ROLE OF AMMONIUM AND GLUTAMINE TRANSPORT SYSTEMS IN NITROGEN CONTROL OF NITROGENASE IN NITROGEN-FIXING CYANOBACTERIA

BY

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This thesis is dedicated to my

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I certify that the thesis entitled "**Role of Ammonium and Glutamine Transport Systems in Nitrogen Control of Nitrogenase in Nitrogen-fixing Cyanobacteria**" submitted by Mr. **Reddy Shetty Prakasham** for the degree of **Doctor of Philosophy** of the **North-Eastern Hill University, Shillong**, embodies the record of original investigation carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any other degree of this or any other University.

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STATEMENT

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(R. PRAKASHAM)

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ABBREVIATIONS

ADP = Adenosine 5'-diphosphate

ATP = Adenosine 5'-triphosphate

ATS = Ammonium transport system

BSA = Bovine serum albumin

C = Carbon

Chl = Chlorophyll

cm = Centimeter

cm^3 = Milliliter

DCMU = Dichlorophenyl dimethylurea

dm^3 = Liter

DNA = Deoxyribonucleic acid

e^- = Electron

FMN = Flavin mononuclotide

GOGAT = Glutamate synthase

GS = Glutamine synthetase

h = Hour(s)

Het^+ = Heterocystous

Het^- = Non heterocystous

HEPES = 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid

LDH = Lactate dehydrogenase

MA = Methylamine

min = Minute(s)

mm^3 = Microliter

MSX = L-Methionine-DL-sulphoximine

N = Nitrogen

NAD = Nicotinamide adenine dinucleotide

NADH = Nicotinamide adenine dinucleotide reduced

NADP = Nicotinamide adenine dinucleotide phosphate

NADPH = Nicotinamide adenine dinucleotide phosphate reduced

Nif^+ = Nitrogen fixing

Nif^- = Non nitrogen fixing

NIR = Nitrite reductase

NR = Nitrate reductase

OD = Optical density

PK = Pyruvate kinase

POPOP = 1,4-Bis[2-(5-phenyloxazolyl)]benzene

PPO = 2,5-Diphenyloxazole

PS = Photosystem

RNA = Ribonucleic acid

TCA = Trichloroacetic acid

$TPMP^+$ = Triphenyl methylphosphonium

$\Delta\psi$ = Transmembrane electrical potential

1. INTRODUCTION AND LITERATURE REVIEW

1.1. General:

Nitrogen is one of the essential elements as cellular constituent of living organisms. Although, 78% of the earth's atmosphere consists of molecular nitrogen, most organisms cannot utilize this as nitrogen source. Instead, they require combined nitrogen. The commonest nitrogen source for plants and microbes is ammonia. In modern agriculture, for high yield, chemical fertilizers are used on a large scale to serve as nitrogen source for crop plants. Chemical fertilizer production requires enormous amounts of energy. In addition, considerable amounts of energy, time and labour is needed for distribution and application of these factory produced fertilizers at the field level. Due to this, prices of chemical fertilizers is constantly on increase with a widening gap between supply and demand. However, there are some prokaryotic microorganisms which can convert molecular nitrogen into ammonia under normal physiological conditions. A thorough understanding of the process of biological nitrogen fixation will help to devise strategies resulting in an alternative to the chemical nitrogen fertilizers. Hence, there is a world-wide attempt, by the scientific community, to study the physiological, biochemical and genetic aspects of N_2-fixing organisms. Among such organisms, cyanobacteria are of special interest because of their simple growth requirements, their diazotrophic nature, and their being capable of carrying out

oxygenic photosynthesis (Singh, 1961; Sprent, 1979; Stewart, 1980; Whitton & Carr, 1982; Gallon & Chaplin, 1987; Rai, 1990).

1.2. Cyanobacteria:

Cyanobacteria are widely distributed in both aquatic and terrestrial habitats (Fogg *et al.*, 1973). They occur freely (free-living) as well as in symbiotic associations (Stewart *et al.*, 1983; Gallon & Chaplin, 1987; Rai, 1990). They are photosynthetic prokaryotes with a higher plant type oxygenic photosynthesis and share characteristics of gram-negative bacteria (Stanier, 1977; Stanier & Cohen-Bazire, 1977; Stewart, 1980). They produce two types of metabolically active cells namely, vegetative cells and heterocysts. The third type of cells, akinetes, are metabolically less active and serve as perennating bodies (Nichols & Adams, 1982; Rai *et al.*, 1985; 1988). Various details of cyanobacteria have been extensively reviewed in the recent past (Stanier *et al.*, 1978; Rippka *et al.*, 1979; Bothe *et al.*, 1980; Gallon, 1980; Stewart, 1980; Stewart *et al.*, 1980; 1982; Bothe, 1982; Carr & Whitton, 1982; Gallon & Chaplin, 1987; Hallenbeck, 1987; Rai, 1990).

1.2.1. Vegetative cells:

The vegetative cells have photosynthetic machinery essentially like chloroplasts, which carry out higher plant type oxygenic photosynthesis using water as ultimate source of reductant (Doolittle, 1979; 1982; Ho & Krogmann, 1982). Photosynthetic components are located on a system of thylakoid

membranes which are topographically and functionally distinct from plasma membrane. They are usually distributed near periphery of the cell and often oriented parallel to the cell wall (Fogg *et al.*, 1973; Long & Whitton, 1973). Chlorophyll **a** is the principal light harvesting pigment, while phycobiliprotein complexes are accessory pigments of PS II. These pigments are located on thylakoid membrane (Cohen-Bazire & Bryant, 1982; Ho & Krogmann, 1982; Morschel & Rhiel, 1987). Under conditions of N-deficiency the accessory pigments are readily used as nitrogen sources (Allen & Smith, 1969; Stewart *et al.*, 1978; Cohen-Bazire & Bryant, 1982). Vegetative cells produce ATP by oxidative phosphorylation and via cyclic and non-cyclic photophosphorylation. Carbon-di-oxide fixation occurs through Calvin cycle (Stanier, 1977, Stewart, 1977; Allen, 1984).

The major reserve polymers present in cyanobacterial vegetative cells are polysaccharides (glycogen), cyanophycin (N-reserve) and polyphosphates which are mobilized when a need arises (Merrick, 1979; Smith, 1982). Polysaccharide reserves are located along the thylakoid membranes (Ris & Singh, 1961; Jost, 1965).

Vegetative cells of cyanobacteria also accumulate poly-hedral bodies which are store houses of Calvin cycle enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Codd & Stewart, 1976; Allen, 1984).

In non-heterocystous N_2-fixing strains, the vegetative cells also contain nitrogenase (Gallon & Chaplin, 1988). In heterocys-tous forms; however, the nitrogenase is located in heterocysts which are non-photosynthetic and which receive fixed-carbon from neighbouring vegetative cells (Bothe *et al.*, 1984; Stewart *et al.*,

1985; Bergman *et al.*, 1986).

1.2.2. Heterocysts:

In inorganic growth medium devoid of combined nitrogen, 5-10% of the vegetative cells differentiate into specialized structures called heterocysts. Heterocysts are generally larger than vegetative cells and comparatively paler in colour. They are sites of dinitrogen fixation (Fay *et al.*, 1968; Stewart, 1980; Janaki & Wolk, 1982; Bergman *et al.*, 1986) and have undergone a number of structural, biochemical and genetic changes during their development (Wolk, 1982; Golden *et al.*, 1985; Haselkorn *et al.*, 1987). They possess thick envelope comprising of an inner laminated layer, a central homogeneous layer and an outer fibrous layer (Wolk, 1982). The thylakoid membranes are present but they lack PS II activity and have little or no phycobiliproteins (Reinman & Thornber, 1979; Alberte *et al.*, 1980; Stewart, 1980). They are unable to fix CO_2 due to lack of ribulose 1,5-bisphosphate carboxylase (Winkenbach & Wolk, 1973; Codd & Stewart, 1977; Codd *et al.*, 1980; Cossar *et al.*, 1985) and depend on adjacent vegetative cells for provision of carbohydrates (Wolk, 1968; Stewart, 1980; Bothe *et al.*, 1984; Stewart *et al.*, 1985).

The carbon dissimilation, in heterocysts, is mainly through oxidative pentose phosphate pathway (Smith, 1982). The key enzymes of hexose oxidizing pathway, glucose 6-phosphate NADP: oxidoreductase and 6-phosphogluconate NADP: oxidoreductase are present in heterocysts (Winkenbach & Wolk, 1973; Bohme, 1987) at far higher levels than in vegetative cells (Gallon & Chaplin, 1987). Recently, it has been reported that heterocysts contain all

glycolytic pathway enzymes and a low level of pyruvate ferredoxin oxidoreductase (Stewart *et al.*, 1985). Primary assimilation of nitrogenase-derived ammonia occurs in heterocysts. Glutamine is transported to vegetative cells. It is however, debatable whether GOGAT occurs in heterocysts (Hallenbeck, 1987).

Thioredoxins have been implicated in regulation of carbon metabolism in cyanobacteria. They are small proteins which are reduced in light by reduced ferredoxins. Reduced thioredoxin activates Calvin cycle enzymes and inactivates the oxidative pentose phosphate pathway enzyme, glucose 6-phosphate NADP: oxidoreductase. Thioredoxin has been reported to be absent in heterocysts (Rowell *et al.*, 1985a; Bohme, 1987). Thus, glucose 6-phosphate NADP: oxidoreductase remains active in heterocysts even during light.

Heterocysts generate ATP by cyclic photophosphorylation, oxidative phosphorylation (Stewart, 1980; Gallon & Chaplin, 1987) and oxyhydrogen reaction (Hallenbeck & Benemann, 1979; Bothe, 1982). Heterocyst production is inhibited by ammonium (Singh *et al.*, 1983a).

1.3. Nitrogen-metabolism in cyanobacteria:

The common sources of inorganic nitrogen for cyanobacteria are N_2, NO_3^- and NH_3. While all cyanobacteria are capable of utilizing NO_3^- and NH_3, N_2-utilization is limited to those cyanobacteria which are capable of nitrogen-fixation. These include all heterocystous and some non-heterocystous forms.

1.3.1.1. Nitrogen fixation:

The enzyme responsible for the reduction of dinitrogen to ammonia is nitrogenase (Gallon, 1980; Stewart, 1980; Hallenbeck, 1987; Smith *et al.*, 1987a). Some of the nitrogen-fixing cyanobacteria are listed in table 1.1.

Table: **1.1. Nitrogen-fixing cyanobacteria:**

Category	Name of the cyanobacteria
I. Filamentous, heterocystous forms.	*Anabaena* sp.
	Calothrix sp.
	Chlorogleopsis sp.
	Fischerella sp.
	Nodularia sp.
	Nostoc sp.
	Scytonema sp.
II. Filamentous, non-heterocystous forms.	*Oscillatoria* sp.
	Plectonema sp.
	Trichodesmium sp.
III. Unicellular, aerobic forms:	*Gloeothece* sp.
	Synechocystis sp.
IV. Unicellular, microaerobic forms.	*Dermocarpa* sp.
	Pleurocarpa sp.
	Xenococcus sp.

1.3.1.2. Nitrogenase:

There are at least two nitrogenase systems capable of

reducing molecular nitrogen to ammonia: the classical nitrogenase (Mo-nitrogenase) and vanadium-nitrogenase (Smith *et al.*, 1987a). Both enzymes consist of two oxygen-sensitive metalloproteins. While the iron-protein is common in both enzyme systems, the second metalloprotein varies: In case of classical nitrogenase it is a molybdenum-iron protein while in the case of vanadium nitrogenase it is a vanadium-iron protein. The vanadium nitrogenase has only recently been discovered in bacteria (*Azotobacter vinelandii*) and in cyanobacteria (*Anabaena variabilis*) hence only limited data on its nature and physiology are available (Hales *et al.*, 1985; Robson *et al.*, 1986a; 1986b; Gallon & Chaplin, 1987; Smith *et al.*, 1987a; Kentemich *et al.*, 1988).

1.3.1.3. Biochemistry of nitrogenase:

Nitrogenase consists of two iron-sulphur proteins, neither of which is active by itself. One of them is the iron protein which is a dimer of two identical subunits. It has molecular weight of 50,000 - 60,000 Da encoded by *nif* H gene. In general, it contains a single 4Fe-4S cluster, but in cyanobacteria 2Fe-2S cluster has also been reported (Haaker *et al.*, 1985; Howard *et al.*, 1985; Gallon & Chaplin, 1987; Smith *et al.*, 1987a). It has redox potential in the range of -0.24 V to -0.393 V, depending on the source (Smith *et al.*, 1987a).

Molybdenum-iron protein of nitrogenase is a tetramer $\alpha_2\beta_2$ of two different subunits: α subunit encoded by the gene *nif* D has molecular weight of 50,000 Da and β subunit encoded by the gene *nif* K has molecular weight of 60,000 Da (Gallon, 1980; Smith *et al.*, 1987a). It contains 2 Mo atoms, 30 Fe atoms and slightly lower number of sulphur atoms (Watt *et al.*, 1986). It also

possesses two FeMoco centers, four Fe-S clusters of 4Fe-4S type (P centers) and an additional 2 Fe atoms designated as 'S' centers (Orme-Johnson, 1985; Lowe *et al.*, 1985; Shah *et al.*, 1986).

Nitrogenase being a versatile catalyst, uses substrates as varied as N_2, C_2H_2, N_2O, CN^-, H_2, HCN, CH_3CN, C_2H_4 and a number of small triple bonded substrates (Bothe *et al.*, 1982; Lowe *et al.*, 1985; Jensen & Burris, 1986; Gallon & Chaplin, 1987; Smith *et al.*, 1987a).

1.3.1.4. Requirements for nitrogenase activity:

For its activity, the enzyme nitrogenase requires, reductant, ATP and a reducing atmosphere.

Nitrogenase is rapidly inactivated by exposure to oxygen (half life = 40 s in air) (Smith *et al.*, 1987b). In heterocystous cyanobacteria, the heterocysts provide suitable environment for nitrogen-fixation in aerobic conditions, because heterocysts lack photosynthetic oxygen evolution (Reinman & Thornber, 1979; Alberte *et al.*, 1980) and have high respiratory oxygen consumption rate (Haury & Wolk, 1978; Walsby, 1982; Jensen & Cox, 1983; Sprent *et al.*, 1987). Aerobic nitrogen-fixation in non-heterocystous cyanobacteria is made possible by temporal separation of photosynthesis and nitrogen-fixation (Mullineaux *et al.*, 1981). Other cyanobacteria fix nitrogen under microaerobic or anaerobic conditions.

1.3.1.4.1. Provision of reductant:

In most nitrogen-fixing organisms, including cyanobacteria, ferredoxin is believed to be the immediate electron donor to nitrogenase (Stewart, 1980; Bothe *et al.*, 1982; 1984; Schrautemeier & Bohme, 1985; Gallon & Chaplin, 1987). However, under

iron-deficient conditions flavodoxin substitutes for ferredoxin as electron donor (Smillie, 1965; Bothe, 1969; Hallenbeck, 1987; Smith *et al.*, 1987a).

Ferredoxins are iron-sulphur proteins containing one or more iron-sulphur clusters which are involved in electron transfer. Cyanobacteria contain typical soluble plant type ferredoxin with 2Fe-2S (acid-liable) clusters in the prosthetic group (Bothe 1969; Hallenbeck, 1987; Smith *et al.*, 1987a). Flavodoxins are the simplest flavoproteins containing one molecule of FMN in the prosthetic group per protein molecule. In catalysis, they act as electron carriers shuttling between the fully reduced and semi-quinone forms (Bothe, 1969; Klugkist *et al.*, 1986; Smith *et al.*, 1987a).

At present, the mechanism whereby ferredoxin is reduced is a controversial subject. However, several electron generating systems are reported. The possible reductant generating enzymic pathways are pyruvate: ferredoxin oxidoreductase (Leach & Carr, 1971; Smith *et al.*, 1987a), glucose 6-phosphate NADP: oxidoreductase, 6-phosphogluconate NADP: oxidoreductase and isocitrate NADP: oxidoreductase (Apte *et al.*, 1978; Schrautemeier & Bohme, 1984; 1985; Bohme, 1987). The enzymes glucose 6-phosphate NADP: oxidoreductase and isocitrate NADP: oxidoreductase are sensitive to high concentration of $NADPH_2$ and negatively regulated by light through thioredoxin (Duggan & Anderson, 1975; Grossman & McGowan, 1975; Schaeffer & Stanier, 1978; Cossar *et al.*, 1984). However, very low $NADPH_2$/NADP ratio (between 1 - 3) is reported in N_2-fixing organisms (Gallon & Chaplin, 1987). Under such conditions, reduction of ferredoxin (midpoint electrical potential -0.42 V) by $NADPH_2$ (midpoint electrical potential -0.32 V) is thermodinami-

cally unfavourable. Therefore, Haaker *et al* (1980) and Hawkesford *et al* (1981) suggested the involvement of $\Delta\psi$ component of proton motive force for transfer of electrons to nitrogenase by reversed electron flow (Stewart *et al*., 1982). In heterocystous cyanobacteria, hydrogen is another possible source of reductant for nitrogen fixation, through the action of uptake hydrogenase (Smith *et al*., 1987a). In the light, hydrogen may generate the reduced ferredoxins by donating electrons to photosynthetic electron transport chain prior to PS I (Bothe *et al*., 1984; Klugkist *et al*., 1986; Smith *et al*., 1987a).

1.3.1.4.2. Provision of ATP:

N_2-fixation is a highly ATP dependent process. Hydrolysis of ATP triggers electron transfer from iron protein to molybdenum-iron protein (Smith *et al*., 1987a; Cordewener *et al*., 1988). In general, 2 Mg-ATP molecules are required for transfer of one electron. However, recent studies indicate that, ATP/e^- ratio is close to 4 (Cordewener *et al*., 1988). In heterocysts, ATP is generated in the light via cyclic photophosphorylation and by the action of uptake hydrogenase, whereas, in the dark, it is produced via oxidative phosphorylation (Maryan *et al*., 1986) and substrate level phosphorylation (Bottomley & Stewart, 1976).

1.3.1.5. Mechanism of action:

A low redox potential molecule, reduced ferredoxin/flavodoxin (midpoint redox potential -0.44 to -0.45 V), donates electrons to iron protein component of nitrogenase (Bohme, 1987; Smith *et al*., 1987a). The iron protein of nitrogenase has two binding sites for Mg-ATP (Eady, 1986; Watt *et al*., 1986). Binding of Mg-ATP lowers the midpoint redox potential of iron protein from

about -0.3 V to -0.4 V or more making it a powerful reducing species and markedly alters the conformation of iron protein (Smith *et al.*, 1987a). The reduced iron protein complexes with the oxidized form of molybdenum-iron protein (Lowe *et al.*, 1985). The molybdenum-iron protein component has midpoint redox potential of 0.0 to -0.29 V when it binds with FeMoco (Watt *et al.*, 1986). It has two binding sites for iron protein (Postgate, 1982; Smith *et al.*, 1987a). The transfer of electrons from reduced iron protein to molybdenum-iron protein is rapid and irreversible (Lowe *et al.*, 1985). This reduction of molybdenum-iron protein is coupled with the simultaneous hydrolysis of Mg-ATP (Burgess, 1985; Mortenson *et al.*, 1985; Cordewener *et al.*, 1988). This hydrolysis of Mg-ATP occurs only when the iron-protein complexes with molybdenum-iron protein of nitrogenase in the presence or absence of external electron donor (Gallon & Chaplin, 1987). As soon as the electrons are transferred to molybdenum-iron protein, the nitrogenase complex dissociates (Burgess, 1985). Then the electron flow proceeds to substrate, which is thought to be reduced in three two-electron steps (Smith *et al.*, 1987a). On the whole, 25% of the available electrons reduce the protons and evolve hydrogen (Simson & Burris, 1984; Smith *et al.*, 1987a).

1.3.1.6. Regulation of nitrogenase by ammonia:

Nitrogenase activity, in all N_2-fixing organisms, is known to be affected by various combined-nitrogen sources and oxygen (Stewart & Lex, 1970; Neilson & Nordlund, 1975; Rippka & Stanier, 1978; Jones & Monty, 1979; Brill, 1980; Roberts & Brill, 1981; Bognar *et al.*, 1982; Thomas *et al.*, 1982; Paparao & Singh, 1983; Singh *et al.*, 1983b; Yoch *et al.*, 1983; Collins & Brill, 1985;

Bohme, 1986; Reich *et al.*, 1986; 1987; Stewart *et al.*, 1987).

Nitrogenase activity is quickly and reversibly inhibited by the addition of ammonia (Haaker *et al.*, 1980; Salminen, 1981; Ludden *et al.*, 1984). Different phenomena have been described with respect to regulation of nitrogenase activity *in vivo*. For example, in *Rhodospirillum rubrum* the inhibition is due to covalent modification of iron-protein component of nitrogenase (Pope *et al.*, 1985; Zumft, 1985; Hallenbeck, 1987). Whereas, in bacteriods of *Pisum sativum* (L) it is due to uncoupling effect (Salminen, 1981). In *A. vinelandii* Haaker *et al* (1980) have shown that inhibition of nitrogenase by ammonia is due to deenergization of the membrane by ammonium resulting in the inhibition of the supply of reductant for nitrogen-fixation.

In cyanobacteria however, such fast nitrogenase switch-off is not observed except at higher pH. At the alkaline environment ammonia enters in the cyanobacterium by diffusion and leads to an immediate inactivation of nitrogenase and this inactivation is thought to be due to uncoupling effect (Reich *et al.*, 1986; 1987).

In vivo nitrogenase synthesis is regulated, in various nitrogen-fixing bacteria and cyanobacteria, upon the addition of ammonia and/or a product of ammonia assimilation (Gallon & Chaplin, 1987; Hallenbeck, 1987). The mechanism is not same in all nitrogen-fixing organisms. For example, in *Klebsiella pneumoniae* and *Rhodopseudomonas capsulata*, *nif* L gene product acts as a repressor for other *nif* genes (Hallenbeck *et al.*, 1982; Collins & Brill, 1985; Hallenbeck, 1987; Smith *et al.*, 1987a). However, it appears that ammonia itself is not the repressor of nitrogenase synthesis; it must be assimilated at least to gluta-

mine. The evidence comes from the glutamine auxotroph of *R. capsulata* where derepression of nitrogenase is found in the presence of ammonia (Wall & Gest, 1979). Whereas, in *R. rubrum*, glutamine and asparagine repressed the nitrogenase synthesis (Neilson & Nordlund, 1975). However, in *K. pneumoniae* regulation of nitrogenase synthesis is thought to be related with glutamine synthetase. It is found that a mutation of structural gene for GS (*gln* A) often caused a repression of nitrogenase synthesis (Gallon & Chaplin, 1987). While in *Clostridium pasteurianum* ammonia degraded the mRNA responsible to code for nitrogenase (Daesch & Mortenson, 1972).

In cyanobacteria, repression of nitrogenase synthesis is known to be influenced by ammonia and/or its derivatives. Nitrogenase synthesis and heterocyst development is repressed by ammonia (Stewart, 1980). However, presence of MSX, an inhibitor of GS, prevents the repression of nitrogenase synthesis by ammonia (Stewart and Rowell, 1975). Thus, it appears that ammonia needs to be assimilated before nitrogenase synthesis can be repressed (Stewart *et al.*, 1985). In *Gloeocapsa*, a product of ammonia assimilation, is thought to be repressor of nitrogenase synthesis (Thomas *et al.*, 1982).

Recently, ammonia *per se* has been suggested to be involved in repression of nitrogenase synthesis in cyanobacteria (Singh *et al.*, 1983b; Turpin *et al.*, 1984; Mackerras and Smith, 1986).

1.3.2. Nitrate assimilation:

Virtually all cyanobacteria tested so far, are capable of

using nitrate as nitrogen source. Diazotrophic cyanobacteria prefer nitrate to N_2. Exogenous nitrate is first transported into the cell where it is reduced to ammonia by the action of nitrate reductase (NR) and nitrite reductase (NIR) enzyme systems. Such nitrate reduction in cyanobacteria is linked to photosynthesis (Manzano *et al.*, 1976; Lara *et al.*, 1987).

In general, nitrate uptake system in diazotrophic cyanobacteria is of two types: a) nitrate inducible/ammonium-repressible. Such a system is found in the cyanobacterium, *Anabaena* 7120, where nitrate uptake activity develops in nitrate medium only and presence of ammonium represses it (Meeks *et al.*, 1983; Rai & Bergman, 1986). b) ammonium repressible/derepressible. Such a system is exemplified by the cyanobacterium *Anabaena cycadeae*, where nitrate uptake activity is present both in N_2- and NO_3^--medium; presence of ammonia represses it (Bagchi *et al.*, 1985b).

A genetic link between glutamine synthetase and the nitrate uptake system has been reported in the cyanobacterium *A. cycadeae* (Singh *et al.*, 1985a). The glutamine auxotroph of *A. cycadeae* has been shown to posses several fold higher nitrate uptake level than the wild type strain (Singh *et al.*, 1985a).

The kinetics of the development of nitrate uptake system studies indicated that uptake system has a regulatory role in the development of nitrate reductase system in *A. cycadeae* (Bagchi *et al.*, 1985a). In this cyanobacterium it was found that nitrate uptake system development is independent to that of nitrate reductase system development.

In heterocystous filamentous cyanobacteria, the nitrate

uptake and reductase systems are found to be associated with vegetative cells only and have been shown to be absent in akinetes and in heterocysts (Rao *et al.*, 1984; Kumar *et al.*, 1985; Rai & Bergman, 1986). Absence of nitrate metabolism in heterocysts results in the abolition of competition for molybdenum cofactor and reductant between nitrogenase and nitrate reductase (Kumar *et al.*, 1985; Rai & Bergman, 1986).

In *Nostoc muscorum*, nitrate transport is energy-dependent; its ATP requirement has been shown Rai by *et al* (1981a). In *Anabaena* 7120, nitrate and nitrite share a common transport system (Rai & Bergman, 1986). Nitrate reductase in cyanobacteria is found to be ferredoxin dependent (Kumar *et al.*, 1985; Rai & Bergman, 1986). In general, nitrate reductase in cyanobacteria is of two types; a) nitrate inducible/ammonia repressible. In *Anabaena cylindrica* it is nitrate inducible while in *Anacystis nidulans* it is ammonia repressible (Ohmori & Hattori, 1970; Herrero *et al.*, 1981). b) ammonia repressible/derepressible. Such a regulatory system is found in *Anabaena* 7120: In this cyanobacterium the nitrate reductase apoprotein undergoes repression/derepression control while the Mo-cofactor is constitutive. The apoprotein synthesis is repressed in NH_4^+-medium whereas derepressed in N_2- and NO_3^--medium (Kumar *et al.*, 1985; Rai, 1990).

1.3.3. Ammonia assimilation:

The product of nitrogen-fixation and nitrate or nitrite assimilation is ammonia, which is subsequently assimilated into organic compounds via glutamine synthetase and glutamate synthase pathway (GS-GOGAT pathway) (Dharmawardene *et al.*, 1973; Wolk *et*

al., 1976; Miflin & Lea, 1976; Stewart, 1980; Orr & Haselkorn, 1982; Stewart *et al.*, 1983; 1987; Rai *et al.*, 1986a).

Heterocysts, the sites of ammonia production by nitrogen-fixation, have a GS concentration nearly two-fold higher than that in the vegetative cells (Bergman *et al.*, 1985). The cyanobacterial GS is a dodecameric enzyme consisting of 12 identical subunits. Each subunit has relative molecular mass of 50,000 (Sampaio *et al.*, 1979; Stewart *et al.*, 1987). GS activity is essentially irreversible under normal physiological conditions; it has a high affinity for ammonium (Km = 0.02 mM) (Miflin & Lea 1976), is inhibited by methionine sulphoximine (MSX) (Stewart & Rowell, 1975; Gallon, 1980; Stewart *et al.*, 1985; 1987) and hydroxylysine (Ladha *et al.*, 1978; Stewart *et al.*, 1987), and is deactivated by ammonia or darkness in cyanobacteria (Rowell *et al.*, 1979). In symbiosis, the cyanobionts contain low GS-GOGAT activities which may be responsible for liberation of nitrogenase derived ammonia (Rai *et al.*, 1980; 1981b; 1984; Stewart *et al.*, 1983; 1987; Joseph & Meeks, 1987).

Glutamate synthase (GOGAT) in cyanobacteria converts glutamine to glutamate and requires reduced ferredoxin as a reductant (Ohmori, 1981; Stewart *et al.*, 1983). GOGAT has been reported to be absent in heterocysts (Thomas *et al.*, 1977; Rai *et al.*, 1982) however, some workers suggest GOGAT to be present in heterocysts at a low activity (Gupta & Carr, 1981; Bothe *et al.*, 1984). Azaserine is an inhibitor of this enzyme (Hartman, 1973; Ohmori *et al.*, 1985).

1.4. Ammonium transport:

Besides molecular nitrogen, ammonia is the most wide spread nitrogenous compound on earth available for utilization by various organisms (Kleiner, 1981; 1985a). Among all inorganic nitrogen sources ammonia is a preferred nitrogen source for all nitrogen-fixing organisms including cyanobacteria (Stewart, 1980; Gibson, 1984; Mackerras & Smith, 1986). When it is available in excess, it represses nitrogenase as well as nitrate uptake, NR and ammonium transport (Zumft & Castillo, 1978; Bothe, 1982; Stewart *et al.*, 1983; 1987; Singh *et al.*, 1983b; Gibson, 1984; Avissar, 1985; Bagchi *et al.*, 1985b; Mackerras & Smith, 1986).

Ammonia is a weak base and occurs in two forms; ammonia (NH_3) and ammonium (NH_4^+) (Kleiner, 1981; 1985a). Ammonium has dissociation constant of 9.25. The neutral molecule, ammonia, is a weak base and protonates to give ammonium at physiological pH (Henderson, 1971; Kleiner, 1985a).

Biological membranes show little or no permeability for ammonium, suggesting a need for specific ammonium transport carrier. The existence of specific carrier for ammonium have been inferred from the following criteria:

1. Metabolism of any compound starts with its passage across biomembranes. It is generally assumed that if a molecule exist as a neutral and ionic species- like weak acid and base, biomembranes are rather permeable towards the neutral form but less for ions, which normally requires a carrier (Henderson, 1971; Tien, 1974; Kleiner, 1985a).
2. Biomembranes are rather highly permeable towards unprotonated form of ammonia. In such cases, no specific transport system

is likely to exist for ammonium because of rapid equilibrium between ammonia and ammonium. However, when a pH difference occurs across biomembranes, unequal distribution of non-permeating ions is observed, indicating occurrence of specific carrier system (Henderson, 1971).

3. In the absence of Δ pH, microorganisms show ammonium and methylammonium gradients across biomembranes at the expense of energy. Accordingly, such a membrane system should have reduced permeability towards ammonium in order to avoid futile cycle (Kleiner, 1985a).
4. Saturation kinetics.
5. Distinct pH profile studies.
6. Specific inhibitor studies.
7. Genetic and metabolic control studies and
8. Studies on transport-deficient mutants.

Thus, in summary, ammonium transport across biomembranes, is a carrier mediated process which equilibrate the ions across the membrane according to an electric field. This implies that ammonium and not ammonia is the transported species. However, it is yet to be discovered whether it is uniport channel or mobile carrier (Kleiner, 1985a). Ammonium transport system (ATS) has a role in the retention of internally generated ammonia from nitrogen fixation and nitrate reduction (Kleiner, 1985b; Kerby *et al.*, 1987).

1.4.1. Ammonium transport in bacteria:

Nitrogen-fixing bacteria provide a suitable model system to study ATS, because no enzyme system would be expected to be

repressible by the ubiquitous nitrogen source, N_2 (Kleiner, 1985a). ATS has been found to occur in several diazotrophic bacteria (Barnes & Zimniak, 1981; Alef & Kleiner, 1982a; 1982b; Hartman & Kleiner, 1982; Ivanovsky *et al.*, 1982; Gober & Kashket, 1983; Glenn & Dilworth, 1984; Genthner & Wall, 1985; Hara *et al.*, 1985; Holtel & Kleiner, 1985; Howitt *et al.*, 1986). In most of the bacteria, ATS has been characterized by using methylammonium as ammonium analogue. The apparent Km value for methylammonium uptake ranges from 2 to 140 μM (Alef & Kleiner, 1982a; 1982b; Hartman & Kleiner, 1982; Kleiner, 1982; Muzzucco & Benson, 1984; Genthner & Wall, 1985; Pargent & Kleiner, 1985; Howitt *et al.*, 1986) and is competitively inhibited by the addition of ammonium; Ki value ranges from 1 to 15 μM, indicating that both ammonium and methylammonium are transported by same carrier (Alef & Kleiner, 1982a; Kleiner, 1982; Gober & Kashket, 1983; Muzzucco & Benson, 1984; Kleiner, 1985a; Howitt *et al.*, 1986). In *Paracoccus denitrificans*, two different carriers, depending on growth conditions, have been reported for methylammonium uptake (Holtel & Kleiner, 1985; Kleiner, 1985a). Most bacteria have capacity to accumulate methylammonium upto 100 fold against a concentration gradient across the membranes. The intracellular ammonium pool ranges from 0.2 to 2.9 mM (Booth & Hamilton, 1980; Gordon & Moore, 1981; Kleiner, 1981; 1982; 1985a).

Ammonium transport show a distinct pH profile having optimum pH between 6.4 to 6.8 (Kleiner, 1982; Gober & Kashket, 1983; Genthner & Wall, 1985; Howitt *et al.*, 1986). Ammonium transport in all bacterial species, is found to be an energy dependent process. Most studies, dealing with energetics of ammonium

transport, have revealed that transmembrane electrical potential, is the driving force for ammonium transport (Barnes & Zimniak, 1981; Kleiner & Fitzke, 1981; Kleiner, 1982; 1985a). It is found that ammonium accumulation requires transmembrane electrical potential of -70 mV (Kleiner, 1982; 1985a). However, the involvement of ATP in ammonium accumulation is not ruled out in *A. vinelandii* (Gordon & Moore, 1981; Moore & Gordon, 1984).

In most species the synthesis of ammonium of carrier is subjected to nitrogen control, while the activity may be regulated either by glutamine or by ammonia (Kleiner, 1982; 1985a). High levels of ammonium in the medium repress the ammonium carrier in most of the organisms (Alef & Kleiner, 1982a; Hartman & Kleiner 1982; Bogdahn *et al.*, 1983; Muzzucco & Benson, 1984; Holtel & Kleiner, 1985). This nitrogen control of ammonium transport is thought to be mediated by the *ntr* regulatory system (Streicher *et al.*, 1974; Magasanik, 1982; Kleiner, 1985a). Recent studies on *Escherichia coli* strains containing different *ntr* gene mutants revealed that *ntr* A & C gene products are required to derepress the ammonium carrier, while, *ntr* B gene product plays a role in repression (Genthner & Wall, 1985). Addition of glutamine strongly decreases the methylammonium uptake (Ivanovsky *et al.*, 1982; Jayakumar & Barnes, 1984; Hara *et al.*, 1985) which is thought not to be regulated by intracellular glutamine pool (Kleiner & Castroph, 1982; Jayakumar & Barnes, 1984). MSX also inhibits the ammonium transport, which is probably due to its regulatory binding site at the carrier (Kleiner & Castroph, 1982; Kleiner *et al.*, 1983).

1.4.2. Ammonium transport in cyanobacteria:

The importance of ATS lies in the uptake of exogenous ammonium and for the retention of ammonium produced during nitrogen fixation (Kleiner, 1985b; Kerby *et al.*, 1987). Although, ATS in nitrogen fixing bacteria has been studied extensively (Silver, 1978; Kleiner, 1981; 1985a), in cyanobacteria, work on ATS commenced very recently. Rai *et al* (1984) were the first to characterize the ATS in *A. variabilis* and *Anabaena azollae*. Subsequently, it has been characterized in several cyanobacteria (Boussiba *et al.*, 1984a; Kashyap & Johar, 1984a; 1984b; Kashyap & Singh, 1985; Singh *et al.*, 1985b; 1986; 1987; Kerby *et al.*, 1986; 1987; Rai *et al.*, 1986a; 1986b; Stewart *et al.*, 1987).

In most studies, the ammonium analogue, methylammonium, has been used to characterize ATS in cyanobacteria, since methylammonium uses the same transport system as ammonium (Boussiba *et al.*, 1984b; Rai *et al.*, 1984; Singh *et al.*, 1985**b**; 1987; Kerby *et al.*, 1986; 1987). However, it has also been characterized by using ammonium (Kashyap & Johar, 1984a; Kashyap & Singh, 1985).

Ammonium transport, in most of the cyanobacteria so far tested, is found to be biphasic with a rapid initial phase followed by a relatively slower second phase. The first phase, lasting 2-3 minutes, is independent of methylammonium metabolism, while, the second phase is dependent on methylammonium metabolism via GS (Boussiba *et al.*, 1984b; Rai *et al.*, 1984; Singh *et al.*, 1985b; 1986; 1987; Kerby *et al.*, 1986; 1987; Boussiba & Gibson, 1987).

Ammonium uptake at pH 9.0 is much higher than pH 7.0; at this pH ammonia could diffuse into cells and be concentrated by protonation as a consequence of the pH difference (Δ pH) (Rai *et*

al., 1984; Kerby *et al.*, 1986; 1987). At pH 7.0, MSX showed an inhibitory effect on second uptake phase, which is thought to be due to MSX inhibition of GS (Rai *et al.*, 1984; Boussiba & Gibson, 1985). However, studies on a GS mutant, *A. cycadeae*, indicated that second phase of ammonium transport may not necessarily be linked to GS activity but may represent a separate ATS, suggesting two ATS in cyanobacteria. They are: an MSX-insensitive rapid ATS and an MSX-sensitive slower ATS (Singh *et al.*, 1985b). Studies on a MSX mutant of *Anabaena doliolum* have revealed that MSX has two inhibitory targets in cyanobacteria, one at the transport level and other at the GS activity level (Singh *et al.*, 1986). MSX does not show any inhibitory effect on ammonium transport at pH 9.0 (Turpin *et al.*, 1984).

Kinetics of concentration-dependent ammonium transport studies revealed two ATS in *N. muscorum*: a high affinity ATS at low external ammonium concentration ranging from 1 - 35 μM with a Km of 11 μM; and a low affinity ATS at the higher external ammonium concentration ranging from 35 - 300 μM with a Km of 66 μM. Both systems are inhibited by methylammonium (Kashyap & Johar, 1984a; Kashyap & Singh, 1985). The internal pool of free methylammonium is found to be 1.25 mM in *A. cycadeae* and 1.4 mM in *A. variabilis* (Rai *et al.*, 1984; 1986b).

Ammonium transport, in *A. variabilis* & *A. cycadeae*, is an active and energy dependent process. It is inhibited by the addition of CCCP & $TPMP^+$, indicating $\Delta\psi$ is the driving force for ammonium transport (Rai *et al.*, 1984; 1986a; Kerby *et al.*, 1987; Singh *et al.*, 1987). In *A. nidulans* ammonium transport has been shown to be ATP-dependent (Boussiba *et al.*, 1984a; Kashyap &

Singh, 1985).

Ammonium transport in *A. variabilis* and *A. cycadeae* is ammonium repressible (Rai *et al.*, 1986b; Boussiba & Gibson, 1987; Singh *et al.*, 1987). Repression of ammonium transport is caused by ammonium itself, whereas, derepression requires *de novo* protein synthesis (Rai *et al.*, 1986b; Singh *et al.*, 1987). Genetic control of ATS has been inferred from studies on the methylamine, streptomycin and MSX mutants of *N. muscorum* and *A. cycadeae* (Kashyap & Johar, 1984a; Singh *et al.*, 1985b). In symbiotic cyanobacterium *A. azollae*, both MSX-insensitive and MSX-sensitive ATS are present whereas, in the cyanobiont of cycad root nodules, only the MSX-insensitive rapid ATS is present (Rai *et al.*, 1984; 1986a).

1.4.3. Cyclic retention of ammonia:

When N_2-fixing bacteria or cyanobacteria are grown under N_2- and NO_3^--media a high internal and a low external ammonia level is observed (Booth & Hamilton, 1980; Gordon & Moore, 1981; Kleiner, 1981; 1985b; Rai *et al.*, 1984; 1986a). This concentration gradient must result in outward diffusion of ammonia (Henderson, 1971; Kleiner, 1981; 1985a). However, such a diffusion is not observed in N_2- and NO_3^--grown conditions, because of the derepression of ammonium carrier. This carrier is responsible for recovery of ammonia, which is diffused from an internal pool, as ammonium and is an energy requiring process (Rai *et al.*, 1984; 1986b; Kashyap & Singh, 1985; Singh *et al.*, 1987). This energy expenditure is to balance the energy dependent export of protons (Kleiner, 1985a; 1985b). Thus, a constant ammonia excretion/

absorption is found under ammonia limited conditions. This cyclic ammonia/ammonium retention is also evidenced from; a) repression of ammonium carrier by ammonia and b) ammonium transport mutants, which constantly release ammonia to the medium (Kashyap & Johar, 1984b; Kleiner, 1985a). It is assumed that every ammonia molecule passes the futile cycle about 6 times before it is being assimilated (Kleiner, 1985a; 1985b). Based on the average H^+/ATP stiochiometry (3), it is found that 2 ATP molecules are expended for cyclic retention of each ammonia molecule (Kleiner, 1985a; 1985b). An outline of the cyclic retention of ammonia, proposed by Kleiner (1985b), is given in Fig 1.1.

1.5. Glutamine & glutamate transport:

Glutamine and glutamate are products of ammonia assimilation which regulate the heterocyst differentiation and aerobic nitrogen fixation in cyanobacteria (Neilson & Nordlund, 1975; Stewart, 1980; Stewart *et al.*, 1983; 1987; Rai *et al.*, 1984; Rai, 1990).

Relatively, little is known about amino acid transport in cyanobacteria (Kleiner, 1985a). In unicellular cyanobacterium *A. nidulans* amino acid transport is an energy dependent process (Leekaden & Simonis, 1979; Labarre *et al.*, 1987). Chapman & Meeks (1983), working with filamentous, heterocystous cyanobacterium, *A. variabilis* showed that glutamate and glutamine are transported via two transport systems (a high affinity & a low affinity system) with Km values of 13.8 & 100 μM and 1.3 & 1.1 mM, respectively. However, glutamine transport studies in symbiotic *Nostoc* sp. of

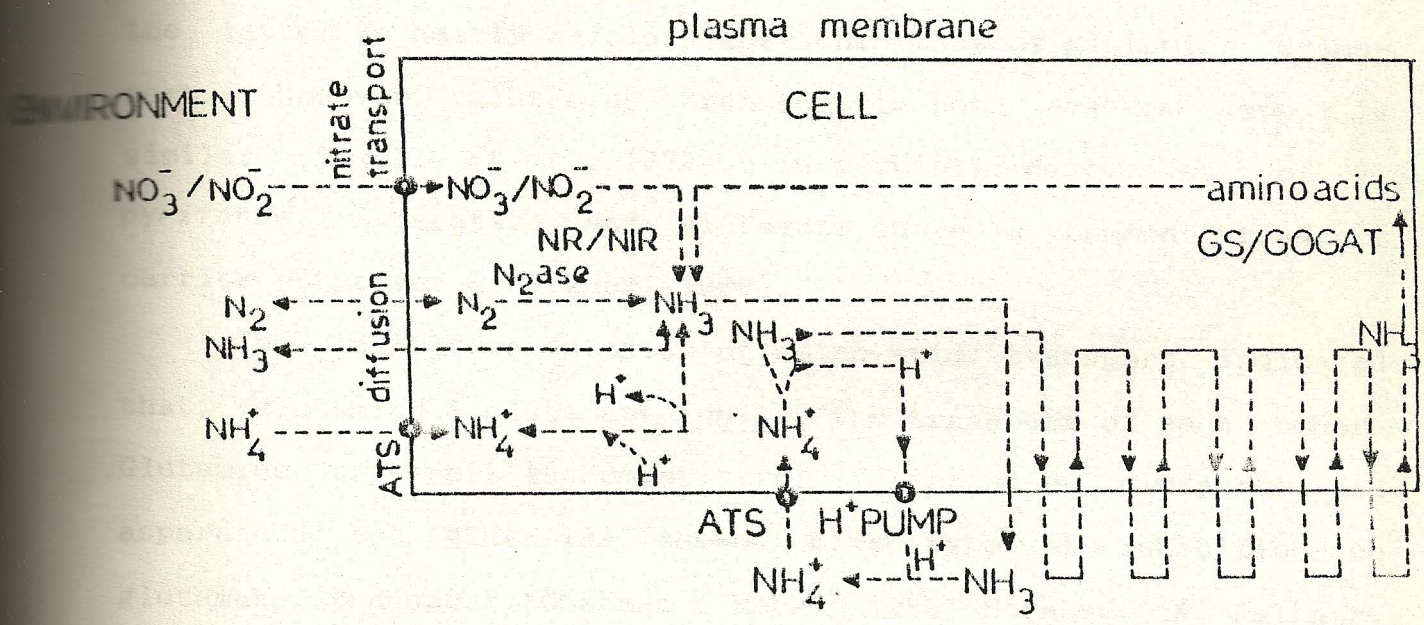

Fig 1.1 Proposed cyclic retention of ammonia, import of exogenous inorganic nitrogen and its fate. The idea of cyclic retention of ammonia is taken from bacterial systems (Kleiner, 1985b). ATS, ammonium transport system; GS, glutamine synthetase; GOGAT, glutamate synthase; NR, nitrate reductase, NIR, nitrite reductase; N_2ase, nitrogenase.

Geosiphon pyriforme indicated only one transport system with much higher affinity (Km = 0.1 to 1.3 μM) (Strasser & Falkner, 1986).

Comparative studies on glutamate & glutamine transport in *A. variabilis* and *A. cylindrica* indicated that glutamate transport in the latter is nearly 3-fold higher than that of glutamine transport. However, glutamine transport in both *Anabaena* spp. is similar (Rowell *et al.*, 1977). In symbiotic *Nostoc* sp. of *G. pyriforme*, asparatate and glutamate share a common transport carrier (Strasser & Falkner, 1986).

The inhibition studies of amino acid transport indicated that, glutamine & glutamate inhibit the transport of each other. Glutamine transport is competitively inhibited by glutamate and asparatate but glutamine showed mixed type of inhibition on glutamate transport (Chapman & Meeks, 1983; Strasser & Falkner, 1986).

1.6. Photobiological production of ammonia by cyanobacteria and their application in field:

Cyanobacteria have long been used as biofertilizers due to their dual function: Photosynthesis and nitrogen fixation (Singh, 1961; Jenkinson, 1977; Reynaud & Roger, 1978; Stewart *et al.*, 1979; 1987; Stewart, 1980). The input of fixed nitrogen by cyanobacteria ranges from a few Kg of $N.ha^{-1}.a^{-1}$ to 100 Kg of $N.ha^{-1}.a^{-1}$ (Fogg *et al.*, 1973; Stewart *et al.*, 1979; Venkatraman, 1980). However, the current use of cyanobacteria in rice-fields has serious limitations, because, normal cyanobacteria use fixed nitrogen present in the field, instead of fixing nitrogen, thus

becoming weed for the crop (Musgrave *et al.*, 1982; Stewart *et al.*, 1983; 1987). So, the strains which would liberate more ammonia and fix N_2 at higher rate would be better for biofertilizer use or for use in photobiological production of ammonia (Stewart *et al.*, 1987). Therefore, it is obvious that, we should produce suitably modified strains, maximizing nitrogen fixation, minimizing GS-GOGAT activities and/or abolishing ATS. Such modifications are well known in the cyanobacterial symbionts (Rai *et al.*, 1984; 1986b; Kleiner, 1985b; Nierzwicki-Bauer & Haselkorn, 1986). Free-living cyanobacteria have been used for ammonia production by inhibiting the GS enzyme either by MSX or hydroxylysine (Stewart & Rowell, 1975; Stewart & Rodgers, 1977; Ladha *et al.*, 1978; Stewart *et al.*, 1987) and immobilizing them in alginate (Musgrave *et al.*, 1982; 1983a; 1983b; Kerby *et al.*, 1983; Muallem *et al.*, 1983). Inhibition of GS enzyme by MSX makes the cyanobacterium a glutamine auxotroph which requires glutamine for growth (Kerby *et al.*, 1985; Rowell *et al.*, 1985b; Spiller *et al.*, 1986). However, suitable ammonia liberating strains can be obtained by partially inhibiting the GS activity (about 90 - 95%) or by manipulating the ATS so that most of the nitrogenase-derived ammonia is liberated and still the organism survives by assimilating part of the ammonia (Stewart *et al.*, 1987).

1.7. The present study:

Ammonium transport system is necessary for uptake of exogenous ammonium and retention of the nitrogenase derived ammonia (Kleiner, 1985b; Kerby *et al.*, 1987). Hence, modifi-

cation/abolition of this system would lead to leakage of nitrogenase derived ammonia and lack of intracellular accumulation of exogenous ammonia. This type of strains would be useful in production of ammonia without the need of GS-inhibitor, MSX, which is toxic and expensive. These can also be used in field where they would liberate much more of the fixed ammonia than normal cyanobacteria. Furthermore, ATS mutants would be much better than GS-mutants because:

1. GS mutants would be glutamine auxotrophs. Hence, their survival in field would be difficult and their maintenance in the laboratory would be expensive.
2. ATS mutants would fix nitrogen even in the presence of exogenous ammonium because nitrogenase will remain active. This is because exogenous ammonia would not accumulate in the cell in absence of ATS and hence nitrogenase will not be repressed.
3. ATS mutants will still assimilate enough ammonia to sustain themselves albeit at a slower growth rate. This is even more advantageous since, less fixed-C would be required for biomass and growth, diverting more photosynthate for nitrogen fixation.

However, before ATS can be manipulated a thorough knowledge of ATS in cyanobacteria is needed. With this in view the present study was undertaken to characterize the ATS in *Nostoc* ANTH and *Anabaena* 7120 and to understand its role in physiology and biochemistry of the nitrogen fixing cyanobacteria.