattern of weakly fluorescent cells; these are likely to represent an early stage of heterocyst differentiation because they express a *hetR–gfp* reporter but, unlike heterocysts, they divide to produce unusual small cells when filaments are incubated at low light levels for several days (Xu and Wolk, 2001). It was also found that a *hetC–gfp* reporter showed the strongest GFP fluorescence in proheterocysts and heterocyst (Xu and Wolk, 2001).

1.9 *hetL* overexpression stimulates heterocyst formation

Overexpression of the *hetL* gene strongly stimulates heterocyst formation in a *patS*-overexpression strain and in the wild type (Liu and Golden, 2002). The predicted HetL protein is composed almost entirely of pentapeptide repeats (~45) with a consensus of A(D/N)L*X, where * is a polar amino acid; approximately 40 *Anabaena* genes contain this repeat motif. In wild-type *Anabaena, hetL* expression from a heterologous promoter induced Mch development in nitrate- and ammonium-containing medium. *hetL* overexpression causes heterocyst development in an *ntcA* null mutant but not in a *hetR* mutant, indicating that it affects early developmental decisions. A *hetL* null mutant showed normal heterocyst development and diazotrophic growth.

1.10 How pattern of heterocyst regulated

1.10.1 PatS influences the initial heterocyst pattern

The *patS* gene encodes a small peptide inhibitor of heterocyst differentiation and plays an important role in the control of pattern formation (Yoon and Golden, 1998; 2001). It is thought that PatS works by lateral inhibition, in such a way that PatS produced by a differentiating cell inhibits the differentiation of its neighbors to establish a pattern of single heterocysts along chains of vegetative cells. A *patS–gfp* reporter strain revealed pairs and small clusters of *patS*-expressing cells during the early stage of heterocyst differentiation, and PatS signaling is likely to be involved in the resolution of these clusters to single heterocyst (Fig. 5). Differentiating cells were inhibited by PatS during the period of 6–12 hours after heterocyst induction, when groups of differentiating cells began to be resolved to a single proheterocyst. Increased transcription of *patS* during development was found to coincide with expression from a new transcription start-site, 39 bases upstream from the first translation initiation codon. A *patS* mutant grown for several days under nitrogen-fixing conditions showed partial restoration of the normal heterocyst pattern, presumably because of a gradient of nitrogen compounds or other molecules produced by heterocysts.

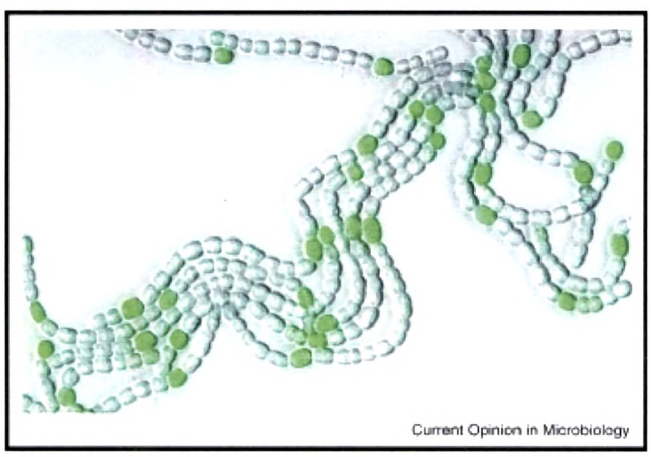

Fig. 5 Filaments of *Anabaena* PCC 7120 containing a plasmid carrying a *patS–gfp* reporter 27 hours after nitrogen step-down (Golden and Yoon, 2003).

1.10.2 Role of HetN in influencing the maintenance of heterocyst pattern

The *hetN* gene was originally proposed to be involved in controlling pattern formation (Black and Wolk, 1994); however, the surrounding locus is required for producing heterocyst glycolipids (Bauer *et al.,* 1997). Recent work shows that *hetN* is expressed primarily in heterocysts and suggests that it is involved in the maintenance of heterocyst pattern by suppressing the formation of new heterocysts adjacent to previously formed heterocyst (Callahan and Buikema, 2001).

1.10.3 Role of Calcium in Heterocyst differentiation

The important role of Ca^{2+} is to acts as intracellular second messenger in prokaryotes, as well as in eukaryotes (Torrecilla *et al.,* 2004). It plays essential role in various physiological processes in prokaryotes that incorporate chemotaxis, motility, pathogenesis, the cell cycle, control of the initiation of replication, quorum sensing, and spore and fruiting body formations.

Ca^{2+} play direct role in signaling in cyanobacteria. *hetR* encodes an unusual serine-type protease that has calcium-modulated activity (Zhou *et al.,* 1998; Dong *et al.,* 2000). Alteration in calcium signal correlated with early arrest of heterocyst differentiation as evidenced by the absence of patterned loss of phycobiliportein fluorescence. Therefore, it can be correlated with calcium transient signaling for proteolysis of phycobiliproteins to supply the amino acid for the heterocyst specific protein synthesis and must play an important role. Calcium specific drug treatments also evidenced that heterocyst differentiation was arrest in early steps. Thus, calcium transient in response to nitrogen deprivation plays a important role in differentiation process.

1.11 Regulation of heterocyst formation during late phase

1.11.1 Heterocyst-specific expression of *patB*

PatB contains an N-terminal domain with two putative 4Fe-4S centers and a C-terminal domain containing a DNA-binding motif. The original frame shift mutation in *patB*, which results in the truncation of the C-terminal domain, showed poor diazotrophic growth and caused filaments to accumulate more heterocysts than normal, resulting in an abnormal multiple contiguous heterocysts (Mch) pattern (Liang *et al.*, 1993). Recent work has shown that a *patB* deletion mutant is completely defective for diazotrophic growth and that a *patB–gfp* reporter fusion shows heterocyst-specific expression at 16 hours after nitrogen step-down (Jones *et al.*, 2003). PatB mutants show normal initial pattern of heterocyst spacing along the filament but later on, it differentiate in to excess heterocyst after several days in absence of combined nitrogen. Similar to the original mutant, a mutant defective in the N-terminal ferredoxin-like domain has Mch phenotype. GFP reporter fusions of PatS and HetR show normal patterns of expression in a *patB* frameshift mutant, thus, indicating that PatB must function downstream of them. Additionally, PatB might be a redox-sensitive transcription factor that is required in the late stages of heterocyst differentiation. A *patB* deletion mutant suffers an almost complete cessation of growth and nitrogen fixation within 24 h of combined nitrogen source removal

1.11.2 HepA and HepK/DevR two-component system

The outermost layer of the heterocyst envelope consists of polysaccharide, and several genes that are required for its synthesis have been identified, including *hepA*. HepA is a component of an ABC transporter and *hepA* expression is dependent on DNA sequences upstream of *hepC*. Four novel DNA-binding proteins were identified that bind to these sequences; inactivation of the genes for two of them, *abp2* and *abp3*, blocked expression of *hepC* and *hepA*, and prevented formation of the heterocyst envelope glycolipid layer and diazotrophic growth (Koksharova and Wolk, 2002).

The *hepK* gene is required for *hepA* expression (Zhu *et al.,* 1998). HepK was predicted to be a sensory protein-histidine kinase (Zhu *et al.,* 1998). The *devR* gene from *N. punctiforme* is required for development of mature heterocysts (Campbell *et al.,* 1996). DevR can be phosphorylated and was predicted to be a response regulator component of a phosphorelay regulatory pathway (Hagen and Meeks, 1999). It has now been shown that in *Anabaena* PCC 7120, HepK has protein-histidine autokinase activity and that DevR is its cognate response regulator. This suggests that they compose part of a two-component system that regulates heterocyst polysaccharide biosynthesis (Zhou and Wolk, 2003). An *Anabaena* PCC 7120 *devR* inactivated strain, similar to *hepK* mutants, failed to produce the heterocyst envelope polysaccharide layer (Zhou and Wolk, 2003). As expected, a *hepK–gfp* transcriptional fusion showed strongest reporter expression in differentiating proheterocysts (Zhou and Wolk, 2003).

1.11.3 Differentiation and maturation

The *devBCA* gene cluster and *hglK* are involved in glycolipid transport and assembly, essential for heterocyst envelope formation and mutants are blocked at an early stage of development, suggesting that completion of the envelope might be a developmental checkpoint (Black *et al.,* 1995; Fiedler *et al.,* 1998). Further support for this type of morphological checkpoint is provided by recent data, which demonstrate that heterocyst maturation requires HcwA, an autolysin, presumably required for restructuring the peptidoglycan layer (Zhu *et al.,* 2001). It was also found that *pbpB*, which encodes a putative penicillin-binding protein, is required for aerobic nitrogen fixation (Lazaro *et al.,* 2001).

The gene *alr0117* is also necessary for the formation of the polysaccharide layer, which is suggested by the complementation of the mutant and activation of the gene for formation of polysaccharide layer formation (Ning and Xu, 2004). *alr0117* encodes a two-component histidine kinase with a transmembrane segment at its 5'-terminus. *alr0117* may control multiple genes that are apparently unrelated to each other during heterocyst development. Heterocysts are required to have fix dinitrogen in an oxygen-containing milieu (Fox^+) phenotype to for nitrogen fixation (Fan *et al.,* 2005). Complementation and other analysis provide evidence that at least *all5343*, *all5347*, *alr5348*, *asr5350-alr5353* and

alr5356, but not *asr5349*, are required for a Fox^+ phenotype. Electron microscopy showed a role of *all5345* through *all5347* in normal deposition of the envelope glycolipids.

1.12 Organization of *nif* genes in cyanobacteria

Most of the genes involved in nitrogen fixation (*nif* genes), originally described in *Klebsiella*, have also been detected in other diazotrophic organisms. The structural genes of the nitrogenase complex, *nifH*, *nifD* and *nifK*, represent one of the most highly conserved gene groups in bacteria. On the basis of sequence similarity to *Klebsiella* DNA probes, *nifHDK* and *nifS* were cloned and mapped in *Anabaena* sp. PCC 7120. Subsequent sequence analysis of adjacent genes and comparison to other *nif* genes from *Klebsiella*, *Azotobacter* and *Rhizobium* species led to the current picture of *nif* gene organization in *Anabaena* (Haselkorn *et al.*, 1991).

1.12.1 Developmentally regulated genome rearrangements in heterocysts

Heterocyst differentiation in *Anabaena* 7120 is accompanied by developmentally regulated genome rearrangements that affect *fdxN*, *nifD* and *hupL* gene expression. These rearrangements of the vegetative cell genome occur during late stages of differentiation at about the same time that the nitrogen fixation genes begin to be transcribed. All the DNA elements (*fdxN* is interrupted by a 55 kb DNA element, the *nifD* gene by an 11-kb element and the *hupL* gene by 10.5 kb of chromosomal DNA) become excised upon heterocyst differentiation (Carrasco *et al.*, 1995). The corresponding genes encoding these site-specific recombinases are *xisF*, *xisA* and *xisC*, respectively, which are located within the excised DNA elements. *xisF* alone is not sufficient but require *xisH* and *xisI* for the heterocyst specific excision of the *fdxN* element (Ramaswamy *et al.*, 1997). *Anabaena* 7120 mutants for *xisA* or *xisF* formed heterocysts but did not grow on nitrogen-free media (Carrasco *et al.*, 1994). The *hupL* rearrangement in *Anabaena* 7120 was independently found by pulsed-field electrophoresis and by comparison of the restriction pattern of vegetative cell and heterocyst DNA (Metveyev *et al.*, 1994). The 10.5 kb *hupL* element is not present in *Anabaena* 29413 (T. Happe and H. Böhme, unpublished). In contrast to *Anabaena* 7120, the closely related

cyanobacterium *Anabaena* 29413 does not contain the *fdxN* element in the *nif1* region; only the *nifD* element is present. In the *nif2* region, the *nifD* element and the *fdxN* gene are absent Both *Pseudoanabaena* and *Fischerella* lack the 11-kb element (Wolk *et al.*, 1994).

1.12.2 Functions of *nif* genes in *Anabaena*

The function of many *nif* genes in *Anabaena* has still not been determined, but a possible function can be inferred by analyzing analogous genes described in other diazotrophic bacteria. The first operon on the left includes the genes *nifB*, *fdxN*, *nifS* and *nifU*, which are required for biosynthesis of the iron-molybdenum (FeMo)- or the iron-vanadium (FeV) cofactor, but *fdxN*, *nifS* and *nifU* of *Anabaena* 29413 were not essential for nitrogen fixation to take place (Lyons and Thiel, 1995; Masepohl *et al.*, 1997a). The *glbN* gene of *Nostoc commune* was discovered between *nifS* and *nifU*. It encodes cyanoglobin, the only known prokaryotic myoglobin that might scavenge for oxygen or act as a component of the membrane-associated, microaerobically induced terminal oxidase. Cyanoglobin was only detected in *Nostoc*, when, in addition to microaerobiosis, the cells were starved of nitrogen (Potts *et al.*, 1992). The next operon to *nifB,S,U* consists of *nifH*, *nifD* and *nifK* (Fig. 2a). The *nifH* gene encodes the dinitrogenase reductase, a homodimer (2 x 30 kDa) with one [4Fe-4S]-cluster at the interface; *nifD* and *nifK* encode the α- and β-subunits of dinitrogenase, respectively, an $\alpha_2\beta_2$ tetramer of 240 kDa associated with two FeMo-cofactors and two P-clusters. Because NifE and NifN show significant structural similarity to NifD and NifK, respectively, it has been suggested that NifE and NifN generate the scaffold on which the FeMo-cofactor is assembled. NifE and NifN also form $\alpha_2\beta_2$ tetramer that binds the NifB cofactor, a small iron-sulfur-cluster protein and a precursor of the FeMo-cofactor. The precise function of the *nifX* gene in cyanobacterial nitrogen fixation remains to be determined.

The *nifW* gene is necessary for full stability or processing of the FeMo-protein. The functions of *hesA* and *hesB* are not known, although insertional inactivation of *hesA* impairs nitrogen fixation by approximately 55% (Borthakaur *et al.*, 1990). The *fdxH* gene, which is transcribed late during heterocyst development together with the nitrogenase genes, encodes

a unique [2Fe-2S]-ferredoxin, which is a specific electron donor for nitrogenase *in vitro* (Bohme and Haselkorn, 1988). Inactivation of *fdxH* led to a delay in nitrogen fixation, showing that FdxH is necessary for the magnitude of maximum nitrogenase activity and optimal growth under nitrogen-fixing conditions, but that *fdxH* is not essential for diazotrophic growth (Masepohl *et al.*, 1997b). The *nifV*, *nifZ* and *nifT* genes are separated from the main *nif* gene region in *Anabaena* 7120. The *nifV* gene encodes homocitrate synthase and homocitrate is an integral component of the FeMo-cofactor. The functions of *nifZ* and *nifT* are not clear; inactivation of *nifV* in *Anabaena* 7120 led to mutant strains that were still capable of diazotrophic growth but nitrogenase activity was reduced by about 30–40% (Stricker *et al.*, 1997). The *nifJ* gene encoding a pyruvate:flavodoxin oxidoreductase, is not closely linked to other *nif* genes of *Anabaena* 7120. In *Klebsiella*, NifJ functions to degrade pyruvate and generate reduced flavodoxin (NifF) as a specific electron donor to nitrogenase. An *Anabaena* 7120 a *nifJ* mutant was unable to grow on medium depleted of both iron and combined nitrogen. However, this strain was capable of diazotrophic growth when iron was present (Bauer *et al.*, 1993). No equivalent of the *nifF* gene has been found in *Anabaena* (Masepohl *et al.*, 1997b). Induction of the nitrogenase complex is accompanied by the induction of the hydrogen uptake system. The *hupL* gene encodes the large subunit of a membrane-bound [NiFe]-uptake hydrogenase and uses molecular hydrogen, a byproduct of nitrogenase activity (Carrasco *et al.*, 1995). To improve the efficiency of nitrogen fixation, hydrogen becomes oxidized in a respiratory, ATP-forming reaction. Wolk and co-workers used transposon mutagenesis, based on a Tn5-derivative bearing *luxA,B* (encoding luciferase) of *Vibrio fischeri* as a transcriptional reporter, to identify mutants that exhibit enhanced luciferase activity after removal of ammonia from the medium. Visualization of gene activation in single cells was made possible using constructs in which the promoter region of P*nifHDK* and P*rbcLS* was fused to *luxA,B* (Elhai and Wolk, 1990). Among the first genes to be activated by nitrogen deprivation (within 0.5 h) were the *nirAnrtABCDnarB* genes of the *nir* operon, encoding the structural genes for nitrite reductase (*nirA*), nitrate permease (*nrtABCD*) and nitrate reductase (*narB*). *Anabaena* strains carrying a mutation in *nirA, nrtC* or *nrtD* remained competent to make heterocysts and fix nitrogen (Cai and Wolk, 1997).

1.12.3 Alternative nitrogenase systems

Anabaena 29413 grown in anaerobic conditions expresses a second Mo-dependent nitrogenase system (*nif2*) in all vegetative cells some hours after induction and long before heterocysts begin to develop. In contrast to the *nif1* system of heterocysts, which functions under both anaerobic and external aerobic conditions and is developmentally regulated, the *nif2* system is expressed in all cells only under anaerobic conditions and is regulated by environmental factors (Schrautemeier *et al.*, 1995; Thiel *et al.*, 1995). *Anabaena* 29413 has a very similar *nif1* and *nif2* gene arrangement (Thiel *et al.*, 1997). The environmentally regulated *nif2* system lacks *fdxN*, but contains the *fdxB* gene downstream of *fdxH2*. The *fdxB* gene encodes a 2[4Fe-4S]-ferredoxin of unknown function (Schrautemeier *et al.*, 1995) and is similar to the corresponding gene from *Rhodobacter*.

In addition to the *nif1* and *nif2* genes, which encode nitrogenase-1 and -2, respectively, and which require the same FeMo-cofactor, *Anabaena* 29413 also contains *vnf* genes encoding a V-dependent nitrogenase (Thiel, 1993), as found in *Azotobacter vinelandii*. The alternative, V-nitrogenase-encoding *vnfDGK* genes of *Anabaena* 29413 are organized much like those of *Azotobacter* spp. However, the gene for the δ subunit of the V-nitrogenase, *vnfG*, is fused to the *vnfD* gene in *Anabaena* 29413. Two genes, *vnfE* and *vnfN*, which are similar to *vnfEN* genes of *A. vinelandii* were found downstream from *vnfDGK* in *Anabaena* 29413 (Thiel, 1993). Insertional inactivation of the *vnfN* gene produced a mutant that grew poorly on a medium where vanadium replaced molybdenum (Thiel, 1996).

1.12.4 Genes involved in the regulation of nitrogen fixation

Upon deprivation of combined nitrogen, photosynthesizing vegetative cells differentiate to form N_2-fixing heterocysts. This requires the coordinated regulation of many genes. These changes in gene expression involve modification of the transcription apparatus. In *Anabaena sigA* encodes the major sigma factor and two other nitrogen-regulated sigma factors *sigB* and *sigC* were isolated. However, inactivation of either *sigB* or *sigC* genes had

showed normal heterocyst differentiation and nitrogen fixation (Brahmasha and Haselkorn, 1992). A new group 2 sigma-factor gene, *sigD*, has been cloned and the *sigD* defective mutant strain had impaired diazotrophic growth and the appearance of heterocyst was delayed (Khudyakov and Golden. 2001).

Development of heterocyst requires inductions of many genes which are dependent on HetR (Cai and Wolk, 1997), as well as NtcA, because NtcA required for *hetR* induction, heterocyst development and impairment of expression of HetR dependent genes. Heterocyst formation is required to fix atmospheric dinitrogen and *hetR* overexpression leads to heterocyst formation even in the presence of nitrate but cells remain inactive for nitrogen fixation it becomes active without combined nitrogen (Buikema and Haselkorn, 2001). Expression analysis *ntcA* of *Anabaena* PCC 7120 on different nitrogen sources showed, *ntcA* produces multiple transcripts under nitrogen limiting conditions (Ramasubramanian, *et al.,* 1994; 1996; Wei, *et al.,* 1994; Herrero *et al.,* 2001). mRNA's corresponding to four transcription sites from -49, -136, -180 and -190 were isolated. A 0.8 kb transcript was present in both condition in absence and presence of the combine nitrogen sources regardless the source after growth of either 3 or 6 days, while 1.4 kb (longer) transcript was seen in the presence of combined nitrogen sources from 6 days sample. A 1 kb transcript was present in the 3 day (log phase) and 6 day (late-log-early-stationary phase) sample in absence of combine nitrogen source. This suggests the *ntcA* transcription increases upon nitrogen depletion and binding activity is higher in heterocyst.

Both positive and negative regulations of NtcA are subject to the presence of ammonia, lack of ammonia leads to activation of the genes involved in the heterocyst formation, nitrogen fixation and *ntcA* gene itself while the *gor* and *rbcLS* genes are repressed (Wisen *et al.,* 2004). The biosynthesis of the proteins controlled by NtcA; under these conditions shows high response to changes in NtcA concentration. This sensitivity may initiate a rapid heterocyst differentiation and subsequent N_2 fixation. 2-OG added to the wild type *Anabaena* sp. strain PCC 7120, led to higher frequency of heterocysts and earlier commitment to heterocyst (Li *et al.,* 2003). Physiological role of 2-OG is to regulate the proportion of the heterocyst relative to the vegetative cells as per the need of the filaments.

During heterocyst formation from the point of starvation of the combined nitrogen 2-OG accumulates and there is an imbalance in the carbon/nitrogen. In heterocyst, glutamine is synthesized with the help of nitrogenase and glutamine synthetase, heterocyst does not contain the enzyme glutamate synthase (Martin-Figueroa *et al.*, 2000). Glutamine can then be used in vegetative cells for the synthesis of glutamate using 2-OG as the carbon skeleton, thus the imbalance in C/N along the filaments is less, so that fewer heterocysts are induced. Changes in the intracellular pool of 2-OG can also change the expression of genes dependent on NtcA (Muro-pastor *et al.*, 2001; Vazques-Bermudez *et al.*, 2003).

Affinity of NtcA to bind its target DNA fragment, such as promoter region of *glnA* was enhanced by 2-OG (Vazquez-Bermudez *et al.*, 2002; Tanigawa *et al.*, 2002). Combined nitrogen depletion led to increase in intracellular level of 2-OG rapidly to threshold concentration and it binds to and activate the transcription factor NtcA. This signaling effect of 2-OG amplified through positive autoregulation of NtcA (Herrero *et al.*, 2001). It supported by the observation that the presence of 2-OG leads to more heterocyst differentiation even in the presence of NO_3^-.

1.13 Gene rearrangement in *Anabaena* sp. stain PCC 7120 for nitrogen fixation

Heterocyst differentiation involves an orderly pattern of gene expression that involves up to 1000 different genes, constituting 15-25% of the entire genome (Adams & Duggan, 1999; Lynn *et al.*, 1986). Heterocyst differentiation in some cyanobacteria is accompanied by developmentally regulated DNA rearrangements results in the removal of segments of DNA from the genome that accompanies some sort of developmental shift of differentiation process. In *Anabaena* sp. Strain PCC 7120, origin of developmentally regulated DNA rearrangements is unknown, but it has been suggested that they may be of viral origin (Haselkorn, 1992).

Developmentally regulated gene rearrangement of three different genes, *nifD, fdxN* and *hupL* was reported in the *Anabaena* PCC 7120 (Lammers *et al.*, 1986, ref). DNA elements disrupting these genes were cloned in plasmids and their rearrangements were also demonstrated in *E. coli.* When *E. coli* containing entire *nifD* gene carrying *nifD* element was used for rearrangement it showed 0.3 % excision frequency after overnight growth (Lammers *et al.*, 1986). Rearranged plasmid DNA showed that nature of the excision event is identical to that of *Anabaena* PCC 7120 where *nifD* element was lost from the substrate plasmid. The *nifD* element contained *xisA* gene that is responsible for excision. Golden and Weist (1988) constructed the *xisA* mutant using site-directed inactivation of the *xisA* gene in *Anabaena* PCC 7120 chromosome. Although the *xisA* mutant produced heterocysts and correctly excises the 55kb *fdxN* element, they failed to excise the 11kb element and were unable to fix nitrogen. This indicates that *xisA* is required for the *nifD* rearrangement in *Anabaena* heterocysts and that the DNA rearrangement is necessary for proper expression of the nitrogen fixing genes. Brusca *et al.* (1990) constructed pAM461, a substrate plasmid, that contained the left and right borders of *nifD* element but did not contain an intact *Anabaena* PCC 7120 open reading frame larger than 180bp. pAM461 plasmid did not rearrange in the absence of a complementary plasmid expressing the *xisA* gene. This result suggests that in *E. coli* the *nifD* element can rearrange properly in the absence of any other *Anabaena* gene products.

Excision of *nifD* element belongs to site-specific recombination. Since, many site-specific recombinations involve accessory proteins, excision of the *nifD* element in *E. coli* might also involves accessory proteins of *E. coli* in the excision event (Lammers *et al.*, 1986). Excision of the *nifD* element in *E. coli* was considered not dependent on RecA since *E. coli* MT8820TR contains a *recA* mutation. Additionally, these experiments did not show much variation in the excision frequency when the *E. coli* was given heat shock or grown under different nature of nitrogen sources. This result was surprising as the excision occurs in *Anabaena* PCC 7120 only in the heterocysts, which are formed only under nitrogen starvation conditions.

Further excision studies with *E. coli recA*$^-$ showed the rearrangement frequency 0.3% after overnight growth, like *E. coli* strain MT8820TR which is *recA*$^-$ (Karunakaran 2000). But the excision frequency was increased to 1% after 24 h of growth. Changing media condition from the rich medium to minimal medium, further increased the rearrangement to 10% in *recA*$^+$ *E. coli* strain. *Klebsiella pneumoniae* NifA protein has same binding site like NtcA of *Anabaena* sp. strain PCC 7120 and xisA putative promoter region contains three binding sites for this proteins. Interestingly, rearrangement frequency in the presence of *nifA* gene increased to 50% and 75% respectively, in *recA*$^-$ and *recA*$^+$ *E. coli* strains. Using *xisA* promoter with β Galactosidase transcriptional fusion, the activity of *xisA* promoter monitored in the above conditions. Presence of XisA protein was found to results in the specific loss of plasmids containing its target sites in *E. coli* and the extent of plasmid loss was directly correlated with excision frequency. This suggested XisA protein may also have endonuclease activity, in addition to site specific recombinase activity, which could account for the its toxic effects of the XisA protein.

1.14 Objectives of the present work

Earlier, increase in frequency of rearrangement of *nifD* gene in *E. coli* showed that excision frequency is subjective to many gears like few genes, *recA*, *nifA* and also by medium in which *E. coli* strains grow. This exposition was directed towards perceptive of the excision of *nifD* element of *Anabaena* sp. stain PCC 7120 using *E. coli* model system. What are the other accessory elements which play a role in the excision of *nifD* element of *Anabeana* sp strain PCC 7120? Why *xisA* transcript or protein has not been detected so far? What is the role of N terminal domain and C terminal domain in the rearrangement? What is the promoter activity in presence of the factors which influence the rearrangement? Wheather XisA can be purified using transcriptional fusion strategy? If so, how XisA protein can be purified and can be assayed for endonuclease activity?

To answer all these questions we worked on following objectives

(i) To monitor the role of physiological conditions on the excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli*

(ii) To study the effect of NtcA on the excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli.*

(iii) To study the role of N terminal domain of XisA protein in the rearrangement.

(iv) Expression of the site specific recombinase XisA as a MBP::XisA fusion protein by transcriptional fusion.

(v) Expression and partial purification of XisA and its *in vitro* analysis.