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

NORTH-EASTERN HILL UNIVERSITY LIBRARY

By

JYOTIRMOY BHATTACHARYA

DEPARTMENT OF BIOCHEMISTRY

SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENT
OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

NORTH - EASTERN HILL UNIVERSITY
SHILLONG, INDIA 2001.

Thesis

EHU LIBRARY
No. 103560
10-8-07
22/07/08
Da
Cl
Sub.He
Enter b
Transcribed by

DS
589.460413
BHA

The North-Eastern Hill University

Department Of Biochemistry

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**.

Jyotirmoy Bhattacharya
Candidate

R.Sharma
Head
Department of Biochemistry

A.N.Rai
Supervisor

Dedicated to
my beloved parents and granny

LIST OF CONTENTS

Pages

Acknowledgements VIII
Abbreviations IX-X
List of Tables XI-XII
List of Figures XIII- XIV

1.0 General Introduction : 1-19

1.1 Cyanobacteria
1.2 Vegetative Cells
1.3 Heterocysts and N_2–fixation
1.4 Inorganic N metabolism
  1.4.1 Nitrate and nitrite metabolism
  1.4.2 Ammonium transport and assimilation
1.5 Amino acid nutrition and transport
1.6 Akinete differentiation and germination
1.7 Cyanobacteria in symbiosis
  1.7.1 Symbiotic diversity/competence
  1.7.2 Metabolic status of the cyanobiont
1.8 Artificial symbiosis
1.9 Present study

2.0 Materials and Methods : 20-30
2.1 Organisms
2.2 Culture methods
  2.2.1 Culture medium

2.2.2 Sterilization
2.2.3 Culture conditions
2.3 Growth parameters
2.3.1 Chlorophyll *a*
2.3.2 Protein estimation
2.3.2.1 Extraction of protein
2.3.2.2 Estimation of protein
2.3.2.3 Procedure
2.4 Oxygen exchange
2.5 Enzyme assays
2.5.1 Nitrogenase
2.5.2 Glutamine synthetase
2.5.2.1 Extraction of enzyme
2.5.2.2 Glutamine synthetase transferase
2.5.3 Nitrate reductase
2.5.4 Nitrite reductase
2.5.5 Nitrite estimation
2.5.5.1 Reagents
2.5.5.2 Procedure
2.6 Uptake experiments
2.6.1 Nitrate and nitrite uptake
2.6.1.1 Reagents
2.6.1.2 Procedure
2.6.2 Ammonium and amino acid uptake
2.7 Colonization of *Nostoc* ANTH and its mutants with rice plants.
2.8 Chemicals

3.0 **Isolation and characterization of a chlorate-resistant mutant (ClO-*R*) of the symbiotic cyanobacterium, *Nostoc* ANTH: Heterocyst**

formation and N_2-fixation in presence of nitrate, and evidence for separate nitrate and nitrite transport systems. 31-48
3.1 Introduction
3.2 Materials and methods
3.2.1 Strains and culture conditions
3.2.2 Isolation of the ClO-*R* mutant
3.2.3 Analysis of growth, heterocyst frequency and nitrogenase activity
3.2.4 Nitrate and nitrite uptake assays
3.2.5 Nitrate reductase, nitrite reductase and glutamine synthetase (transferase) activity
3.2.6 Ammonium transport assay
3.3 Results and discussion
3.3.1 Growth and N_2-fixation of parent and mutant
3.3.2 Activities of nitrate uptake, nitrate reductase, nitrite uptake and nitrite reductase
3.3.3 Glutamine synthetase activity
3.3.4 Ammonium uptake activity

4.0 **Colonization of rice (*Oriza sativa* L.) roots, N_2-fixation and nitrogen transfer by cyanobacterium *Nostoc* ANTH and its mutants.** 49-73
4.1 Introduction
4.2 Materials and methods
4.2.1 Strains and culture conditions
4.2.2 Isolation of the PQ-*R* mutant
4.2.3 Colonization experiment
4.2.4 Determination of natural ^{15}N abundance ($\delta^{15}N$)
4.2.5 Growth measurement
4.2.6 Chlorophyll *a* determination and measurement of root dry weights

4.2.7 Oxygen exchange
4.2.8 Heterocyst frequency and nitrogenase activity
4.2.9 Superoxide dismutase activity
4.2.10 Scanning electron micrography
4.3 Results and discussion
4.3.1 Characterization of the paraquat-resistant (PQ-*R*) mutant
4.3.2 Colonization ability of *Nostoc* ANTH and its mutants
4.3.3 Adsorption kinetics studies
4.3.4 N_2-fixation by *Nostoc* ANTH and its mutant strains under free-living and associated conditions
4.3.5 Utilization of sugars by *Nostoc* ANTH and its mutants
4.3.6 Evidence of N-transfer from associated *Nostoc* strains to the rice plants

5.0 **Nitrogen uptake and assimilation in the cyanobacterium *Nostoc* ANTH, a symbiotic isolate from *Anthoceros*.** **74-97**
5.1 Introduction
5.2 Materials and methods
5.2.1 Strains and culture conditions
5.2.2 Isolation of the Het^-Nif^- mutant
5.2.3 Growth, heterocyst frequency and nitrogenase activity
5.2.4 Nitrate reductase and glutamine synthetase (transferase) activities
5.2.5 Ammonium and amino acid transport assays.
5.3 Results and discussion

5.3.1 Growth, heterocyst frequency and nitrogenase activity
5.3.2 Activities of nitrate reductase and glutamine synthetase
5.3.3 Ammonium uptake activity
5.3.4 Uptake activities of glutamine, asparagine, arginine and alanine

6.0 **Summary** **98-100**
References **101-120**
Curriculum vitae and Publication XV

ACKNOWLEDGEMENTS

I express my sincere gratitude to my esteemed mentor **Prof. A.N. Rai** for his excellent and endless guidance throughout this project, constant encouragement and for teaching me scientific writing. I would like to record my thanks to **Prof. H.N.Singh** for introducing me to **Prof. A.N. Rai**. I must admit that I was fortunate enough to work under his excellent supervision.

I thank **Dr. A.K.Singh** for many helpful scientific discussions we had together. I must say that you have been of tremendous help to me in this project.

I thank **Prof. R. Sharma, Prof. R.N. Sharan, Dr. Anis Alam** and **Dr. Don K. Syiem** for sharing their scientific knowledge with me.

I am grateful to **Judy, Mani baboo Singh, Sumanta, Bany, Bijoy Das, Omarlin, Raj Kumar, Nonibala, Rima, Vrinda** and other colleagues at the Department of Biochemistry and many others for extending their pleasant cooperation and assistance to me during this study.

Special thanks are due to my beloved **parents, maternal uncles** and **aunts, brother, sister** and **Sanjay** for enormous love and care they have shown to me.

I also acknowledge the financial support from **CSIR** provided to me during this study.

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

LIST OF TABLES

Table 3.1 Growth (Gr), heterocyst frequency (HF) and nitrogenase activity (N_2ase) of *Nostoc* ANTH and its ClO-*R* mutant as a function of nitrogen sources.

Table 3.2 Nitrate and nitrite uptake activity in *Nostoc* ANTH and ClO-*R* mutant grown in N_2-, NO^-_3- or NH^+_4-media.

Table 3.3 Nitrate reductase, nitrite reductase and glutamine synthetase (GS) transferase activities of *Nostoc* ANTH (parent) and its ClO-*R* mutant in different nitrogen media.

Table 4.1 Growth (Gr), heterocyst frequency (HF), nitrogenase (N_2ase) activity and O_2 evolution rates of *Nostoc* ANTH and its PQ-*R* mutant in presence and absence of paraquat.

Table 4.2 Colonization of rice roots by *Nostoc* ANTH and its mutants.

Table 4.3 Nitrogenase activity of the *Nostoc* ANTH and its mutants under free-living and associated conditions.

Table 4.4 Effect of glucose and fructose on heterocyst frequency (HF) and nitrogenase activity (N_2ase) of *Nostoc* ANTH and its mutants.

Table 4.5 $\delta^{15}N$ values of N_2-fixing cultures of *Nostoc* and of rice plants co-cultivated in a peralite with or without association with *Nostoc* ANTH or its mutants (PQ-*R* or ClO-*R*).

Table 5.1 Growth (Gr), heterocyst frequency (HF) and nitrogenase activity (N_2ase) of *Nostoc* ANTH and its Het^{-}Nif^{-} mutant as a function of nitrogen sources.

Table 5.2 Nitrate reductase (NR) and glutamine synthetase (GS) transferase activities of *Nostoc* ANTH and its Het^{-}Nif^{-} mutant as a function of nitrogen sources.

Table 5.3 ^{14}C-methylammonium uptake activity in parent and its Het^{-}Nif^{-} mutant grown in N_2-, NO_3^-- or NH_4^+-media.

Table 5.4 Effect of DCMU, DCCD and CCCP on glutamine, asparagine, arginine and alanine uptake by *Nostoc* ANTH.

LIST OF FIGURES

Fig 2.1 STRR 1A-PCR-based DNA fingerprints of *Nostoc* sp., *Nostoc* sp. (PR: Paraquat-resistant mutant), *Nostoc* sp. (AR: Azetidine-2-carboxylate-resistant mutant), *Nostoc* ANTH and *Nostoc* PCC 9229.

Fig 2.2 Comparison of nucleotide sequences of $tRNA^{Leu}$ (UAA) intron from cyanobacterium *Nostoc* ANTH and *Nostoc* sp.

Fig 3.1 Effect of nitrate on nitrogenase activity of *Nostoc* ANTH and its ClO-*R* mutant.

Fig 3.2 ^{14}C-methylammonium uptake in *Nostoc* ANTH (A) and its ClO-*R* mutant (B) under N_2- (▲), nitrate- (■) and ammonium-grown conditions (●).

Fig 4.1 Association of *Nostoc* ANTH with roots of a rice plant after 4 d (A) and 7 d (B) of co-culture in 10-fold diluted N_2-medium. 2,4-D treated rice roots showing para-nodule formation (C).

Fig 4.2 Scanning electron micrographs of root surfaces of rice plants colonized by *Nostoc* ANTH.

Fig 4.3 Adsorption of *Nostoc* ANTH to rice roots (Strain IET-13783).

Fig 5.1 ^{14}C-glutamine uptake in *Nostoc* ANTH and its Het^-Nif^- mutant.

Fig 5.2 ^{14}C-asparagine uptake in *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant.

Fig 5.3 ^{14}C-arginine uptake in *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant.

Fig 5.4 ^{14}C-alanine uptake in *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant.

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

UNIVERSITY LIBRARY
Date
103569

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.

CHAPTER 2

MATERIALS AND METHODS

2.1 Organisms :

The cyanobacterium *Nostoc* sp. used in the present study was a soil-isolate from North-Eastern Hill University campus, Meghalaya. tRNA^{Leu} (UAA) intron sequence analysis (done in the laboratory of Prof. P. Lindblad, Uppsala University, Sweden) as well as PCR fingerprints using STRR 1A primer (done in the laboratory of Prof. B. Bergman, Stockholm University, Sweden) were performed to determine the identity of this *Nostoc* strain. The results (Fig. 2.1 and 2.2) show that this *Nostoc* strain is virtually identical to *Nostoc* ANTH isolated by us earlier from *Anthoceros* (Prakasham and Rai, 1991). Both strains showed identical fingerprints (Fig 2.1) and tRNA^{Leu} (UAA) intron sequences of both strains were similar except for a difference of just two bases (at 111 and 139) (Fig 2.2). For full details of protocols for STRR-1A PCR and tRNA^{Leu} (UAA) intron sequence analysis, see Rasmussen and Svenning (1998) and Paulsrud and Lindblad (1998), respectively.

Isolation procedures of nitrogenase-derepressed, herbicide-resistant and non-N_2-fixing mutants of *Nostoc* ANTH are detailed in materials and methods sections of chapters 3, 4 and 5, respectively.

2.2 Culture methods :

2.2.1 Culture medium :

The cyanobacterium *Nostoc* ANTH was grown from axenic stock cultures in N_2-medium (BG-11_0 medium; Rippka *et al*, 1979). The concentrations of macronutrients in N_2-medium were (mM): $K_2HPO_4.3H_2O$, 0.18; Na_2CO_3, 0.19; $MgSO_4.7H_2O$, 0.30; $CaCl_2.2H_20$, 0.25; EDTA (disodium salt), 0.003; Citric acid, 0.029; Ferric ammonium citrate, 0.030. The concentrations of micronutrients in N_2-medium were (μM): H_3BO_3, 46; $MnCl_2.4H_2O$, 9.2; $ZnSO_4.7H_2O$, 0.77; $Na_2MoO_4.2H_2O$, 1.6; $CuSO_4.5H_2O$, 0.32; $Co(NO_3)_2.6H_2O$, 0.17. As and when required, the N_2-medium was supplemented with combined nitrogen sources such as 1 mM L-amino acids, 5 mM sodium nitrate (nitrate-medium) or 2 mM ammonium chloride (ammonium-medium). The medium was always buffered with equimolar concentration of HEPES. The pH of the medium was adjusted to 7.5 before autoclaving.

2.2.2 Sterilization :

All glasswares and nutrient media were autoclaved at 121 °C (15 psi) for 15 min. The heat labile chemicals were sterilized by ultrafiltration using Whatman membrane filters of pore size 0.45 μm. Such chemicals were then added to previously sterilized nutrient medium.

2.2.3 Culture conditions :

The cyanobacterium *Nostoc* ANTH and its mutants were maintained on agar slants as well as in liquid BG-11_0 or BG-11 (nitrate or ammonium)-medium. Culture room temperature was maintained at 25 °C and light was provided at a photon fluence rate of 50 μmol.m^{-2}.s^{-1}on the surface of the vessels.

2.3 Growth parameters :

The growth of cyanobacterium *Nostoc* ANTH and its mutants were measured using the following parameters:

2.3.1 Chlorophyll *a* :

Chlorophyll *a* was measured using the method described by Mackinney (1941). 5 ml samples were extracted in equal volume of 100 % methanol for 10 min. The Chlorophyll *a* concentration was measured spectrophotometrically in the supernatant of centrifuged extracts at 663 nm by using the formula: Chlorophyll *a* (μg.ml^{-1}) = Absorbance at 663 nm x 12.63.

2.3.2 Protein estimation :

Protein content was measured according to Lowry *et al*, (1951) as per details given below:

2.3.2.1 Extraction of protein :

5 ml of cyanobacterial culture was centrifuged and the pellet was resuspended in 1 ml of distilled water. The cells were disrupted by ultrasonication using a Soniprep

150 (MSE) fitted with a microprobe. The supernatant was collected after centrifugation at 3000 rpm for 5 min and used for protein determination.

2.3.2.2 **Estimation of protein :**

Reagents :

A: 2 % Na_2CO_3 in 0.1 N NaOH.

B: 1 % sodium potassium tartarate solution.

C: 0.5 % $CuSO_4$ solution.

D: 100 ml of reagent A mixed with 1 ml each of reagent B and C (freshly prepared before use).

E: 1 N Folin and Ciocalteu's phenol reagent.

F: Standard protein solution: Bovine Serum Albumin (BSA) solution was prepared in the range of 10-100 $\mu g.ml^{-1}$.

2.3.2.3 **Procedure :**

To 1 ml of cyanobacterial protein extract, 5 ml of reagent D was added and mixed gently. This was incubated for 10 min at room temperature and then 0.5 ml reagent E was added rapidly. After 30 min the mixture was centrifuged and the absorbance of the supernatant was read at 750 nm. A calibration curve was prepared by using BSA solution as standard for determination of cyanobacterial protein content.

2.4 **Oxygen exchange :**

Oxygen evolution and consumption was measured by using a Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirring (Rank Brothers, England). Measurement involved: adding 3 ml cyanobacterial culture to the sample chamber of the non-polarized electrode and allowing each sample to equilibrate for 5 min with stirring. The electrode was then polarised and the linear rate of oxygen evolution was obtained in light supplied by a 100 W tungsten filament bulb, which was shielded from the sample by water bath acting as heat filter. Oxygen consumption was measured in dark with the chamber wrapped with aluminium foil.

2.5 Enzyme assays :

2.5.1 Nitrogenase :

Nitrogenase activity was measured using acetylene reduction assay (Stewart *et al*, 1967). 8 ml of cyanobacterial culture was placed in 18 ml serum stoppered vials. Acetylene gas was injected to a final concentration of 10 % (v/v) of air phase in the vials. The vials were incubated in light (photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$) at 25 °C. After 1 h, 1 ml gas sample was analyzed for ethylene produced by using a Tracor 540 gas chromatograph fitted with a porapak T column (stainless steel column 6' x 1/8" packed with porapak T mesh size 80/100) and a flame ionization detector.

2.5.2 Glutamine synthetase :

2.5.2.1 Extraction of enzyme :

Cultures were harvested by centrifugation, washed twice in 50 mM Tris-HCl buffer, pH 7.5 and resuspended in the same buffer. The cells were treated for 10 min with alkyltrimethylammonium bromide (CTAB) at a final concentration of 100 $\mu g.ml^{-1}$ (Frias *et al.*, 1994). Glutamine synthetase transferase activity was then assayed *in situ* using CTAB permeabilized cells.

2.5.2.2 Glutamine synthetase transferase :

This was essentially as described by Sampio *et al.*, (1979) except that CTAB permeabilized cells were used. The reaction mixture contained in a final volume of 3 ml, 1 ml enzyme extract (CTAB-permeabilized cell suspension), 40 μmol Tris-HCl buffer pH 7, 3 μmol $MnCl_2$, 20 μmol Potassium arsenate, 0.4 μmol ADP (Na^+salt), 60 μmol hydroxylamine and 30 μmol glutamine. The reaction mixture was incubated in the dark for 10 min at 30 °C. The reaction was terminated by the addition of 2 ml of stop mixture (4 ml of $FeCl_3$, 1 ml of 24 % TCA, 0.5 ml of 6 N HCl and 6.5 ml of water). The absorbance of the supernatant was read at 540 nm after 10 min of centrifugation at 2000 rpm. The concentration of ϒ-glutamyl hydroxamate formed was estimated from a standard curve that was prepared in the range of 0-0.2 $\mu mol.ml^{-1}$ ϒ-glutamyl hydroxamate.

2.5.3 Nitrate reductase :

Nitrate reductase (NR) activity was measured *in situ* (Manzano *et al*, 1976) using CTAB permeabilized cells. 5 ml of cyanobacterial culture was taken and centrifuged. The pellet was thoroughly washed with and resuspended in NR buffer (50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.3 M sucrose, 1 mM KNO_3, 1 mM EDTA and 5 mM $MgCl_2$). CTAB was added at a final concentration of 100 $\mu g.ml^{-1}$ and the suspension incubated for 10 min at room temperature with vigorous shaking. The reaction mixture contained in addition to permeabilized cells in a final volume of 1 ml: 20 mM KNO_3, 100 mM glycine-KOH (pH 10.5), 4 mM methyl viologen, 10 mM sodium dithionite freshly dissolved in 0.1 ml of 0.23 M $NaHCO_3$. After 7 min of incubation in dark at 30 °C, the reaction was terminated by adding 0.2 ml of 1 M zinc acetate. Subsequently nitrite formed was determined by the method of Snell and Snell (1949).

2.5.4 **Nitrite reductase :**

Nitrite reductase (NR) activity was measured *in situ* using CTAB permeabilized cells (Arizmendi and Serra, 1990). 5 ml of cyanobacterial culture was centrifuged and the pellet was thoroughly washed with buffer containing 50 mM Tris (pH 7.5). To such cyanobacterial suspension CTAB was added at a final concentration of 100 $\mu g.ml^{-1}$ and incubated for 10 min with vigorous shaking. The reaction mixture contained in addition to permeabilized cells in a final volume of 1 ml: 2.5 mM KNO_2, 90 mM Tris-HCl (pH 7.5), 3 mM methyl viologen, 20 mM sodium dithionite freshly prepared in 0.3 M $NaHCO_3$. After 5 min of pre-incubation at room temperature, the reaction was carried out at 30 °C for 10 min and stopped by vigorous shaking to oxidize excess reductant. Subsequently remaining nitrite was determined by the method of Snell and Snell (1949).

2.5.5 **Nitrite estimation :**

Nitrite was estimated colorimetrically as described by Snell and Snell (1949).

2.5.5.1 **Reagents :**

A: 1 % (w/v) sulphanilamide in 3 M HCl.

B: 0.02 % (w/v) N-(1-Napthyl ethylene diamine dihydrochloride) in distilled water.

C: Potassium nitrite solution was prepared in the range of 10-100 nmol.ml^{-1}. This was used as standard.

2.5.5.2 **Procedure :**

To 1 ml of sample, 1 ml of sulphanilamide and 1 ml of 1-Napthyl ethylene diamine dihydrochloride was added. The solution was mixed thoroughly and the absorbance was read at 540 nm after 15 min. A calibration curve was prepared by using potassium nitrite solution as standard for estimation of nitrite.

2.6 Uptake experiments :

2.6.1 Nitrate and nitrite uptake :

Nitrate/nitrite uptake by cyanobacterial cultures was measured by following the disappearance of nitrate/nitrite (100 μM) from the external medium. The concentration of nitrite was determined by the method of (Snell and Snell, 1949) as described previously. Nitrate concentration was measured by following the method of Cawse (1967).

2.6.1.1 **Reagents :**

A: 2 % sulphamic acid.

B: 6.67 % $HClO_4$

C: Sodium nitrate solution was prepared in the range of 100-500 nmol.ml^{-1}. This was used as standard.

2.6.1.2 **Procedure :**

To 1 ml of sample, 1 ml of sulphamic acid and 3 ml of $HClO_4$ was added. The solution was mixed thoroughly and the absorbance was read at 210 nm. A calibration curve was prepared by using sodium nitrate solution as standard for estimation of nitrate. Further details on nitrate/nitrite uptake assays are given in chapter 3.

2.6.2 **Ammonium and amino acid uptake :**

^{14}C-methylammonium an analogue of ammonium, was used as a probe to estimate the ammonium uptake, as described by Rai *et al.*, (1984). Further details are given in chapter 3. Glutamine, arginine, asparagine and alanine uptakes were measured by using ^{14}C-labeled glutamine, arginine, asparagine and alanine as detailed in chapter 5.

2.7 **Colonization of *Nostoc* ANTH and its mutants with rice plants :**

Experimental details about colonization and transmission electron microscopic (TEM) studies are given in chapter 4.

2.8 **Chemicals :**

All chemicals were purchased from Sigma chemical company, U.S.A.

Fig 2.1. STRR 1A-PCR-based DNA fingerprints of *Nostoc* sp., *Nostoc* sp. (PR: Paraquat-resistant mutant), *Nostoc* sp. (AR: Azetidine-2-carboxylate-resistant mutant), *Nostoc* ANTH and *Nostoc* PCC 9229.

These fingerprints were obtained using short tandemly repeated repetitive sequences (STRR 1A) as primer and whole filaments of *Nostoc* as templates. The STRR 1A primer used here had the following sequence: 3′-CCCCTRACCCCTRACC-5′. Note that the fingerprint of *Nostoc* sp. and its mutants are similar to that of *Nostoc* ANTH and quite distinct from *Nostoc* PCC 9229 (included here as control).

Lane M represents DNA molecular weight standards (bp).

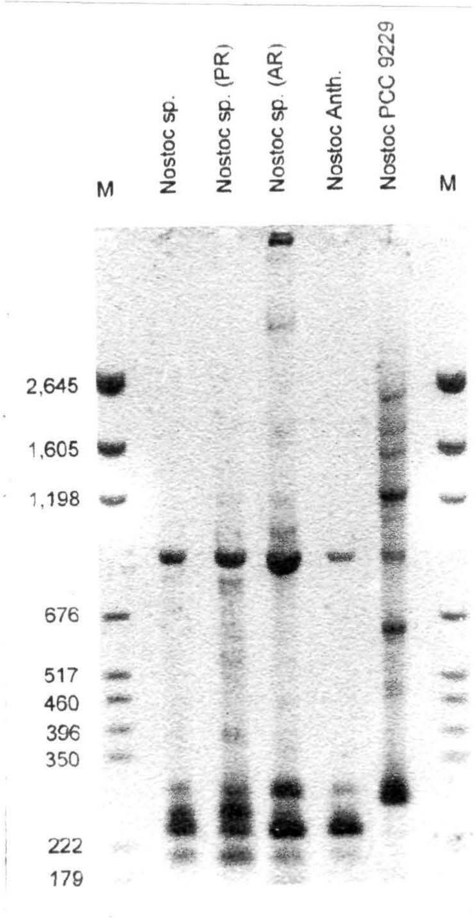


Fig 2.2. Comparison of nucleotide sequences of tRNA^{Leu} (UAA) intron from cyanobacterium *Nostoc* ANTH and *Nostoc* sp.

The bold letters (111 and 139) indicate the difference of bases between *Nostoc* ANTH and *Nostoc* sp.

Sample				
Nostoc ANTH	AAATAATTGA	GCCTTAAAGA	AGAAATTCTT	30
Nostoc sp.	AAATAATTGA	GCCTTAAAGA	AGAAATTCTT	30
Nostoc ANTH	TAAGTGGATG	CTCTCAAACT	CAGGGAAACC	60
Nostoc sp.	TAAGTGGATG	CTCTCAAACT	CAGGGAAACC	60
Nostoc ANTH	TAAATCTGTT	CGCAGACATG	GCAATCCTGA	90
Nostoc sp.	TAAATCTGTT	CGCAGACATG	GCAATCCTGA	90
Nostoc ANTH	GCCAAGCCCA	AGATAATTGG	**A**AAGGTGCAG	120
Nostoc sp.	GCCAAGCCCA	AGATAATTGG	**G**AAGGTGCAG	120
Nostoc ANTH	AGACTCGACG	GGAGCTAC**C**C	TAACGTCAAG	150
Nostoc sp.	AGACTCGACG	GGAGCTAC**T**C	TAACGTCAAG	150
Nostoc ANTH	ACGAGGGTAA	AGAGAGAGTC	CAATTCTCAA	180
Nostoc sp.	ACGAGGGTAA	AGAGAGAGTC	CAATTCTCAA	180
Nostoc ANTH	AGCCATTAGG	CAGTAGCGAA	AGCTGCGGGA	210
Nostoc sp.	AGCCATTAGG	CAGTAGCGAA	AGCTGCGGGA	210
Nostoc ANTH	GAATG			215
Nostoc sp.	GAATG			215

CHAPTER 3

Isolation and characterization of a chlorate-resistant mutant (ClO-*R*) of the symbiotic cyanobacterium, *Nostoc* ANTH: Heterocyst formation and N_2-fixation in presence of nitrate, and evidence for separate nitrate and nitrite transport systems.

3.1 Introduction :

Cyanobacteria are a diverse group of O_2-evolving photosynthetic prokaryotes. Many of them are N_2-fixers and potential biofertilizers, particularly for rice paddies. However, cyanobacterial biofertilizer falls short of supplying total nitrogen requirements of the crop and chemical nitrogen fertilizers are often used as supplements. Such fertilizer applications adversely affect N_2-fixation by the cyanobacteria (Singh, 1961; Rai *et al*, 2000; Whitton, 2000). Indeed combined nitrogen sources such as nitrate and ammonia inhibit and repress nitrogenase as well as heterocyst formation in cyanobacteria. In the cyanobacterial cell, the nitrate is reduced to nitrite by Ferredoxin (Fd)-dependent nitrate reductase. The nitrite is then reduced to ammonia by nitrite reductase. Finally, the ammonia is assimilated by glutamine synthetase. Both ammonia *per se* and a product of ammonia assimilation via GS are thought to be the actual repressors (Gallon and Chaplin, 1987; Flores and Herrero, 1994; Herrero *et al*, 2001). Heterocysts are the sites of aerobic N_2-fixation in heterocystous cyanobacteria (Bergman *et al*, 1986). Ammonia and nitrate both repress heterocyst formation and thereby N_2-fixation but while ammonia represses nitrogenase in pre-existing heterocysts, nitrate is unable to do so because heterocysts lack nitrate uptake and reductase (Kumar *et al*, 1985; Rai and Bergman, 1986; Renstrom-Kellner *et al*, 1990). Cyanobacterial genes involved in nitrate and nitrite uptake and assimilation have been identified. They are in an operon (*nir-nrtABCD-narB*) that is regulated by nitrogen availability and are co-transcribed (see Herrero *et al*, 2001). It has been suggested that nitrate and nitrite are transported through a common permease (Luque *et al*, 1994) but a nitrite transport system quite distinct from the common nitrate/nitrite transporter, has also been reported (Maeda and Omata, 1997).

Nostoc is a filamentous heterocystous cyanobacterium having a versatile modes of C and N metabolism, and a wide range of ecological habitats (Potts, 2000). *Nostoc* is also the most common cyanobiont occurring in symbiosis with plants ranging from algae to angiosperms (Rai *et al*, 2000). In such symbioses, the *Nostoc* provides fixed-nitrogen to the plant. In recent past some successful attempts have been made to create artificial associations between crop plants, particularly rice and wheat and *Nostoc* or *Anabaena* strains (Ganter *et al*, 1995; Kamuru *et al*, 1997; Svircev *et al*, 1997). It has been reasoned that symbiotically competent *Nostoc* strains may be particularly suited for such associations (Rai *et al*, 2000). One such strain is *Nostoc* ANTH, which forms symbioses with *Anthoceros*. In preliminary experiments, this strain showed a tendency to colonise roots of rice plants (Rai *et al*, 1996).

From ecological and agricultural point of view, the cyanobacterial mutants derepressed for N_2-fixation in presence of nitrate and/or ammonium are likely to be more useful in associative symbioses with wheat and rice plants (Kamuru *et al*, 1997; Rai *et al*, 2000). Using the nitrate analogue chlorate as a selection agent a spontaneous chlorate-resistant (ClO-*R*) mutant was isolated during the present study. We report here that this mutant is capable of heterocyst formation and N_2-fixation in presence of nitrate. The mutant lacks nitrate uptake and induction of nitrate reductase activity by nitrate. However, this mutant is able to transport and assimilate nitrite, indicating that there is a transport system for nitrite, which is distinct from that for the nitrate.

3.2 Materials and methods :

3.2.1 **Strains and culture conditions :** Axenic clonal cultures of the diazotrophic cyanobacterium *Noctoc* ANTH and its chlorate-resistant (ClO-*R*) mutant were grown in batch cultures using $BG11_0$ (N_2-medium) (Rippka *et al,* 1979). N_2-medium supplemented with 5 mM sodium nitrate or 2 mM ammonium chloride is referred to as NO^-_3-medium and NH^+_4-medium, respectively. The NO^-_3- and NH^+_4-media were always buffered with equimolar concentration of HEPES. The pH was adjusted to 7.5 using NaOH before autoclaving. The cultures were routinely maintained at 25 °C with a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$.

3.2.2 **Isolation of the ClO-*R* mutant :** As a first step towards isolation of ClO-*R* mutants, the survival of cyanobacterium *Nostoc* ANTH was checked at increasing concentrations of potassium chlorate. A concentration of 60 mM chlorate was found to be the lethal dose for *Nostoc* ANTH, both in liquid as well as solid media. Spontaneously occurring ClO-*R* mutants of cyanobacterium *Noctoc* ANTH were obtained by plating approximately $2.4x10^7$ colony-forming units on solid N_2-medium containing 60 mM chlorate. After 3 weeks of incubation, the few surviving colonies, which remained were picked up and transferred to plates containing 60 mM chlorate. The ClO-*R* mutants arose at a frequency of $1.3x10^{-7}$. One such mutant was further characterized with regard to growth, heterocyst frequency, N_2-fixation and uptake and assimilation of nitrate, nitrite and ammonium.

3.2.3 **Analysis of growth, heterocyst frequency and nitrogenase activity :** Growth was measured as increase in concentration of Chl *a* (Mackinney, 1941). Heterocyst frequency was calculated as percentage of total cells by light microscopic observations after 96 h of incubation in different nitrogen media (Bagchi and Singh, 1984). The acetylene reduction assay was used to measure nitrogenase activity (Stewart *et al*, 1967).

3.2.4 **Nitrate and nitrite uptake assays :** The N_2-, NO^-_3- and NH^+_4 -grown cultures of the parent and mutant strains were harvested during the exponential growth phase, washed and resuspended in N_2-medium adjusted to pH 8.6 with 1 mM HEPES-NaOH buffer. Uptake of nitrate and nitrite was measured by determining the rates of their depletion from the external medium. Uptake was started by addition of $NaNO_3$ (100 μM) or KNO_2 (100 μM) to the cell suspension. The choice of 100 μM external concentration was based on earlier studies in *Anabaena* sp. PCC 7120 and *Synechococcus* sp. strain PCC 7942 (Frias *et al*, 1997; Maeda and Omata, 1997). Where needed, dicyclohexylcarbodimide (DCCD, 10 μM) was added to the cell suspension 15 min prior to the addition of nitrate or nitrite. Samples were withdrawn after 3 h of incubation, the cells removed by rapid centrifugation and the cell-free supernatants were analyzed for residual nitrate or nitrite. Nitrate concentration was measured by its absorbance at 210 nm in acid solution (Cawse, 1967) and nitrite concentration was measured by the method of Snell and Snell (1949).

3.2.5 **Nitrate reductase, nitrite reductase and glutamine synthetase (transferase) activity :** Glutamine synthetase (transferase) was measured according to (Sampio *et al*, 1979). Nitrate reductase (Manzano *et al*, 1976) and nitrite reductase activities (Arizmendi and Serra, 1990) were measured in cells permeabilized with alkyltrimethylammonium bromide and dithionite-reduced methyl viologen was used as reductant. Protein concentration was determined by the method of Lowry *et al*, (1951).

3.2.6 **Ammonium transport assay :** This was done using the radioactive analogue of ammonium, ^{14}C-methylammonium. The NO^-_3 -grown cultures of the parent and mutant strains were harvested during the exponential growth phase, washed in N_2-medium and then transferred to N_2-, NO^-_3- and NH^+_4 -media. After 48 h cells were harvested, washed and resuspended in 10 mM HEPES-NaOH buffer (pH 7.0) to a concentration of 5 µg Chl *a*.ml^{-1}. After equilibrating for 10 min at 25 °C and a photon fluence rate of 50µmol.m^{-2}.s^{-1}, ^{14}C-methylammonium was added to a final concentration of 50 µM (sp. activity 172 kBq.µmol^{-1}). At different time intervals, 400 µl samples were taken out rapidly and the cells were separated from their bathing medium by centrifugation through silicon-oil DC 550/dinonyl phthalate (40/60, v/v) into perchloric acid/water (15/85, v/v) (Scott and Nicholls, 1980). The ^{14}C in perchloric acid fraction was measured using a liquid scintillation counter (model 1801, Beckman Instruments). Non-specific binding of ^{14}C-methylammonium was determined by measuring its incorporation in toluene-treated cells (Rai *et al*, 1984). This value was always subtracted from the value obtained for toluene-untreated sample.

3.3 Results and discussion :

3.3.1 Growth and N_2-fixation of parent and mutant : A comparison of the parent and its ClO-*R* mutant with regard to their growth, heterocyst formation and nitrogenase activity when using different nitrogen sources is presented in Table 3.1. The parent strain grew better at the expense of ammonium followed by that of nitrite, nitrate and N_2. The growth of ClO-*R* mutant was found to be similar to that of its parent in presence of ammonium, nitrite and N_2. However, the mutant grew at a slower rate than its parent in nitrate-supplemented medium. This slower growth of the mutant in nitrate-medium may have resulted due to an impairment of nitrate uptake and/or its utilization as a nitrogen source.

The parent strain produced heterocysts and nitrogenase activity in N_2-medium but not in nitrate, nitrite or ammonium-containing media. Similarly, the ClO-*R* mutant lacked heterocysts and N_2-fixation in ammonium and nitrite-supplemented media. However, in contrast to the parent strain, the mutant developed heterocysts and nitrogenase activity in nitrate-media.

Fig 3.1 shows effects of nitrate addition on nitrogenase activity of the parent and mutant strains. Nitrogenase activity of the parent strain declined progressively as the incubation time in nitrate-medium increased and it reached to a value close to zero by 96 h. On the other hand, the nitrogenase activity in the mutant strain was unaffected and remained similar to that of N_2-grown cultures. In summary, the data suggests that the mutant could be defective in utilization of nitrate as a nitrogen source as a result of which it continues to form heterocysts and fix N_2 in nitrate-medium. These results are consistent with the earlier findings that mutants altered in their ability to assimilate nitrate may have their ability to form heterocyst and nitrogenase activity unaffected (Martin-Nieto *et al*, 1991; Cai and Wolk, 1997; Frias *et al*, 1997).

3.3.2 **Activities of nitrate uptake, nitrate reductase, nitrite uptake and nitrite reductase :** In order to determine the cause of nitrogenase activity remaining derepressed in nitrate-medium, nitrate/nitrite uptake and reductase and GS activities were measured in the parent and mutant strains (Table 3.2 and 3.3). The N_2–grown parent strain showed a nitrate uptake rate of 2.8 μmol.min^{-1}.mg^{-1} Chl *a*. This activity increased in nitrate-grown cells to 3.6 μmol.min^{-1}.mg^{-1} Chl *a* but was repressed in ammonium-grown cells (0.4 μmol.min^{-1}.mg^{-1} Chl *a*). The ATPase inhibitor, DCCD strongly inhibited nitrate uptake. These observations suggest a nitrate-inducible, ammonium-repressible nitrate uptake system, which is energy-dependent. The rate of nitrate uptake in the mutant strain was found to be less than 8 % of that in the parent strain. The effects of ammonium and DCCD on nitrate uptake by the mutant strain were similar to that in the case of the parent strain. These results suggest that the mutant is defective in nitrate uptake. In contrast the mutant had significant levels of nitrite uptake activity (60 % of that in the parent). As in the case of the parent, the nitrite uptake was ammonium-repressible and sensitive to DCCD. Furthermore, since the assays were done under alkaline conditions (pH 8.6), the uptake could not be attributed to passive diffusion of nitrous acid (Martin-Nieto *et al*, 1989; Maeda and Omata, 1997).

The nitrate reductase activity of the parent strain was 60 % higher in presence of nitrate than in N_2–grown cells, indicating that the enzyme may be induced/stabilized by nitrate (Table 3.3). N_2–grown cultures of the ClO-*R* mutant showed a nitrate reductase activity similar to that in the N_2–grown parent strain. However, there was no change in activity of nitrate reductase when the mutant cells were transferred to the nitrate-medium. Thus the mutant lacked induction of nitrate reductase by nitrate. Ammonium caused a strong repression of nitrate reductase activity both in the *Nostoc* ANTH (parent) and its ClO-*R* mutant. In contrast to nitrate reductase, nitrite reductase activity was similar in the parent and mutant strains (Table 3.3). Furthermore, in both cases the nitrite reductase activity was ammonium-repressible.

In cyanobacteria, genes encoding the nitrite reductase (*nirA*), the multicomponent transporter for nitrate and nitrite (*nrtABCD*), and nitrate reductase (*narB*) proteins have been identified and characterized on the basis of phenotypes exhibited by various classes of nitrate and/or nitrite uptake and assimilation defective mutants. These genes are organised in a cluster and co-transcribed as a single operon (*nirA-nrtABCD-narB*) in *Anabaena* sp. strain PCC 7120 and *Synechococcus* sp. strain PCC 6803 (Luque *et al*, 1992, 1993, 1994; Cai and Wolk, 1997; Frias *et al*, 1997; Herrero *et al*, 2001). The gene *nrtA* encodes a single 45 kDa nitrate and nitrite binding cytoplasmic membrane lipoprotein. The *nrtB*, *nrtC* and *nrtD* encode proteins that resemble the ATP-binding proteins of ABC transporters (Luque *et al*, 1994; Maeda and Omata, 1997). A gene-cassette insertion into the *nir-nrtA* region in *Anabaena* sp. strain PCC 7120 resulted in impaired nitrite and nitrate reductase activity thus suggesting that these clustered genes are co-transcribed as a single operon (Frias *et al*, 1997). There are also reports of independent regulation of *nirA* and *narB* (Luque *et al*, 1992; Cai and Wolk, 1997). In addition, products of *narA* and *narC* have been implicated in nitrate reduction and product of *nirB* in nitrite reduction (Kuhlemier *et al*, 1984; Suzuki *et al*, 1995).

The ClO-*R* mutant isolated during the present study showed normal levels of nitrite reductase activity, lacked nitrate uptake activity, and showed upto 60 % lower nitrate reductase activity and 40 % lower nitrite uptake activity. These, together with the fact that the mutant formed heterocysts and nitrogenase activity in nitrate-containing medium but not in nitrite-medium, indicate that nitrite uptake and assimilation in the mutant are intact while nitrate uptake and reductase are affected. Therefore, it seems highly likely that the mutant was a product of a lesion in one of the genes encoding nitrate-permease (*nrtABCD*) complex and not in *narB* or any common gene regulating the expression of nitrate and nitrite uptake and reductases in cyanobacteria. However, this does not preclude the possibility of a coordinated expression of nitrate and nitrite assimilation genes in cyanobacterium *Nostoc* ANTH. The ClO-*R* mutant was similar to the *Synechococcus* sp. strain PCC 7942 (FM16) mutant (Madueno *et al*, 1988) in the sense that both lacked nitrate uptake activity but retained significant levels of nitrate reductase activity. However, in contrast to FM16, the nitrate reductase activity of the ClO-*R* mutant was subject to ammonium repression. Another unique aspect of the mutant was that it had significant levels of nitrite uptake activity (inspite of a complete lack of nitrate uptake). This suggested that in the cyanobacterium *Nostoc* ANTH there is an uptake system for nitrite quite distinct from that for nitrate. These results are in agreement with the conclusions of Maeda and Omata (1997) suggesting a nitrite transport system quite distinct from the common transport system for nitrate and nitrite in *Synechococcus* sp. strain PCC 7942. These conclusions were based on observations that mutants (*nrtABCD* and *nrtA* deleted) lacking nitrate uptake activity showed only partial defect in nitrite uptake activity (Maeda and Omata, 1997). Such findings are contrary to the view of Luque *et al* (1994), who had reported that nitrate and nitrite share a common transport system since, *nrtD* mutants isolated by them were fully defective in the uptake of both nitrate and nitrite.

3.3.3 **Glutamine synthetase activity :** The activity of the primary-ammonium-assimilating enzyme GS was determined in N_2-, nitrate- and ammonium-grown cells of the parent and mutant strains (Table 3.3). The N_2- and nitrate-grown cells of both the parent and mutant strains showed comparable levels of GS activity. Thus, the acquisition of resistance to chlorate had no effect on GS activity. However, in ammonium-grown cells of both the parent and mutant strains, GS activity was repressed by 50 %. These results on *Nostoc* ANTH are consistent with the earlier findings of ammonium-repressible nature of glutamine synthetase in other cyanobacteria (Merida *et al.*, 1991; Frias *et al*, 1994).

3.3.4 **Ammonium uptake activity :** The status of ammonium uptake was studied in *Nostoc* ANTH and its mutant as a function of inorganic nitrogen sources using ^{14}C-methylammonium (an analogue of ammonium) (Prakasham and Rai, 1991; Shehawy and Kleiner, 1999). As shown in Fig 3.2, N_2–grown parent and mutant strains showed a biphasic pattern of methylammonium uptake. Such a methylammonium uptake pattern was marked by an initial rapid phase lasting for 60 s, followed by a slower second phase, which remained linear during the next 8 min of the experimental period. The rates of methylammonium uptake were similar in the parent and mutant strains. The methylammonium uptake activities during first and second phase were 55 and 7.3 $nmol.mg^{-1}$ Chl *a*.min^{-1}, respectively. However, the ammonium- and nitrate-grown cells of the parent strain and the ammonium-grown cells of the mutant strain showed negligible methylammonium uptake activity. In contrast to the parent strain, the ClO-*R* mutant had a fully derepressed methylammonium uptake activity under nitrate-grown condition. The pattern and rate of methylammonium uptake in the nitrate-grown mutant was nearly similar to that in N_2–grown cells. This is as expected since the ClO-*R* mutant was defective in nitrate uptake and assimilation; hence, nitrate was unable to affect the methylammonium uptake activity. These results are consistent with the ammonium-repressible nature of the methylammonium uptake in *Nostoc* ANTH (Prakasham and Rai, 1991) and the decreased expression of ammonium transport genes (*amt*) in nitrate- and ammonium-grown cells of *Synechocystis* sp. PCC 6803 (Montesinos *et al*, 1998). Overall, it is concluded that the ammonium transport system is intact in the mutant but its activity is unaffected by nitrate. These results also indicate that *ntcA*-mediated nitrogen regulatory system remains intact in the mutant since N_2-fixation, heterocyst differentiation, GS activity and ammonium transport, which are also controlled by *ntcA* remain unaffected (Herrero *et al*, 2001).

The results presented here indicate that *Nostoc* ANTH has a distinct nitrite transport system and that the ClO-*R* mutant of *Nostoc* ANTH can fix N_2 in presence of high concentrations of nitrate, which otherwise inhibits N_2-fixation. The mutant lacks nitrate uptake and has a 60 % lower nitrate reductase activity in nitrate-medium but the GS and ammonium transport activity remain unaffected. Thus the lack of inhibitory effect of nitrate on N_2-fixation was due to lack of nitrate uptake and not due to lack of enzymes for its assimilation (nitrate reductase and glutamine synthetase) or the lack of ammonium transport system for retention of ammonia. Such a mutant could serve as a better source of biofertilizer in rice-paddies since nitrate can be used as a chemical nitrogen fertilizer supplement without affecting N_2-fixation. Furthermore, the mutant is able to colonise rice roots as effectively as the parent *Nostoc* ANTH, following co-culture of the mutant with rice seedlings. This property of the mutant may be particularly helpful in transfer of the fixed nitrogen directly to rice plants (see chapter 4).

Table 3.1. Growth (Gr), heterocyst frequency (HF) and nitrogenase activity (N_2ase) of *Nostoc* ANTH and its ClO-*R* mutant as a function of nitrogen sources.

6 d old N_2-grown cultures were used as initial source of inoculum. The growth (μg Chl *a*.ml^{-1}), frequency of heterocysts (per 100 vegetative cell) and nitrogenase activity (nmol C_2H_4 formed.μg^{-1} Chl *a*.h^{-1}), were determined after 96 h of incubation in media containing different nitrogen sources as indicated below. Chl *a* concentration at the start of incubation (0 time) was 0.27 μg Chl *a*.ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Growth-medium	Parent			ClO-*R*		
	Gr	HF	N_2ase	Gr	HF	N_2ase
$BG11_0$	2.0 ± 0.1	5.8 ± 0.3	11 ± 0.8	1.9 ± 0.3	5.6 ± 0.2	10 ± 0.7
$BG11_0$+5mM NO^-_3	2.2 ± 0.1	0.0	0.0	1.9 ± 0.1	5.2 ± 0.5	11 ± 1.3
$BG11_0$+5mM NO^-_2	2.3 ± 0.1	0.0	0.0	2.4 ± 0.2	0.0	0.0
$BG11_0$+2mM NH^+_4	2.6 ± 0.2	0.0	0.0	2.5 ± 0.1	0.0	0.0

Fig 3.1. Effect of nitrate on nitrogenase activity of *Nostoc* ANTH and its ClO-*R* mutant.

6 d old N_2-grown cultures were harvested axenically, washed with sterile $BG11_0$-medium and inoculated into fresh N_2- ($BG11_0$) and nitrate- ($BG11_0$+5 mM $NaNO_3$) media. At time intervals indicated, samples were withdrawn and nitrogenase activity measured. Symbols: N_2-medium, parent (●), nitrate-medium, parent (▲), N_2-medium, mutant (o), nitrate-medium, mutant (Δ). The values presented are means from two independent experiments, each with two replicates.

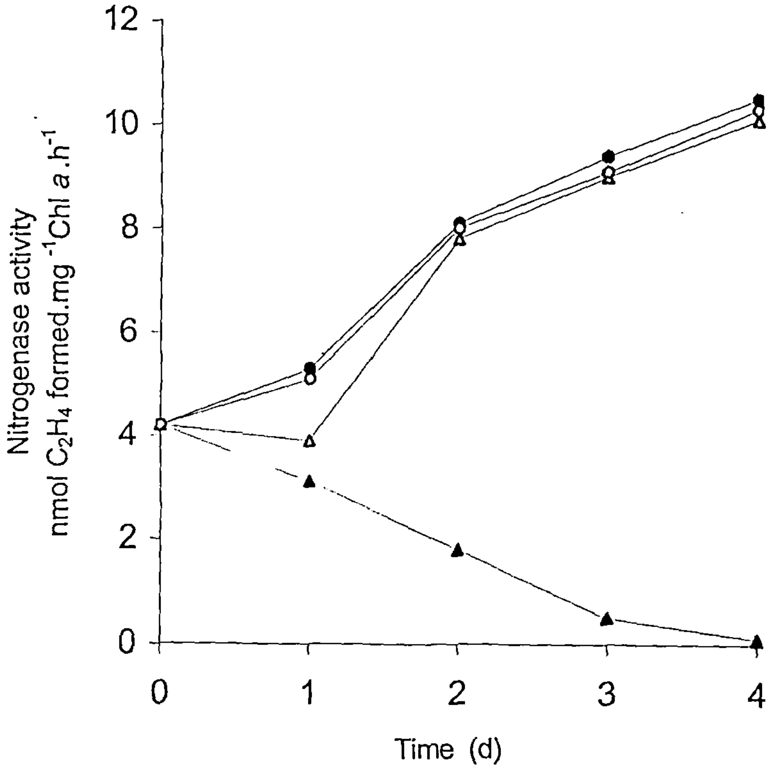


Table 3.2. Nitrate and nitrite uptake activity in *Nostoc* ANTH and ClO-*R* mutant grown in N_2-, NO^-_3- or NH^+_4-media.

Exponentially growing cultures of both parent and mutant strains were washed and resuspended in N_2-medium (5-7 µg Chl *a*.ml^{-1}). Nitrate or nitrite were added to a final concentration of 100 µM and their disappearance from the medium measured in presence and absence of DCCD (10 µM). See materials and methods for more details. The uptake rates are expressed as µmol substrate taken up.min^{-1}.mg^{-1} Chl *a*. The values presented are means ± SE from two independent experiments, each with two replicates.

Growth-medium	Addition	Nitrate uptake		Nitrite uptake	
		Parent	ClO-*R*	Parent	ClO-*R*
$BG11_0$	None	2.8 ± 0.2	0.2 ± 0.1	24 ± 1.1	14 ± 0.8
$BG11_0$	DCCD	0.0	0.0	0.5 ± 0.2	0.4 ± 0.1
$BG11_0$+5mM NO^-_3	None	3.6 ± 0.1	0.3 ± 0.1	29 ± 0.9	17 ± 1.0
$BG11_0$+5mM NO^-_3	DCCD	0.0	0.0	1.3 ± 0.2	0.7 ± 0.2
$BG11_0$+2mM NH^+_4	None	0.4 ± 0.1	0.0	0.2 ± 0.1	0.0
$BG11_0$+2mM NH^+_4	DCCD	0.0	0.0	0.0	0.0

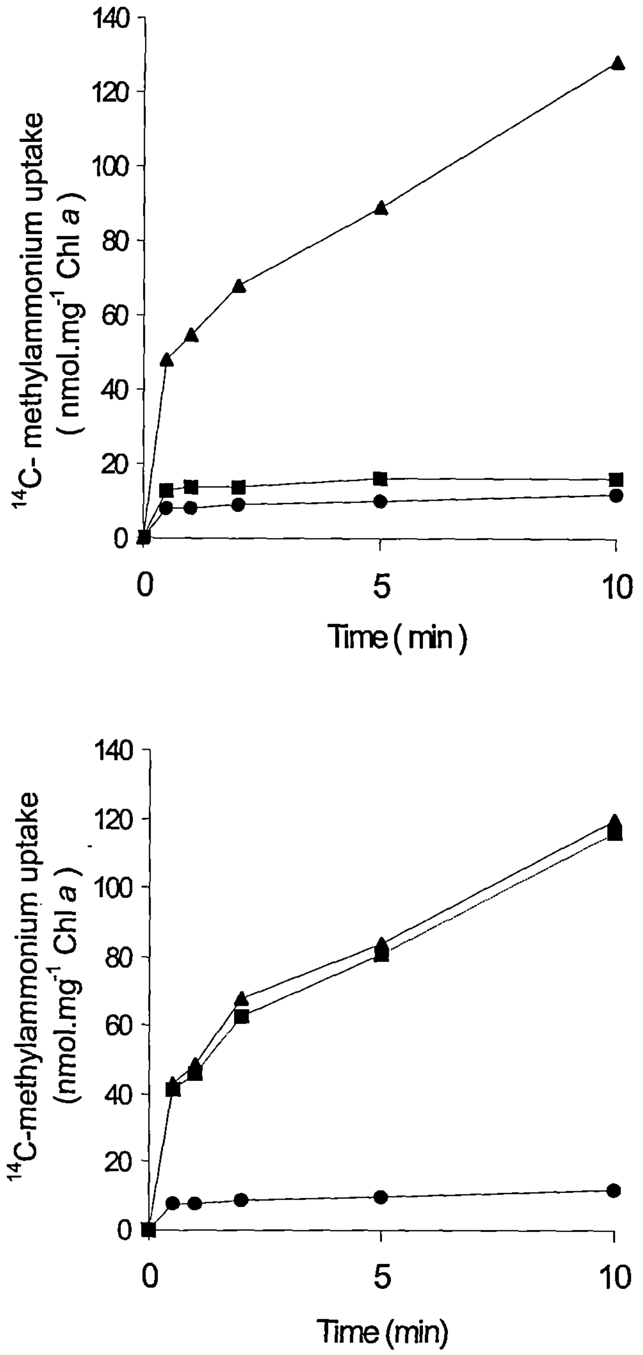
Table 3.3. Nitrate, nitrite reductase and glutamine synthetase (GS) transferase activities of *Nostoc* ANTH (parent) and its ClO-*R* mutant in different nitrogen media.

The nitrate reductase (NR) activity (nmol NO^-_2 formed.min^{-1}.mg^{-1} protein), nitrite reductase (nmol NO^-_2 consumed.min^{-1}.mg^{-1} protein) and glutamine synthetase (GS) transferase activity (nmol γ-glutamyl hydroxamate formed.min^{-1}.mg^{-1} protein) were determined after 48 h of incubation in different media as indicated below. The values presented are means ± SE from two independent experiments, each with two replicates. Nd: not determined.

Growth-medium	Parent			ClO-*R*		
	NR	NiR	GS	NR	NiR	GS
$BG11_0$	1.9 ± 0.1	540 ± 22	701 ± 2.8	1.8 ± 0.1	516 ± 10	692 ± 13
$BG11_0$+5mM NO^-_3	4.7 ± 0.6	Nd	690 ± 9.0	1.9 ± 0.1	Nd	686 ± 16
$BG11_0$+2mM NH^+_4	0.8 ± 0.1	230 ± 13	359 ± 4.0	0.4 ± 0.2	211 ± 6	363 ± 2.0

Fig3.2. ^{14}C-methylammonium uptake in *Nostoc* ANTH (A) and its ClO-*R* mutant (B) under N_2- (▲), nitrate- (■) and ammonium-grown conditions (●).

Nitrate-grown exponential cultures were washed and transferred to different nitrogen media and incubated for 48 h. Thereafter, the cells were washed, resuspended in HEPES buffer and used for ^{14}C-methylammonium uptake as described in materials and methods. The values presented are means from two independent experiments, each with two replicates.



CHAPTER 4

Colonization of rice (*Oriza sativa* L.) roots, N_2-fixation and nitrogen transfer by cyanobacterium *Nostoc* ANTH and its mutants.

4.1 Introduction :

Nitrogen is one of the essential elements determining productivity of plants including crops such as rice. Unfortunately, no plant species including the rice plant is able to reduce atmospheric nitrogen into a form utilisable for their growth. Therefore, chemical nitrogen fertilizers are used in order to meet nitrogen requirements of crops. It has been well documented that N_2-fixing cyanobacteria, which are one of the major constituents of microbial flora in wetland rice-fields, can effectively serve as a nitrogen biofertilizer and reduce our dependency on chemical nitrogen fertilizers (Singh, 1961; Roger and Ladha, 1992). However, such free-living cyanobacteria release nitrogen only on turn over of their biomass and the nitrogen released in soil may not be fully and directly available to the plants. Therefore, in order to ensure direct and continuous transfer of fixed-nitrogen to rice plants creation of artificial associations involving N_2-fixing cyanobacteria and rice plants seems a promising prospect (Whitton, 2000; Rai *et al*, 2000).

Cyanobacteria, particularly the species of *Nostoc* enter into symbiosis with many plants and provide fixed-nitrogen to them (Adams, 2000; Rai *et al*, 2000). In recent years there have been several attempts to create new N_2-fixing associations between N_2-fixing cyanobacteria (some *Nostoc* and *Anabaena variabilis* strains) and cereals such as rice and wheat (Obreht *et al*, 1993; Svircev *et al*, 1997; Gantar and Elhai, 1999). However, rice-fields are often rich in herbicides and chemical nitrogen fertilizers that limit the photoautotrophic growth and N_2-fixing capacity of cyanobacteria (Whitton, 2000). Therefore, herbicide-resistant and/or nitrogenase-derepressed (N_2-fixation in presence of combined-nitrogen sources) cyanobacterial strains would be far better for use in artificial associations with cereal crops (Modi *et al*, 1991; Spiller *et al*, 1993; Kamuru *et al*, 1997). An ammonia-excreting nitrogenase-derepressed mutant of *Anabaena variabilis* has been shown to fix N_2 at higher rates in association with wheat and rice plants, and transfer fixed-nitrogen to the host plants (Spiller *et al*, 1993; Spiller and Gunasekaran, 1994; Kamuru *et al*, 1997). However, there are no available reports regarding the use of any herbicide-resistant cyanobacterial mutant for creation of beneficial associations with wheat or rice plants.

Paraquat, a bipyridilium class of herbicide, is a known inducer of photooxidative stress in photosynthetic organisms including cyanobacteria (Bagchi *et al*, 1991). Paraquat mediated toxicity has been attributed to the production of active oxygen species such as superoxide and its subsequent peroxide products by reaction between oxygen and PS1 reduced paraquat (Carroll *et al*, 1988; Bagchi *et al*, 1991; Herbert *et al*., 1992; Tichy and Vermaas, 1999). The stress symptoms include a severe reduction in nitrogenase activity and growth, accompanied by extensive photobleaching and cell lysis (Bagchi *et al*, 1991; Tichy and Vermaas, 1999). Several mechanisms for detoxification of oxygen radicals, like increased production of superoxide dismutase

(SOD) and peroxidases have been suggested to confer paraquat tolerance (Bagchi *et al*, 1991; Herbert *et al.*, 1992; Tichy and Vermaas, 1999).

The objective of the present study was to isolate and characterize a paraquat-resistant (PQ-*R*) mutant of cyanobacterium *Nostoc* ANTH and to create N_2-fixing associations with rice plants using *Nostoc* ANTH and its mutants. The diazotrophic cyanobacterium *Nostoc* ANTH was chosen for rice colonization experiments because this strain is more likely to succeed in forming such associations in view of its already existing natural symbiotic competency. The data presented here indicate successful colonization of rice roots by *Nostoc* ANTH and its herbicide-resistant mutant. In the association, N_2-fixation and transfer of fixed-nitrogen from associated cyanobacteria to the rice plants are reported. Another mutant of *Nostoc* ANTH (a chlorate-resistant mutant) capable of N_2-fixation in presence of nitrate was found to be equally effective in colonization, associative N_2-fixation and nitrogen transfer.

4.2 Material and methods :

4.2.1 Strains and culture conditions : Axenic clonal cultures of the diazotrophic cyanobacterium *Nostoc* ANTH and its paraquat-resistant mutant (*PQ-R*) were grown in batch cultures using BG11$_0$ (N_2-medium) (Rippka *et al*, 1979). N_2-medium supplemented with 5 mM sodium nitrate, 1 mM ammonium chloride or 1 mM urea was referred to as NO^-_3-medium, NH^+_4-medium and urea-medium, respectively. The NO^-_3-, NH^+_4- and urea-media were always buffered with equimolar concentration of HEPES. For heterotrophic growth, the N_2-medium was supplemented with 10 μM DCMU and 2 mM filter-sterilized D-glucose, sucrose or fructose. The pH was adjusted to 7.5 using NaOH before autoclaving. The cultures were routinely maintained at 25 °C with a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$.

4.2.2 Isolation of the PQ-*R* mutant : A concentration of 0.06 μM of paraquat was found to be the lethal dose for cyanobacterium *Nostoc* ANTH, irrespective of nitrogen source provided for growth. Spontaneously occurring PQ-*R* mutants of *Nostoc* ANTH was obtained by plating approximately $4x10^8$ colony-forming units on solid N_2-medium containing 0.4 μM paraquat. After 3 weeks of incubation, the few surviving colonies, which remained were picked up and transferred to plates containing 0.4 μM paraquat. The PQ-*R* mutants arose at a frequency of $1.9x10^{-8}$. One such mutant was characterized with regard to growth, heterocyst frequency, N_2-fixation, O_2-evolution and rice root colonization activities.

4.2.3 **Colonization experiment :** The rice seeds of IET 13783 variety, procured from Indian Council of Agricultural Research, Shillong were used for colonization experiments. Rice seeds were surface sterilized by washing with distilled water, then in 1 % (v/v) sodium hypochlorite solution for 5 min. Such seeds were thoroughly rinsed with sterile distilled water and the germination was carried out on autoclaved peralite in plastic containers. The peralite was irrigated with a ten-fold dilution of autoclaved BG-11 medium ($BG11_0$+nitrate: Rippka *et al*, 1979). The experiments were carried out in a growth chamber at 28 °C, saturating relative humidity and a 12 h light/dark cycle. Light intensity was 50 $\mu mol.m^{-2}.s^{-1}$.

10 d old seedlings of rice were uprooted from peralite, roots washed with distilled water, and suspended in 15 ml capacity culture tubes containing 10 ml of 10-fold diluted BG-11 medium ($BG11_0$+nitrate, ammonium or urea) or $BG11_0$–medium. *Nostoc* inocula were added to a final concentration of 1 µg Chl $a.ml^{-1}$. The cyanobacteria used for inoculation of the media were grown for 4 d in batch cultures, harvested by centrifugation and the filaments washed by repeated centrifuging and resuspending in fresh $BG11_0$–medium. The co-cultivation was carried out at 28 °C with the plant roots exposed to light (50 $\mu mol.m^{-2}.s^{-1}$) or kept in darkness by wrapping aluminium foils around the culture tube. After 4 d of co-culture, the seedlings were taken out and roots excised and washed to remove loosely associated cyanobacteria, and used for assessing colonization (µg Chl $a.g^{-1}$root dry wt), and associative N_2-fixation (N_2 ase activity). Short term experiments were carried out to assess adsorption of *Nostoc* ANTH to the roots. Tests for root colonization were also carried out in peralite. For this, the cyanobacterial suspension was added to the peralite in which seedlings

were growing. Alternatively, seedlings were uprooted, dipped in cyanobacterial suspension for 30 min and then transplanted in peralite. During co-cultivation the peralite was irrigated with 10-fold diluted $BG11_0$–medium.

In another set of experiment, the synthetic auxin 2,4-Dichlorophenoxy acetic acid (2,4-D) was added into the co-culture medium to a final concentration of 1 mg l^{-1} to induce paranodules and assess any increase in colonization by *Nostoc* ANTH. Throughout the experiment saturating relative humidity and a 12 h light/12 h dark conditions were maintained.

4.2.4 **Determination of natural ^{15}N abundance (δ^{15}N) :** 10 d old rice seedlings were co-cultured with *Nostoc* ANTH, its ClO-*R* mutant or PQ-*R* mutant as detailed above. After 4 d these were washed to remove loosely associated cyanobacteria and then transplanted on peralite. The plants were kept in 12 h light/12 h dark cycle and irrigated with 10-fold diluted $BG11_0$–medium. After 3 weeks of growth, the plants were uprooted, cleaned and dried. The dried plants were finely ground and the powdered sample used for $^{15}N/^{14}N$ ratio analysis. In a parallel experiment rice seedlings were grown in peralite without associating with cyanobacterial samples of N_2-fixing cultures of *Nostoc* and symbiotic tissues from *Gunnera-Nostoc* and from *Zamia-Nostoc* symbiosis were also analysed for comparison. *Gunnera* and *Zamia* plants were taken from the green house. ^{15}N abundance was determined using a Europa Scientific Isotope Ratio Mass Spectrometer. Plants from one container (5 plants) were combined as one sample. Following formula was used for calculation of δ^{15}N:

$\delta^{15}N\ (‰) = [(R_{sample}/R_{standard})-1] \times 100$

where, R= $^{15}N/^{14}N$ ratio and the standard is the dinitrogen of air, defined as having a δ value of zero.

4.2.5 **Growth measurement :** Growth was measured as increase in concentration of Chlorophyll *a*.

4.2.6 **Chlorophyll *a* determinations and measurement of root dry weights :** Chl *a* was extracted into methanol in the darkness at 4 °C. The absorbance at 663 nm was measured and Chl *a* concentration calculated according to Mackinney (1941). Roots from which the Chl *a* of associated cyanobacteria has been extracted, were dried at 80 °C for 72 h and weighed to determine their dry weight.

4.2.7 **Oxygen exchange :** Rate of oxygen evolution of parent and its PQ-*R* mutant was measured polarographically at 25 °C and at a photon fluence rate of 50 $\mu mol.m^{-2}s^{-1}$ by using a Clark type oxygen electrode.

4.2.8 **Heterocyst frequency and nitrogenase activity :** Heterocyst frequency was calculated as percentage of total cells by light microscopic observations. Nitrogenase activity was measured by following acetylene reduction assay (Stewart *et al*, 1967).

4.2.9 **Superoxide dismutase activity :** Superoxide dismutase activity (SOD) was assayed by following the method of Beyer and Fridovich (1987). 5 ml of cyanobacterial culture was centrifuged and the pellet was thoroughly washed with buffer containing 50 mM potassium phosphate and 0.1 mM EDTA (pH 7.8). To such cyanobacterial suspension CTAB was added at a final concentration of 25 μg.ml^{-1} and incubated for 10 min with vigorous shaking. The reaction mixture contained in addition to permeabilized cells in a final volume of 1 ml: 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 57 μM nitro blue tetrazolium (NBT), 9.9 mM methionine and 0.9 μM riboflavin. The mixture was irradiated for 7 min by means of a 100 W tungsten filament bulb. The absorbance of the supernatant was read at 560 nm after 5 min of centrifugation at 5000 rpm. One unit of SOD activity is the amount that decreases the rate of nitro blue tetrazolium reduction by 50 %.

4.2.10 **Light and scanning electron micrography :** The root surfaces and freshly cut transverse sections were examined with an Olympus BX60 microscope. Cross sections of roots colonized by *Nostoc* ANTH were prepared for scanning electron microscopy (SEM) by sectioning with razor blade. Pieces of roots were air-dried, mounted on stubs, coated with gold and examined with a Cambridge Stereoscan 260 SEM.

4.3 Results and discussion :

4.3.1 Characterization of the paraquat-resistant (*PQ-R*) mutant : A comparison of the parent and its PQ-*R* mutant with regard to their diazotrophic growth, heterocyst formation, nitrogenase and O_2-evolution activities is presented in Table 4.1. Paraquat at a concentration of 0.4 μM was completely inhibitory to the diazotrophic growth of *Nostoc* ANTH. The heterocyst formation, nitrogenase and O_2-evolution activities were completely inhibited by paraquat. The parent strain showed light-stimulated O_2-uptake instead of the O_2-evolution in presence of paraquat. The PQ-*R* mutant grew diazotrophically irrespective of the presence or absence of paraquat although such growth was marginally slower (approximately 20 % less) than that of its parent strain growing in absence of paraquat. Since the mutation to paraquat resistance permits the growth of the mutant at inhibitory concentrations of paraquat it was of interest to determine the effect of paraquat on other metabolic functions. O_2-evolution activity of the mutant in presence and absence of paraquat was impaired by nearly 35 % when compared to its parent grown in absence of paraquat. However, no significant difference was observed on heterocyst formation and nitrogenase activity of the mutant in absence and presence of paraquat.

Paraquat accepts an electron from PS1 and reduces O_2 to O_2^- (superoxide anion) and its further detoxification by superoxide dismutase results in production of hydrogen peroxide. The superoxide anion is a potent inhibitor of cyanobacterial growth and nitrogenase activity in presence of light. An increased tolerance to paraquat toxicity has been positively correlated with increased expression of superoxide dismutases and peroxidases (Herbert *et al*, 1992; Tichy and Vermaas, 1999). The negative effect of paraquat on nitrogenase activity of paraquat-treated cells can be partially mitigated by exclusion of air, absence of light or inhibition of photosynthesis by DCMU (Tozum *et al*, 1979; Bagchi *et al*, 1991). Such observations suggest that paraquat toxicity is greatly enhanced by atmospheric oxygen as well as the photosynthetically generated oxygen. Indeed under similar conditions the parent strain (*Nostoc* ANTH) showed less inhibition of nitrogenase activity by paraquat. Furthermore, the superoxide dismutase activity of both the parent and its mutant strain remained similar. This activity was 12.3 ± 1.8 units. mg^{-1} protein. Thus, the resistance to paraquat in the PQ-*R* mutant seems to be due to a decrease in the rate of photosynthetic O_2-evolution. This could also explain the reduced growth. These results are similar to those obtained from paraquat-tolerant mutants of the fern *Ceratopteris*, which failed to show any increase in levels of antioxidative enzymes including superoxide dismutase (Carroll *et al*, 1988). Since, the growth and N_2-fixation of PQ-*R* mutant was insensitive to paraquat, this strain was further tested to see if it could associate with rice roots and carry out associative N_2-fixation and nitrogen transfer.

4.3.2 **Colonization ability of *Nostoc* ANTH and its mutants :** The rice seedlings were co-cultured with *Nostoc* ANTH and its mutants (PQ-*R* and ClO-*R*) in N_2- and in nitrate-supplemented media in presence and absence of light. The parent and its mutants were able to form tight association with the rice seedlings: maximal association was observed in submerged portion of rice roots and the basal parts of shoots. The colonization of rice roots by these *Nostoc* strains occurred in light as well as in darkness, and in nitrogen-

4.3.3 **Adsorption kinetics studies :** As in the case of wheat (Ganter *et al.,* 1991 a,b), hormogonia are important for adherence to rice roots during initial colonization. The process of adsorption of *Nostoc* ANTH and its mutants to the roots of rice plants was followed during a 6 h period. The kinetics of adsorption of both the parent and its mutants to the roots of rice plants was found to be essentially similar and biphasic in nature. The results have been presented for the parent strain only (Fig. 4.3). The first rapid phase lasted for 30 min followed by a slower second phase during the remaining experimental period. The rate of adsorption during the first phase turned out to be 70 % higher than that during the second phase and prolonged incubation led to saturation of root surfaces, thereby reducing further adsorption. Similar adsorption kinetics was observed by Spiller *et al.,* (1993) in case of the adsorption of *Anabaena variabilis* mutants to the roots of wheat plants. It is apparent that *Nostoc* ANTH and its mutants are able to adsorb tightly to the roots of rice within hours. This can be of practical use in rice cultivation.

Based on these observations, rice seedlings were dipped in cyanobacterial suspension for 30 min and thereafter planted in peralite to see if this method of cyanobacterial inoculation would lead to successful colonization. Observation of plants after one week of growth on peralite, confirmed successful colonization of rice roots. Thus, during actual rice cultivation in the field, when seedlings are uprooted for transplantation in rice paddies, cyanobacterial inocula could be adsorbed on to the roots by simply keeping the seedlings in a pool of cyanobacterial suspension for a few hours.

4.3.4 **N_2-fixation by *Nostoc* ANTH and its mutant strains under free-living and associated conditions :** The *Nostoc* ANTH and its PQ-*R* mutant were compared with regard to nitrogenase activity under free-living and rice root associated conditions (Table 4.3). In nitrate-supplemented co-culture medium, the nitrogenase activity of the free-living parent and its PQ-*R* mutant decreased markedly, the effect being more pronounced in the darkness. On the other hand the ClO-*R* mutant showed nitrogenase activity which was similar in N_2- and nitrate-media. All *Nostoc* strains showed enhanced levels of nitrogenase activity when associated to the rice roots. Both under light and in darkness, nitrate was less inhibitory to N_2-fixation by cyanobacteria associated to the rice roots. The cyanobacterial cells, which remained unassociated (free-living) in co-cultures, had a lower nitrogenase activity than those in associated cells. Furthermore, presence of nitrate and absence of light (darkness) had a more pronounced effect on nitrogenase activity of unassociated cells compared to those, which were associated to the roots. These results indicate that high cyanobacterial N_2-fixation rates could be achieved during both light and dark periods of rice cultivation cycle even in presence of nitrate fertilizers. Thus, rice plants can derive their nitrogen requirements from chemical nitrogen fertilizers as well as from root associated cyanobacteria. These results indicate that, as in cyanobacterial-wheat associations (Ganter *et al*, 1991 a, 1995) the plant and/or its products influenced nitrogenase activity of the associated *Nostoc* strains. The nitrogenase activities of unassociated (free-living) *Nostoc* appear lower than those reported for laboratory cultures of heterocystous cyanobacteria. However, it should be noted that the present measurements were done on young inocula (4 d old) in co-cultures and the nutrient medium used ($BG11_0$)was ten-fold diluted.

4.3.5 **Utilization of sugars by *Nostoc* ANTH and its mutants :** In many natural symbioses, the C requirements of the cyanobionts are taken care of by the host plant (Rai *et al*, 2000; Wouters *et al*, 2000). The observation that the rice root associated strains of *Nostoc* ANTH showed higher nitrogenase activity and that significant levels of N_2-fixation occurred in darkness indicates that the plant may provide fixed C to the associated *Nostoc* strains. We tested the heterotrophic N_2-fixation ability of *Nostoc* ANTH and its mutants (PQ-*R* and ClO-*R*) to see if exogenous sugars can support nitrogenase activity in these strains (Table 4.4). The heterocyst frequency and nitrogenase activity of *Nostoc* ANTH and its mutants were higher when grown in light with glucose or fructose as the carbon source than when grown without glucose or fructose. Addition of DCMU (a PSII inhibitor) or incubation of the strains in dark resulted in a severe decline in nitrogenase activity. However, when glucose or fructose were added to the medium there was less of a decline in nitrogenase activity in darkness. These results imply that sugar metabolism is unaffected in the mutants and that glucose and fructose can sustain N_2-fixation by *Nostoc* ANTH and its mutants in absence of photosynthesis. In other words the rice root associated *Nostoc* ANTH and its mutants can carry out N_2-fixation in darkness by receiving fixed C from rice plants (e.g., glucose or fructose). These results are consistent with earlier reports, which suggested that glucose or fructose can act as a source of reductant and energy for dark N_2-fixation in *Anabaene variabilis* associated with wheat roots (Spiller *et al*, 1993).

More recently it has been shown that fructose increases *hetR* expression in the *Gunnera* cyanobiont, *Nostoc* PCC 9229 (Wouters *et al*, 2000).

4.3.6 Evidence of N-transfer from associated *Nostoc* strains to the rice plants : It is well established that there is little or no discrimination between ^{15}N and ^{14}N during biological N_2-fixation. Therefore, diazotrophy provides an input of fixed-nitrogen with natural ^{15}N abundance similar to that in atmospheric N_2 (i.e., $\delta^{15}N$ closer to 0 $^o/_{oo}$). Other forms of nitrogen have relatively higher natural ^{15}N abundance (higher $\delta^{15}N$) due to isotope discrimination. Thus, plants receiving nitrogen through biological N_2-fixation tend to have lower $\delta^{15}N$ values than those utilizing fixed-nitrogen by means other than fixation of N_2. This difference of natural ^{15}N abundance among plants can be used for assessing the contribution of biological N_2-fixation in a diazotrophic association (Shearer and Kohl, 1987; Bergerson *et al*, 1988; Handlay and Raven, 1992).

In the present study natural ^{15}N abundance of rice plants grown with or without cyanobacteria was used to evaluate cyanobacterial contribution to the nitrogen budget of rice plants (Table 4.5). $\delta^{15}N$ values of control plants grown without combined nitrogen and in absence of cyanobacteria showed a $\delta^{15}N$ value of 5.14 $^o/_{oo}$. The $\delta^{15}N$ values of rice plants grown in association with *Nostoc* ANTH or its mutants were significantly lower (1.72-1.79 $^o/_{oo}$). This indicates that significant amounts of nitrogen derived directly from N_2-fixation was provided by *Nostoc* strains to the rice plants. Values of $\delta^{15}N$ in N_2-fixing cultures of *Nostoc* ANTH and in symbiotic tissues of plants in symbiotic associations with *Nostoc* ranged from –0.69 to 0.15. In these systems N_2 is the sole source of nitrogen (i.e., N_2-fixation meets the total nitrogen requirements).

The results presented in this study indicate that the paraquat-resistant nature of the PQ-*R* mutant was an outcome of a decrease in photosynthetic O_2-evolution activity and not due to increase in the superoxide dismutase activity. Both the cyanobacterium *Nostoc* ANTH and its PQ-*R* and ClO-*R* mutants colonized the root surfaces of rice plants. Occasionally they occupied intercellular spaces. The colonization was tight and biphasic in nature and short time of co-culture is sufficient to provide maximum cyanobacterial inocula to rice plants. Following co-culture, the nitrogenase activity of rice root associated cyanobacteria was always higher than the free-living/non-associated cyanobacteria, irrespective of the presence and absence of nitrate or light. Thus, associated cyanobacteria can fix N_2 day and night even in the presence of nitrate fertilizers. Lower $\delta^{15}N$ values of plants grown in association with *Nostoc* ANTH or its mutants clearly indicate transfer of N_2–derived nitrogen to the rice plants.

Table 4.1. Growth (GR), heterocyst frequency (HF), nitrogenase (N_2ase) activity and O_2 evolution rates of *Nostoc* ANTH and its PQ-*R* mutant in presence and absence of paraquat.

6 d old N_2-grown cultures were used as initial source of inoculum. The growth (μg Chl *a*.ml^{-1}), frequency of heterocysts (per 100 vegetative cell), nitrogenase activity (nmol C_2H_4 formed.μg^{-1} Chl *a*.h^{-1}) and O_2 evolution (nmol O_2 evolved.μg^{-1} Chl *a*.h^{-1}) were determined after 4 d of incubation in N_2-medium with (+) or without (-) 0.4 μM paraquat. Chl *a* concentration at the start of incubation (0 time) was 0.3 μg Chl *a*.ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

* represents O_2 consumption.

Parameters	Parent		PQ-*R* mutant	
	+Paraquat	-Paraquat	+Paraquat	-Paraquat
Growth (GR)	0.0	2 ± 0.1	1.6 ± 0.2	1.7 ± 0.2
HF	0.0	5 ± 0.7	5.2 ± 0.3	5 ± 0.47
N_2ase activity	0.0	10 ± 1	8.6 ± 0.1	8.1 ± 0.2
O_2 evolution	-43.0*	224 ± 5	145 ± 2.3	141 ± 8

Table 4.2 . Colonization of rice roots by *Nostoc* ANTH and its mutants.

10 d old rice seedlings were co-cultured with the cyanobacterium *Nostoc* ANTH, *Nostoc* ANTH PQ-*R* mutant and *Nostoc* ANTH ClO-*R* mutant. After 4 days of co-culture, the seedlings were taken out, roots excised and washed to remove loosely associated cyanobacteria and the associated cyanobacteria is quantified (μg Chl *a*.g^{-1}root dry wt). See materials and methods for more details. The values presented are average from 5 plants.

Strains	Colonization of rice roots			
	Light		Dark	
	NO^-_3- medium	N_2-medium	NO^-_3- medium	N_2 -medium
Parent	682	528	630	465
PQ-*R* mutant	750	571	640	642
ClO-*R* mutant	721	564	629	638

Fig 4.1. Association of *Nostoc* ANTH with roots of rice plant after 4 d (A) and 7 d (B) of co-culture in 10-fold diluted N_2-medium. 2,4-D treated rice roots showing paranodules (C). Note the patches of *Nostoc* ANTH on the surface of rice roots. Bars: A and C, 0.35 mm; B, 4 mm.

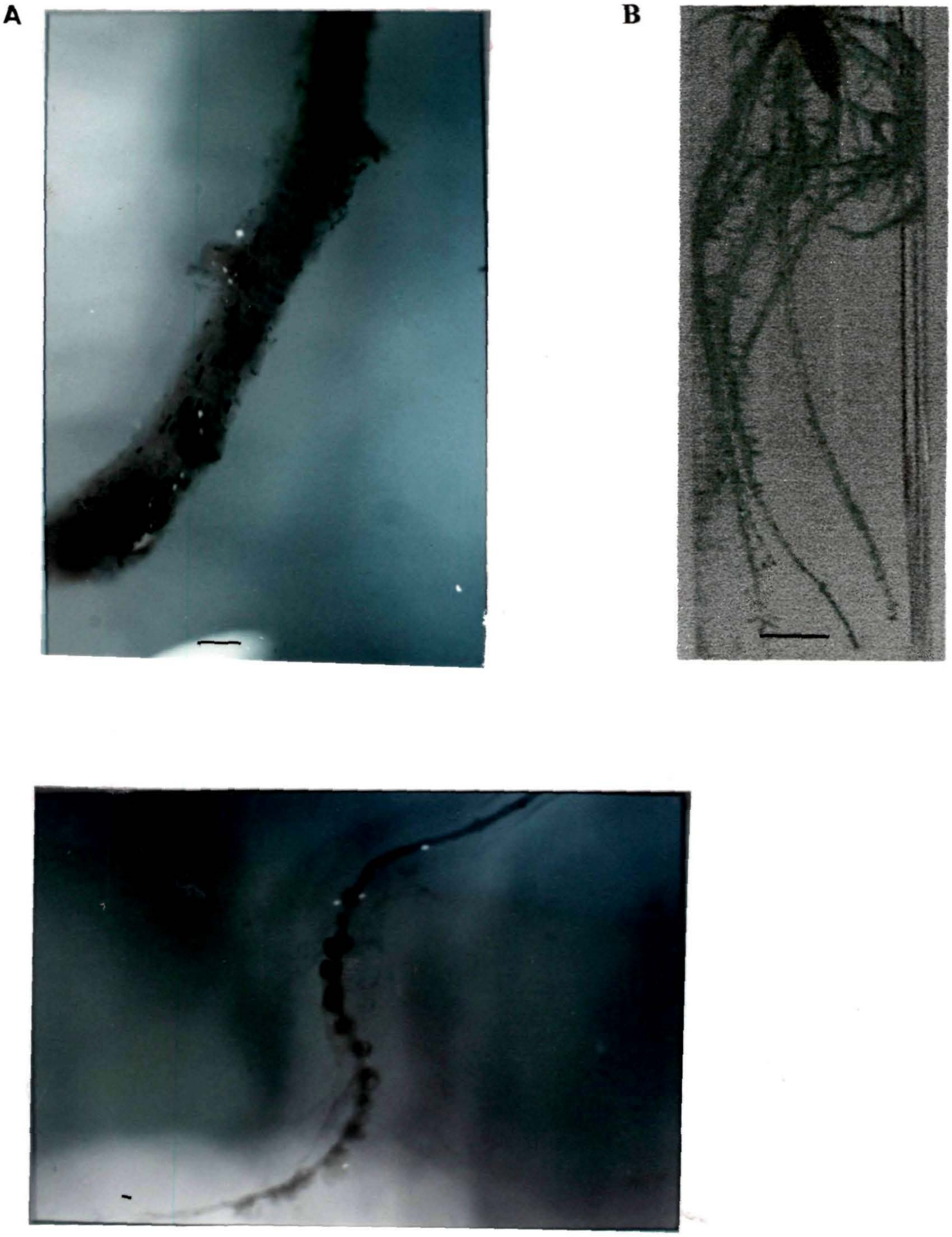
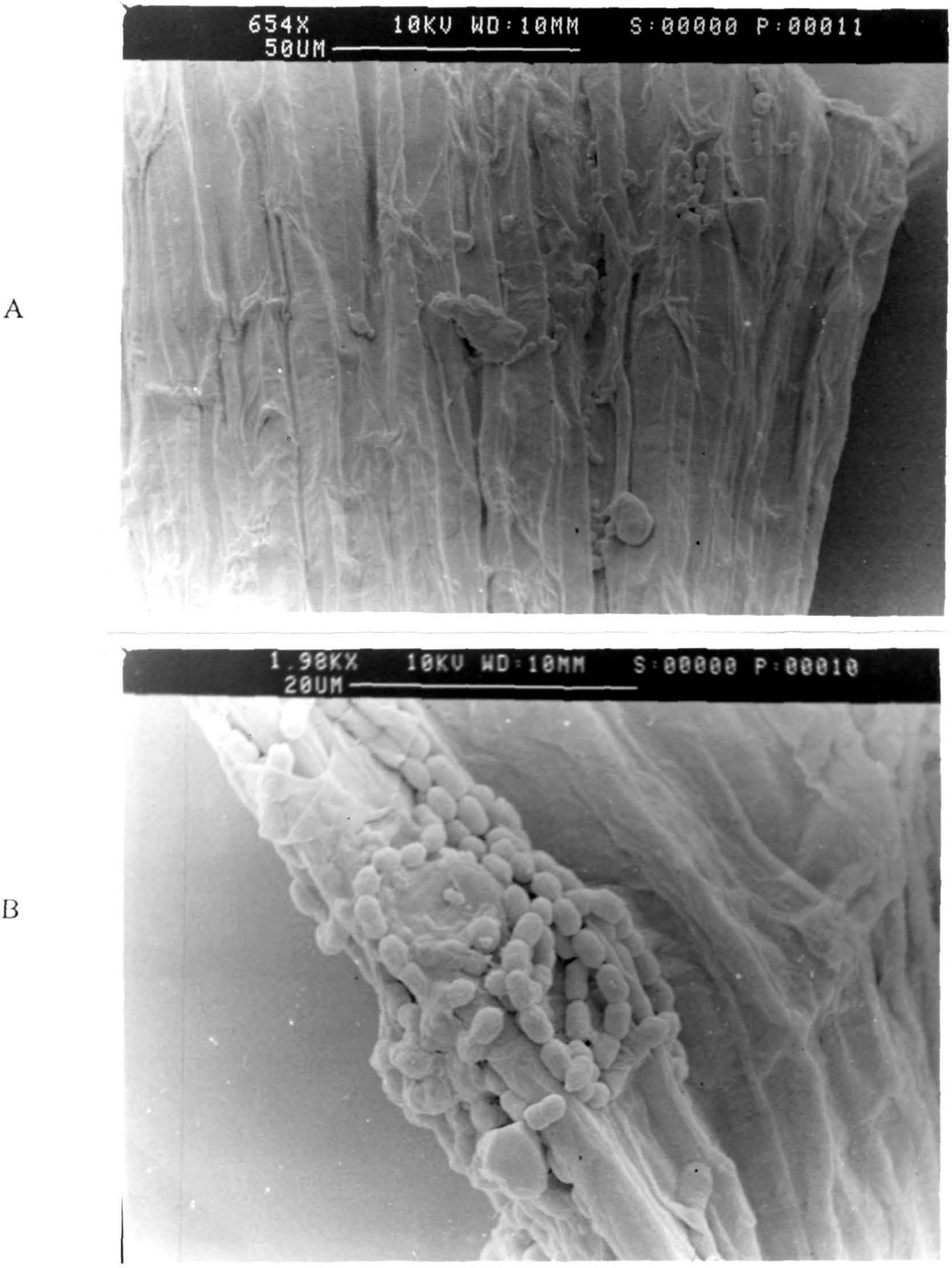
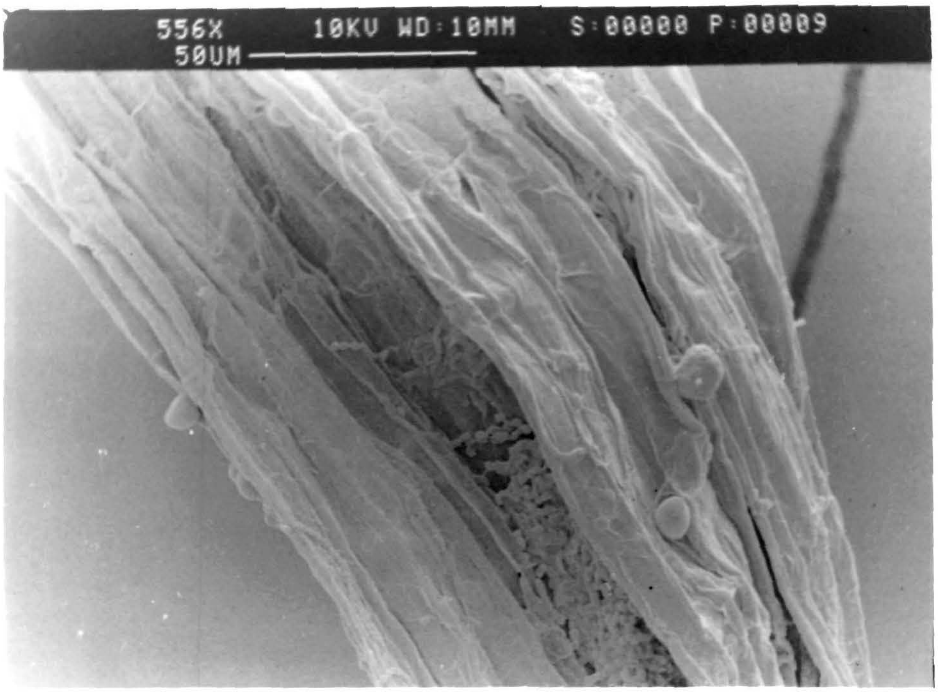


Fig 4.2. Scanning electron micrographs of root surfaces of rice plants colonized by *Nostoc* ANTH.

A. *Nostoc* ANTH hormogonia at root surface during initial stages of colonization. B. Heterocystous filaments (as patches) of *Nostoc* ANTH colonizing the root-surface. C. Filaments in crevices of rice roots. D. A cross section of the rice root showing penetration of *Nostoc* ANTH between loosely arranged cells. Bars: A, 50 μm; B, 20 μm; C, 50 μm; D, 10 μm.



C


556X 10KV WD:10MM S:00000 P:00009
50UM

D

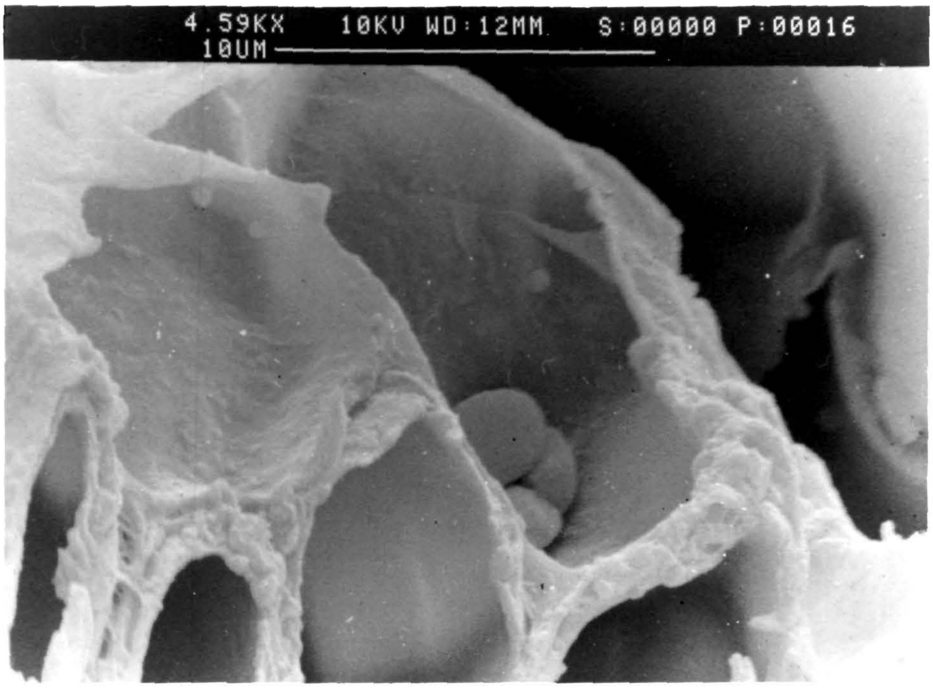

4.59KX 10KV WD:12MM S:00000 P:00016
10UM

Fig 4.3. Adsorption of *Nostoc* ANTH to rice-roots (Strain IET-13783).

4 d old N_2-fixing culture of *Nostoc* ANTH was centrifuged and resuspended in 10-fold diluted $BG11_0$–medium to a final concentration of 1 μg Chl *a*.ml^{-1}. Root portions of 10 d old rice seedlings were submerged in this suspension. At differnt time intervals plants were taken out, their roots excised, and Chl *a* and dry weight determined. Values given are means of estimates from 5 plants for each time period. The values did not vary by more than 10 % from the mean.

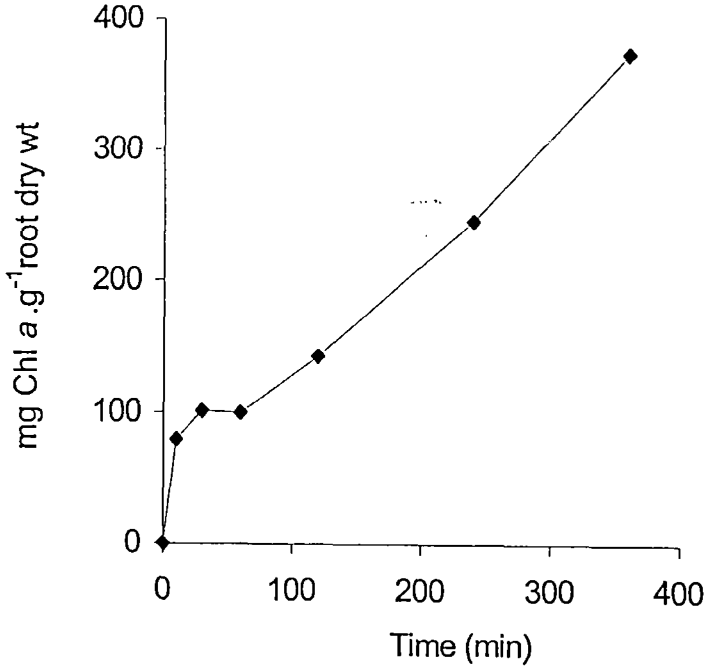


Table 4.3. Nitrogenase activity of the *Nostoc* ANTH and its mutants under free-living and associated conditions.

After co-culture of the rice seedlings with *Nostoc* ANTH or its mutants (PQ-*R* and ClO-*R*), the nitrogenase activity of free-living (unassociated cells in the medium) and associated cyanobacteria (those tightly bound to the rice roots) were measured by acetylene reduction. Experiments were carried out in N_2- and NO^-_3-media, both under light and in darkness. The nitrogenase activity is expressed as nmol C_2H_2 reduced.h^{-1}.μg^{-1} Chl *a*. See materials and methods for more details. The values presented are average from 5 plants.

Strains	Nitrogenase activity			
	Light		Dark	
	NO^-_3-medium	N_2-medium	NO^-_3-medium	N_2-medium
Nostoc ANTH				
Free-living	0.16	4.85	0.20	0.40
Associated	1.46	9.95	1.02	1.47
PQ-*R* mutant				
Free-living	0.15	4.80	0.18	0.45
Associated	1.60	10.1	1.27	1.50
ClO-*R* mutant				
Free-living	4.36	4.7	0.22	0.39
Associated	4.21	9.3	1.34	1.57

Table 4.4. Effect of glucose and fructose on heterocyst frequency (HF) and nitrogenase activity (N_2ase) of *Nostoc* ANTH and its mutants.

6 d old N_2-grown cultures were harvested axenically, washed with sterile N_2-medium and inoculated into fresh N_2-medium with or without 2 mM glucose and 2 mM fructose in presence or absence of 10 μM DCMU. The heterocyst frequency (per 100 vegetative cells) and nitrogenase activity (nmol C_2H_2 reduced.h^{-1}.μg^{-1} Chl *a*) were determined after 4 d of incubation in different media as indicated below. Chl *a* concentration at the time of incubation was 0.25 μg Chl *a*.ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Growth-medium	Parent		PQ-*R* mutant/ClO-*R* mutant	
	HF	N_2ase	HF	N_2ase
N_2-medium (BG11$_0$)	5 ± 0.3	14 ± 1.1	5 ± 0.4	12 ± 0.8
BG11$_0$+DCMU	0.0	0.0	0.0	0.0
BG11$_0$+Glucose	7 ± 0.5	18 ± 1.6	7 ± 0.6	19 ± 0.3
BG11$_0$+Glucose+DCMU	2 ± 0.2	3 ± 0.2	2 ± 0.1	2 ± 0.3
BG11$_0$+fructose	6 ± 0.1	15 ± 1.6	7 ± 0.2	14 ± 0.6
BG11$_0$+fructose+DCMU	3 ± 0.2	3 ± 0.4	2 ± 0.3	3 ± 0.1

Table 4.5. $\delta^{15}N$ values of N_2-fixing cultures of *Nostoc* and of rice plants co-cultivated in a peralite with or without association with *Nostoc* ANTH or its mutants (PQ-*R* or ClO-*R*).

For comparison, $\delta^{15}N$ values of *Gunnera* and *Zamia* symbiotic tissues (wherein the *Nostoc* cyanobiont resides, fixes N_2 and provides fixed-nitrogen to the host) were also determined. Values are means of 3 replicates, from two experiments ± SEM.

Samples	$\delta^{15}N$ values ($^{o}/_{oo}$)
Rice plants	5.14 ± 0.25
Rice plants grown in association with *Nostoc* ANTH	1.72 ± 0.31
Rice plants grown in association with *Nostoc* ANTH mutants	1.79 ± 0.27
Gunnera species:*Nostoc* containing gland tissue	-0.46
Zamia species:coralloid roots	0.75
N_2–fixing cultures of *Nostoc* ANTH	-0.69

CHAPTER 5

Nitrogen uptake and assimilation in the cyanobacterium *Nostoc* ANTH, a symbiotic isolate from *Anthoceros*.

5.1 Introduction :

Most N_2-fixing cyanobacteria are capable of photoautotrophic growth at the expense of a variety of combined nitrogen sources. The commonest forms of utilizable nitrogen sources are ammonia, nitrate, nitrite, urea and amino acids such as glutamine, arginine and asparagine (Herrero and Flores, 1990; Flores and Herrero, 1994; Herrero *et al.*, 2001). However, the relative efficiency with which such amino acids support cyanobacterial growth varies greatly and requires further study. For example, *Synechocystis* PCC 6803 grows as well on arginine as on nitrate, but *Anabaena* sp. PCC 7120 grow more slowly on arginine than on nitrate (Flores and Muro-Pastor, 1990; Herrero and Flores, 1990).

Repression-derepression or induction system of control is known to regulate cyanobacterial nitrogen assimilation. While much information exists on nitrogen regulation of N_2-fixation, and of uptake and assimilation of nitrate, nitrite and ammonium, there is little information about nitrogen regulation of amino acid uptake, amino acid-nitrogen nutrition and the associated metabolic changes (Prakasham *et al.*, 1991; Singh *et al.*, 1991; Herrero *et al.*, 2001). Available reports indicate that arginine is a weak repressor of nitrogenase and nitrate reductase in *Anabaena* sp PCC 7120 whereas nitrate reductase activity is induced by arginine in *Oscillatoria chalybea*

(Herrero and Flores, 1990; Bednarz and Schmidt, 1991). Similarly, the effect of glutamine was found to be variable on heterocyst formation and nitrogenase activity in *Anabaena variabilis*; it repressed nitrogenase activity but not heterocyst formation (Thiel and Leone, 1986).

Amino acid uptake has been characterized in *Anabaena variabilis*, *Anabaena* sp PCC 7120 and *Synechocystis* strain PCC 6803 in some detail (Chapman and Meeks, 1983; Labarre *et al*, 1987; Flores and Muro-Pastor, 1988, 1990; Herrero and Flores, 1990; Montesinos *et al*, 1995, 1997). There are three high affinity amino acid transport systems in *Anabaena* sp PCC 7120; 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. Three amino acid transport systems have also 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; Flores and Muro-Pastor, 1990). Amino acid transport defective mutants of *Anabaena* sp PCC 7120 excrete amino acids in the extracellular medium indicating that amino acid transporters are necessary for uptake and cellular retention of amino acids (Montesinos *et al*, 1995, 1997). A clearer understanding of amino acid uptake processes and use of amino acids as nitrogen sources should help develop cyanobacterial strains useful for photobiological production of amino acids.

Nostoc ANTH forms symbiosis with *Anthoceros* and provides fixed-nitrogen to its host in the form of ammonia. It utilizes nitrate, nitrite and ammonia as nitrogen sources but has not been characterized with regard to amino acid uptake and nutrition. To study the latter aspects in this cyanobacterium, a mutant defective in heterocyst formation and N_2-fixation (Het $^-$ Nif $^-$) was isolated. Amino acid-nitrogen nutrition, the associated metabolic changes and amino acid uptake was compared in this mutant and its parent (*Nostoc* ANTH). The results presented here indicate that glutamine and asparagine served as better sources of nitrogen for growth, followed by arginine and alanine. Heterocyst formation, and activities of nitrogenase and nitrate reductase were completely repressed by glutamine and asparagine but only partially repressed by arginine and alanine. The GS activity however, remained unaffected in presence of these amino acids. Glutamine, asparagine, arginine, alanine and ammonium uptake activities were higher in the Het $^-$ Nif $^-$ mutant than its parent. The uptake of these amino acids was biphasic, energy-dependent and required *de novo* protein synthesis. Unlike ammonium uptake, the uptake of glutamine, asparagine, arginine and alanine was not inhibited by nitrate or ammonium. Evidence is also provided that *Nostoc* ANTH lacks the second nitrogenase (Nif 2).

5.2 Materials and methods :

5.2.1 Strains and culture conditions : Axenic cultures of the diazotrophic cyanobacterium *Noctoc* ANTH and its Het $^-$ Nif $^-$ mutant were grown in batch cultures using BG11$_0$ (N_2-medium) (Rippka *et al*, 1979). As and when required, the N_2-medium was supplemented with combined nitrogen sources such as 5 mM sodium nitrate (nitrate-medium), 2 mM ammonium chloride (ammonium-medium), or 1 mM amino acid (glutamine, asparagine, arginine or alanine). The medium was always buffered with equimolar concentration of HEPES. The pH of the medium was adjusted to 7.5 before autoclaving. The cultures were routinely maintained at 25 °C with a photon fluence rate of 50 μ mol. $m^{-2}.s^{-1}$.

5.2.2 Isolation of the Het $^-$ Nif $^-$ mutant : The Het $^-$ Nif $^-$ mutant was isolated from nitrosoguanidine (NTG) mutagenized cultures of the cyanobacterium *Noctoc* ANTH. Exponentially growing cultures were sonicated (MSE Soniprep) to yield short filaments (approximately 3-4 cells per filament) and then treated with 100 $\mu g.ml^{-1}$ of NTG for 90 min in 10 mM citrate buffer (pH 6). This treatment resulted in 95 % killing. The mutagenised cultures were washed and incubated in ammonium-medium for 4 d to permit segregation and expression of mutations. These ammonium-grown cells were washed and incubated for 2 d in N_2-medium and subsequently treated with 60 $\mu g.ml^{-1}$ of penicillin for 24 h to kill N_2-fixing cells. The survivors (5.6×10^5 colony-forming units) were plated on solid N_2-medium containing 100 μM nitrate. Colonies appeared after one week of incubation. The colonies that were unable to grow and produce heterocysts and nitrogenase activity in N_2-medium, i.e., the Het $^-$ Nif $^-$ mutants arose at a frequency of 4.1x 10^{-4}. One such mutant was further characterized with regard to uptake and assimilation of ammonium and amino acids.

5.2.3 **Growth, heterocyst frequency and nitrogenase activity :** Growth was measured as increase in concentration of Chl *a* (Mackinney, 1941). Heterocyst frequency was calculated as percentage of total cells by light microscopic observations after 72 h of incubation in different nitrogen media (Bagchi and Singh, 1984). The acetylene reduction assay was used to measure nitrogenase activity (Stewart *et al*, 1967). To check the presence of a second nitrogenase, which has been reported to be active in vegetative cells of *Anabaena variabilis* under anaerobic conditions (Thiel *et al.*, 1995; Thiel and Pratte, 2001), nitrogenase activity was measured in aerobic as well as in anaerobic cultures. The nitrate -grown cultures of the parent and its Het $^-$ Nif $^-$ mutant were harvested during the exponential growth phase, washed and incubated aerobically in N_2-medium, or anaerobically in N_2-medium supplemented with 5 mM glucose. Anaerobic conditions were achieved by treating cyanobacterial cultures with 20 µM DCMU (inhibitor of photosynthetic O_2- evolution) for 30 min in serum stoppered vials. The vials were then properly flushed with a gas mixture of N_2 : CO_2 (95 : 5 % v/v).

5.2.4 **Nitrate reductase and glutamine synthetase (transferase) activities :** Glutamine synthetase (transferase) was measured according to Sampio *et al.*, (1979). Nitrate reductase was measured in cells permeabilized with alkyltrimethylammonium bromide using dithionite-reduced methyl viologen as reductant (Manzano *et al*, 1976). Protein concentration was determined by the method of Lowry *et al*, (1951).

5.2.5 **Ammonium and amino acid transport assays :** Ammonium transport assay was done using the radioactive analogue of ammonium, ^{14}C-methylammonium, as described in Chapter 3. Glutamine, arginine, asparagine and alanine uptakes were measured by using ^{14}C-labeled glutamine (sp. activity 256 kBq.µmol^{-1}), arginine (sp. activity 65 kBq.µmol^{-1}), asparagine (sp. activity 63 kBq.µmol^{-1}) and alanine (sp. activity 71 kBq.µmol^{-1}). The nitrate-grown cultures of the parent and its Het$^-$ Nif$^-$ mutant were harvested during the exponential growth phase, washed in N_2-medium and then incubated in N_2-medium, NO^-_3-medium, NH^+_4 –medium and in N_2-medium supplemented with 1 mM glutamine, arginine, asparagine, or alanine for 48 h . The Het$^-$ Nif$^-$ mutant was incubated for only 24 h in N_2-medium because the mutant lacked growth in N_2-medium. After incubation in different nitrogen-media the cells were harvested, washed and resuspended in 10 mM HEPES-NaOH buffer (pH 7.0) to a concentration of 5 µg Chl *a*.ml^{-1}. The ^{14}C-labeled glutamine, arginine, asparagine and alanine were added to a final concentration of 50 µM. The uptake experiments were carried out at 25 ^{0}C and a photon fluence rate of 50 µmol.m^{-2}.s^{-1}. Where needed, dicyclohexylcarbodimide (DCCD, 50 µM), dichlorophenyl dimethylurea (DCMU, 10 µM) and carbonyl cyanide chlorophenyl hydrazone (CCCP, 25 µM) were added to the cell suspension 30 min prior to the addition of labeled amino acids and were present during the experiments. At different time intervals, 400 µl samples were taken out rapidly and the cells were separated from their bathing medium by centrifugation through silicon-oil DC 550/dinonyl phthalate (40/60, v/v) into perchloric acid/water (15/85, v/v) (Scott and Nicholls, 1980). The ^{14}C in perchloric acid fraction was measured using a liquid scintillation counter (Model 1801, Beckman Instruments). Non-specific binding of ^{14}C-methylammonium, glutamine, asparagine, arginine or alanine was determined by measuring its incorporation in toluene-treated cells (Rai *et al*, 1984; Prakasham and Rai, 1991). This value was always subtracted from the value obtained for toluene-untreated sample.

5.3 Results and discussion :

5.3.1 Growth, heterocyst frequency and nitrogenase activity: *Nostoc* ANTH and its nitrosoguanidine (NTG) induced mutant were characterized with regard to growth, heterocyst frequency and nitrogenase activity as a function of different nitrogen sources in the growth media (Table 5.1). The parent strain (*Nostoc* ANTH) grew well in N_2-medium. Nitrate and ammonium were equally good as sources of nitrogen for the growth of the parent strain. The photoautotrophic growth of the Het $^-$ Nif $^-$ mutant in nitrate and ammonium-media was similar to its parent strain. However, in contrast to the parent strain, the mutant did not grow in N_2-medium. The mutant lacked heterocysts and did not exhibit any nitrogenase activity under aerobic growth conditions. Furthermore, nitrogenase activity remained undetectable even under anaerobic conditions in the mutant. In the parent strain, a nitrogenase activity of 13.6 nmol C_2H_4 formed.μg^{-1} Chl *a*.h^{-1} was detectable after 20 h of nitrogen starvation under anaerobic conditions. This coincided with the appearance of heterocysts. These results indicate that in *Nostoc* ANTH, nitrogenase is present exclusively in heterocysts and that the second nitrogenase (Nif 2), which is reported to be expressed in vegetative cells of *Anabaena variabilis* under anaerobic conditions (Thiel *et al*, 1995; Thiel and Pratte, 2001) is lacking in *Nostoc* ANTH. Thus, the mutant's inability to grow in N_2-medium can be attributed to the lack of heterocyst and nitrogenase because of which it could not utilize N_2 as nitrogen source for growth. The Het $^-$ Nif $^-$ mutant provided us an experimental system to assess the ability of different amino acids to serve as sole nitrogen sources for growth of *Nostoc* ANTH.

Out of the 20 amino acids tested, only 4 (glutamine, asparagine, arginine and alanine) supported the growth of *Nostoc* ANTH. This was evident from the fact that glutamine and asparagine repressed heterocyst formation and N_2-fixation in *Nostoc* ANTH, and yet the growth was as good or better than that in the N_2-fixing conditions. Similar levels of growth was found in the Het $^-$ Nif $^-$ mutant in media supplemented with asparagine or glutamine.

Arginine and alanine were utilized as nitrogen sources by the mutant but the growth rate was poor. Arginine and alanine supported a better growth rate of the parent strain because heterocyst formation and N_2-fixation was not fully repressed (i.e. the parent strain was still using N_2 as nitrogen source, atleast partly). Thus, arginine and alanine were not as good a nitrogen source as the glutamine and asparagine. Other amino acids (glutamate, aspartate, proline, lysine, histidine, serine, phenylalanine, glycine, cysteine, cystine, valine, tryptophan, threonine, tyrosine, isoleucine and leucine) did not serve as a source of nitrogen for *Nostoc* ANTH. This was evident from the fact that these amino acids did not support the growth of the Het $^-$Nif $^-$ mutant.

The heterocyst frequency and nitrogenase activity in the parent strain was repressed in glutamine- and asparagine-supplemented media. This is similar to the effects of nitrate and ammonium on heterocyst formation and nitrogenase activity. In contrast, only partial repression of heterocyst frequency and nitrogenase activity was observed in arginine- and alanine-grown parent strain. These results indicate that glutamine and asparagine serves as best nitrogen sources in the cyanobacterium *Nostoc* ANTH. Our results agree with the glutamine-mediated inhibition of nitrogenase observed in *Anabaena variabilis* but in contrast to *Anabaena variabilis* glutamine also inhibited the heterocyst formation in *Nostoc* ANTH (Thiel and Leone, 1986). Furthermore, comparatively poor nitrogen sources like arginine and alanine were a poor repressor of heterocyst formation and nitrogenase activity. These observations are in agreement with earlier observations in *Anabaena* PCC 7120 (Ownby, 1977; Herrero and Flores, 1990).

5.3.2 **Activities of nitrate reductase and glutamine synthetase (GS)** : The nitrate reductase and glutamine synthetase activities of the Het $^-$ Nif $^-$ mutant were similar to its parent strain (Table 5.2). The nitrate reductase activity in both the parent and its mutant strain was nitrate inducible/ammonium repressible. This activity was also repressed in the glutamine- and asparagine-grown parent and its mutant strain, whereas only a weak repression was observed in presence of arginine and alanine. The GS activity was partially repressed in ammonium-grown parent and its mutant strain. However, no significant repression of GS activity was observed either in the parent or its mutant strain in presence of glutamine, asparagine, arginine and alanine (i.e., the activity remained nearly similar to those in N_2-grown cultures).

These results indicate that the Het $^-$ Nif $^-$ mutant was unaffected in nitrate and ammonium assimilation as opposed to heterocyst formation and nitrogenase activity and therefore, the mutant is not defective in the global nitrogen regulatory gene *ntcA* (Herrero *et al,* 2001). However, the mutant phenotype may have resulted due to functional impairment of a common gene like *hetR*, which regulates the formation of heterocysts and nitrogenase (Buikema and Haselkorn, 1991 a). Secondly, nitrate reductase activity was strongly inhibited by glutamine and asparagine but only partially by arginine and alanine; therefore, the growth of *Nostoc* ANTH in the presence of arginine and alanine may depend on simultaneous N_2-fixation. This may explain the poor growth of the Het $^-$ Nif $^-$ mutant, compared to its parent, in arginine- and alanine-supplemented media (compare Table 5.1 and 5.2). Our results are in agreement with partial repression of nitrate reductase activity by arginine in *Anabaena* PCC 7120 (Herrero and Flores, 1990), in contrast to the arginine induction of nitrate reductase activity in *Oscillatoria chalybea* (Bednarz and Schmidt, 1991). Thirdly, GS activity remained virtually unaffected in presence of glutamine, asparagine, arginine and alanine as has been reported earlier in *Nostoc muscorum* (Singh *et al*, 1991).

5.3.3 **Ammonium uptake activity** : Ammonium transport system has been characterized in *Nostoc* ANTH using ^{14}C-methylammonium and found to be ammonium repressible (Prakasham and Rai, 1991). In the present study, the N_2-grown parent (*Nostoc* ANTH) showed an ammonium uptake rate of 7 nmol.min^{-1}.mg^{-1} Chl *a*. In N_2-medium, the rate of ammonium uptake in the mutant, was highly derepressed, being nearly two-fold higher than that in its parent strain. Since the mutant cannot use N_2 as nitrogen source, incubation of the mutant in N_2-medium must have led to nitrogen starvation and therefore the high rate of ammonium transport activity. The ammonium uptake activity was inhibited in presence of nitrate or ammonium both in the parent and in the mutant strains (Table 5.3). Overall the data shows that ammonium as well as nitrate repress the ammonium transport activity and that N starvation leads to increased ammonium transport activity.

5.3.4 **Uptake activities of glutamine, asparagine, arginine and alanine :** Since glutamine, asparagine, arginine and alanine were utilized as nitrogen sources for growth by *Nostoc* ANTH and its Het$^-$ Nif$^-$ mutant, uptake of such amino acids were studied as a function of different nitrogen sources in the media. Glutamine uptake was biphasic in nature in both the parent and its mutant strain grown in N_2- and glutamine-media (Fig 5.1). The initial rapid phase lasted for less than 2 min followed by a slower second phase during the remaining experimental period. The N_2-grown parent strain showed glutamine uptake rates of 55 and 11 nmol.min^{-1}.mg^{-1} Chl *a*, during first and second phase, respectively. The N_2-grown Het$^-$Nif$^-$ mutant exhibited glutamine uptake rates of 70 and 27 nmol.min^{-1}.mg^{-1} Chl *a*, during first and second phase, respectively. The activity and pattern of glutamine uptake, remained unaffected in nitrate- and ammonium-grown cells of both the parent and its mutant strain (data not shown). On the other hand, the glutamine uptake rates were higher in glutamine-grown parent and its mutant strain. The observed glutamine uptake rates in parent were 93 and 44 nmol.min^{-1}.mg^{-1} Chl *a* and in mutant 95 and 45 nmol.min^{-1}.mg^{-1} Chl *a*, during first and second phase, respectively. This increase in the rate of glutamine uptake was significantly inhibited by chloramphenicol (an inhibitor of protein synthesis) in glutamine-grown parent strain. The Het$^-$Nif$^-$ mutant also showed a similar inhibition of glutamine uptake in presence of chloramphenicol.

These results indicate that glutamine uptake in the cyanobacterium *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant are biphasic, glutamine-inducible and requires *de novo* protein synthesis. Unlike ammonium uptake, the uptake of glutamine was not repressed by nitrate or ammonium, indicating that the glutamine uptake system is not under strict nitrogen control but may be regulated by the nitrogen status of the cell. This is consistent with the lack of nitrate and ammonium inhibition of leucine uptake in *Anabaena variabilis* (Thiel, 1988) and arginine uptake in *Anabaena* PCC 7120 (Herrero and Flores, 1990). The higher glutamine uptake rate observed in the Het $^-$ Nif $^-$ mutant may have resulted because of nitrogen starvation. A similar increase in the rate of glutamine uptake was reported in the Het $^-$ Nif $^-$ mutants of *Anabaena variabilis* and this increase correlated well with higher levels of glutamine in glutamine-grown cells compared to ammonium- or N_2-grown cells (Thiel and Leone, 1986).

The N_2-grown parent strain showed asparagine uptake rates of 30 and 11 $nmol.min^{-1}.mg^{-1}$ Chl *a*, during first and second phase, respectively. In N_2-medium, the Het $^-$ Nif $^-$ mutant exhibited asparagine uptake rates of 78 and 33 $nmol.min^{-1}.mg^{-1}$ Chl *a*, during first and second phase, respectively. The rates of asparagine uptake, both during the first and second phase, increased in the asparagine-grown parent strain to 56 and 29.2 $nmol.min^{-1}.mg^{-1}$ Chl *a*, respectively. A similar response was found in the case of asparagine-grown Het $^-$ Nif $^-$ mutant, where the asparagine uptake rates increased to 89 and 37 $nmol.min^{-1}.mg^{-1}$ Chl *a*, during first and second phase, respectively (Fig 5.2).

The N_2-grown parent strain showed arginine uptake rates of 34 and 21 $nmol.min^{-1}.mg^{-1}$ Chl *a*, during first and second phase, respectively. In N_2-medium, Het $^-$ Nif $^-$ mutant took up arginine at a rate of 60 and 31 $nmol.min^{-1}.mg^{-1}$ Chl *a*, during first and second phase, respectively. This rate of arginine uptake increased in the arginine-grown parent strain to 115 and 51 $nmol.min^{-1}.mg^{-1}$ Chl *a*, and in its mutant strain to 135 and 50 $nmol.min^{-1}.mg^{-1}$ Chl *a*, during first and second phase, respectively (Fig 5.3).

The N_2-grown parent strain showed alanine uptake rates of 25 and 8 nmol.min^{-1}.mg^{-1} Chl *a*, during first and second phase, respectively. In N_2-medium, Het $^-$ Nif $^-$ mutant exhibited alanine uptake rates of 53 and 28 nmol.min^{-1}.mg^{-1} Chl *a*, during first and second phase, respectively. This rate of alanine uptake increased in the alanine-grown parent strain to 50 and 24 nmol.min^{-1}.mg^{-1} Chl *a* and in its mutant strain to 59 and 29 nmol.min^{-1}.mg^{-1} Chl *a*, during first and second phase, respectively (Fig 5.4). The increased rate of alanine uptake by alanine-grown cells was inhibited by chloramphenicol, indicating a requirement for protein synthesis. Similar response was observed regarding asparagine and alanine uptake also (data not shown).

Nitrate- and ammonium-grown cells of both the parent and mutant strains showed amino acid uptake rates which was similar to those observed in N_2-grown cells. Thus, as in the cae of glutamine uptake, the uptakes of asparagine, arginine and alanine were also biphasic, substrate-inducible and not repressed by nitrate or ammonium. The induction of amino acid uptake by the substrate required *de novo* protein synthesis. In N_2-medium, the Het $^-$ Nif $^-$ mutant strain showed a much higher rate of amino acid uptake than the parent because of nitrogen starvation caused by its inability to use N_2 as nitrogen source. However, both the mutant and the parent strains had similar uptake rates when grown in media containing the amino acids.

The effect of DCMU (an inhibitor of non-cyclic photosynthetic electron transport), DCCD (an inhibitor of F_0 part of ATP) and CCCP (an uncoupler of photosynthesis) were studied on the uptake rates of glutamine, asparagine, arginine and alanine in the parent (Table 5.4). The protonophore CCCP caused a strong inhibition of glutamine, asparagine, arginine and alanine uptake; a similar inhibition was also observed in the presence of the ATPase inhibitor, DCCD. The inhibitor of photosynthetic O_2-evolution, DCMU, partially inhibited the uptake of glutamine, asparagine, arginine and alanine. These inhibitors had a similar effect on uptake of amino acids by the Het $^-$ Nif $^-$ mutant (data not shown). The results indicate that the cellular uptake of amino acids in *Nostoc* ANTH and its mutant is energy dependent. Our results are in agreement with energy-dependent amino acid uptake in *Anabaena* sp. PCC 7120 and *Anabaena variabilis* (Thiel, 1988; Herrero and Flores, 1990; Montesinos *et al*, 1995).

To conclude, the cyanobacterium *Nostoc* ANTH and its Het $^-$ Nif $^-$ mutant can grow at the expense of glutamine, asparagine, arginine and alanine as a source of fixed-nitrogen. However, the relative efficiency with which these amino acids supported cellular growth varied. Glutamine and asparagine repressed N_2-fixation and served as a sole source of fixed-nitrogen for growth. Arginine and alanine were a comparatively poorer source of nitrogen for growth and did not fully repress N_2-fixation or heterocyst formation. The poor growth in arginine and alanine media was not due to poor uptake rates since the uptake rates for these amino acids were not significantly different from those for glutamine or asparagine. The GS activity remained unaffected during cultivation in media containing any one of the four amino acids tested. The uptake of amino acids was substrate-inducible, requiring *de novo* synthesis, energy-dependent and biphasic in nature. Nitrate and ammonium repressed ammonium uptake but did not repress uptake of amino acids. In N_2-medium, the uptake of ammonium and amino acids in the Het $^-$ Nif $^-$ mutant were significantly higher than its parent strain. The mutational loss of diazotrophy in the mutant resulted in nitrogen starvation when N_2 was the sole source of nitrogen leading to highly derepressed ammonium and amino acid transport systems. Evidence is also provided that *Nostoc* ANTH lacks the second nitrogenase (Nif 2).

Table 5.1. Growth (Gr), heterocyst frequency (HF) and nitrogenase activity (N_2ase) of *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant as a function of nitrogen sources.

4 d old nitrate-grown cultures were used as initial source of inoculum. The growth (μg Chl *a*.ml^{-1}), frequency of heterocysts (per 100 vegetative cell) and aerobic nitrogenase activity (nmol C_2H_4 formed.μg^{-1} Chl *a*.h^{-1}) were determined after 72 h of incubation in media containing different nitrogen sources as indicated below. Chl *a* concentration at the start of incubation (0 time) was 0.29 μg Chl *a*.ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Growth-medium	Parent			Het$^-$Nif$^-$ mutant		
	Gr	HF	N_2ase	Gr	HF	N_2ase
BG11$_0$	1.2 ± 0.1	5.3 ± 0.2	12 ± 0.6	0.1 ± 0.0	0.0	0.0
BG11$_0$+5 mM NO^-_3	1.3 ± 0.1	0.0	0.0	1.2 ± 0.1	0.0	0.0
BG11$_0$+2 mM NH^+_4	1.4 ± 0.1	0.0	0.0	1.5 ± 0.1	0.0	0.0
BG11$_0$+1 mM Glutamine	2.1 ± 0.2	0.2	0.1	2.0 ± 0.2	0.0	0.0
BG11$_0$+1 mM Asparagine	1.9 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	1.7 ± 0.1	0.0	0.0
BG11$_0$+1 mM Arginine	1.2 ± 0.1	2.6 ± 0.3	6.1 ± 0.3	0.9 ± 0.1	0.0	0.0
BG11$_0$+1 mM Alanine	0.9 ± 0.1	2.9 ± 0.1	6.9 ± 0.2	0.6 ± 0.1	0.0	0.0

Table 5.2. Nitrate reductase (NR) and glutamine synthetase (GS) transferase activities of *Nostoc* ANTH and its Het^-Nif^- mutant as a function of nitrogen sources.

The nitrate reductase (NR) activity (nmol NO^-_2 formed.min^{-1}.mg^{-1} protein) and glutamine synthetase (GS) transferase activity (nmol γ-glutamyl hydroxamate formed.min^{-1}.mg^{-1} protein) were determined after 24 h of incubation in media containing different nitrogen sources as indicated below. Chl *a* concentration at the start of incubation was 4 μg Chl *a*.ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Growth-medium	Parent		Het^-Nif^- mutant	
	NR	GS	NR	GS
$BG11_0$	1.8 ± 0.11	610 ± 7	2.1 ± 0.03	648 ± 4
$BG11_0$+5 mM NO^-_3	4.2 ± 0.15	598 ± 3	4.8 ± 0.11	593 ± 7
$BG11_0$+2 mM NH^+_4	0.2 ± 0.10	376 ± 6	0.4 ± 0.18	371 ± 8
$BG11_0$+1 mM Glutamine	0.3 ± 0.06	595 ± 8	0.5 ± 0.10	582 ± 4
$BG11_0$+1 mM Asparagine	0.5 ± 0.04	581 ± 7	0.4 ± 0.16	594 ± 6
$BG11_0$+1 mM Arginine	0.9 ± 0.01	601 ± 4	1.1 ± 0.02	598 ± 2
$BG11_0$+1 mM Alanine	1.2 ± 0.06	587 ± 3	1.4 ± 0.21	585 ± 7

Table 5.3. ^{14}C-methylammonium uptake activity in parent and its Het $^{-}$ Nif $^{-}$ mutant grown in N_2-, NO^{-}_3- or NH^{+}_4-media.

Nitrate-grown exponential cultures were washed and transferred to N_2-, NO^{-}_3- or NH^{+}_4-media and incubated for 48 h. Thereafter, the N_2-, NO^{-}_3- or NH^{+}_4-grown cells were washed, resuspended in HEPES buffer and used for ^{14}C-methylammonium uptake as described in materials and methods. The N_2-grown cells in case of Het $^{-}$ Nif $^{-}$ mutant refer to the nitrate-grown cells, which were subjected to nitrogen starvation for 24 h in N_2-medium; this is because the mutant does not grow in N_2-medium.

The uptake rates were calculated from the linear uptake between 2 and 10 min after ^{14}C-methylammonium addition. The uptake rates are expressed as nmol.mg^{-1}Chl *a*. min^{-1}. The values presented are means from two independent experiments ± standard error (SE), each with two replicates.

Growth-medium	^{14}C-methylammonium uptake	
	Parent	Het $^{-}$Nif $^{-}$ mutant
N_2- medium ($BG11_0$)	7 ± 0.48	13 ± 0.12
NO^{-}_3-medium ($BG11_0$ + 5 mM NO^{-}_3)	0.6 ± 0.07	0.7 ± 0.01
NH^{+}_4-medium ($BG11_0$ + 5 mM NH^{+}_4)	0.3 ± 0.03	0.2 ± 0.06

Fig 5.1. ^{14}C-glutamine uptake in *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant.

Nitrate-grown exponential cultures were washed and transferred to N_2- ($BG11_0$) and glutamine- ($BG11_0$+ 1 mM glutamine)-media and incubated for 48 h. To one set of glutamine-medium chloramphenicol (1 μg.ml^{-1}) was added at the beginning of incubation. The N_2-grown cells in case of Het$^-$Nif$^-$ mutant refer to the nitrate-grown cells, which were subjected to nitrogen starvation for 24 h in N_2-medium; this is because the mutant does not grow in N_2-medium. After the incubation, the N_2- and glutamine-grown cells were washed, resuspended in HEPES buffer and used for ^{14}C-glutamine uptake as described in materials and methods. The values presented are means from two independent experiments, each with two replicates. Symbols: N_2-medium, parent (□), glutamine-medium, parent (O), glutamine-medium + chloramphenicol, parent (▲), N_2-medium, mutant (■) and glutamine-medium, mutant (●).

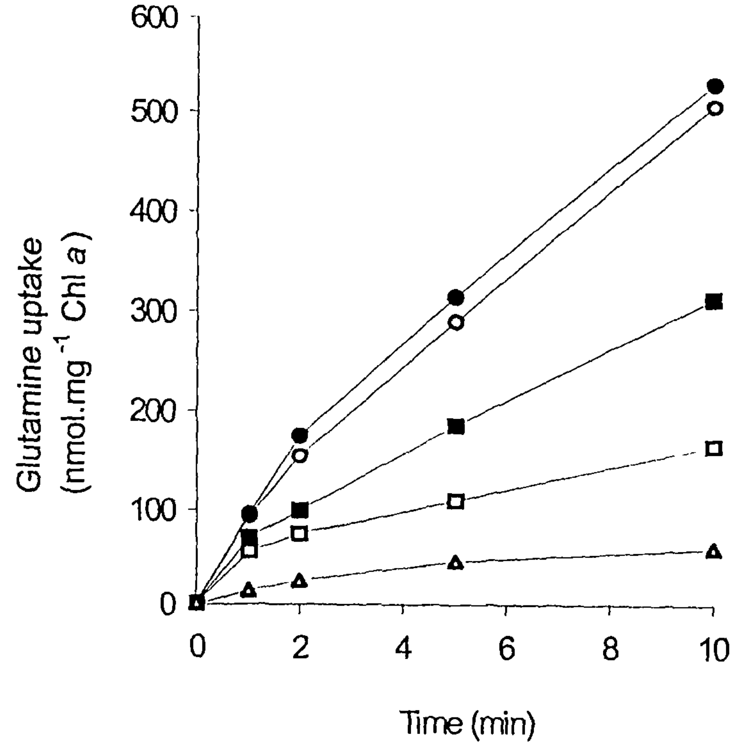


Fig 5.2. ^{14}C-asparagine uptake in *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant.

Nitrate-grown exponential cultures were washed and transferred to N_2- (BG11$_0$) and asparagine- (BG11$_0$+ 1 mM asparagine)-media and incubated for 48 h. The N_2-grown cells in case of Het$^-$Nif$^-$ mutant refer to the nitrate-grown cells, which were subjected to nitrogen starvation for 24 h in N_2-medium; this is because the mutant does not grow in N_2-medium. Thereafter, the N_2- and asparagine-grown cells were washed, resuspended in HEPES buffer and used for ^{14}C-asparagine uptake. The values presented are means from two independent experiments, each with two replicates. Symbols: N_2-medium, parent (□), asparagine-medium, parent (O), N_2-medium, mutant (■) and asparagine-medium, mutant (●).

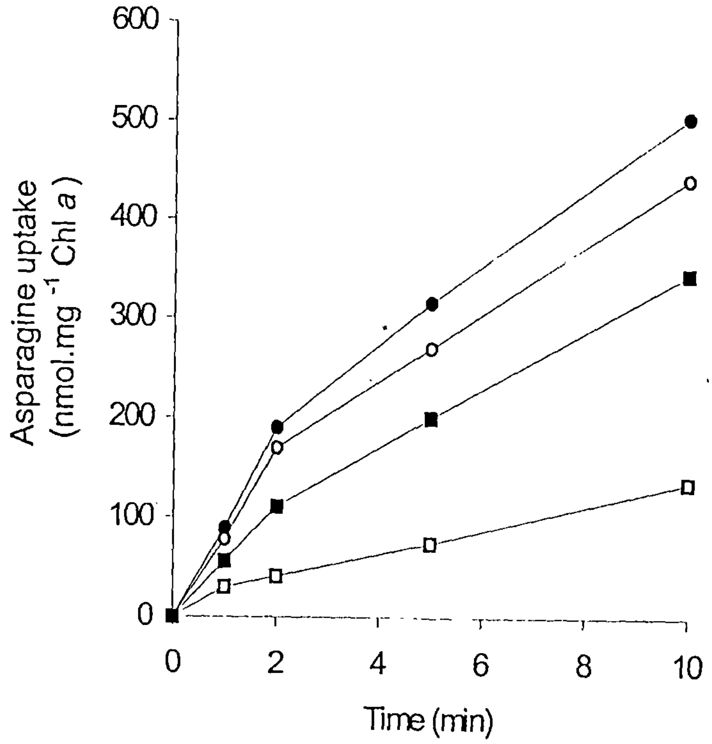


Fig 5.3. ^{14}C-arginine uptake in *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant.

Nitrate-grown exponential cultures were washed and transferred to N_2- (BG11$_0$) and arginine- (BG11$_0$+ 1 mM arginine)-media and incubated for 48 h. Thereafter, the N_2- and arginine-grown cells were washed, resuspended in HEPES buffer and used for ^{14}C-arginine uptake. The N_2-grown cells in case of Het$^-$ Nif$^-$ mutant refer to the nitrate-grown cells, which were subjected to nitrogen starvation for 24 h in N_2-medium; this is because the mutant does not grow in N_2-medium. The values presented are means from two independent experiments, each with two replicates. Symbols: N_2-medium, parent (□), arginine-medium, parent (O), N_2-medium, mutant (■) and arginine-medium, mutant (●).

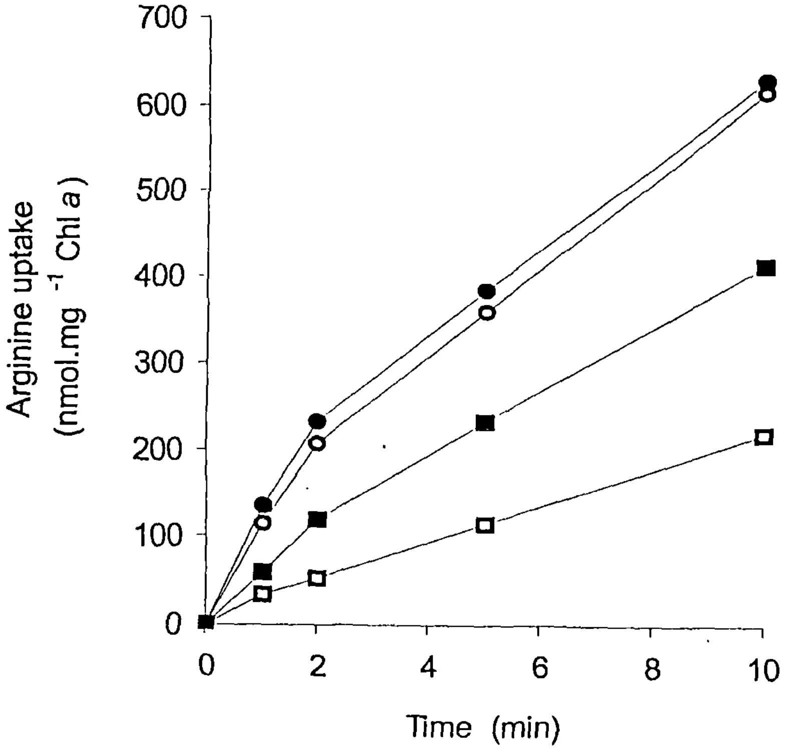


Fig 5.4. ^{14}C-alanine uptake in *Nostoc* ANTH and its Het$^-$Nif$^-$ mutant.

Nitrate-grown exponential cultures were washed and transferred to N_2- ($BG11_0$) and alanine- ($BG11_0$+ 1 mM alanine)-media and incubated for 48 h. Thereafter, the N_2- and alanine-grown cells were washed, resuspended in HEPES buffer and used for ^{14}C-alanine uptake. The N_2-grown cells in case of Het$^-$ Nif$^-$ mutant refer to the nitrate-grown cells, which were subjected to nitrogen starvation for 24 h in N_2-medium; this is because the mutant does not grow in N_2-medium. The values presented are means from two independent experiments, each with two replicates. Symbols: N_2-medium, parent (□), alanine-medium, parent (O), N_2-medium, mutant (■) and alanine-medium, mutant (●).

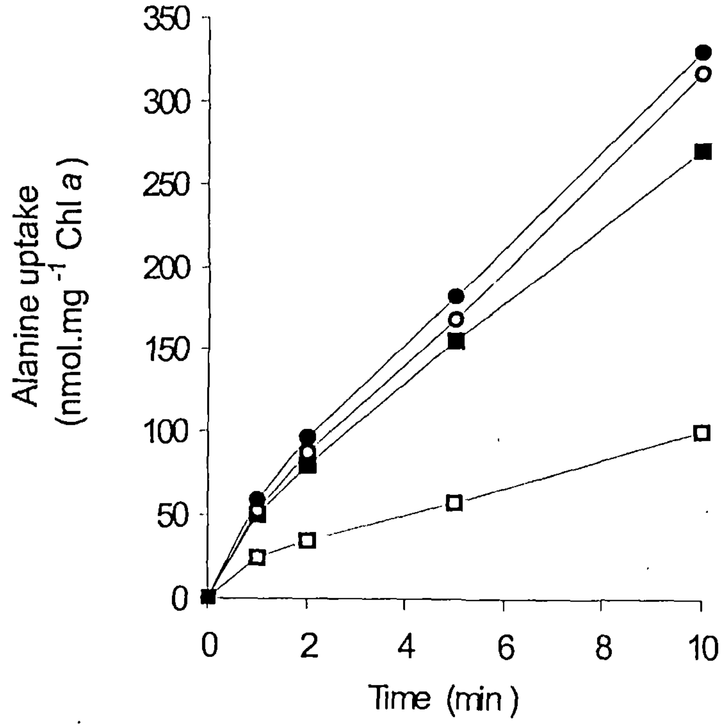


Table 5.4. Effect of DCMU, DCCD and CCCP on glutamine, asparagine, arginine and alanine uptake by *Nostoc* ANTH.

Nitrate-grown exponential cultures were washed and transferred to glutamine-, asparagine-, arginine- and alanine-media and incubated for 48 h. Thereafter, the cells were washed and resuspended in HEPES buffer. ^{14}C-gluamine, ^{14}C-asparagine, ^{14}C-arginine and ^{14}C-alanine were added after 30 min of incubation in light with or without DCMU (10 μM), DCCD (50 μM) and CCCP (25 μM).

The uptake rates were calculated from the linear uptake between 2 and 10 min after ^{14}C incorporation into the cells. The uptake rates are expressed as nmol.mg^{-1}Chl *a*. min^{-1}. The values presented are means from two independent experiments ± standard error (SE), each with two replicates.

Treatment	Uptake rates			
	Glutamine	Asparagine	Arginine	Alanine
Control	44 ± 0.2	29 ± 0.5	51 ± 0.5	24 ± 0.1
Control + DCMU	21 ± 1.0	15 ± 0.1	26 ± 0.8	10 ± 0.8
Control + DCCD	4.1 ± 0.6	7.2 ± 1.3	9.0 ± 1.1	3.0 ± 0.2
Control + CCCP	3.8 ± 0.4	4.0 ± 0.2	5.3 ± 0.1	1.8 ± 0.1

CHAPTER 6

SUMMARY

The present study incorporates details of mutational construction and characterization of herbicide resistant and nitrogenase derepressed mutants of the diazotrophic cyanobacterium *Nostoc* ANTH. *Nostoc* ANTH and its mutants were further characterized with regard to their ability to associate with rice plants, associative nitrogen-fixation and nitrogen transfer. An attempt has also been made to characterize amino acid uptake, amino acid-nitrogen nutrition and the associated metabolic changes with a view to understand overall nitrogen metabolism in this strain for future development of cyanobacterial strains useful for photobiological production of amino acids. The findings are summarized below:

1. Using the nitrate analogue chlorate as a selection agent a spontaneous chlorate resistant (ClO-*R*) mutant of *Nostoc* ANTH was isolated and characterized with regard to growth, heterocyst frequency, nitrogen-fixation and uptake and assimilation of nitrate, nitrite and ammonium. The results indicate that the ClO-*R* mutant of *Nostoc* ANTH can fix atmospheric nitrogen in presence of high concentrations of nitrate, which otherwise inhibits N_2-fixation. The mutant lacks nitrate uptake and has a 60 % lower nitrate reductase activity in nitrate-medium but the GS and ammonium transport activity remain unaffected. Thus the lack of inhibitory effect of nitrate on N_2-fixation was due to

lack of nitrate uptake and not due to lack of enzymes for its assimilation (nitrate reductase and glutamine synthetase) or the lack of ammonium transport system for retention of ammonia. Although, the nitrate uptake is lacking in the mutant, it is able to transport and assimilate nitrite, indicating that there is a transport system for nitrite, which is distinct from that for the nitrate. Such a mutant could serve as a better source of biofertilizer in rice-paddies since nitrate can be used as a chemical nitrogen fertilizer supplement without adversely affecting N_2-fixation.

2. Using the herbicide, paraquat, a spontaneous paraquat-resistant (PQ-*R*) mutant of *Nostoc* ANTH was isolated. Characterization of the PQ-*R* mutant revealed that its paraquat-resistant nature was an outcome of a decrease in photosynthetic O_2-evolution activity and not due to increase in the superoxide dismutase activity. The growth and N_2-fixation of PQ-*R* mutant was insensitive to paraquat.

3. The cyanobacterium *Nostoc* ANTH and its PQ-*R* and ClO-*R* mutants colonized the root surfaces of rice plants. Occasionally they occupied intercellular spaces. The attachment to root was tight and biphasic in nature. A short time of co-culture was sufficient for attachment of *Nostoc* ANTH inocula to the rice roots. Following co-culture, the nitrogenase activity of rice root associated cyanobacteria was always higher than the free-living/non-associated cyanobacteria. Sugars from the plant, e.g., Glucose and/or fructose can sustain N_2-fixation by rice root associated *Nostoc* ANTH and its mutants in absence of photosynthesis (i.e., in darkness or in presence of DCMU). Thus, associated cyanobacteria can fix N_2 day and night even in the presence of nitrate fertilizers. Lower $\delta^{15}N$ values of plants grown in association with *Nostoc* ANTH or its mutants clearly indicate transfer of N_2–derived nitrogen to the rice plants.

4. Amino acid-nitrogen nutrition, associated metabolic changes and amino acid uptake was compared in a mutant defective in heterocyst formation and N_2-fixation (Het $^-$ Nif $^-$) and its parent (*Nostoc* ANTH). *Nostoc* ANTH and its Het $^-$ Nif $^-$ mutant can grow at the expense of glutamine, asparagine, arginine and alanine as a source of fixed-nitrogen. However, the relative efficiency with which these amino acids supported cellular growth varied. Glutamine and asparagine repressed N_2-fixation and served as a sole source of fixed-nitrogen for growth. Arginine and alanine were a comparatively poorer source of nitrogen for growth and did not fully repress N_2-fixation or heterocyst formation. The poor growth in arginine and alanine media was not due to poor uptake rates since the uptake rates for these amino acids were not significantly different from those for glutamine or asparagine. The GS activity remained unaffected during cultivation in media containing any one of the four amino acids tested. The uptake of amino acids was substrate-inducible, requiring *de novo* synthesis, energy dependent and biphasic in nature. Nitrate and ammonium repressed ammonium uptake but did not repress uptake of amino acids. In N_2-medium, the uptake of ammonium and amino acids in the Het $^-$ Nif $^-$ mutant were significantly higher than its parent strain. The mutational loss of diazotrophy in the mutant resulted in nitrogen starvation when N_2 was the sole source of nitrogen leading to highly derepressed ammonium and amino acid transport systems. I did not find any evidence of the second nitrogenase (Nif 2) in vegetative cells of *Nostoc* ANTH.

REFERENCES

Adams DG. (2000) Symbiotic interactions. In: The ecology of cyanobacteria. Whitton BA and Potts M eds. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 523-561.

Adams DG, Duggan PS. (1999) Heterocyst and akinete differentiation in cyanobacteria. New Phytol. 144, 3-33.

Arizmendi JM, Serra JL (1990) Purification and some properties of the nitrite reductase from the cyanobacterium *Phormidium laminosum*. Biochim Biophys Acta. 1040, 237-244.

Bagchi SN, Singh HN. (1984) Genetic control of nitrate reduction in the cyanobacterium *Nostoc muscorum*. Mol Gen Genet. 193, 82-84.

Bagchi SN, Rai AN, Singh HN. (1985 a) Regulation of nitrate reductase in cyanobacteria. Repression-derepression control of nitrate reductase apoprotein in the cyanobacterium *Nostoc muscorum*. Biochim Biophys Acta. 838, 370-373.

Bagchi SN, Rai UN, Rai AN, Singh HN. (1985 b) Nitrate metabolism in the cyanobacterium *Anabaena cycadeae*: Regulation of nitrate uptake and reductase by ammonia. Physiol Plant. 63, 322-326.

Bagchi SN, Ernst A, Boger P. (1991) The effect of activated oxygen species on nitrogenase of *Anabaena variabilis*. Z.Naturforsch. 46c, 407-415.

Bauer CC, Ramaswamy KS, Endley S, Scappino LA, Golden JW, Haselkorn R. (1997) Suppression of heterocyst differentiation in *Anabaena* PCC 7120 by a cosmid carrying

103560
Date..............

wild-type genes encoding enzymes for fatty acid synthesis. FEMS Microbiol Lett. 151, 23-30.

Bergman B, Rai AN. (1989) The *Nostoc-Nephroma* symbiosis: localization, distribution pattern and levels of key proteins involved in nitrogen and carbon metabolism of the cyanobiont. Physiol plant. 77, 216-224.

Bergman B, Lindblad P, Pettersson A, Renstrom E, Tiberg E. (1985) Immuno-gold localization of glutamine synthetase in a nitrogen-fixing cyanobacterium (*Anabaena cylindrica*). Planta. 166, 329-334.

Bergman B, Lindblad P, Rai AN. (1986) Nitrogenase in free-living and symbiotic cyanobacteria: Immunoelectron microscope localization. FEMS Microbiol Lett. 35, 75-78.

Bergman B, Rai AN, Johansson C, Soderback E. (1992) Cyanobacterium-plant symbiosis. Symbiosis. 14, 67-81.

Bergman B, Matvayev A, Rasmussen U. (1996) Chemical-signalling in cyanobacterium-plant symbiosis. Trends in Plant Science. 1, 191-197.

Bergman B, Gallon JR, Rai AN, Stal L.J. (1997) Nitrogen fixation by non-heterocystous cyanobacteria. FEMS Microbiol Rev. 19, 139-185.

Berjerson FJ, Peoples MB, Turner GL. (1988) Isotopic discriminations during the accumulation of nitrogen by soybeans. J Plant Physiol. 15, 407-420.

Bednarz J, Schmid GH. (1991) Induction of nitrate reductase activity by arginine in the filamentous cyanobacterium *Oscillatoria chalybea*. Z. Naturforsch. 46c, 591-596.

Beyer WF Jr, Fridovich I. (1987) Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. Anal Biochem. 161, 559-566.

Black TA, Wolk C.P. (1994) Analysis of a het ⁻ mutation in *Anabaena* sp. strain PCC 7120 implicates a secondary metabolite in the regulation of heterocyst spacing. J Bacteriol. 176, 2282-2292.

Black TA, Cai Y, Wolk CP. (1993) Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. Mol Microbiol. 9, 77-84.

Black K, Buikema WJ, Haselkorn R. (1995) The *hglK* gene is required for localisation of heterocyst-specific glycolipids in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol. 177, 6440-6448.

Bohme H, Haselkorn R (1988) Molecular cloning and nucleotide sequence analysis of the gene coding for heterocyst ferredoxin from the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol Gen Genet. 214, 278-285.

Bottomley PJ, Stewart WDP. (1976) ATP pools and transients in the blue-green alga, *Anabaena cylindrica*. Arch Microbiol. 108, 249-258.

Boussibba S, Gibson J. (1987) Regulation of methylammonium/ammonium transport in the unicellular cyanobacterium *Synechococcus* R-2 (PCC 7942). FEMS Microbiol Lett. 43, 289-293.

Boussibba S, Resch CM, Gibson J. (1984) Ammonium uptake and retention in some cyanobacteria. Arch. Microbiol. 138, 287-292.

Bryant DA. (1994) The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

BuikemaWJ, Haselkorn R. (1991a) Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol. 179, 258-266.

BuikemaWJ, Haselkorn R. (1991b) Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. Genes Dev. 5, 321-330.

CaiY, Wolk CP. (1997) Nitrogen deprivation of *Anabaena* sp. strain PCC 7120 elicits rapid activation of a gene cluster that is essential for uptake and utilization of nitrate. J Bacteriol. 129, 258-266.

Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ. (1997) *Trichodesmium*, a globally significant marine cyanobacteria. Science. 276, 1221-1229.

Carr NG, Whitton BA. (1982) The biology of cyanobacteria. Blackwell Scientific Publications, Oxford, UK.

Carroll EW, Schwarz OJ, Hickok LG. (1988) Biochemical studies of paraquat-tolerant mutants of the fern *Ceratopteris richardii*[1]. Plant Physiol. 87, 651-654.

Cawse PA. (1967) The determination of nitrate in soil solution by ultraviolet spectrophotometry. Analyst. 92, 311-315.

Chapman JS, Meeks JC. (1983) Glutamine and glutamate transport by *Anabaena variabilis*. J Bacteriol. 156, 122-129.

Cohen MF, Meeks JC. (1997) A hormogonium regulating locus, *hrmUA*, of the cyanobacterium *Nostoc punctiforme* strain ATCC 29133 and its response to an extract of a symbiotic partner *Anthoceros punctatus*. Mol Plant-Microbe Inter. 10, 280-289.

Daday A, Smith GD. (1987) The hydrogenase-nitrogenase relationship in a symbiotic cyanobacterium isolated from *Macrozamia communis* L. Johnson. Aust J Plant Physiol. 14, 319-324.

Dean DR, Jacobson MR. (1992) Biochemical genetics of nitrogenase. In: Biological nitrogen fixation. Stacey G, Burris RH and Evans HJ eds. Chapman and Hall, New York, USA. pp. 763-834.

Durner J, Bohm I, Knorzer OC, Boger P. (1996) Proteolytic degradation of dinitrogenase reductase from *Anabaena variabilis* (ATCC 29413) as a consequence of ATP depletion and impact of oxygen. J Bacteriol. 178, 606-610.

Ernst A, Black T, Cai Y, Panoff JM, Tiwari DN, Wolk CP. (1992) Synthesis of nitrogenase in the mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst development or metabolism. J Bacteriol. 174, 6025-6032.

Fernandez-Pinas F, Leganes F, Wolk CP. (1994) A third genetic locus required for the formation of heterocysts in *Anabaena* sp. strain PCC 7120. J Bacteriol. 176, 5277-5283.

Fillat MF, Borrias WE, Weisbeek PJ. (1991) Isolation and overexpression in *Escherichia coli* of the flavodoxin gene from *Anabaena* PCC 7119. Biochem J. 280, 187-191.

Flores E, Herrero A. (1994) Assimilatory nitrogen metabolism and its regulation. In: The molecular biology of cyanobacteria. Bryant DA ed. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 487-517.

Flores E, Muro-Pastor MI. (1988) Uptake of glutamine and glutamate by dinitrogen fixing cyanobacterium *Anabaena* sp. PCC 7120. FEMS Microbiol Lett. 56, 127-130.

Flores E, Muro-Pastor MI. (1990) Mutational and kinetic analysis of basic amino acid transport in the cyanobacterium *Synechococcus* sp. strain PCC 6803. Arch Microbiol. 154, 521-527.

Forchhammer K, Tandeau de Marsac N. (1994) The P_{II} protein in the cyanobacterium *Synechococcus* sp. strain PCC 7942 is modified by serine phosphorylation and signals the cellular nitrogen-status. J Bacteriol. 176, 84-91.

Forchhammer K, Tandeau de Marsac N. (1995) Functional analysis of the phosphoprotein P_{II} (*glnB* gene product) in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J Bacteriol. 177, 2033-2040.

Fredriksson C. (1996) Nitrogenase localization reveals cell differentiation in filamentous, non-heterocystous cyanobacteria. PhD thesis, Stockholm University, Sweden.

Frias JE, Merida A, Herrero A, Martin-Nieto J, Flores E. (1993) General distribution of the nitrogen control gene *ntcA* in cyanobacteria. J Bacteriol. 175, 5710-5713.

Frias JE, Flores E, Herrero A. (1994) Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol Microbiol. 14, 823-832.

Frias JE, Flores E, Herrero A. (1997) Nitrate assimilation gene cluster from the heterocyst forming cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol. 179, 477-486.

Gallon JR. (1992) Reconciling incompatible nitrogen fixation and oxygen. New Phytol. 122, 571-609.

Gallon JR, Chaplin AE. (1987) An introduction to nitrogen fixation. Cassell Educational, London, UK.

Ganter M, Elhai J.. (1999) Colonization of wheat para-nodules by the N_2-fixing cyanobacterium *Nostoc* sp. strain 2S9B. New Phytol.141, 373-379.

Ganter M, Kerby NW, Obrcht Z, Rowell P. (1991 a) Colonization of wheat (*Triticum vulgare* L.) 1. A survey of soil cyanobacterial isolates forming associations with roots. New Phytol. 118, 447-483.

Ganter M, Kerby NW, Rowell P. (1991 b) Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria: 11. An ultrastructural study. New Phytol. 118, 485-492.

Ganter M, Kerby NW, Rowell P, Obreht Z, Scrimgeour C. (1995) Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria. 1V. Dark nitrogenase activity and effect of cyanobacteria on natural ^{15}N abundance in the plants. New Phytol. 129, 337-343.

Golden JW, Robinson SJ, Haselkorn R. (1985) Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena*. Nature. 314, 419-423.

Golden JW, Mulligan ME, Haselkorn R. (1987) Different recombination site specificity of two developmentally regulated genome rearrangements. Nature. 327, 526-529.

Handley LL, Raven JA. (1992) The use of natural abundance of nitrogen isotopes in plant physiology and ecology. Plant Cell and Environ. 15, 965-985.

Herbert SK, Samson G, Fork DC, Laudenbach DE. (1992) Characterization of damage to photosystem I and II in a cyanobacterium lacking detectable iron superoxide dismutase activity. Proc Natl Acad Sci USA. 89, 8716-8720.

Herdman M. (1987) Akinetes: structure and function. In: The cyanobacteria. Fay P and Van Baalen C eds. Elsevier, Amsterdam, The Netherlands. pp. 227-250.

Herdman M. (1988) Cellular differentiation: Akinetes. In: Methods in enzymology, Vol. 167. Packer L and Glazer AN eds. Academic Press, London, UK. pp. 222-232.

Herrero A, Flores E. (1990) Transport of basic amino acids by the dinitrogen -fixing cyanobacterium *Anabaena* sp. PCC 7120. J Biol Chem. 265, 3931-3935.

Herrero A, Muro-Pastor MA, Flores E. (2001) Nitrogen control in cyanobacteria. J Bacteriol. 183, 411-425.

Janson S, Rai AN, Bergman B. (1993) The marine lichen *Lichinia confinis* (O.F.Mull.) C. Ag.: ultrastructure and localization of nitrogenase, glutamine synthetase, phycoerythrin and ribulose 1,5-bisphosphate carboxylase/oxygenase in the cyanobiont. New Phytol. 124, 149-160.

Janson S, Rai AN, Bergman B. (1995) Intracellular cyanobiont *Richellia intracellularis*: ultrastructure and immunolocalization of phycoerythrin, nitrogenase, Rubisco and glutamine synthetase. Marine Biol. 124, 1-8.

Jiang F, Mannervik B, Bergman B. (1997) Evidence for redox regulation of the transcription factor NtcA, acting both as an activator and a repressor, in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol. 327, 513-517.

Kamuru F, Albrecht SL, Allen LH, Shanmugam KT. (1997) Growth and accumulation of ^{15}N in rice inoculated with the parent and a nitrogenase-derepressed strain of *Anabaena variabilis*. Appl Soil Ecology. 5,189-195.

Kentemich T, Haverkamp G, Bothe H. (1991) The expression of a third nitrogenase in the cyanobacterium *Anabaena variabilis*. Z. Naturforsch. 46c, 217-222.

Khudyakov I, Wolk CP. (1997) *het*C, a gene encoding for a protein similar to bacterial ABC protein exporters, is involved in early regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. J Bacteriol. 179, 6971-6978.

Kim J, Rees DC. (1994) Nitrogenase and biological nitrogen fixation. Biochemistry. 33, 387-397.

Kleiner D. (1985) Energy expenditure for cyclic retention of NH_3/NH^+_4 during N_2-fixation by *Klebsiella pneumoniae*. FEBS Lett. 187, 237-239.

Kuhlemeier CJ, Teeuwsen VJP, Janssen MJT, Van Arkel GA. (1984) Cloning of a third nitrate reductase gene from the cyanobacterium *Anacystis nidulans* R2 using a shuttle gene library. Gene. 31, 109-116.

Kumar AP, Rai AN, Singh HN. (1985) Nitrate reductase activity in isolated heterocysts of the cyanobacterium *Nostoc muscorum*. FEBS Lett. 179, 125-128.

Labarre J, Thuriaux P, Chauvat F. (1987) Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803. J Bacteriol. 169, 4668-4673.

Leganes F. (1994) Genetic evidence that *hepA* gene is involved in the normal deposition of the envelope of both heterocysts and akinetes in *Anabaena variabilis* ATCC 29413. FEMS Microbiol Lett. 123, 63-68.

Leganes F, Fernandez-Pinas F, Wolk CP. (1994) Two mutations that block heterocyst differentiation have no effect on akinete differentiation in *Nostoc ellipsosporum*. Mol Microbiol. 12, 679-684.

Liang J, Scappino L, Haselkorn R. (1992) The *patA* gene product which contains a region similar to *cheY* of *E.coli*, controls heterocyst pattern formation in the cyanobacterium *Anabaena* sp. strain PCC 7120. Proc Natl Acad Sci USA. 89, 5655-5659.

Liang J, Scappino L, Haselkorn R. (1993) The *patB* gene product, required for growth of the cyanobacterium *Anabaena* sp. strain PCC 7120 under nitrogen-limiting conditions, contains Ferredoxin and helix-turn-helix domains. J Bacteriol. 175, 1697-1704.

Lin S, Henze S, Lundgren P, Bergman B, Carpenter EJ. (1998) Whole cell immunolocalization of nitrogenase in marine diazotrophic cyanobacterium, *Trichodesmium* spp. Appl and Environ Microbiol. 64, 3052-3058.

Lindblad P, Bergman B. (1990) The cycad-cyanobacterial symbiosis. In: Handbook of symbiotic cyanobacteria. Rai AN ed. CRC Press, Boca Raton, FL, USA. pp. 43-63.

Liotenberg S, Campbell D, Castets AM, Houmard J, Tandeau de Marsac N. (1996) Modification of the P_{II} protein in response to carbon and nitrogen availability in filamentous cyanobacteria. FEMS Microbiol Lett. 144, 185-190.

Lowry GH, Rosenbrough J, Farr AL, Randell RJ. (1951) Protein measurement with Folin-phenol reagent. J Biol Chem. 244, 4436-4440.

Luque I, Herrero A, Flores E, Madueno F. (1992) Clustering of genes involved in nitrate assimilation in the cyanobacterium *Synechococcus*. Mol Gen Genet. 232, 7-11.

Luque I, Flores E, Herrero A. (1993) Nitrite reductase gene from *Synechococcus* sp. PCC 7942: homology between cyanobacterial and higher-plant nitrite reductase. Plant Mol Biol. 22, 1201-1205.

Luque E, Flores E, Herrero A. (1994) Nitrate and nitrite transport in the cyanobacterium *Synechococcus* sp. PCC 7942 are mediated by the same permease. Biochim Biophys Acta. 1184, 296-298.

Mackinney G. (1941) Absorption of light by chlorophyll solutions. J Biol Chem. 140, 315-322.

Madueno F, Borrias WE, Van Arkel GA, Guerrero MG. (1988) Isolation and characterization of *Anacystis nidulans* R2 mutants affected in nitrate assimilation: Establishment of two new mutant types. Mol Gen Genet. 213, 223-228.

Maeda S, Omata T. (1997) Substrate-binding lipoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in the transport of nitrate and nitrite. J Biol Chem. 272, 3036-3041.

Maldener I, Fiedler G, Ernst A, Fernandez-Pinas F, Wolk CP. (1994) Characterization of *devA*, a gene required for the maturation of proheterocysts in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol. 176, 7543-7549.

Manzano C, Candau P, Gomez-Moreno C, Relimpio AM, Losada M. (1976) Ferredoxin dependent photosynthetic reduction of nitrate and nitrite in particles of *Anacystis nidulans*. Mol Cell Biochem. 10, 161-169.

Martin-Nieto J, Herrero A, Flores E. (1989) Regulation of nitrate and nitrite reductases in dinitrogen-fixing cyanobacteria and Nif$^-$ mutants. Arch Microbiol. 151, 475-478.

Martin-Nieto J, Herrero A, Flores E. (1991) Control of nitrogenase mRNA levels by products of nitrate assimilation in the cyanobacterium *Anabaena* sp. strain PCC 7120. Plant Physiol. 97, 825-828.

Maryan PS, Eady RR, Chaplin AE, Gallon JR. (1986) Nitrogen fixation by *Gloeothece* sp. PCC 6909: Respiration and not photosynthesis supports nitrogenase activity in the light. J Gen Microbiol. 132, 789-796.

Meeks JC. (1990) Cyanobacterium-bryophyte associations. In: Handbook of symbiotic cyanobacteria. Rai AN ed. CRC Press, Boca Raton, FL, USA. pp. 43-63.

Merida A, Flores E, Florencio FG. (1991) Regulation of glutamine synthetase activity in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 6803 by the nitrogen source: Effect of ammonium. J Bacteriol. 173, 4095-4100.

Merrick MJ, Edwards RA. (1995) Nitrogen control in bacteria. Microbiol Rev. 59, 604-622.

Modi DR, Singh AK, Rao KS, Chakravarty D, Singh HN. (1991) Construction of multiple herbicide resistant ammonia excreting strains of cyanobacterium *Nostoc muscorum*. Biotechnol Lett. 13, 393-398.

Montesinos ML, Herrero A, Flores E. (1995) Amino acid transport required for diazotrophic growth in the cyanobacterium *Anabaena* sp strain PCC 7120. J Bacteriol. 177, 3150-3157.

Montesinos ML, Herrero A, Flores E. (1997) Amino acid transport in taxonomically diverse cyanobacteria and identification of two genes encoding elements of a neutral amino acid permease putatively involved in recapture of leaked hydrophobic amino acids. J Bacteriol. 179, 853-862.

Montesinos ML, Muro-Pastor AM, Herrero A, Flores E. (1998) Ammonium/Methyl ammonium permeases of a cyanobacterium- Identification and analysis of three nitrogen-regulated *amt* genes in *Synechocystis* sp. strain 6803. J Biol Chem. 273, 31463-31470.

Obreht Z, Kerby NW, Gantar M, Rowell P. (1993) Effects of root associated N_2-fixing cyanobacteria on growth and nitrogen content of wheat (*Triticum vulgare* L.) seedlings. Biol Fertil Soils. 15, 68-72.

Omata T, Andriesse X, Hirano A. (1993) Identification and characterization of a gene cluster involved in nitrate transport of the cyanobacterium *Synechococcus* sp. PCC 7942. Mol Gen Genet. 236, 193-202.

Ownby JD. (1977) Effects of amino acids on methionine-sulphoximine-induced heterocyst formation in *Anabaena*. Planta. 136, 277-279.

Paulsrud P, Lindblad P. (1998) Sequence variation of the tRNA^{leu} (UAA) intron as a marker for genetic diversity and specificity of symbiotic cyanobacteria in some lichens. Appl Environ Microbiol. 64, 310-315.

Pennell RI. (!992) Cell surface arabinogalactan proteins, arabinogalactans and plant development. In: Perspectives in Plant Cell Recognition. Callow JA and Green JR eds. Cambridge University Press, Cambridge, UK. pp. 105-121.

Potts M. (2000) *Nostoc*. In: The ecology of cyanobacteria. Whitton BA and Potts M eds. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 465-504.

Prakasham R, Rai AN. (1991) Evidence for the occurrence of specific methylammonium transport system in the cultured cyanobiont of *Anthoceros punctatus-Nostoc* association. J Gen Microbiol. 137, 1783-1788.

Prakasham R, Singh AK, Singh HN, Rai AN. (1991) Inorganic nitrogen regulation of glutamate uptake in the cyanobacterium *Nostoc muscorum*. Physiol Plant. 82, 257-260.

Rai AN. (1990) Handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, Florida, USA.

Rai AN, Bergman B. (1986) Modification of NO^-_3 metabolism in heterocysts of the N_2-fixing cyanobacterium *Anabaena* 7120 (ATCC 27893). FEMS Microbiol Lett. 36, 133-137.

Rai AN, Rowell P, Stewart WDP. (1984) Evidence for an ammonium transport system in free-living and symbiotic cyanobacteria. Arch Microbiol. 137, 241-246.

Rai AN, Rao VV, Singh HN. (1988) Metabolic changes associated with akinete germination in the cyanobacterium *Anabaena doliolum*. New Phytol. 109, 133-138.

Rai, AN, Borthakur M, Singh S, Bergman B. (1989) *Anthoceros-Nostoc* symbiosis: Localization of nitrogenase, glutamine synthetase, phycoerythrin and Ribulose-1,5-bisphosphate carboxylase/oxygenase in the cyanobiont and the cultured (free-living) isolate *Nostoc* 7801. J Gen Microbiol. 135, 385-395.

Rai AN, Borthakur M, Bergman B. (1992) Nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110. J Gen Microbiol. 138, 481-491.

Rai AN, Borthakur M, Paul D. (1996) Symbiotic cyanobacteria : biotechnological applications. J Sci Indus Res. 55, 742-752.

Rai AN, Soderback E, Bergman B. (2000) Cyanobacterium-plant symbiosis. New Phytol. 147, 449-481.

Ramaswamy KS, Endley S, Golden JW. (1996) Nitrate reductase activity and heterocyst suppression on nitrate in *Anabaena* sp strain PCC 7120 require *moeA*. J Bacteriol. 178, 3893-3898.

Rasmussen U, Svenning MM. (1998) Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repititive sequences. Appl Environ Microbiol. 64, 265-272.

Rasmussen U, Johansson C, Bergman B. (1994) Early communication in the *Nostoc-Gunnera* symbiosis : plant induced cell differentiation and protein synthesis in the cyanobacterium. Mol Plant-Microbe Inter. 6, 696-702.

Rasmussen U, Johansson C, Renglin A, Bergman B. (1996) A molecular characterization of the *Gunnera-Nostoc* symbiosis : comparison with *Rhizobium-* and *Agrobacterium*-plant interactions. New Phytol. 133, 391-398.

Rao VV, Rai AN, Singh HN. (1984) Metabolic activities of the akinetes of the cyanobacterium *Anabaena doliolum* : oxygen exchange, photosynthetic pigments and enzymes of nitrogen metabolism. J Gen Microbiol. 130, 1299-1302.

Renstrom-Kellner E, Rai AN, Bergman B. (1990) Correlation between nitrogenase and glutamine synthetase expression in the cyanobacterium *Anabaena cylindrica*. Physiol Plant. 80, 12-19.

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol. 111, 1-61.

Roger PA, Ladha JK. (1992) Biological N_2-fixation in wetland rice fields : estimation and contribution to nitrogen-balance. Plant and Soil. 141, 41-55.

Sampio MJAM, Rowell P, Stewart WDP. (1979) Purification and some properties of glutamine synthetase from the nitrogen fixing cyanobacterium *Anabaena cylindrica* and *Nostoc* sp. J Gen Microbiol. 111, 181-191.

Scott ID, Nicholls DG. (1980) Energy transduction in intact synaptosomes. Influence of plasma-membrane depolarization on the respiration and membrane potential of internal mitochondria determined *in situ*. Biochem J. 186, 21-33.

Shearer G, Kohl DH. (1989) Natural ^{15}N abundance as a method of estimating the contribution of biologically fixed nitrogen to N_2-fixing systems : potential for non-legumes. In: Nitrogen fixation with non-legumes. FA Skinner *et al* eds. Kluwer Acadamic Publishers, Dordrecht, The Netherlands. pp. 289-299.

Shehawy RM, Kleiner D. (1999) Ammonium (methylammonium) transport by heterocysts and vegetative cells of *Anabaena variabilis*. FEMS Mcrobiol Lett. 181, 303-306.

Singh AK, Singh HN, Rai AN. (1991) Evidence for a role of glutamine synthetase in assimilation of amino acids as nitrogen source in the cyanobacterium *Nostoc muscorum*. Biochem International. 25, 887-894.

Singh DT, Rai AN, Singh HN. (1985) Methylammonium (ammonium) uptake in a glutamine auxotroph of the cyanobacterium *Anabaena cycadeae*. FEBS Lett. 186, 51-53.

Singh DT, Modi DR, Singh HN. (1986) Evidence for glutamine synthetase and methylammonium (ammonium) transport system as two distinct primary targets of methionine sulphoximine inhibitory action in the cyanobacterium *Anabaena doliolum*. FEMS Mcrobiol Lett. 37, 95-98.

Singh DT, Ghosh R, Singh HN. (1987) Physiological characterization of the ammonium transport system in the free-living diazotrophic cyanobacterium *Anabaena cycadeae*. J Plant Physiol. 127, 231-239.

Singh RN. (1961) Role of blue green algae in nitrogen economy of Indian agriculture. Indian Council of Agricultural Research, New Delhi, India.

Singh RK, Stevens SE Jr. (1992) Cloning of the *nifHDK* genes and their organization in the heterocystous cyanobacterium *Mastigocladus laminosus*. FEMS Microbiol Lett. 94, 227-234.

Smith AJ. (1982) Modes of cyanobacterial carbon metabolism. In: The Biology of cyanobacteria. Carr NG and Whitton BA eds. Blackwell and Scientific Publications, Oxford, UK. pp. 47-86.

Snell FD, Snell CT. (1949) Colorimetric methods of analysis. Vol 3. D. Van Nostrand Co. New York, USA. pp. 804-805.

Soderback E, Lindblad P, Bergman B. (1990) Developmental patterns related to nitrogen fixation in the *Nostoc-Gunnera magellamica* Lam. symbiosis. Planta. 182, 355-362.

Spiller H, Gunasekaran M. (1994) Ammonium-excreting mutant strain of the cyanobacterium *Anabaena variabilis* supports growth of wheat. Appl Microbiol Biotechnol. 33, 477-480.

Spiller H, Stallings W Jr., Woods T, Gunasekaran M. (1993) Requirement for direct association of ammonium-excreting *Anabaena variabilis* mutant (SA-1) with roots for maximum growth and yield of wheat. Appl Microbiol Biotechnol. 40, 557-566.

Stewart WDP, Lex M. (1970) Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain 594. Arch Microbiol. 73, 250-260.

Stewart WDP, Fitzgerald GP, Burris RH. (1967) *In situ* studies on N_2-fixation using acetylene reduction technique. Proc Natl Acad Sci USA. 58, 2071-2078.

Strasser P, Falkner G. (1986) Characterization of the glutamate/aspartate transport system in a symbiotic *Nostoc* sp. Planta. 168, 381-385.

Suzuki T, Horie N, Sugiyama T, Omata T. (1995) Identification and characterization of two nitrogen-regulated genes of the cyanobacterium *Synechococcus* sp. strain PCC 7942 required for maximum efficiency of nitrogen assimilation. J Bacteriol. 177, 290-296.

Svircev Z, Tamas I, Nenin P, Drobac A. (1997) Co-cultivation of N_2-fixing cyanobacteria and some agriculturally important plants in liquid and sand cultures. Appl Soil Ecol. 6, 301-308.

Tandeau de Marsac N, Houmard J. (1993) Adaptation of cyanobacteria to environmental stimuli : new step towards molecular mechanisms. FEMS Microbiol Rev. 104, 119-190.

Thiel T. (1988) Transport of leucine in the cyanobacterium *Anabaena variabilis*. Arch Microbiol. 149, 466-470.

Thiel T. (1993) Characterization of genes for an alternative nitrogenase in the cyanobacterium *Anabaena variabilis*. J Bacteriol. 175, 6276-6286.

Thiel T, Leone M. (1986) Effect of glutamine on growth and heterocyst differentiation in the cyanobacterium *Anabaena variabilis*. J Bacteriol. 168, 769-774.

Thiel T, Pratte B. (2001) Effect on heterocyst differentiation of nitrogen fixation in vegetative cells of the cyanobacterium *Anabaena variabilis* ATCC 29413. J Bacteriol. 183, 280-286.

Thiel T, Lyons EM, Erker JC, Ernst A. (1995) A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium. Proc Natl Acad Sci. 92, 9358-9362.

Tichy M, Vermaas W. (1999) In vivo role of catalase-peroxidase in *Synechocystis* sp. strain PCC 6803. J Bacteriol. 181, 1875-1882.

Tozum SRD, Gallon JR. (1979) The effects of methyl viologen on *Gloeocapsa* sp. LB795 and their relationship to the inhibition of acetylene reduction (Nitrogen fixation) by oxygen. J Gen Microbiol. 111, 313-326.

Tsinoremas NF, Castets AM, Harrison MA, Allen JF, Tandeau de Marsac N. (1991) Photosynthetic electron transport controls nitrogen assimilation in cyanobacteria by means of posttranslational modification of the *glnB* gene product. Proc Natl Acad Sci USA. 88, 4565-4569.

Tumer NE, Robinson SJ, Haselkorn R. (1983) Different promoters for the *Anabaena* glutamine synthetase gene during growth with molecular or fixed nitrogen. Nature. 306, 337-342.

Van Rhizn P, Vanderleyden J. (1995) The *Rhizobium*-plant symbiosis. Microbiol Rev. 59, 124-142.

Wei TF, Ramasubramanium TS, Golden JW. (1994) *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J Bacteriol. 176, 4473-4482.

Weisshar H, Boger P. (1983) Nitrogenase activity of the non-heterocystous cyanobacterium *Phormidium faveolavum*. Arch Microbiol. 136, 270-274.

Whitton BA. (2000) Soils and Rice-fields. In: The ecology of cyanobacteria. Whitton BA and Potts M eds. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 233-255.

Whitton BA, Potts M. (2000) The ecology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Wolk CP. (1996) Heterocyst formation. Annu Rev Genet. 30, 59-78.

Wolk CP, Ernst A, Elhai J. (1994) Heterocyst metabolism and development In: Molecular genetics of cyanobacteria. Bryant DA ed. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 769-823.

Wouters J, Janson S, Bergman B. (2000) The effects of exogenous carbohydrates on nitrogen fixation and *hetR* expression in *Nostoc* PCC 9229 forming symbiosis with *Gunnera*. Symbiosis. 28, 63-76.

Yoon HS, Golden JW. (1998) Heterocyst pattern formation controlled by a diffusible peptide. Science. 282, 935-938.

Zhou R, Cao Z, Zhao J. (1998 a) Characterization of HetR turnover in *Anabaena* sp. strain PCC 7120. Arch Microbiol. 169, 417-423.

Zhou R, Wei X, Jiang N, Li H, Dong Y, Hsi K-L, Zhou J. (1998 b) Evidence that HetR is an unusual serine-type protease. Proc Natl Acad Sci USA. 95, 4959-4963.

CURRICULUM VITAE

Name : Jyotirmoy Bhattacharya

Date and place of birth : 02-08-1972, Jabalpur, Madhya Pradesh

Nationality : Indian

Sex : Male

Address : Department of Biochemistry,
North-eastern Hill University,
Shillong-793022, Meghalaya, India.
: e-mail- `jyotirmoyb@hotmail.com`

Academic Qualifications :

M.Sc. (Bioscience, 1995) : RD University, Jabalpur
First Division

B.Sc. (1993) : RD University, Jabalpur
Second Division.

Intermediate (1990) : CBSE
First Division

High School : CBSE
First Division

Fellowship Awarded : CSIR, JRF NET (1996-1998)
: CSIR, SRF NET (1998-2001)

Publication : One, J.Gen.Appl.Microbiol, 46, 225-229, (2000)

Manuscripts to be submitted : Three (from this thesis)

J. Gen. Appl. Microbiol., **46**, 225–229 (2000)

Short Communication

Evidence for the involvement of a common genetic determinant in the control of thallium-resistant (Tl^{+}-R) phenotype and salinity-tolerant phenotype in the cyanobacterium *Nostoc muscorum*

Surendra Singh,* Jyotirmoy Bhattacharya,[1] and Hriday N. Singh[1]

Department of Biological Sciences, Rani Durgavati University, Jabalpur-482 001 (M.P.), India
[1]Department of Biochemistry, North-Eastern Hill University, Shillong-793 022 (Meghalaya), India

(Received January 22, 1998; Accepted June 23, 2000)

Key Words——cyanobacterium; K^{+}-uptake; mutation; N_2-fixation; osmoprotectant; proline uptake; salinity tolerance; thallium-resistance

The modern agriculture ecosystem is facing a growing problem of salinity stress adversely affecting its metabolism and productivity. Organisms adapted to grow in natural habitats of varying salinity stresses are known to do so because of their salinity-inducible genetic ability to produce a variety of compatible salinity protectants such as trehalose, sucrose, sorbitol, mannitol, proline, and glycine betaine (Bartels and Nelson, 1994; Bohnert and Jensen, 1996; Csonka, 1989). Identification and isolation of salinity-tolerant genes have led to their successful use in producing genetically engineered microbes and plants for growth and propagation in a natural saline environment (Bohnert and Jensen, 1996). The molecular mechanism of salinity tolerance in cyanobacteria in comparison with that in enterobacterial systems is studied very little and thus is virtually unknown (Singh et al., 1996).

K^{+} is reported to function as a primary intracellular osmotic signal for adaptation to salinity stress in *Escherichia coli* and *Salmonella typhimurium* through the activation of their salinity-inducible genes (Higgins et al., 1987; Mahan and Csonka, 1983). Cyanobacteria are known to grow in a saline environment because of their inherent genetic ability to produce salinity protectants in response to salinity stress (Reed and Stewart, 1985). No critical studies have been done, however, to examine the primary nature of a cyanobacterial osmotic signal, whether it is K^{+}, Na^{+}, or nonexistent.

In bacterial systems, analogues of salinity protectants have been used to analyze the biochemical basis of their salinity tolerance. The application of this approach led to the identification of L-proline as a salinity protectant in the cyanobacterium *Nostoc muscorum* (Singh et al., 1996). In this study, a similar approach was followed in examining the role of K^{+} in the regulation of cyanobacterial salinity tolerance. Thallium (Tl^{+}), the toxic analogue of K^{+}, used in the past for studying K^{+}-transport in bacterial systems (Damper et al., 19~~97~~79), was also used here as a selective agent for isolating spontaneously occurring thallium-resistant (Tl^{+}-R) mutants of the cyanobacterium *N. muscorum*. One such mutant was then examined for K^{+} transport, Na^{+} transport, proline content, and salinity tolerance. The results reported here suggest a definite role of intracellular K^{+} in the adaptation of the cyanobacterium to salinity stress.

The exponentially growing axenic clonal cultures of

---

[*] Address reprint requests to: Dr. Surendra Singh, Department of Biological Sciences, Rani Durgavati University, Jabalpur-482 001 (M.P.), India.

N. muscorum maintained in diazotrophic Chu-10 growth medium (Gerloff et al., 1950) at 28±2°C and at a photon fluence rate of 50 µmol $m^{-2} s^{-1}$ were employed for the isolation of spontaneously occurring mutant resistance to the growth inhibitory action of thallium chloride (TlCl) according to a previously reported method (Singh et al., 1994).

A measurement of $^{22}Na^+$ (500 µm, specific activity 7.4 MBq ml^{-1}) and of ^{14}C-proline (50 µM, specific activity 7.5 kBq ml^{-1}) uptake was conducted in the reaction mixture by the method reported previously (Rai et al., 1984).

The K^+ uptake was measured under stressed and unstressed conditions by using atomic absorption spectroscopy (AAS). Ten millimolar HEPES-NaOH buffer (pH 7.5) were used as a medium to estimate K^+ uptake by exponentially growing cyanobacterial cultures under given growth conditions. The experimental samples were equilibrated with the unstressed or stressed buffer medium for 30 min before the addition of KCl at a final concentration of 5 ppm (127.9 µm). The stressed salinity condition of 106-osmolarity (nmol/kg water) was created by the addition of 60 mM NaCl to the buffer medium. The K^+ uptake was estimated by determining the K^+ content of various supernatants at given time intervals by AAS and thus subtracting these values from the control value.

The content of protein, chlorophyll *a*, and intracellular proline was measured as described previously (Lowry et al., 1951; Mackinney, 1941; Singh et al., 1996).

The cyanobacterium *N. muscorum* did not survive a TlCl dose of 0.2 µM under diazotrophic growth conditions. Spontaneous mutants resistant to growth inhibitory action of 1.0 µM TlCl were isolated and characterized in respect to mutation frequency, salinity survival, Na^+ and K^+ uptake activity, proline uptake activity, and intracellular level of proline. A Tl^+-R mutant arose with a frequency of about 1×10^{-6}, a finding suggesting the involvement of a single mutational event in the spontaneous generation of Tl^+-R mutant phenotype in *N. muscorum*. The salinity (NaCl) survival characteristics of the parent and mutant *N. muscorum* are given in Fig. 1. The general comparative kinetics of the parent and the mutant salinity survival curves clearly show that the mutant is more highly sensitive to salinity stress than its parent. The mutation to Tl^+-R phenotype is thus found to be simultaneously accompanied by a significant decrease in the salinity tolerance level of the cyanobacterium. The cyanobacterial thallium-sensitive (Tl^+-S)/Tl^+-R genetic determinant clearly appears to play a regulatory role in its adaptation to salinity stress.

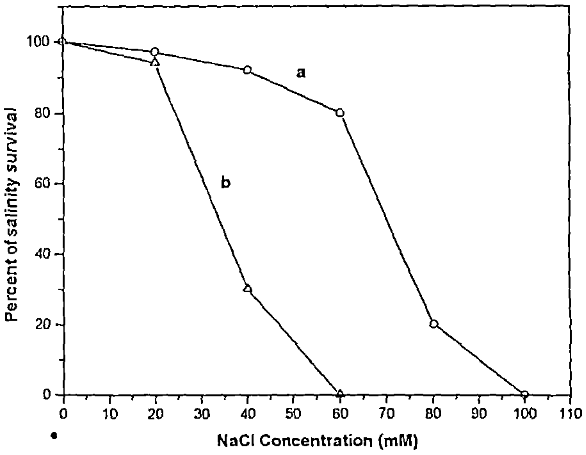


Fig. 1. Salinity survival characteristics of *N. muscorum* parent and its Tl^+-R mutant strain.

The NaCl survival curves for the two strains of *N. muscorum*. (a) Parent strain on NaCl-containing diazotrophic medium; (b) Tl^+-R strain on NaCl-containing diazotrophic medium. The inoculated plates were incubated for 2 weeks in the growth chamber and examined for the number of surviving colonies growing on each plate. The percentage survival was determined by treating the total number of surviving colonies on control plates as 100%. The mean values from three independent experimental determinations are shown ±SEM where these exceed the dimension of the symbols.

A role of Na^+ and K^+ has been implicated in the regulation of cyanobacterial response to salinity/osmotic stress (Reed and Stewart, 1985). Therefore the characteristics of Na^+ uptake of both the parent and the mutant were studied under normal growth conditions, and the results of these studies are shown in Fig. 2. The two cyanobacterial strains seem almost similar in respect to their Na^+-uptake process, a finding suggesting that mutation to the Tl^+-R phenotype does not influence the Na^+ uptake process in the cyanobacterium. The same series of experiments was repeated with a salinity stress of 50 mM, giving an osmolarity of 103 (nmol/kg water) for 3 h. A salinity stress of this strength also did not significantly influence the Na^+ uptake characteristics of the two strains (data not shown).

Similarly, both strains were examined for K^+ uptake characteristics under stressed and unstressed conditions (Fig. 3). In comparison with the parent *N. muscorum*, its mutant strain seems to completely lack the

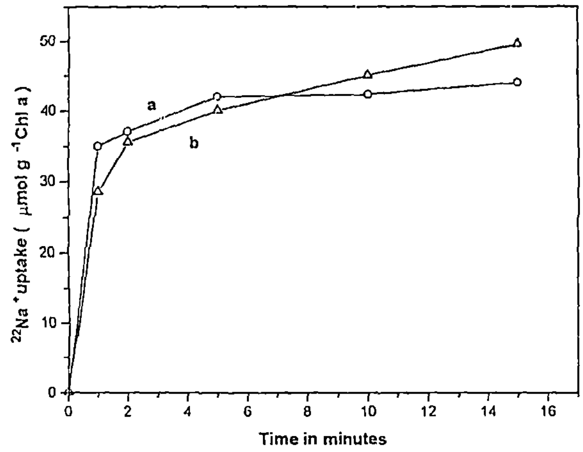


Fig. 2. ^{22}Na$^+$ uptake activity of parent and Tl$^+$-R mutant strain.

A comparison of ^{22}Na$^+$ uptake activity in (a) parent *N. muscorum* and (b) Tl$^+$-R strain. The mean values from three independent experimental determinations are shown ±SEM where they exceed the dimensions of the symbols.

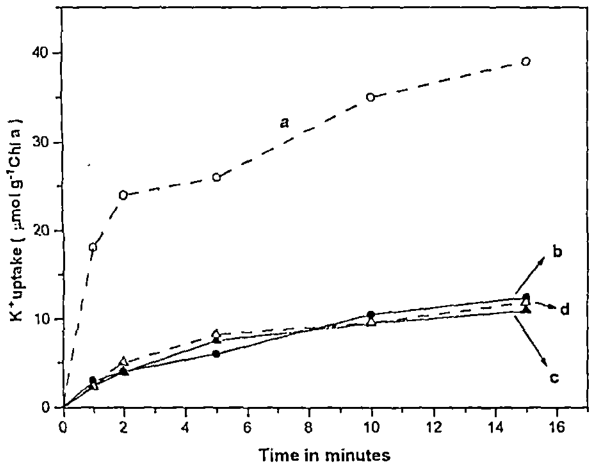


Fig. 3. K$^+$ uptake activity of two cyanobacterial strains (parent and Tl$^+$-R strain) with NaCl and without it.

K$^+$ uptake activity in cultures of (a) parent *N. muscorum* under NaCl stress; (b) parent strain without stress; (c) Tl$^+$-R strain without stress; (d) Tl$^+$-R strain under NaCl stress. The mean values from three independent experimental determinations are shown ±SEM where they exceed the dimensions of the symbols.

Table 1. Intracellular levels of proline in parent *N. muscorum* and its Tl$^+$-R mutant strain.

Treatment	Parent strain	Tl$^+$-R strain
N_2 medium	10.5	11.1
N_2 medium+NaCl	12.2	12.4
N_2 medium+Proline	30.6	20.4
N_2 medium+NaCl+Proline	101.4	30.9

The source of inocula for the given experiments were a 6-day-old diazotrophically grown culture of the two strains. The intracellular level of proline (µmol proline g^{-1} protein) was determined in liquid nitrogen broken cultures of the parent and its Tl$^+$-R mutant following their incubation for 3 h in the NaCl-stressed (50 mM) or unstressed diazotrophic or proline (1.0 mM) growth medium by the acid ninhydrin method, as described in Singh et al. (1996). Proline and NaCl were added to the experimental cultures at the start of the experiment. Each value is an average of these independent experiments.

salinity-inducible K$^+$ uptake system. Evidently the Tl$^+$-S phenotype appears to be related to salinity-inducible K$^+$ uptake phenotype and the Tl$^+$-R phenotype to the loss of such an inducible K$^+$ uptake process. Because the mutation from Tl$^+$-S phenotype to Tl$^+$-R phenotype results in the loss of salinity-inducible K$^+$ uptake process, and because Tl$^+$-R phenotype also exhibits loss in the level of salinity tolerance, it is concluded that the salinity-inducible K$^+$ uptake process in the cyanobacterium functions in its adaptation to salinity stress.

It has already been shown that one mode of salinity protection in *N. muscorum* is by the overproduction of exogenously supplied or endogenously produced proline (Singh et al., 1996). Therefore the next relevant experiments were conducted to examine the intracellular level of proline in both parent and mutant strains of *N. muscorum* under stressed and unstressed conditions and in the absence or presence of exogenous proline.

As shown in Table 1, the intracellular level of proline in diazotrophically grown cultures of both strains remained almost similar under unstressed conditions. However, the intracellular proline level in exogenously supplied cultures of the parent strain showed respectively about a 2.5-fold and an 8.3-fold rise under unstressed and stressed conditions. In comparison, a similar parallel experiment with mutant strain showed no great difference in the intracellular proline content of unstressed and stressed cultures. Apparently, salinity-inducible proline accumulation as evident in the parent strain is deficient in the mutant strain. These findings show that mutation to Tl$^+$-R phenotype in the cyanobacterium *N. muscorum* is accompanied by the loss of its ability to overaccumulate exogenous proline in response to salinity stress. The next series of experiments were therefore planned to check the uptake of

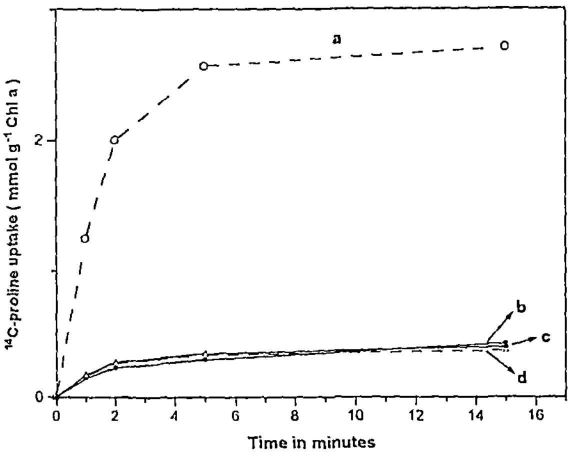


Fig. 4. ^{14}C-proline uptake activity of parent *N. muscorum* and its Tl^+-R mutant strain under stressed and unstressed conditions.

Comparison of ^{14}C-proline uptake activity in cultures of (a) parent *N. muscorum* under NaCl stress; (b) parent *N. muscorum* under unstressed condition; (c) Tl^+-R under unstressed condition; (d) Tl^+-R strain under NaCl stress. The mean values from three independent experimental determinations are shown ±SEM where they exceed the dimensions of the symbols.

^{14}C-proline by the two cyanobacterial strains under stressed and unstressed conditions. As shown in Fig. 4, the parent strain, as expected, showed a more sizable increase in proline uptake activity under stressed conditions than under unstressed ones. In comparison, the mutant strain remained similar in proline uptake activity under both the conditions. The mutation to Tl^+-R in cyanobacterium *N. muscorum* clearly seems to have inactivated its salinity-inducible component of the proline uptake process.

Trk and kdp, respectively, are the known low-affinity and high-affinity system of K^+ transport in *E. coli* and other enterobacteria, either of which function in the uptake and accumulation of K^+ leading to an acquisition of salt tolerance under salinity stress (Rhoads and Epstein, 1978). The salinity inducible K^+ uptake and accumulation is the primary signal essential for an overaccumulation of secondary osmolytes, such as glycine betaine or proline, that protect the bacterial systems against salinity stress (Csonka and Hanson, 1991; Meury et al., 1985). Studies on the role of K^+ as a primary osmolyte in cyanobacterial salinity adaptation have shown that upshock treatment leads to an immediate uptake of Na^+ during the first 2 min, followed by uptake of K^+ during the next 20 min, during which Na^+ is extruded (Reed and Stewart, 1985). Because mutation to Tl^+-R phenotype in *N. muscorum* results in the deficiency only of salinity-inducible K^+ uptake without influencing the Na^+ uptake system, and because Tl^+-R mutant also exhibits a sizable loss of the salinity tolerance level, it is suggested that K^+ uptake and accumulation is an essential prerequisite for the adaptation of *N. muscorum* to salinity stress.

An overaccumulation of compatible organic solutes is a known consequence of salinity-inducible K^+ uptake in enterobacteria (Csonka and Hanson, 1991; Jasper, 1978). K^+ is known to function as a primary signal for the activation of genes that confer adaptation to salt stress in bacteria by promoting increased synthesis or uptake of compatible solutes such as proline (Csonka, 1989). A previous report states that a similar mechanism also remains operative in cyanobacterial adaptation to salt stress (Singh et al., 1996). The present finding, however, that Tl^+-R mutant exhibits physiological pleiotropy suffering simultaneous loss of salinity-inducible K^+ uptake process and proline-uptake process points out a close physiological linkage between salinity-inducible K^+ transport and salinity-inducible proline transport leading to an overaccumulation of proline and an acquisition of salinity tolerance, as already reported for the cyanobacterium *N. muscorum* (Singh et al., 1996). Tl^+-R mutant shows a defect in the salinity-inducible uptake of K^+ and proline. Further experiments are needed to analyze the nature of genetic determinant responsible for the observed close physiological linkage between the two phenotypes of the pleiotropic mutant.

To conclude, the present findings suggest that Tl^+-R genetic determinant is the cause of defective salinity-inducible K^+ uptake activity and proline uptake activity. The Tl^+-R genetic determinant also confers extreme salt sensitivity to the cyanobacterium. Because K^+ has been shown to function as a primary osmolyte responsible for controlling the production and overaccumulation of various salinity protectants in the bacterial system, it is suggested that also in *N. muscorum* the process of salt adaptation depends on the intracellular magnitude of the salinity-inducible primary osmolyte K^+, which is found to function in the control of salinity-inducible proline uptake and accumulation leading to the acquisition of salt tolerance in the cyanobacterium.

Acknowledgments

Financial assistance from the Department of Science and Technology (DST) to H.N.S. and from the Council of Scientific and Industrial Research (C.S.I.R.) to S.S. and J.B. is gratefully

acknowledged.

References

Bartels, D. and Nelson, D. (1994) Approaches to improve stress tolerance using molecular genetics. *Plant Cell Environ.*, **17**, 659–667.

Bohnert, H. J. and Jensen, R. C. (1996) Strategies for engineering water stress tolerance in plants. *TIB TECH Rev.*, **14**, 89–97.

Csonka, L. N. (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.*, **53**, 121–147.

Csonka, L. N. and Hanson, A. D. (1991) Prokaryotic osmoregulation: Genetics and physiology. *Annu. Rev. Microbiol.*, **45**, 569–606.

Damper, P. D., Epstein, W., Rosen, B. P., and Sorensen, E. N. (1997) Thallous ion is accumulated by potassium transport systems in *E. coli* *Biochemistry*, **18**, 4165–4169.

Gerloff, G. C., Fitzgerald, G. P., and Skoog, F. (1950) The isolation, purification and culture of blue green algae. *Am. J. Bot.*, **115**, 193–205.

Higgins, C. F. J., Cairney, D. A., Stirling, L., Sutherland, L., and Booth, I. R. (1987) Osmotic regulation of gene expression: Ionic strength as an intracellular signal? *TIBS*, **72**, 339–344.

Jasper, P. (1978) Potassium transport system of *R. capsulata*. *J. Bacteriol.*, **133**, 1314–1322.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with Folin-phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Mackinney, G. (1941) Absorption of chlorophyll solutions. *J. Biol. Chem.*, **140**, 315–322.

Mahan, M. J. and Csonka, L. N. (1983) Genetic analysis of the proBA gene of *S. typhimurium*: Physical and genetic analysis of the cloned proB$^+$A$^+$ genes of *E. coli* and a mutant allele that confers overaccumulation and enhanced osmotolerance. *J. Bacteriol.*, **156**, 1248–1262.

Meury, J., Robin, A., and Monnier-Champeix, P. (1985) Turgor-controlled K$^+$ fluxes and their pathway in *E. coli*. *Eur. J. Biochem.*, **151**, 613–619.

Rai, A. N., Rowell, P., and Stewart, W. D. P. (1984) Evidence for an ammonium transport system in free living and symbiotic cyanobacteria. *Arch. Microbiol.*, **137**, 241–246.

Reed, R. H. and Stewart, W. D. P. (1985) Evidence for turgor-sensitive K$^+$ influx in the cyanobacterium *A. variabilis* ATCC 29413 and *Synechocystis* PCC 6714. *Biochim. Biophys. Acta*, **812**, 155–162.

Rhoads, D. B. and Epstein, W. (1978) Cation transport in *E. coli*. IX. Regulation of K$^+$ transport. *J. Gen. Physiol.*, **72**, 283–295.

Singh, A. K., Chakravarthy, D., Singh, T. P. K., and Singh, H. N. (1996) Evidence for a role of L-proline as a salinity protectant in the cyanobacterium *Nostoc muscorum*. *Plant Cell Environ.*, **19**, 490–494.

Singh, S., Negi, S., Bharati, N., and Singh, H. N. (1994) Common nitrogen control of Cs$^+$ uptake, Cs$^+$ toxicity and ammonium (methylammonium) uptake in the cyanobacterium *Nostoc muscorum*. *FEMS Lett.*, **117**, 245–248.

NEHU LIBRARY
Acc 103560
Date 10-8-07
Class by
Sub.Heading by