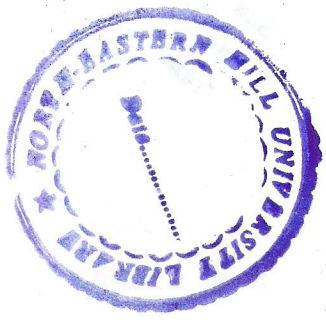
STUDIES ON NITROGEN - NUTRITION IN A HIGHLY SPORULATING *NOSTOC* SPECIES AND ITS USE IN AGRICULTURAL BIOTECHNOLOGY



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STATEMENT

I, **Jyotirmoy Bhattacharya**, hereby declare that the subject matter of this thesis is the record of work done by me, that the content of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North-Eastern Hill University for the degree of **Doctor of Philosophy** in **Biochemistry.**

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Dedicated to

my beloved parents and granny

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
Bq	Becquerel
C	Carbon
°C	Degree centigrade
C_2H_2	Acetylene
C_2H_4	Ethylene
Chl	Chlorophyll
d	Day (s)
g	Gram
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid
h	Hour (s)
Het	Heterocyst
kDa	Kilodalton
M_r	Molecular weight
l	Litre
min	Minute (s)
ml	Millilitre
mM	Millimolar
µmol	Micromole
µM	Micromolar
μm^2	Micro meter square
Mo	Molybdenum
MSX	Methionine sulphoximine
N	Nitrogen
N_2	Dinitrogen
nar	Nitrate reductase

nir	Nitrite reductase
nm	Nanometer
nmol	Nanomole
nrt	Nitrate/Nitrite transport
ntc	Nitrogen control
PCR	Polymerase chain reaction
PS	Photosystem
psi	Pounds per square inch
tRNA	Transfer ribonucleic acid
TCA	Trichloroacetic acid
rpm	Revolution per minute
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
s	Second (s)
W	Watt
wt	Weight
v	Volume
%	Percent
$^{0}/_{00}$	Parts per million

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CHAPTER 1

GENERAL INTRODUCTION

Modern day agriculture faces the serious challenge of increasing the productivity to meet the food demands of the ever-growing human population. Current agricultural practices rely heavily on the use of chemical nitrogen fertilizers, herbicides and pesticides to achieve higher crop yields. Production of chemical nitrogen fertilizers is a highly energy-intensive and costly process, based on fossil fuel. This siphons out a large proportion of national budgets, especially in developing countries, and excessive use of chemical fertilizers contributes to environmental pollution. It is in this context, that a worldwide scientific consensus is emerging for replacing chemical nitrogen fertilizers with biological ones that are cost effective, renewable and eco-friendly.

Biological N_2-fixation is a process, which converts molecular nitrogen into ammonia. Although, atmospheric nitrogen constitutes about 78 % of the earth's total atmosphere, not very many organisms are known which can directly utilize such a vast nitrogen reserve for sustaining their cellular nitrogen requirements. However, some prokaryotic organisms have the capacity to fix atmospheric nitrogen. Among them, cyanobacteria are prominent because of their simple growth requirements and a higher plant type oxygenic photosynthesis (Gallon and Chaplin, 1987; Rai, 1990; Bryant, 1994; Whitton and Potts, 2000).

1.1 Cyanobacteria :

Cyanobacteria are one of the oldest and most diverse group of Gram-negative photosynthetic prokaryotes. They are known to occupy a wide range of ecological habitats (water, soil and air) including hotsprings, brackish water, usar soils and deserts (see Singh, 1961; Carr and Whitton, 1982; Bergman *et al.*, 1997; Whitton and Potts, 2000). In addition, N_2-fixing cyanobacteria form symbiotic associations with a variety of plants and animals (Rai, 1990; Rai *et al.*, 2000; Adams, 2000). Cyanobacterial species include unicellular, filamentous, branched-filamentous and non-filamentous colonial forms (Rippka *et al.*, 1979). Many filamentous cyanobacteria are also known to possess multiple cellular differentiation alternatives which include vegetative cells (sites for photosynthesis), heterocysts (sites for N_2-fixation), akinetes/spores (perennating bodies) and motile trichomes called hormogonia (Tandeau de Marsac and Houmard, 1993; Adams and Duggan, 1999).

1.2 Vegetative cells :

Vegetative cells house the entire photosynthetic machinery in cyanobacteria. The Chlorophyll *a* protein complexes, the photosynthetic reaction centres, the carotenoids and the electron transport system are all contained within the thylakoids. The light harvesting accessory pigments (phycobilliproteins) are located in phycobillisomes attached to the surface of thylakoids, which are also used as nitrogen sources under conditions of N-deficiency (Tandeau de Marsac and Houmard, 1993;

Bryant, 1994). In addition to this, the vegetative cells also contain a number of inclusion/storage bodies such as cyanophycean starch (glycogen) as C reserve, carboxysomes which contain ribulose 1,5-bisphosphate carboxylase/oxygenase, (Rubisco), cyanophycin (a polymer of aspartate and arginine) as N-reserve, and polyphosphate bodies. Vegetative cells produce ATP by oxidative phosphorylation as well as by photophosphorylation coupled to cyclic and non-cyclic photosynthetic electron transport chain. Carbon dioxide fixation occurs through Calvin cycle (see Carr and Whitton, 1982).

1.3 Heterocysts and N_2-fixation :

In absence of combined nitrogen 5-10 % of the vegetative cells of heterocystous filamentous cyanobacteria differentiate into morphologically distinct cells called heterocysts. These are regularly spaced and contain the enzyme nitrogenase, which converts molecular nitrogen into ammonia (Flores and Herrero, 1994; Wolk *et al.*, 1994; Adams and Duggan, 1999).

The nitrogenase enzyme complex consists of two different proteins: Mo-Fe protein (dinitrogenase) and Fe-protein (dinitrogenase reductase). The dinitrogenase is a $\alpha_2\beta_2$ tetramer (M_r 226.8 kDa) and its α and β subunits are encoded by the *nifD* and *nifK* genes respectively. It also contains two molecules of Mo-Fe cofactor. The dinitrogenase reductase (M_r 66 kDa) is a dimer of two identical subunits encoded by *nifH* gene. Some brilliant studies involving genetic and immunological experiments have revealed the cyanobacterial nitrogenase protein to be similar to other N_2-fixing organisms (Bergman

et al., 1986, 1997; Dean and Jacobson, 1992; Flores and Herrero, 1994; Kim and Rees, 1994).

N_2-fixation is a highly energy intensive process requiring ATP, reductants and low oxygen concentration. ATP requirements are fulfilled by generation of ATP by photophosphorylation, oxidative phosphorylation, substrate level phosphorylation and/or uptake hydrogenase activity (Bottomley and Stewart, 1976; Maryan *et al.*, 1986; Daday and Smith, 1987). Oxidative pentose phosphate pathway is a major source of reductant in heterocysts (Smith, 1982; Wolk *et al.*, 1994). Ferredoxin (a product of *fdxH* gene) is known to be the immediate electron donor to nitrogenase (Bohme and Haselkorn, 1988) where as flavodoxin can take on this role under iron-deficient conditions (Fillat *et al.*, 1991).

The enzyme nitrogenase shows extreme sensitivity to oxygen which is manifested both at the level of synthesis and activity (Gallon and Chaplin, 1987; Gallon, 1992; Rai *et al.*, 1992; Durner *et al.*, 1996). In filamentous heterocystous cyanobacteria, where heterocysts are the sites of N_2-fixation, several structural, biochemical and genetic changes take place in order to maintain a microaerobic interior. Such changes include synthesis of multilayered cell envelope, loss of PS II activity, presence of uptake hydrogenase, and high rates of respiration (Wolk *et al.*, 1994). The non-heterocystous cyanobacterial diazotrophs protect nitrogenase from oxygen damage by resorting to temporal separation of N_2-fixation and photosynthesis (Gallon, 1992; Bergman *et al.*, 1997). Some of them, e.g. *Plectonema boryanum* and *Phormidium*, fix N_2 under anaerobic or microaerobic conditions only (Stewart and Lex, 1970; Weisshar and Boger, 1983; Rai *et al.*, 1992). In one non-heterocystous cyanobacterium,

Trichodesmium sp., there is spatial separation of N_2-fixation and photosynthesis (Fredricksson, 1996; Capone *et al.*, 1997; Lin *et al.*, 1998).

The chromosome region harboring *nifHDK* genes in *Anabaena* sp. PCC 7120, undergoes DNA rearrangements during differentiation of vegetative cell into heterocysts. In heterocysts *nifHDK* is contiguous but in vegetative cells an 11 kb DNA fragment interrupts *nifD* gene (Golden *et al.*, 1985). A second rearrangement involving deletion of 55 kb fragment located in *fdxN* gene (bacterial type ferredoxin gene whose function is not known in cyanobacteria) has also been shown to occur during heterocyst differentiation (Golden *et al.*, 1987). These rearrangement events involve site-specific excisases encoded by *xisA* and *xisF*, respectively. The only exception to this characteristic *nif* gene rearrangement is found in *Mastigocladus laminosus*, where the structural *nif* genes are contiguous both in vegetative cells and heterocysts (Singh and Stevens, 1992).

The above mentioned nitrogenase enzyme is the conventional Mo-dependent nitrogenase (Nif 1) which functions in heterocysts only. Recently another Mo-dependent nitrogenase (Nif 2) have been shown to function in vegetative cells of *Anabaena variabilis* ATCC 29413 under anaerobic conditions (Thiel *et al.*, 1995; Thiel and Pratte, 2001). Cyanobacteria are also known to possess alternative nitrogenases, a Vanadium-dependent nitrogenase encoded by *vnfDGK* genes and an Fe-only-nitrogenase in *Anabaena variabilis* (Kentemich *et al.*, 1991; Thiel, 1993).

Genes responsible for regulation of heterocyst formation have started to be characterized (see Wolk *et al.*, 1994; Wolk, 1996; Adams and Duggan, 1999). In response to nitrogen step-down, an autoregulatory gene, *hetR*, is induced in regularly

spaced cells (proheterocysts) within 2-3.5 h. The HetR protein is an unusual serine type protease, which may be degrading the repressor of genes to be switched on and activators of genes to be switched off during heterocyst differentiation (Zhou *et al.*, 1998 a,b). The presence of functional *hetR* is necessary for heterocyst development and its overexpression leads to formation of multiple contiguous heterocysts (Buikema and Haselkorn, 1991 a,b; Black *et al.*, 1993). Two more genes, named *hetP* and *hetC*, are required for heterocyst development. Mutational inactivation of *hetP* blocks heterocyst differentiation and strains carrying extra copies of *hetP* form multiple contiguous heterocysts in absence of combined nitrogen sources (Fernandez-Pinas *et al.*, 1994; Khudyakov and Wolk, 1997). Another gene known as *devA* may be required for the transport of nutrients into heterocysts (Maldener *et al.*, 1994). Formation of functional heterocysts is also linked with morphological changes during differentiation. The formation of heterocyst envelope (innermost glycolipid layer) requires *hetM* for its synthesis and *hglk* encodes a protein needed for the transport of heterocyst glycolipids in *Anabaena* PCC 7120 (Black *et al.*, 1995; Bauer *et al.*, 1997). Three genes, *hepA*, *hepB* and *hepC*, are required for the synthesis or stabilization of the heterocyst envelope (Wolk, 1996). Fox $^-$ mutants have recently been isolated which show defect in heterocyst envelope (ineffective oxygen barrier) and impaired N_2-fixation ability under aerobic conditions but not under anaerobic conditions (Ernst *et al.*, 1992). Genes involved in heterocyst pattern formation have been identified as *patA* and *patB*. Mutations in *patA* gene result in the formation of heterocysts only at the ends of filaments (Liang *et al.*, 1992). *PatB* mutants have been reported to grow slowly under diazotrophic growth conditions because of delayed heterocyst development but they

grow normally under nitrogen sufficient conditions (Liang *et al.*, 1993). Involvement of a secondary metabolite has been suggested in regulation of heterocyst spacing (Black and Wolk, 1994). More recently Yoon and Golden, (1998) have identified a diffusible polypeptide which controls heterocyst pattern formation. In addition to this, operation of nitrogen regulatory events in cyanobacteria, similar to other diazotrophs, have been reported recently. One such gene termed *ntcA* has been found to be wide spread in cyanobacteria (Frias *et al.*, 1993; Herrero *et al.*, 2001). The *ntcA* gene encodes a transcription activator protein named NtcA (a cyclic AMP-binding protein) required for turning on the expression of genes that are subject to ammonium repression. NtcA from *Anabaena* PCC 7120 interacts *in vitro* with promoter regions of *xisA* (an excisase gene required for heterocyst formation), *glnA* (encoding glutamine synthetase), *rbcLS* (encoding Rubisco), *nifH* (encoding dinitrogenase reductase) and *ntcA* (encoding NtcA itself) (Frias *et al*, 1994; Wei *et al.*, 1994). The importance of this gene is exemplified by a *ntcA* mutant of *Anabaena* PCC 7120, which neither forms heterocyst nor shows nitrogenase activity and requires ammonium for growth (Frias *et al.*, 1994). NtcA also binds to the promoter region of *gor* gene (encoding glutathione reductase), thus implying that in addition to its response to cellular nitrogen status, it also responds to cellular redox changes which are essential for heterocyst development and provision of microaerobic condition inside the heterocysts (Jiang *et al.*, 1997). In enterobacterial systems, well-defined *ntr* genes have been shown to control nitrogen assimilation (Merrick and Edwards, 1995). The *glnB* gene product, P_{II} protein, is involved in nitrogen control in both unicellular and filamentous cyanobacteria (Tsinoremas *et al.*, 1991; Liotenberg *et al.*, 1996). The P_{II} protein is a central signal transmitter of cellular

nitrogen status in enterobacteria. The ratio of α-ketoglutarate to glutamine indicates the nitrogen status of a cell. A bifunctional enzyme Uridylyl transferase/Uridylyl removing enzyme senses this ratio, and uridylylates P_{II} protein under nitrogen limitation (high ratio of α-ketoglutarate/glutamine) or deuridylylates the uridylylated P_{II} protein under nitrogen sufficient conditions (low ratio of α-ketoglutarate/glutamine) (Merrick and Edwards, 1995). Unlike the enterobacterial P_{II} protein, the cyanobacterial P_{II} protein is modified by phosphorylation at a seryl residue. Furthermore, the phosphorylation of P_{II} protein seems to be regulated by the cellular status of N and C in cyanobacteria (Forchhammer and Tandeau de Marsac, 1994, 1995; Liotenberg *et al.*, 1996).

1.4 Inorganic nitrogen metabolism :

1.4.1 Nitrate and Nitrite metabolism :

In cyanobacteria, nitrate and nitrite are taken up through a high affinity transport system sensitive to DCCD (an inhibitor of bacterial ATPases), but diffusion of nitrous acid can also contribute to net nitrite uptake under acidic conditions (Luque *et al.*, 1994; Maeda and Omata, 1997). Assimilation of nitrate and nitrite takes place in the vegetative cells by sequential action of nitrate reductase and nitrite reductase resulting in ammonium formation (Flores and Herrero, 1994). Both processes have been reported to be genuinely photosynthesis-dependent (Manzano *et al.*, 1976).

Nitrate uptake and assimilation is absent in heterocysts thus eliminating competition between nitrogenase and nitrate reductase for Mo-cofactor and reductant (Kumar *et al.*, 1985; Rai and Bergman, 1986). Structural genes for nitrite reductase (*nirA*), nitrate/nitrite uptake (*nrtABCD*) and nitrate reductase (*narB*), have been found to

be co-transcribed as a single operon in *Synechococcus* sp. strain PCC 7942 and *Anabaena* sp. strain PCC 7120 (Omata *et al*, 1993; Cai and Wolk, 1997; Frias *et al*, 1997). Mutants impaired in nitrate metabolism (defective in nitrate transport and/or assimilation) exhibit derepressed heterocyst formation and nitrogenase activity in nitrate-containing medium (Martin-Nieto *et al.*, 1991; Cai and Wolk, 1997; Frias *et al*, 1997). Inactivation of *moeA* gene, required for the synthesis of molybdenum-containing cofactor molybdopterin in *Anabaena* PCC 7120, leads to a loss of nitrate reductase activity and forms heterocyst in the presence of nitrate (Ramaswamy *et al.*, 1996). Expression of the nitrate assimilation operon requires NtcA (Herrero *et al.*, 2001).

Presence of exogenous ammonium represses the synthesis of nitrate/nitrite uptake and reductases. Ammonium repression of nitrate/nitrite uptake and assimilation has been shown to result from the negative action of glutamine or its metabolite rather than by a direct effect of the ammonium *per se* (Flores and Herrero, 1994). In absence of ammonium nitrate uptake and reductase are derepressed (Bagchi *et al.*, 1985 a, b; Cai and Wolk, 1997). Furthermore, nitrate is reported to cause induction of nitrate reductase (Bagchi *et al.*, 1985 b).

1.4.2 Ammonium transport and assimilation :

Ammonium is a preferred nitrogen source for many microorganisms. ^{14}C methylammonium (an ammonium analogue) has been used extensively for characterization of ammonium transport systems (ATS) in cyanobacteria (Rai *et al.*, 1984; Boussiba *et al.*, 1984; Boussiba and Gibson, 1987; Shehawy and Kleiner, 1999). Ammonium transport in cyanobacteria has been found to be biphasic with an initial

rapid phase lasting for 2-3 minutes (MSX insensitive and independent of methylammonium metabolism) followed by a slower second phase (MSX sensitive, methylammonium metabolism dependent) (Singh *et al.*, 1985, 1986, 1987). ATS studies on a GS defective mutant of *Anabaena cycadeae* showed that the second phase of ammonium transport may be a separate ATS thus suggesting occurrence of two ATS (Singh *et al.*, 1985). Recently, in *Synechocystis* sp. PCC 6803 three putative *amt* (ammonium transport) genes have been characterised by insertional inactivation studies. Expression of the *amt* genes was found to be under nitrogen control (being derepressed under nitrogen depleted conditions and repressed under nitrogen replete conditions). One of the genes (*amt1*) is a high affinity transporter (K_s for methylammonium 2.7 μM) and it is expressed at higher levels than the other two genes. Transcription of *amt1* gene has also been shown to be activated by the nitrogen control transcription factor, NtcA (Montesinos *et al.*, 1998). The importance of ATS lies in the uptake of exogenous ammonium and retention of ammonium produced during N_2-fixation (Kleiner, 1985). Ammonium generated by N_2-fixation, nitrate/nitrite assimilation or exogenous supply is incorporated mainly by Glutamine synthetase-Glutamate synthase (GS-GOGAT) pathway. The enzyme GS (*glnA* gene product) in cyanobacteria varies depending on nitrogen nutrition (Merida *et al.*, 1991; Flores and Herrero, 1994). The level of GS protein under N_2-fixing conditions is higher than that under ammonium grown conditions. These reports are consistent with increase in *glnA* mRNA synthesis and GS activities under nitrogen depleted conditions. There are two promoters for *glnA*: an *E.coli* type promoter and a *nif*-like promoter (Tumer *et al.*, 1983). Under N_2-fixing conditions the transcription of *glnA* occurs mainly through the *nif*-like promoter. Unlike

enterobacteria, regulation of GS protein in cyanobacteria is not controlled by adenylylation /deadenylylation (Merida *et al.*, 1991).

1.5 Aminoacid nutrition and transport :

Studies on nitrogen nutrition of cyanobacteria have largely focussed on inorganic nitrogen sources. Comparatively fewer studies have explored the utilization of amino acids as nitrogen source and their transport mechanisms. Arginine, asparagine and glutamine are known to serve as nitrogen sources (Thiel and Leone, 1986; Herrero and Flores, 1990; Singh *et al.*, 1991; Flores and Herrero, 1994). On the other hand, some other amino acids like glutamate, histidine and lysine are reported to be growth inhibitory (Chapman and Meeks, 1983; Flores and Muro-Pastor, 1990; Prakasham *et al.*, 1991).

Three amino acid transport systems have been reported in *Synechocystis* strain PCC 6803, one specific for basic amino acids and glutamine, one specific for neutral amino acids excluding glutamine and another one specific for glutamine and glutamate (Labarre *et al.*, 1987). There is a high- and a low-affinity transport system for both glutamate and glutamine (Chapman and Meeks, 1983) but a single transport system for leucine (Thiel, 1988) in *Anabaena variabilis* ATCC 29413. A common transport system for glutamate and aspartate has been reported in *Nostoc* sp. (Strasser and Falkner, 1986). A common transport system for glutamine and glutamate has also been demonstrated in *Anabaena* PCC 7120 (Flores and Muro-Pastor, 1988). In addition, *Anabaena* PCC 7120 has also been shown to possess three high affinity amino acid transport systems (ATP dependent): one for basic amino acids (Herrero and Flores,

1990) and two for neutral amino acids (Montesinos *et al.*, 1995). There are two low affinity transport systems as well: one for basic (Herrero and Flores, 1990) and another for acidic (Montesinos *et al.*, 1995) amino acids. Amino acid transport defective mutants show impaired diazotrophic growth as a result of excretion of some amino acids in the extracellular medium (Montesinos *et al.*, 1995, 1997). Recently, two neutral amino acid transport genes *natA* and *natB* have been identified in *Synechocystis* PCC 6803 and their homologues have also been found in some other cyanobacteria like *Anabaena* sp. strain PCC 7120, *Anabaena* sp. strain PCC 7937, *Nostoc* sp. strain PCC 7413, and *Nostoc* sp. strain PCC 7107 (Montesinos *et al.*, 1997).

1.6 Akinete differentiation and germination :

Formation of thick-walled akinetes/ spores (resting cells) is one of the survival strategies frequently employed by cyanobacteria under unfavourable growth conditions. Phosphate and light limitation have been implicated as triggers for akinete development (Herdman, 1987, 1988; Adams and Duggan, 1999). Akinetes are generally larger than vegetative cells and they contain multilayered extracellular envelope. Metabolic changes associated with akinete differentiation in *Anabaena doliolum* includes reduction in respiratory activity and loss of nitrogenase, nitrate reductase, glutamine synthetase and photosynthetic activities along with photosynthetic pigments (Rao *et al.*, 1984). During germination (when favourable growth conditions resume) all the metabolic activities related to C and N metabolism sequentially reappear allowing the cells to continue their normal growth and multiplication (Rai *et al.*, 1988). Genetic regulation of akinete formation in cyanobacteria is far from clear. Presence of

functional *hetR* has been shown to be essential for both akinete formation as well as heterocyst formation (Leganes *et al*, 1994; Wolk *et al*, 1994). Similarly, formation of akinetes and heterocysts also share another common gene, *hepA*, which encodes envelope polysaccharides (Leganes, 1994). These recent developments are an indicator towards a common developmental regulation of heterocyst and akinete formation, although, specific developmental links between the two processes still remains to be explored.

1.7 Cyanobacteria in symbiosis :

Cyanobacteria form N_2-fixing symbioses with a wide range of plants which include diatoms, lichenised fungi, liverworts, hornworts, water fern (*Azolla*), cycads, and the angiosperm *Gunnera* (see Rai, 1990; Bergman *et al.*, 1996; Rai *et al.*, 2000; Adams, 2000). Possession of such a wide host range makes them the most promising of all N_2-fixing symbionts for extending the host range to include crop plants.

During the past few decades there has been a considerable upsurge of interest to understand various aspects of symbiosis involving cyanobacteria and higher plants. These include symbiont diversity/competence, initiation and development process, specific recognition and signalling process, structural/functional modifications, and nutrient exchange.

1.7.1 Symbiotic diversity/competence :

Symbiosis demands a sophisticated means of cross talk between the partners involved. Although cyanobacterial symbioses cover a wide host range, only a restricted

number of cyanobacterial species and a limited number of plants from different plant groups develop into symbiosis. The symbiotic competence of diazotrophic cyanobacteria is apparently restricted to heterocystous species. Strains belonging to the *Nostoc* sp. (Section IV, cyanobacteria, Rippka *et al.*, 1979) have been shown to reconstitute the symbiosis under laboratory conditions. Such competency results from the ability of *Nostoc* to form motile hormogonia (transient motile units) that are essential for the infection process (Meeks, 1990; Rai *et al.*, 2000). Mutants of the symbiotic *Nostoc* PCC 73302 producing increased number of hormogonia have a higher ability to infect *Anthoceros*. Two open reading frames (*hrmA* and *orfU*) have been discovered whose transcription is under activation control of the hormogonium-inducing factor of *Anthoceros* (Cohen and Meeks, 1997). A heat-labile (M_r <12 kDa) compound has been identified as the hormogonium-inducing signal in the viscous mucilage secreted by the stem glands of *Gunnera* (Rasmussen *et al.*, 1994). Such reports are suggestive of the crucial role played by plant signals/chemicals at the level of cyanobacterial infection. In addition, the acidic mucilage of *Gunnera* appears to contain signalling compounds which acts on compatible *Nostoc* strains. One of them induces the synthesis of two polypeptides of 40 kDa and 65 kDa, and is known to affect cyanobacterial gene expression (Rasmussen *et al.*, 1994, 1996). This plant-mediated control of cyanobacterial infection process seems analogous to rhizobial symbioses, wherein flavanoids encoded by legumes are known to induce symbiosis-specific genes in *Rhizobium*.

Production of specific signalling molecules by the cyanobiont has also been suggested to take place during the infection period. One category of such signalling

molecules is functionally similar to nodule-inducing factors (nod factors) released by rhizobia in response to plant flavonoids (Van Rhizn and Vanderleyden, 1995). Recently similarities between certain *nod* genes (the *nod* box, *nodEF* and *nodNM* genes) and genomic regions of cyanobacteria infecting *Gunnera* (Rasmussen *et al.*, 1996) have been shown. Cyanobionts are also known to release carbohydrate rich arabinogalactan proteins (Bergman *et al.*, 1996), which are thought to act as signalling molecules (Pennel, 1992).

1.7.2 Metabolic status of the cyanobiont :

In symbioses, the cyanobiont undergoes many structural-functional modifications conducive to nutrient exchange between them and their host plants. A certain degree of uniformity is evident in the pattern of such changes described below.

a) **Growth and morphological changes :** In symbiotic tissues, there is a significant reduction in growth and cell division of the cyanobiont compared to its free-living state. The host and the cyanobiont grow in synchrony and the cyanobiont population is maintained as a constant proportion of the host biomass. The morphological changes involve increase in cell size, altered cell shape, lack of polyphosphate granules, fewer carboxysomes, less sheath material and glycogen, thinner cell walls and altered thylakoids arrangements (Rai, 1990; Rai *et al.*, 2000).

b) Photosynthesis and C metabolism : Cyanobionts which occur in symbiosis with heterotrophic hosts retain their photosynthetic ability whereas those in symbioses

with phototrophic hosts become functionally non-photosynthetic, despite having retained their photosynthetic pigments. These metabolic modifications make the cyanobionts dependent on the host for their fixed C requirements (see Rai *et al.*, 2000).

c) Heterocysts and N_2-fixation : In free-living conditions heterocysts (sites of N_2-fixation) constitute about 5-10 % of the total cell population whereas under symbiotic conditions the frequency of heterocysts increases considerably accompanied by altered spacing pattern. In *Gunnera*, 60-80 % of the cyanobiont cells located in mature symbiotic tissue become heterocysts and a heterocyst frequency of 30-40 % has been reported in other cyanobacteria-plant symbioses (Rai, 1990; Bergman *et al.*, 1992; Rai *et al.*, 2000). Overexpression of *hetR* causes similar increases in heterocyst frequency in the free-living *Anabaena* PCC 7120 (Buikema and Haselkorn, 1991 b). Rate of N_2-fixation has also been shown to increase in tandem with the heterocyst frequency (Bergman *et al.*, 1992). However, N_2-fixation rates correlate only to the frequency of single heterocysts and not multiple contiguous heterocysts which is often the case in symbiosis (Lindblad and Bergman, 1990; Soderback *et al.*, 1990).

d) Glutamine synthetase (GS) : GS is the primary ammonia-assimilating enzyme in diazotrophic cyanobacteria. Under free-living conditions, the concentration and activity of GS is two-fold higher in heterocysts than that in vegetative cells (Bergman *et al.*, 1985). This is in sharp contrast to the situation in cyanobionts

(except in cycads) where the level of GS in heterocysts is similar to that in vegetative cells (Bergman and Rai, 1989; Rai *et al.*, 1989; Lindblad and Bergman, 1990; Janson *et al.*, 1993, 1995). This decrease in GS of heterocysts results in ammonia leakage from the cyanobiont (see Rai *et al.*, 2000). In case of cycads where GS levels are not affected in the cyanobiont, the cyanobiont releases amino acids. Such nitrogen releases meet the nitrogen requirements of the host.

1.8 Artificial Symbiosis :

The importance of cyanobacteria in rice cultivation has been extensively documented (Singh, 1961; Whitton and Potts, 2000) and both free-living cyanobacteria as well as *Azolla* are in use as biofertilizers (Roger and Ladha, 1992). Biofertilizer potential of diazotrophic cyanobacteria has started attracting attention of scientific as well as farming community in view of the high cost and potential harms involved in the use of chemical nitrogen fertilizers. However, diazotrophic cyanobacteria are not known to liberate ammonia under normal conditions (unless GS activity or ATS are impaired) for use by crop plants and the nitrogen compounds are only available to plants after the standing cyanobacterial biomass perish. This makes their use very limited in terms of nitrogen supply to the crop plants. In symbiotic associations involving cyanobacteria (as described in previous section), the cyanobionts show an increased heterocyst frequency and nitrogenase activity coupled with ammonia release due to decreased level of GS. Such nitrogen release by cyanobionts meets the nitrogen requirement of the host plant. These natural properties of symbiotic cyanobacteria can be effectively exploited by

creation of artificial associations between symbiotically competent strains of diazotrophic cyanobacteria and crop plants. This would ensure direct and continuous nitrogen transfer from cyanobionts to their host crop plants. Although, creation of artificial symbiosis may be a long term research effort, recent progress made in understanding the specific signalling events between the symbionts coupled with the expanding knowledge of structural, biochemical and molecular changes underlying such processes might enable us to develop a desirable alternative to the use of chemical fertilizers. Recent reports indicate that some strains of *Nostoc* and one ammonia-excreting strain of *Anabaena variabilis* can associate with wheat and rice plants and provide them with fixed nitrogen in the form of ammonia (Spiller *et al.*, 1993; Spiller and Gunasekaran, 1994; Ganter *et al.*, 1995; Kamuru *et al.*, 1997; Ganter and Elhai, 1999).

1.9 Present study :

Cyanobacterial biofertilizer technology suffers from some serious inherent drawbacks. Free-living cyanobacteria release nitrogen only on turn over of their biomass, survival of cyanobacterial inoculum is adversely affected by the use of herbicides and pesticides, and chemical nitrogen fertilizers repress N_2-fixation. Therefore, it has become imperative to manipulate cyanobacterial strains making them more efficient in terms of their biofertilizer potential. An ideal cyanobacterial biofertilizer strain needs to have the following attributes:

a) Resistance to herbicides and pesticides that adversely affect the cyanobacterial growth in rice fields.

b) Derepression of diazotrophy in presence of chemical nitrogen fertilizers.

c) Sporulation ability to aid better survival in the field.

d) Efficient nitrogen release to the crop plants.

For this present study, I have selected a *Nostoc* species, which sporulates profusely. The work presented in this thesis includes mutational construction and characterization of herbicide/pesticide resistant and nitrogenase derepressed mutants of *Nostoc* species. Furthermore, such mutants were characterized with regard to their ability to associate with rice plants, associative N_2-fixation and nitrogen transfer. An attempt has also been made to characterize the uptake and assimilation of some amino acids in the chosen *Nostoc* strain.