CHAPTER 6

Nitrogen Metabolism in Cyanobacteria

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Although, molecular nitrogen is abundant in the earth's atmosphere, it gets incorporated into the biosphere only through assimilation by microorganisms and plants. Wide range of nitrogenous compounds in inorganic ionic forms such as nitrate, nitrite or ammonia as well as simple organic forms like urea, amino acids and some nitrogen containing bases etc. can be metabolized by various organisms. In addition, some prokaryotes are capable of fixing atmospheric nitrogen using nitrogenase enzyme. Among them cyanobacteria are ancient and morphologically diverse group of gram negative eubacteria, many of which combine two seemingly mutually exclusive processes–O_2-evolving photosynthesis and O_2-sensitive nitrogenase dependant nitrogen fixation.

The two processes are separated either temporally in non-heterocystous forms (where alternating cycles of nitrogen fixation and photosynthesis take care of the problem) or spatially, in heterocystous forms (in which case, the nitrogen fixation occurs in heterocysts and photosynthesis in the vegetative cells). Thus, cyanobacteria have evolved an efficient way to protect O_2-sensitive nitrogenase from O_2 evolved during photosynthesis.

Even though cyanobacteria are a self sufficient group of microbes that are capable of independent existence, many enter into symbiotic associations with a wide range of plants (from algae to angiosperms) and with some animals (Rai, 1990; Schenk, 1992; Warner, 1992; Loffelhardt and Bonhert, 1994; Meeks, 1998; Rai *et al.,* 1996, 2000). Symbiosis, as a mode of existence, exerts considerable changes on the cyanobiont's metabolism. In cyanobacterial-plant symbioses, the cyanobiont is almost always diazotrophic, fixing and providing fixed nitrogen to its eukaryotic partner (Stewart *et al.*, 1983; Rai, 1990; Rai *et al.,* 1996). This necessitates adapting morphological, physiological and biochemical changes to bring about higher rate of nitrogen fixation.

Many nitrogen fixing cyanobacteria are also capable of metabolizing a wide range of combined nitrogen sources, the most common being nitrate, nitrite, ammonia and urea. Many are also capable of utilizing amino acids such as glutamine, arginine and asparagines as sole

nitrogen source to various degrees (Herrero and Flores, 1990; Flores and Herrero, 1994; Herrero *et al.*, 2001). In these organisms there exists a system of hierarchical preferences in the order in which the various inorganic nitrogen sources are assimilated when present in combination. For example, when ammonium is present in the growth medium, the nitrate assimilating system gets expressed at very low levels.

NITROGEN FIXATION IN HETEROCYSTOUS AND NON-HETEROCYSTOUS FORMS OF CYANOBACTERIA

Morphologically, cyanobacteria can be grouped into various categories (Table 1). In non-heterocystous cyanobacteria, nitrogenase enzyme responsible for catalyzing conversion of molecular nitrogen to ammonium is expressed in all the cells while in heterocystous forms the enzyme is localized in the heterocysts. The differentiation of heterocysts from vegetative cells is a nitrogen-regulated process and is accompanied by morphological, biochemical and genetic changes (Wolk, *et al.*, 1994). The presence of externally available fixed nitrogen sources such as ammonia, nitrate, nitrite, urea and some amino acids represses heterocyst differentiation (Wolk, *et al.*, 1994), while their absence leads to 5-15% of the vegetative cells to differentiate into heterocysts. The enzyme nitrogenase which functions exclusively in heterocysts under aerobic conditions is the conventional Mo-dependent nitrogenase (Nif 1) (Elhai and Wolk, 1990; Thiel *et al.*, 1995) made up of two different proteins. The Mo-Fe protein (dinitrogenase) is a $\alpha_2\beta_2$ tetramer and its α and β subunits are encoded by *nifD* (Lammers and Haselkorn, 1983; Golden *et al.*, 1985) and *nifK* (Mazur and Chui, 1982) genes, respectively. The other component of the nitrogenase complex is Fe-protein (dinitrogenase reductase), which is a dimer of two identical subunits coded by *nifH* gene. There are reports of significant DNA rearrangement during heterocyst differentiation from vegetative cells in *Anabaena* sp. strain PCC 7120. *nifHDK* is contiguous in heterocysts of this cyanobacterium but an interruption of 11kb DNA fragment is found in *nifD* gene in vegetative cells (Golden *et al.*,1985). A second rearrangement is also known to occur in the process of heterocyst differentiation deleting a 55kb DNA fragment located in *fdxN* gene (bacterial type ferredoxin gene whose function is not known in cyanobacteria) (Golden *et al.*, 1987). These rearrangements involve site-specific excisases encoded by *xisA* (Lammers *et al.*, 1986) and *xisF* (Carrasco *et al.*, 1994), respectively. Under anaerobic conditions, *Anabaena variabilis* ATCC 29413 has also been shown to possess another Mo-dependent nitrogenase (*Nif 2*) functioning in the vegetative cells (Thiel *et al.*,1995; Thiel and Pratte, 2001). Two other alternative nitrogenases – one vanadium-dependent nitrogenase encoded by *vnfDGK* genes and one Fe-only-nitrogenase have also been reported in *Anabaena variabilis* (Kentemich *et al.*, 1991; Thiel, 1993). Nitrogenase enzyme shows extreme sensitivity to O_2 exposure which manifests both at the level of synthesis and activity (Weare and Benemann, 1974; Gallon and Chaplin, 1987; Gallon, 1992; Rai *et al.*, 1992). Studies on the strategies for O_2 protection during aerobic nitrogen fixation show that there is a temporal separation between N_2-fixation and photosynthesis (Wolk *et al.*, 1994). Under microaerobic to anaerobic conditions, non-heterocystous cyanobacterium *Plectonema boryanum* also shows a temporal separation of nitrogenase activity and net O_2 evolution due to photosynthesis (Weare and Benemann, 1974). In these cells nitrogenase protein is found to be irreversibly inactivated and degraded on exposure to air and nitrogenase activity regains only

after fresh synthesis of the protein (Rai *et al.*, 1992). Even though the nitrogenase protein is ~15 fold higher in non-heterocystous cyanobacteria than in the heterocystous forms, the nitrogenase activities are found to be similar (Rai, 1998). This indicates that much of the nitrogenase protein in non-heterocystous cyanobacteria is not involved in N_2-fixation (Stal and Bergman, 1990; Rai *et al.*, 1992) and may be involved in reduction of O_2 as in *Azotobacter* (Thorneley and Ashby, 1989).

Table 1 Nitrogen-fixing cyanobacterial species

	Type	*Representative species*
I	Unicellular species showing budding or binary fission:	
	(i) All strains are aerobic N_2-fixers	*Gloeothece, Synechocystis*
	(ii) Some strains are aerobic N_2-fixers	*Synechococcus,Cyanothece*
	(iii) Microaerobic or anaerobic N_2-fixer	*Gloeocapsa*
II	Unicellular species showing multiple fission: All strains show microaerobic/anaerobic N_2-fixation	*Dermocarpa, Pleurocapsa, Chroococcidiopsis, Xenococcus, Myxosarcina*
III	Nonheterocystous filamentous species:	
	(i) Anaerobic/microaerobic N_2-fixers	*Plectonema, Lyngbya Phormidium, Pseudoanabaena*
	(ii) Some strains capable of aerobic fixation	*Oscillatoria, Microcoleus, Trichodesmium*
IV	Heterocystous filamentous aerobic N_2-fixers dividing in one plane	*Anabaena, Nostoc, Scytonema, Cylindrospermum, Nodularia, Calothrix*
V	Heterocystous filamentous aerobic N_2-fixers dividing in more than one plane	*Chlorogleopsis, Fischerella*

The relationship between the heterocysts and the vegetative cells is of mutualistic metabolite interchange. The vegetative cells lack the capability to fix atmospheric nitrogen and hence rely on the heterocysts for supply of fixed nitrogen. On the other hand, the vegetative cells transfer carbohydrates to the heterocysts. The exact nature of the translocated carbohydrate is not yet established although glucose, sucrose, maltose etc. are likely candidates (Table 2). The carbohydrate metabolism in the heterocysts is through oxidative pentose phosphate pathway as well as via glycolytic pathway generating pyruvate (Haselkorn, 1978; Gallon and Chaplin, 1987).

One of the characteristics of heterocystous cyanobacteria is that they enter into symbiotic associations with other plants (Table 3).

In symbiosis, the demand for fixed nitrogen is far higher than in the free-living state as the cyanobionts is almost entirely responsible for provision of the total fixed nitrogen requirement of the whole symbiosis. To meet such a demand, cyanobacteria in symbiosis differentiate into much higher heterocyst frequency (in some cases up to 80%). In free-living conditions, nitrogenase activity of a cyanobacterium shows a linear increase with the increase in heterocyst frequency (Table 4)

Table 2 Effect of carbohydrates on heterocyst frequency and nitrogenase activity in *Nostoc* ANTH

Growth medium	*Heterocyst frequency (%)*	*Nitrogenase activity (nmol C_2H_2 reduced μg^{-1} Chl a h^{-1})*
N_2-medium	13.95	3.09
N_2-medium + 15 mM sucrose	17.50	8.65
N_2-medium + 50 mM fructose	19.02	9.05
N_2-medium + 50 mM glucose	21.64	12.85
N_2-medium + 50 mM maltose	21.02	4.19
N_2-medium + 50 mM lactose	18.77	4.79
N_2-medium + 10 mM galactose	17.00	3.55

Table 3 N_2-fixing symbioses involving cyanobacteria

Host (plants)	*Cyanobacteria*	*Comments*
Angiosperms: *Gunnera*	*Nostoc* sp.	Cyanobacterium is located inside host cell in stem nodules. Symbiosis is intracellular.
Gymnosperms: *Cycads* (e.g. *Cycas, Macrozamia*)	*Nostoc* sp.	Cyanobacterium is located in the cortical zone of the coralloid roots. Symbiosis is intercellular.
Pteridophytes: *Azolla*	*Nostoc* sp. (usually called *Anabaena azollae*)	Cyanobacterium occupies the mucilage filled cavities on the ventral surface of the dorsal lobes of the leaves. Symbiosis is intercellular.
Bryophytes:		
Hornworts: *Anthoceros Notothylus*	*Nostoc*	Cyanobacterium occupies mucilage filled cavities on undersurface of hornwort/liverwort gametophytic thallus. Symbiosis is intercellular.
Liverworts: *Blasia*	*Nostoc*	
Cavicularia	*Nostoc*	
Mosses: *Sphagnum*		Cyanobacterium occupies the hyaline cells of the moss.
Fungi: Lichens	*Nostoc, Scytonema, Fishcerella, Calothrix,* Unicellular cyanobacteria	N_2-fixing lichens consist of two-membered associations between fungi and cyanobacteria or three-membered associations containing a green alga as well. Symbioses are intercellular and are referred as cyanolichens. Of 18,000 documented species of lichens about 8% are cyanolichens.
Algae:		
Marine diatoms (*Rhizosolenis, Hemiaulus*)	*Richelia, Calothrix*	Cyanobacterium is located in the periplasmic space.
Freshwater diatom (*Rhopalodia*)	Unicellular cyanobacteria	Cyanobacterium appears as bluish-green inclusions in the cytoplasm.
Marine sponges: 38 genera of *Calcarea* and *Desmospongia* groups.	*Aphanocapsa Phormidium*	Cyanobacterium occurs inter or intracellularly through out the sponge tissue and/or superficial tissues. Symbioses are mainly restricted to the photic zone only.

Prokaryotes: Various non photosynthetic bacteria	Various nonheterocystous and heterocystous cyanobacteria	Cyanobacteria act as the host. The bacterium occupies the mucilage sheath. Bacteria are clustered around the heterocysts or heterocyst-vegetative cell junctions in heterocystous forms. The symbiont occurs intracellularly in *Pleurocapsa minor*.
Non photosynthetic protists:		
Amoeba (*Paulinella*) Glaucophyta (*Cyanophora*)	*Cyanelles*	Intracellular inclusions resembling unicellular thin-walled cyanobacteria referred as 'cyanelles'.

Table 4 Linear relation between heterocyst frequency and nitrogenase activity of few cyanobacterial species

Cyanobacterial sp.	*Heterocyst frequency (%)*	*Nitrogenase activity (nmol C_2H_2 reduced mg^{-1} dry wt h^{-1})*
Nostoc linckia	4.52	1.21
Anabaena doliolum	4.75	1.52
Anabaena cycadeae	7.69	2.46
Nostoc ANTH	13.95	3.09

Increase in heterocyst frequency of a cyanobiont depends on its role in the symbiosis and on the availability of carbon nutrition in the form of fixed carbon while in symbiosis. In those symbioses where the cyanobionts transfer fixed-N to its partner(s) and in return receive fixed-C, the heterocyst frequency is highest. Cyanobionts supplying fixed-N but not receiving any fixed-C in return come next followed by the cyanobionts that supply both fixed-N and fixed-C to its partner(s). In the last case, the heterocyst frequency of the cyanobionts is almost similar to its free-living counterpart. Microscopic studies have revealed that double and multiple heterocysts occur beyond a heterocyst frequency of 30%. Studies have revealed that the nitrogenase activity of a cyanobiont shows a linear relationship with increase in single heterocysts (as in the case of free-living cyanobacteria) rather than to the total heterocyst frequency. Immunogold studies have shown that all heterocysts including the multiple ones possess nitrogenase protein (Rai *et al.*, 1989). As seen in Table 5, in the case of *Anthoceros punctatus–Nostoc* symbiosis, the old *Nostoc* cyanobiont colonies isolated from the base of the *Anthoceros* gametophyte have maximum heterocyst frequency. Under the microscope they also revealed occurrence of maximum number of double and multiple heterocysts. Yet nitrogenase activity was much higher in the mature colonies where the ratio of single heterocysts to double or multiple heterocysts is much higher. The reason for lower nitrogenase activity in these colonies despite the existence of nitrogenase protein in all heterocysts could be due to restricted availability of carbohydrates from the vegetative cells. In the case of multiple heterocysts, the heterocysts in the middle of a row are not in contact with any vegetative cells at all and those at the end of the chain are connected only to one vegetative cell as against single heterocysts that are connected to two vegetative cells directly on both sides. Hence, the availability of proper amount of photosynthate is a predisposed criterion for efficient nitrogenase activity. The whole focus of the cyanobiont in symbiosis is towards higher rate of nitrogen fixation which creates a much higher demand for

fixed-C as a source of energy and reductant for nitrogenase activity (Rai, 1998). The reductant in the heterocyst for N_2-fixation is ferredoxin, although under iron deficient condition flavodoxin is used as a substitute for ferredoxin (Gallon and Chaplin, 1987). Heterocysts lack photosystem II activity and hence there is no generation of reductant from water in the non-cyclic electron transport. Generation of reductant by cyclic electron transport involving light and photosystem I is possible, provided there is a suitable source of electron. H_2, via the action of hydrogenase is a possible source of reductant for N_2-fixation in heterocysts. Apart from that, by losing photosystem II, heterocysts protect nitrogenase from O_2 exposure that irreversibly inactivates nitrogenase.

Table 5 Correlation between heterocyst frequency and nitrogenase activity with age in the cyanobiont *Nostoc* ANTH isolated from different parts of the *Anthoceros* gametophytic thallus.

Portion of the gametophyte	*Heterocyst frequency (%)*	*Nitrogenase activity (nmol C_2H_2 reduced mg^{-1} dry wt h^{-1})*
Tip (young colonies)	2.91	1.456
Middle (mature colonies)	19.20	2.018
Base (old colonies)	45.80	1.135

NITRATE UPTAKE AND ITS ASSIMILATION IN CYANOBACTERIA

Nitrate is the most abundant form of fixed-N available to microorganisms and plants in nature and in cyanobacteria it is the most common form of nitrogen nutrition. Exogenous nitrate is efficiently transported in to the cyanobacterial cells where it is then reduced first to nitrite and then to ammonia for assimilation by the combined effort of nitrate reductase (NR) and nitrite reductase (NIR) enzymes (Flores and Herrero, 1994). Heterocysts lack both nitrate uptake and nitrate reductase systems but both these processes are present in the vegetative cells (Kumar *et al.*, 1985; Rai and Bergman, 1986). The transport of nitrate into the cyanobacterial cells is energy dependent and needs participation of ATP to drive nitrate transport (Rai *et al.*, 1981; Flores *et al.*, 1983a; Bagchi *et al.*, 1989). Based on studies of positive effect of sodium ions on the intracellular levels of accumulation of nitrate, in nitrate reductase mutants of unicellular *Synechococcus* sp., it has been suggested that there exits a cotransport of nitrate and sodium with a downhill electrochemical gradient for sodium from outside the cells to inside which might be responsible for nitrate being actively taken up into the cells (Rodriguez *et al.*, 1992). Repression of NR in heterocysts is not due to the absence of Mo-cofactor but lack of apoprotein. This may be a strategy to eliminate competition for Molybdenum (Mo) and reductant between the two molybdo-enzymes – nitrogenase and nitrate reductase (Rai, 1998). Rai and Bergman (1986) have also established that the loss of nitrate uptake and NR during heterocyst differentiation is irreversible and it is probably due to genomic rearrangement during heterocyst development resulting in *nif* gene co-transcription. Structural genes for nitrite reductase (*nirA*), nitrate/nitrite uptake (*nrt ABCD*) and nitrate reductase (*nar B*), have been found to be co-transcripted as a single operon in *Synechococcus* sp. strain PCC 7942 and *Anabaena* sp. strain 7120 (Cai and Wolk, 1993; Omata *et al.*, 1993; Frias *et al.*, 1997). Assimilation of externally available nitrate depends on the status of externally available ammonium in the growth medium. Cyanobacterial cultures engaged in active nitrate uptake and assimilation show drastic inhibition of nitrate uptake on exposure to

ammonium (Ohmori *et al.*, 1977; Rai *et al.*, 1981; Flores *et al.*, 1980; Garbisu *et al.*, 1992). This inhibition is reversible and if the ammonium in the medium is exhausted then the nitrate uptake resumes within minutes. It is suggested that ammonium may inhibit nitrate uptake by lowering the ATP concentration which is required for nitrate uptake (Rai *et al.*, 1981; Rai and Singh, 1982). However, it also seems possible that a product of ammonium assimilation via glutamine synthetase has a feedback role in inhibition of nitrate uptake on exposure to externally supplied ammonium (Flores *et al.*, 1983b). Nitrate uptake also shows strict dependence on CO_2-fixation (Manzano *et al.*, 1976; Flores *et al.*, 1983c). Hence, there are two regulatory forces working on nitrate uptake – ammonium show negative regulation while CO_2-fixation shows a positive effect. The two processes have antagonic roles on nitrate uptake. It is possible that CO_2-fixation products combine with the negative effectors generated by ammonium assimilation, neutralizing the negative effectors (Flores *et al.*, 1983c). Furthermore, nitrate is reported to cause induction of nitrate reductase (Bagchi *et al.*, 1985b). Nitrite uptake is shown to take place both actively and passively. There could be a common transport system for both nitrate and nitrite as products of ammonium assimilation via GS also inhibit the nitrite uptake (Flores *et al.*, 1987; Martin-Nieto *et al.*, 1989). Bhattacharya *et al.* (2002) have recently shown that in *Nostoc* ANTH, there is a nitrite uptake system quite distinct from the common nitrate – nitrite uptake system.

AMMONIA UPTAKE AND ITS UTILIZATION IN CYANOBACTERIA

Ammonia occupies a unique biochemical position as it is the only form of inorganic nitrogen that can be directly incorporated into the organic molecules in the living beings. It occupies the status of the obligate intermediate in the utilization of other inorganic forms of nitrogen. Ammonia is the preferred source of nitrogen and it regulates most of the key enzymes of nitrogen metabolism such as nitrogenase, glutamine synthetase and nitrate reductase in living organisms. Ammonium transport system (ATS) in cyanobacteria has been extensively studied using ^{14}C methylammonium (an ammonium analogue) (Boussiba *et al.*, 1984; Rai *et al.*, 1984; Boussiba and Gibson, 1987; Shehawy and Kleiner, 1999). Ammonium uptake in cyanobacteria has been shown to occur in two phases- an initial rapid phase of 2-3 minutes duration that is MSX insensitive and independent of methylammonium metabolism, followed by a slower second phase which is sensitive to MSX exposure and dependant on methylammonium metabolism (Singh *et al.*, 1985, 1986, 1987). Further studies on GS defective mutant of *Anabaena cycadeae* suggest that the second phase of the ammonium transport may be a separate ATS and hence there could be two ATS present (Singh *et al.*, 1985). Recent studies show that in *Synechocystis* sp. PCC 6803 there are three putative *amt* (ammonium transport) genes that are under nitrogen control i.e. these are derepressed under nitrogen depleted conditions and repressed under nitrogen replete conditions. *amt1* gene, responsible for a high affinity transporter with K_s for methylammonium 2.7 μM is expressed at higher rates than the other two *amt* genes. *amt1* transcription is also under control of transcription factor, NtcA (Montesinos *et al.*, 1998). Kleiner (1985) has suggested that the importance of the ATS lies in the uptake of exogenous ammonium and retention of ammonium produced during nitrogen fixation. Ammonium taken up from external sources or generated during nitrite/nitrate assimilation and N_2-fixation is incorporated by glutamine synthetase-gtutamate synthase (GS-GOGAT) pathway into organic forms. The levels of GS (*glnA*

gene product) enzyme vary in cyanobacteria in relation to nitrogen nutrition (Merida *et al.*, 1991; Flores and Herrero, 1994). Under N_2-fixing conditions GS levels are much higher than when the cyanobacterial cells are grown in the ammonium supplemented medium. Similarly, under nitrogen depleted conditions there is increase in *glnA* mRNA synthesis and GS activity. There are two distinct promoters for *glnA* gene: one *E. coli* type promoter and one *nif*-like promoter (Tumer *et al.*, 1983). Under N_2-fixing conditions *glnA* transcription occurs through a *nif*-like promoter. Regulation of GS protein in cyanobacteria is not under control of adenylation/deadenylation (Merida *et al.*, 1991).

AMINO ACIDS NUTRITION AND TRANSPORT IN CYANOBACTERIA

Some nitrogen fixing cyanobacteria are able to utilize amino acids such as glutamine, arginine and asparagines as sole nitrogen source (Herrero and Floris, 1990; Flores and Herrero, 1994; Herrero *et al.*, 2001). On the other hand, amino acids like glutamate, histidine and lysine are reported to be inhibitory to growth (Chapman and Meeks, 1983; Flores and Muro-Pastor, 1990; Prakasham *et al.*, 1991). However, the relative efficiency of different cyanobacteria to utilize them as sole nitrogen source or the degree to which these amino acids can support cyanobacterial growth varies widely. *Synechococcus* PCC 6803 shows comparable growth on arginine to that of nitrate but *Anabaena* sp. PCC 7120 shows slower growth on arginine than on nitrate (Herrero and Flores, 1990; Flores and Muro-Pastor, 1990; Flores and Herrero, 1994; Herrero *et al.*, 2001). There are scanty and contradictory reports about the role of amino acids on cyanobacterial metabolism. Arginine has been reported to be a weak repressor of nitrogenase and nitrate reductase in *Anabaena* sp. PCC 7120 (Herrero and Flores, 1990) while there are reports of arginine inducing NR activity in *Oscillatoria chalybea* (Bednarz and Schmid, 1991). Similarly, glutamine has been found to repress nitrogenase activity but not heterocyst formation (Thiel and Lyons, 1986). At the same time, there are reports of the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7002 being able to utilize a wide range of organic compounds such as urea, most amino acids and purines (especially hypoxanthine and xanthine) as N-sources for growth and other functions (Kapp *et al.*, 1975).

In *Synechocystis* sp. strain PCC 6803 and in *Anabaena* sp. strain 7120 arginine was found to be taken up and concentrated within the cells by means of a transport system specific for basic amino acids (Labarre *et al.*, 1987; Flores and Muro-Pastor, 1990; Herrero and Flores, 1990; Flores and Herrero,1994). Three amino acid transport systems have been reported in *Synechocystis* sp. strain PCC 6803, one specific for basic amino acids and glutamine, one for neutral amino acids excluding glutamine and one specifically for glutamine and glutamate (Labarre *et al.*, 1987). A high affinity and a low affinity transport system for both glutamine and glutamate (Chapman and Meeks, 1983) and a single transport system for leucine (Thiel, 1988) have been reported in *Anabaena variabilis* ATCC 29413. Strasser and Falkner (1986) have reported a common transport system for aspartate and glutamate in *Nostoc* sp. Similarly, a common transport system for both glutamine and glutamate has also been reported in *Anabaena* PCC 7120 (Flores and Muro-Pastor, 1988). In addition, *Anabaena* 7120 possesses three ATP dependant high affinity amino acid transport systems: two for neutral amino acids (Montesinos *et al.*, 1995) and one for basic amino acids (Herrero and Flores, 1990). Further, there are two low affinity transport

systems: one specific for acidic amino acids (Montesinos *et al.*, 1995) and one for basic amino acids (Herrero and Flores, 1990). In *Anabaena* sp. exogenously supplied glutamine is converted to glutamate mainly by the action of GOGAT although glutaminase activity has also been detected (Haystead *et al.*, 1973; Rowell *et* al., 1977; Montesinos *et al.*, 1995).

Scanty information is available on the uptake regulation of methionine transport system in cyanobacteria except for *Prochlorococcus* spp. (Zubkov *et al.*, 2004). Recent research by our group demonstrated a concentration-dependent effect of methionine in *N. muscorum* stimulating its nitrogenase activity at lower concentrations and inhibiting the same at increased concentrations, suggesting a regulatory role of methionine and/or its metabolized product on nitrogen metabolism. The cyanobacterium showed a biphasic pattern of methionine uptake activity that was competitively inhibited by the amino acids alanine, isoleucine, leucine, phenylalanine, proline, valine, glutamine, and asparagines (Singh *et al.*, 2008). It seems that methionine toxicity is exhibited through its influence on internal nitrogen status mainly by affecting nitrogenase activity of the organism. Amino acids that could act as a nitrogen source were also able to protect cyanobacterium against methionine toxicity. However, unlike nitrate and ammonium, nitrogenase activity inhibition by amino acids varied, and the extent of growth protection by amino acids was determined by their ability to act as nitrogen sources. The best utilizable nitrogen sources such as nitrate and amino acids arginine, glutamine, alanine, and proline supported maximum growth and inhibited nitrogenase activity completely followed by other nitrogen sources. Contrary to nitrate and ammonium, amino acids seem to provide protection at two levels, first at the level of entry of methionine into cells and second by meeting intracellular nitrogen requirement. That both mechanisms operate in case of amino acids became clear by following methionine uptake and nitrogenase activity in the presence of nitrate, ammonium, and amino acids in the medium. Nitrogenase activity was inhibited in all combined nitrogen sources containing media, though to varied degrees. However, methionine uptake activity inhibition occurred only in the presence of amino acids. No influence of inorganic nitrogen compounds on methionine uptake activity suggested a methionine transport system independent of nitrate or ammonium transport.

Studies on amino acids uptake and utilization by *Nostoc* ANTH show growth in glutamine, asparagine and arginine supplemented media as sources of fixed-nitrogen (Table 6). Glutamine and asparagine support growth of *Nostoc* ANTH better than arginine. The heterocyst formation, nitrogenase and NR activities are completely repressed by glutamine and asparagine while the arginine exerts only partial inhibition. However, the glutamine synthetase (GS) activity remained unaffected in presence of these amino acids. These observations are similar to that of *Anabaena* PCC 7120 (Herrero and Flores, 1990). Unlike in *Anabaena variabilis* where glutamine inhibits nitrogenase only, in case of *Nostoc* ANTH glutamine inhibits both nitrogenase activity and heterocyst formation (Thiel and Lyons, 1986; Chen *et al.*, 1987; Bhattacharya *et al.*, 2002a, b). The NR activity of *Nostoc* ANTH is nitrate inducible/ammonium repressible and cultures grown in glutamine and asparagines show strong inhibition of NR activity than those grown in arginine (50%). These results agree with partial repression of NR activity in *Anabaena* PCC 7120 by arginine (Herrero and Flores, 1990) but are in contrast to the arginine induction of NR activity in *Oscillatoria chalybea* (Bednarz and Schmid, 1991). The GS activity in *Nostoc* ANTH in presence of glutamine, arginine and asparagines remains nearly similar to those grown in N_2 medium. Similar results have been reported earlier in *N. muscorum* (Singh *et al.*, 1991).

Table 6 Growth, heterocyst frequency (HF), nitrogenase activity (N_2ase), nitrate reductase activity (NR) and glutamine synthetase/transferase activity (GS) of 4 day old *Nostoc* ANTH as a function of different nitrogen sources.

Addition to $BG11_0$ medium (mM)	*Growth*	*HF (%)*	*N_2ase (nmol C_2H_4 formed μg^{-1} Chl a h^{-1})*	*NR (nmol NO_2 formed $min^{-1}mg^{-1}$ protein)*	*GS (nmol γ glutamyl hydroxamate formed min^{-1} mg^{-1} protein)*
Nil	1.2 ± 0.1	5.3 ± 0.2	12 ± 0.6	1.8 ± 0.11	610 ± 7
NO_3 (5)	1.3 ± 0.1	0.0	0.0	4.2 ± 0.15	598 ± 3
NH_4^+ (2)	1.4 ± 0.1	0.0	0.0	0.2 ± 0.10	376 ± 6
Glutamine (1)	2.1 ± 0.2	0.2	0.1	0.3 ± 0.06	595 ± 8
Asparagine (1)	1.9 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.04	581 ± 7
Arginine (1)	1.2 ± 0.1	2.6 ± 0.3	6.1 ± 0.3	0.9 ± 0.01	601 ± 4

Studies on uptake of different amino acids in *Nostoc* ANTH as a function of different nitrogen sources in the media showed that glutamine uptake was biphasic in nature: an initial rapid phase of about 2 min followed by a slower second phase that lasted the rest of the experimental duration. N_2-grown *Nostoc* ANTH showed glutamine uptake rates of 55 and 11 nmol $min^{-1}mg^{-1}$ Chl *a* during the first and the second phase, respectively. The glutamine uptakes rates were much higher (93 and 44 nmol min^{-1} mg^{-1} Chl *a*) in glutamine-grown cultures. This increase in the rate of glutamine uptake was sensitive to chloramphenicol-a protein synthesis inhibitor. The activity and pattern of glutamine uptake in nitrate and ammonium grown cultures were similar to that of N_2-grown cultures.

The N_2-grown cultures showed asparagine uptake rates of 30 and 11 nmol min^{-1} mg^{-1} Chl *a* during first and second phase, respectively, and the rate of asparagine uptake is higher in asparagine-grown cultures (56 and 29.2 nmol min^{-1} mg^{-1} Chl *a*).

The N_2-grown *Nostoc* ANTH cultures showed arginine uptake rates of 34 and 21 nmol min^{-1} mg^{-1} Chl *a* while the rates were 115 and 51 nmol min^{-1} mg^{-1} Chl *a* in arginine grown cultures during first and the second phase of uptake studies, respectively. Hence, the results suggest that the uptake of glutamine, arginine and asparagine in *Nostoc* ANTH are biphasic and substrate–inducible (Bhattacharya *et al.*, 2002b). Also, such substrate induced amino acid uptake requires *de novo* protein synthesis as was evident from the fact that choramphenicol inhibited uptake processes in all the amino acids studied. Unlike ammonium uptake, glutamine, asparagines and arginine uptake are not inhibited by presence of nitrate or ammonium and hence, uptake of these amino acids probably is not under direct control of nitrogen source present in the surrounding, but may be related to the overall nitrogen status of the cell. Such a lack of direct control by nitrate and ammonium has also been seen for leucine uptake in *Anabaena variabilis* (Thiel, 1986) and for arginine uptake in *Anabaena* PCC 7120 (Herrero and Flores, 1990).

Studies involving effects of DCMU, the inhibitor of non-cyclic photosynthetic electron transport, CCCP (an uncoupler) and DCCP (an inhibitor of F_0 fraction of ATPase) on uptake rates of glutamine, asparagine and arginine found that presence of the protonophore CCCP and the ATPase inhibitor DCCD strongly inhibited the uptake of the amino acids studied. The DCMU (an inhibitor of photosynthetic O_2-evolution) partially inhibited the uptake of glutamine, asparagine

and arginine. Hence, the uptake of the above mentioned amino acids are energy dependant as was already reported in the cases of *Anabaena variabilis* and *Anabaena* PCC 7120 (Thiel, 1988; Herrero and Flores, 1990; Montesinos *et al.*, 1995).

UREA UPTAKE AND ASSIMILATION

Urea is continuously fed into the environment through biological activity and through addition as N-fertilizer for agricultural purposes. Many cyanobacteria belonging to different taxonomic groups can utilize urea as their N-source (Kratz and Myers, 1955; Neilson and Larsson, 1980; Rawson, 1985). Urea uptake appears to be carrier mediated and it is taken up intact and actively, using energy derived mainly from cyclic photophosphorylation and then metabolized intracellularly (Healey, 1977; Jahns *et al.*, 1988). Urea transporters in *Anabaena doliolum* and *Anacystis nidulans* have been reported to possess K_m of 250 and 400 μM, respectively at pH 7.0 (Rai and Singh, 1987a). Presence of ammonia in the medium does not seem to inhibit urea uptake, although, simultaneous presence of urea in the medium depresses ammonia uptake (Healey, 1977; Rai and Singh, 1987a). Since intracellular accumulation of ammonia inhibits its own uptake, this inhibition by urea presumably needs hydrolysis of urea taken up by the cells to ammonia (Healey, 1977; Rai and Singh, 1987a). The enzyme urease degrades the intracellular urea in cyanobacteria releasing one molecule of CO_2 and two molecules of NH_4^+ (Berns *et al.*, 1966, Mackerras and Smith, 1986; Singh and Ahmad, 1989). Urease has been purified from *Spirulina maxima* (Carvajal *et al.*, 1982) and from *Anabaena cylindrica* (Argall *et al.*, 1992) and has been shown to require Ni^{2+} ions in the growth medium. Cyanobacterial urease has been speculated to be a scavenger of amino nitrogen from urea produced internally by degradation of purines and arginine (Ge *et al.*, 1990). Cyanobacterial cells possess cyanophycin granules which are used as nitrogen reserves and are mobilized during N-starvation. They consist of multi-L-arginyl-poly (L-aspartic acid) (Simon, 1971; Allen *et al.*, 1980; Allen and Weathers, 1980). Their degradation is carried out by cyanophycinase to aspartic acid-arginine dipeptide, which gets further degraded to free arginine and eventually to urea (Gupta and Carr, 1981a). Gupta and Carr (1981b) have reported the presence of cyanobacterial arginase activity, which may be an indication of urea producing pathways in cyanobacteria.

CONCLUSION

Nitrogen fixing capability of cyanobacteria has catapulted this group of organisms into the midst of intense research. The behavioural changes of cyanobacteria in presence of various fixed nitrogen sources in terms of growth, physiology and different metabolic processes are of great interest as they modify nitrogen fixing ability of the cyanobacteria. The presence of ammonium in the growth media represses the expressions of the proteins involved in the utilization of other nitrogen sources like nitrate or N_2. Apart from being the most preferred source of nitrogen, ammonium regulates various processes in N-metabolism and photosynthesis in cyanobacteria. GS mediated ammonium metabolism seem to be necessary for all the repressive effects on N-metabolism in presence of externally supplied ammonium. This suggests involvement of a common mechanism in these regulations. Use of amino acids as N-nutrition shows that relative efficiency with which

amino acids can support growth in cyanobacteria varies. Amino acid uptake seems to be substrate inducible, energy dependent, requires *de novo* protein synthesis and is biphasic in nature. Nitrate and ammonium repress ammonium uptake but not amino acid uptake.

Studies involving N-metabolism lead to understanding the molecular mechanism involved in N_2-fixation and modifications in this process on exposure to various alternative N-sources. This knowledge may be important in developing high yielding N_2-fixers for N-fertilization in rice fields and viable, useful symbiotic associations of cyanobacteria with crop plants for enrichment of soil.

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