STUDIES ON GENETIC AND OSMOTIC REGULATION OF DIAZOTROPHY, GLUTAMINE SYNTHETASE AND AMMONIA AND AMINO ACID TRANSPORT SYSTEMS

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

LIFE SCIENCES

SCHOOL OF LIFE SCIENCES

UNIVERSITY OF HYDERABAD

HYDERABAD-500 134

INDIA

SEPTEMBER, 1994

REGD. NO. PL - 5938 **KORADA SREENIVASA RAO**

DEDICATED TO MY MOTHER

SMT. KORADA JATLATHALLI

हैदराबाद विश्वविद्यालय
UNIVERSITY OF HYDERABAD
(विश्वविद्यालय की स्थापना संसद 1974 अधिनियम के अनुसार हुई)
(A Central University, established in 1974 by an Act of the Parliament)

SCHOOL OF LIFE SCIENCES

टेलेक्स/Telex : 425-2050 U HYD IN
ग्राम्स/Grams : Central Varsity
दुरभाष 253901, 902, 950, 951, 163
Phone : 253116, 117, 035, 064, 320
फॉक्स/Fax : 91-842--253145

डाकघर : केन्द्रीय विश्वविद्यालय परीसर
P.O. Central University Campus,
गच्चीबौली/Gachibowli
हैदराबाद-500 134 आ प्र (भारत)
HYDERABAD-500 134, A.P. (India)

संख्या :
Ref. No. :

तिथि :
Date : September 28, 1994

CERTIFICATE

This is to certify that the thesis entitled ***"Studies on Genetic and Osmotic Regulation of Diazotrophy, Glutamine Synthetase and Ammonia and Amino Acid Transport Systems"*** *is based on the results of the work done by Mr. K. Sreenivasa Rao for the degree of* ***Doctor of Philosophy*** *under my supervision. This work has not been submitted for any degree or diploma of any other University or Institution.*

Prof. A.R. Reddy
Head
Department of Plant Sciences
HEAD
Deptt. of Plant Sciences
School of Life Sciences
University of Hyderabad
HYDERABAD-500 134. (INDIA)

Prof. H.N. Singh
Supervisor
Prof. H. N. SINGH
School of Life Sciences
University of Hyderabad.
HYDERABAD-500134 (India)

28.9.94
Prof. N.C. Subrahmanyam FNA
Dean
School of Life Sciences
Dean, School of Life Sciences
University of Hyderabad,
Hyderabad-500 134. (India)

DECLARATION

The candidate declares that this work has been carried out by him under the supervision of Prof. H.N. Singh, School of Life Sciences, University of Hyderabad, Hyderabad - 500 134, India and that this thesis work has not been submitted for any degree or diploma of any other University or Institution.

Prof. H.N. Singh
Supervisor

Mr. K. Sreenivasa Rao
Candidate

15.9.94

Prof. H.N. SINGH
School of Life Sciences
University of Hyderabad
HYDERABAD-500 134 (India)

ACKNOWLEDGEMENTS

I take this opportunity to express my indebtedness to my esteemed and magnanimous teacher, Prof. H.N. Singh, for suggesting me this vital problem, for his supervision, constant encouragement and keen interest in the present study.

I am also thankful to Prof. N.C. Subrahmanyam FNA, Dean, School of Life Sciences and Prof. A.R. Reddy, Head, Department of Plant Sciences for providing me the necessary administrative help.

I extend my sincere thanks to Prof. R.P. Sharma, Prof. U.N. Singh, Dr. M. Ramanadham and Dr. M.N.V. Prasad for their help in times of need.

I have no words to acknowledge my indebtedness to my friend and colleague Mr. D. ChakravaLrty who influenced my academic as well as personal life and who shared every bit of my experience here.

I extend my sincere thanks to Drs. A.K. Singh, S.K. Verma, D.R. Modi, R.K. Singh, C.J. Yadav and Mr. Neeraj, Mr. Pramod and Mrs. Sangeetha Negi.

I am overwhelmed with love and pleasure in acknowledging the help rendered by my friends Ms. Shyamala, Mr. Suresh and Mr. Jayananda.

With gratitude, I sincerely acknowledge the cooperation and timely help rendered by Mr. Rajagopalan and Mr. Rajasekhar. The direct and indirect inspiration, which I got from Mr. Kiran, Mr. Vepa, Mr. Ramkumar and Ms. Sailaja is gratefully acknowledged.

My thanks are also due to Sri. E.V.S.N. Murty for his sincere help during the present work. Student community of the University (1991-92 batch), particularly the residents of C-Hostel are gratefully acknowledged for making my stay on the campus a memorable one.

Gratefully, I express deep sense of gratitude and indebtedness to my mother (Late) Smt. Ammaji, whose vision and foresight has been felt within myself throughout the present study. I am grateful my *beloved parents,* my sisters and brothers-in-law, for their inspiration, encouragement, love and affection, which enabled me to overcome the stress and strains during my stay on the campus.

Finally, the financial assistance given by the University Grants Commission (UGC) in the form of Junior and Senior Research Fellowships is gratefully acknowledged.

Korada Sreenivasa Rao

ABBREVIATIONS

ATP	Adenosine triphosphate
pBQ	Para benzoquinone
CFU	Colony Forming Unit
DCMU	3-(3,4-dichlorophenyl)-l,l-dimethyl urea
DMSO	Dimethyl sulfoxide
DCPIP	2,6-Dichlorophenol indophenol
EDA	Ethylene diamine
EDTA	Ethylene diamine tetraacetic acid
GS	Glutamine synthetase
GOGAT	Glutamine-oxo-glutamte amino transferase
x g	multiples of gravitational constant
HEPES	N-2-hydroxyethyl piperazine-N'-ethano sulfonic acid
Het$^-$	Phenotype indicating absence of heterocysts
het	Gene coding for heterocyst differentiation
MSX	L-Methionine-DL-Sulphoximine
MV	Methyl viologen
nif	Genes coding for nitrogen fixation
Nif$^-$	Phenotype indicating inability to fix nitrogen

ntr	Genes coding for overall 'nitrogen regulatory system'
PPT	Phosphinothricin
PS I	Photosystem I
PS II	Photosystem II
RUBISCO	Ribulose bisphosphate Carboxylase
TRICINE	N-tris-(hydroxymethyl)-methyl glycine
TRIS	Tris-(hydroxymethyl)-amino methane
TE	Tris-EDTA buffer
v/v	Percent 'volume in volume' number of cubic centimeters of a constituent in 100 cm^3 of solution
w/v	Percent weight in volume number of grams of constituent in 100 cm^3 of solution

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Nitrogen is the most limiting nutrient of bioproductivity. The success of modern agriculture, depends on the availability of fixed nitrogen and the present source of fixed nitrogen has been fossil fuel-based chemical nitrogen fertilizer factories. In view of the limited availability of fossil fuels, such a technology would soon become less practicable. It has been emphasized that an improvement in the efficiency of biological nitrogen fixation and conferring this ability to other systems such as cereal crops would significantly reduce the dependence on nitrogen fertilizers (Bri[illegible] 1977). Two principal approaches to the problem have been suggest[illegible] (Merrick and Dixon, 1984). The first one involves ma[illegible]pulat[illegible]n of existing association between nitrogen fixing bacteri[illegible] crop plants and the second involves the transferring of [illegible]rogen fixing (*nif*) genes into the plant genome. It is ag[illegible]st this background scientists have intensified their effort[illegible] understand the process of biological ni[illegible]gen fix[illegible]at [illegible]ular level and manipulate it accordingly, s[illegible] to ser[illegible] renewable source of nitrogen in mode[illegible]n agri[illegible]ture. [illegible]e diazotrophs capable of growth [illegible] the simple exper[illegible] of light, [illegible]ter and air have been suggested to be the ideal syste[illegible] as a source of fixed nitrogen in agricultural and non-agricultural ecosystems.

Although surrounded by an enormous reservoir of atmospheric nitrogen, most organisms cannot metabolically utilize this nitrogen source because of its empirical inertness at normal temperature. However, the enzyme nitrogenase has the ability to reduce nitrogen

to ammonia at normal temperature and pressure in diazotrophs. Unfortunately, biological nitrogen fixation is a prokaryotic characteristic restricted to a small number of selected diverse nutritional groups.

The cyanobacteria are an ancient and diverse group of gram negative, oxygenic, photoautotrophic prokaryotes (Stanier and Cohen-Bazire, 1977) growing and multiplying at the expense of light, water and air and contribute greatly to the nitrogen economy of aquatic and terrestrial habitats through their ability to fix atmospheric nitrogen (Fay, 1981). They incorporate simple unicellular organisms (with a frequent tendency for aggregation and colony formation), plain unbranched filamentous forms and strains with a more elaborate branched filamentous structure.

Cyanobacteria also popularly known as blue-green algae, are prokaryotes with oxygenic mode of photosynthesis similar to Chloroplast photosynthesis. Prochlorophyta like cyanobacteria also include prokaryotes with oxygenic mode of photosynthesis. But the basic difference between the two groups of prokaryotes lies in their accessory photosynthetic pigments which are phycobiliproteins in cyanobacteria and chlorophyll *b* in prochlorophyta (Lewin, 1976; Burger-Wiersma et al., 1986). While a majority of cyanobacteria are known to fix nitrogen, critical evidence for the occurrence of this process in prochlorophyta, is still lacking (Cohen-Bazire and Bryant, 1982; Patterson and Withers, 1982; Smith, 1982).

Studies on molecular biology of the members of cyanobacteria and prochlorophyta are important from the point of

view of their being possible ancestors to the present day chloroplasts in eukaryotic algae. In addition, three attributes of cyanobacteria make them a biological system of considerable interest from applied and biotechnological point of view. The first and the most singular attribute is, the diazotrophic mode of nutrition at the simple expense of light, water and air. The second equally important attribute is, the oxygenic mode of photoautotrophy. The modern tools of genetic engineering is being applied to identify, isolate and modify the appropriate photosynthetic genes for their possible engineering into chloroplasts of crop plants for better photosynthetic efficiency and photosynthetic yield in agriculture (Golden et al., 1986; Bryant and de Marsac, 1988; Williams, 1988). The third attribute is related to the ecology of the diazotrophic cyanobacteria under water-logged rice fields of tropical countries. Scientists have repeatedly observed indispensable role of cyanobacterial diazotrophs in nitrogen economy of rice agriculture under conditions with no provision for chemical nitrogen fertilizer (Singh, 1961). It is these observations which lead cyanobacterialogists to use them as a source of biofertilizer and in this regard, it is noteworthy to mention that in India cyanobacterial biofertilizer technology has been in operation at the Indian Agricultural Research Institute (IARI), New Delhi, since last two decades (Venkatraman, 1975). However, the scientific basis of such a biofertilizer based technology is still to be critically analyzed and discussed and accordingly modified for better efficiency and returns. For full realization of the cyanobacterial biofertilizer potential, it is essential to know clearly at molecular level, the genes specific to heterocyst organization, nitrogen fixation, oxygen protection, etc. In addition, equally

important area of research, relates to a clear understanding of the coupling among the process of nitrogen fixation, CO_2 fixation and photosynthesis. Subsequent to the understanding of these aspects at molecular level, one would like to know the genetics of ammonium assimilation, ammonia release, osmotolerance, herbicide resistance and derepression of nitrogen fixation. The following paragraphs would describe our present knowledge about those aspects with a summary of the achievements made during the present study along developing methods and strains of cyanobacteria suitable for use as a biofertilizer at commercial scale.

In a detailed survey, Stewart (1973) classified various diazotrophic cyanobacteria into three major groups based on the physiological parameters. They are :

(i) Non-heterocystous diazotrophic cyanobacteria, such as *Plectonema boryanum* capable of fixing nitrogen only under microaerobic or anaerobic conditions;

(ii) Non-heterocystous diazotrophic cyanobacteria, such as *Aphanothece* and *Gloeocapsa* sp. capable of fixing nitrogen aerobically;

(iii) Filamentous heterocystous cyanobacteria, such as *Nostoc* and *Anabaena* capable of fixing nitrogen aerobically.

The non-heterocystous cyanobacteria, unicellular or filamentous, are known to carry out the antagonistic processes of oxygenic photosynthesis and nitrogen fixation within the same cell. The question has been repeatedly asked about the mechanisms of the oxygen protection of cyanobacterial nitrogenase under such

conditions. A number of recent studies have been directed to understand these aspects at molecular level but it has not yet been possible to understand it completely (Fay, 1992; Gallon, 1992). At one extreme, are forms like *P. boryanum* which do not seem to possess any effective oxygen protective mechanism, thus rendering them to keep fixing nitrogen, only in the absence of oxygen or in a strongly reducing environment.* At the other extreme, are forms like *Trichodesmium* which are capable of carrying nitrogen fixation and oxygenic photosynthesis within the same cell under aerobic conditions. In forms like *Gloeothece,* temporal separation of oxygenic photosynthesis and nitrogenase activity has been suggested as possible mechanism of oxygen protection of cyanobacterial nitrogenase activity (Gallon and Chaplin, 1988). Recent evidences indicate that in *Gloeothece* sp. PC6909, the process of nitrogen fixation and oxygen photosynthesis can occur concurrently within the same cell (Orteg-Calvo and Stal, 1991). Such results suggest that depending on the growth conditions, diazotrophic cells might acquire one or the other mode of oxygen protection, that renders them nitrogen fixing under the given conditions. Not much is known about the conformational protection of cyanobacterial nitrogenase, from oxygen under aerobic conditions in non-heterocystous forms. The limited information available on the organization of *nif* genes in non-heterocystous forms indicates that the nitrogenase structural genes *nif* HDK occur contiguously without suffering any interruption as is characteristic of nitrogenase structural genes in heterocystous forms (Buikema and Haselkorn, 1993).

In heterocystous diazotrophic cyanobacteria heterocyst lacks photosystem II (PS II) activity, Rubisco activity, glutamate

synthase activity and nitrate reductase activity and contains functional synthetic and activity system of nitrogenase (Codd et al., 1980; Peterson et al., 1981; Kumar et al., 1985; Haselkorn et al., 1987). Heterocyst differentiation and nitrogenase synthesis and activity occurs when ammonium grown filaments are transferred to combined nitrogen-free medium for growth. During heterocyst differentiation from vegetative cells a number of structural and biochemical reorganizations occurs in order to render the heterocyst an anaerobic factory for nitrogenase synthesis and activity (Fleming and Haselkorn, 1973, 1974; Kulasooriya et al., 1972; Lang and Fay, 1971; Stewart, 1980). C : N ratio of *Nostoc* or *Anabaena* is known to increase from 4.5 to 8.1 during heterocyst differentiation (Kulasooriya et al., 1972). Recently Buikema and Haselkorn have shown experimental evidences for the operation of *het* R gene in heterocyst differentiation and of *pat* A gene in control of heterocyst spacing pattern (Buikema and Haselkorn, 1991; Liang et al., 1992). Similar genetic elements have also been shown to control heterocyst differentiation and heterocyst pattern formation in *N. muscorum* (Singh et al., 1994). The action and the role of *het* R gene product in heterocyst differentiation has not yet been established, although it possible that it is able to sense cellular C : N ratio that triggers the process of heterocyst formation. A comparative study of the organization of nitrogenase structural genes *nif* HDK on vegetative cells and heterocysts have revealed fundamental differences in that, while in vegetative cells, the *nif* K is separated from the *nif* D by a DNA element of 11 kb, in heterocysts, *nif* D and *nif* K are contiguous lacking the 11 kb DNA element characteristic of *nif* D gene in vegetative cell. Such rearrangement of *nif* genes in heterocysts, has been suggested to be

the reason for the expression of *nif* genes in heterocysts and not in vegetative cells (Golden et al., 1985). A second rearrangement during heterocyst differentiation, resulting in the deletion of a 55 kb long DNA element, occurring adjacent to the *nif* S gene has also been recorded (Golden et al., 1988). The unique organization and rearrangement of *nif* genes in heterocystous cyanobacteria, for functional expression of nitrogenase synthesis and activity is a general characteristic. The single exceptions to this characteristic gene expression in heterocystous nitrogen fixing cyanobacteria are *Fischerella* sp. ATCC27923 (Saville et al., 1987) and *Mastigocladus* (Singh and Stevens, 1992), where the *nif* structural genes are contiguous similar to that seen in non-heterocystous forms. Elhai and Wolk (1990) by the use of reporter gene *lux* fused to cyanobacterial *nif* promt ter have shown clearly that expression of *nif* genes within the heterocyst is developmentally but not environmentally (cf. Helber et al., 1988) regulated.

In *Azotobacter vinelandii* there are three genetically distinct nitrogenases, one containing molybdenum (Mo) called Mo-nitrogenase (Nitrogenase 1), another containing vanadium (V) called V-nitrogenase (Nitrogenase 2) and the third which neither contains Mo or V but iron (Nitrogenase 3) (Bishop et al., 1986). The three nitrogenases are known to be regulated differently in response to the presence of Mo or V. Evidences have been presented for the possible occurrence of V-nitrogenase in *N. muscorum* (Singh et al., 1993a,b) and *A. variabilis* (Kentemich et al., 1988). It would be interesting to know the nature of genetic elements that participate in regulation and organization of the genes for

V-nitrogenase and Mo-nitrogenase in general.

Cyanobacterial nitrogenase like nitrogenases of other prokaryotes is an enzyme capable of producing both ammonia and hydrogen. The loss of precious cellular energy and reductant in the form of hydrogen, during nitrogen fixation is considerable. However, nitrogen fixing cells seem to have evolved a strategy to overcome this wasteful process of hydrogen loss, by producing membrane bound uptake hydrogenase, that functions in recycling of nitrogenase, catalysed by hydrogen production, for reuse as a source of energy or reductant. The concurrent occurrence of nitrogenase activity in nitrogen fixation and uptake hydrogenase activity in hydrogen recycling, has been found to optimize the process of diazotrophy in both free-living and symbiotic forms including cyanobacteria (Evans et al., 1987; Bothe, 1982; Eisbrenner a Bothe, 1979). It is therefore important to know the relation between the physiology of uptake hydrogenase and nitrogenase in heterocystous cyanobacteria, as well as in non-heterocystous diazotrophic forms, as such knowledge is crucial to photoharvest ammonia or hydrogen from any diazotrophic cyanobacteria at commercial scale. In addition such findings will further encourage development of techniques for construction of diazotrophic cyanobacterial strains capable of sustained ammonia excretion during their growth and metabolism.

Glutamine synthetase and glutamate synthase (GS-GOGAT) pathway of ammonia assimilation, is the primary mode of ammonia nutrition in diazotrophic and non-diazotrophic cyanobacteria. Nitrogen regulation of GS activity has been studied in diazotrophic

cyanobacteria and it is known that there are two nitrogen regulated promoters involving transcriptional control of *gln* A gene (Turner et al., 1985). Heterocyst formation, nitrogenase synthesis and activity and ammonium transport system, all exhibit nitrogen control, whose nature and mechanism is still unknown (Haselkorn, 1978; Stewart, 1980; Rai et al., 1984; Singh et al., 1985; Singh et al., 1989). In enterobacteria, well defined *ntr* system of genetic control is known to operate in control of nitrogen assimilation (Merrick, 1988). But in cyanobacteria there is total lack of knowledge on this aspect, at genetic and molecular level. Some studies of genetic nature have demonstrated the participation of the product of *ntr* A gene in control of nitrate reductase, glutamine synthetase and methylammonium transport system in *Synechococcus* sp. PCC7942 (Vega-Palas et al., 1990). Similarly the operation of *ntr*-like regulatory gene product, in positive control of heterocyst formation, nitrogenase activity and ammonium transport system has been demonstrated in *N. muscorum* (Singh et al., 1989). The ratio of α-ketoglutarate to glutamine is known to generate the nitrogen status signal, in an enterobacterial cell. This ratio can legitimately be also called C : N ratio and an increase in it would mean the nitrogen starvation and a decrease in it would mean nitrogen surplus state. Nitrogen signal transduction mechanism has been well characterised at genetic and molecular level in enterobacteria. The sensor molecule of this ratio is a bifunctlonal enzyme called uridylyl transferase (UT)/uridylyl removing (UR) enzyme, which uridylylates P_{II} protein under nitrogen limitation and deuridylylates uridylylated P_{II} protein under nitrogen sufficiency. The uridylylated P_{II} protein is required for the activation of Ntr B protein kinase activity, leading to phosphorylation of Ntr C

protein. The phosphorylated Ntr C (Ntr C-P), is an activator of genes under the control of nitrogen regulated promoters. Thus nitrogen starvation signal, results in the formation of Ntr C-P protein which is a positive regulator of transcription from nitrogen regulated promoters. The nitrogen surplus signal generates deuridylylated form of P_{II} protein that promotes phosphatase activity of Ntr B leading to dephosphorylation of Ntr C-P with the ultimate result in switching on the transcription from various nitrogen regulated operons. Recently the P_{II} protein and its gene *gln* B have been shown to be present in a wide range of cyanobacteria and it has been shown to undergo phosphorylation in the absence of ammonium (nitrogen source) and/or under PS II light, *i.e.*, functioning of PS II is favoured over PS I light. This finding has directly implicated the role of P_{II} protein in co-ordination of oxygenic photosynthesis in inorganic nitrogen metabolism (Tsinoremas et al., 1991; Allen , 1992). Further studies along this line in diazotrophic cyanobacteria will unravel the fundamentals of coupling mechanism between photosynthesis and nitrogen nutrition. These findings suggest a possible occurrence of mutants which would be defective in both oxygenic photosynthesis as well as the nature of inorganic nitrogen nutrition. However, until today no such mutants were reported.

In the general scheme of plan for construction of cyanobacterial strains for use as biofertilizers the following attributes need to be present in them:

(i) Nitrogen fixation derepressed for combined nitrogen sources,

(ii) Ability to excrete ammonia derived from fixed nitrogen,

(iii) Resistance to rice field herbicides,

(iv) Resistance to osmolarity and salinity,

(v) Ability to compete against native forms under field conditions.

Derepressed mutants of *N. muscorum* for heterocyst formation and nitrogenase activity have been reported and techniques have also been developed to produce them under laboratory conditions (Singh and Singh, 1978; Singh et al., 1994). Mutants have also been isolated, specifically defective in ammonium transport system but without the ability to excrete ammonia during diazotrophic growth, thus, ruling out the possible role of ammonium transport system in extracellular liberation of ammonia (Singh et al., 1978; Singh et al., 1994). Mutants defective in GS activity, have normally been found to be ammonia excreting and such mutants have been isolated in diazotrophic cyanobacteria by selecting them for resistance to GS inhibitors like L-methionine DL-sulfoximine (MSX), phosphinothricin (PPT) and ethylene diamine (EDA) (Singh et al., 1983, 1988; Modi et al., 1991; Kerby et al., 1988). Thus in this area of cyanobacterial biofertilizer technology, methods are emerging for the generation of ammonia excreting strains. Similarly genes conferring resistance against DCMU, atrazine, basalin, machete and propanil have been identified and techniques developed for their transfer by the technique of genetic transformation in *N. muscorum* (Singh et al., 1987; Chapter 3 of this thesis).

The problem of salinity/osmolarity has been investigated at a very preliminary level, with a view to simply identify the nature of compatible solutes that occur in cyanobacterial forms naturally adapted to grow in hypersaline/hyperosmotic habitats. In some

freshwater forms the nature of compatible solutes have been identified in response to salinity/osmotic stresses. The general impression from such studies is that these organisms like bacteria and higher plants accumulate glycinebetaine, sucrose, trehalose, glucosyl-glycerol as principal osmolytes in salinity/osmotic adaptation (Mackay et al., 1983; Warr et al., 1988). However, this aspect has been studied in much detail only in enterobacterial forms like *E. coli* and S. *typhimurium* where the osmotic signal appears to be transduced through the *kdp* system of potassium transport and transport system of proline/glycinebetaine accumulation (Lucht and Bremer, 1994). This aspect has not been studied in cyanobacteria in much detail and one would like to know the molecular biology of osmotic signal transduction as well in order to construct strains that can tolerate hypersalinity/hyperosmolarity in agricultural ecosystems.

Scope of the present investigation

The thesis contains results of studies on construction of multiple herbicide resistant ammonia excreting strains of diazotrophic cyanobacterium *Nostoc muscorum*, as described in Chapter 3. The results for the first time suggest the successful application of techniques of genetic transformation and mutation in the production of multiple herbicide resistant ammonia excreting strains of the diazotrophic cyanobacterium *Nostoc muscorum*. *Nostoc muscorum* parent was transformed with the DNA from *Gloeocapsa* sp. containing genes for machete resistance, basal in resistance, atrazine resistance, 3-(3,4 dichlorophenyl)-1, 1-dimethyl urea

(DCMU) resistance and propanil resistance. The transformants were selected for machete resistance and examined for presence of remaining herbicide resistant phenotypes. Evidences suggest that the genetic determinants of all the five herbicide phenotypes to closely linked. Further experiments on photosystem I and photosystem II activities in the presence of the five herbicides suggested to be inhibitory to PS-II activity alone. Such transformants were used to generate their ammonia excreting L-methionine-DL-sulfoximine (MSX)-resistant, Phosphinothricin (PPT)-resistant and Ethylene diamine (EDA)-resistant GS strains. Results suggested that the inhibitor resistant GS is altered in its activity leading to excretion of ammonia in the growth medium. EDA-resistant GS was found to be excreting ammonia more efficiently over other GS strains under photoautotrophic diazotrophic conditions. These results do imply to have a long bearing . the construction of biofertilizer strains.

As a follow up to the work done in Chapter 3 studies were carried out on the mutational analysis of GS response to EDA. Two classes of mutants have been identified. One class of mutants assimilate EDA as a fixed nitrogen source, while the the other class excretes ammonia under diazotrophic growth conditions. A role for the *gln* A allele in control of its mutation frequency to EDA resistant GS phenotype is suggested. Chapter 4 of this thesis has concentrated on these aspects.

Chapter 5 deals with the characterization of methionine transport in the parent and mutant strains resistant to the methionine analogues : ethionine and norleucine, GS inhibitor :

phosphinothricin and to the growth inhibitor azide and its correlation to diazotrophy in the cyanobacterium.

Salinity and osmotic resistant mutants of *Nostoc muscorum* have been isolated and their cross-resistance relationship has been explored at the genetic and biochemical level. Osmotic and salinity modulation of growth, photosynthetic oxygen evolution, nitrogenase activity, GS activity and ammonium transport activity have also been studied in the parent and mutant strains. A preliminary study on the osmoprotective nature of proline is analyzed. The results are described in Chapter 6 of this thesis.

On the whole the thesis is strongly built around a central idea, aiming to construct strains with desirable genetic traits, using the classical methods of mutational manipulation and genetic transformation techniques for their use as a competent and scientifically viable biofertilizer.

CHAPTER 2

MATERIALS AND METHODS

Organisms used

The diazotrophic cyanobacterial strains *Nostoc muscorum*, and *Gloeocapsa* sp. were used in the present study. *N. muscorum* is a photoautotrophic, unbranched filamentous and diazotrophic cyanobacterium, which grows luxuriously in modified Chu No. 10 medium (Gerloff et al., 1950). Under these conditions, 5-6% of the vegetative cells get differentiated into regularly spaced heterocysts along the filament. The combined nitrogen free medium is represented as N2-medium and when it is supplemented with a final concentration of 5 mM KNO_3, 5 mM $NaNO_2$ and 1 mM NH_4Cl (buffered with 50 mM HEPES-NaOH, pH 8.5), represented as NO_3^-, NO_2^- and NH_4^+ media respectively. Addition of NO_3^-, NO_2^- and NH_4^+ inhibits heterocyst formation and nitrogen fixation under diazotrophic growth conditions (Stewart and Singh, 1975).

Gloeocapsa sp. is a unicellular aerobic diazotrophic cyanobacterium growing under photoautotrophic conditions. It was originally isolated from paddy fields and found to be resistant to herbicides, machete and basalin (Singh et al., 1987b). Axenic culture of *Gloeocapsa* sp. was grown in BG-11 medium which is a slight modification of G-11 of Hughes et al. (1958).

Axenic batch cultures of these strains were grown in liquid medium in air-conditioned illuminated culture room at a photon fluence rate of 50 µmol m s and temperature of 28 ± 2 C. Clonal cultures were maintained on agar slants which were prepared by adding 1.2% (v/v) Agar-Agar to the liquid medium prior to autoclaving.

Culture media

The modified Chu No. 10 medium (Gerloff et al., 1950) used for growing the cyanobacterial strains *N. muscorum* had the composition indicated in Table 2.1a & b.

TABLE 2.1a

Macronutrient Stock (grams **100** ml^{-1})

Macro nutrient	Concentration
1 Calcium chloride	1.10
2 Magnesium sulphate	0.50
3 Dipotassium hydrogen phosphate	0.20
4 Sodium silicate	0.88 .
5 Sodium carbonate	0.40
6 (a) Citric acid	0.06
(b) Ferric citrate	0.06

TABLE 2.1b

Hicronutrient stock (grams L^{-1})

	Micro nutrient	Concentration
1	Manganous chloride •	1.81
2	Boric acid	2.86
3	Zinc sulphate	0.222
4	Copper sulphate	0.079
5	Sodium molybdate	0.0177
6	Cobalt nitrate	0.05

5 ml each of the macronutient and 1 ml micronutrient solutions were taken in a final volume of 1 liter double distilled water and pH was adjusted to 8.5-9.0 (with 0.1 N NaOH), prior to autoclaving. NO_3^- , NO_2^- and NH_4^+ were added to the above medium when required.

TABLE 2.2

Composition of BG-11 medium (grams 100 ml^{-1})

	Macronutrient	Concentration
1	Dipotassium hydrogen phosphate	4.0
2	Magnesium sulphate	7.5
3	Calcium chloride	3.6
4	Fe-EDTA	0.1
5	Sodium carbonate	2.0
6	(a) Citric acid	0.6
	(b) Ferric ammonium citrate	0.6

The micronutrient stock used was essentially as that described for modified Chu No. 10 medium.

1 ml of each macronutrient and 1 ml of micronutrient was added in a final volume of 1 liter of double distilled water and the pH was adjusted to 8.5-9.0 before autoclaving. NO_3, NO_2^- and NH_4^+ were added to the above medium where required.

Sterilization

Culture media were sterilized prior to inoculation by autoclaving at 15 lbs sq^{-1} inch pressure for 15 min at 121 °C.

Growth measurement

Cyanobacterial cultures are homogenous in liquid medium and therefore, a direct absorbance change at 663 nm is proportional to its multiplication and can be used for growth measurements, but **growth** also results in an increase of biomass. Therefore parameters like chlorophyll a and protein will also facilitate the measurement of growth in cyanobacteria. The growth in the present study was usually measured at regular intervals by estimating chlorophyll a.

Estimation of Chl a

The chlorophyll content was estimated by using the method of Mackinney (1941) by extracting into 100% methanol. Five ml of the culture was withdrawn and centrifuged at 2,000 RPM for 15 min and the supernatant was discarded. The pellet was dissolved in equal volume of 100% methanol. Then the tubes were kept in hot water bath maintained at 60 °C for 10 min. The solution was again centrifuged at 2,000 RPM for 10 min and the absorbance of the chlorophyll extract was read at 665 nm. The chlorophyll a content was calculated by using the following formula.

$$13.42 \times A_{663} = \mu g \text{ Chlorophyll a } ml^{-1}$$

Estimation of cellular protein

The protein content of the crude extracts was estimated by using the method of Lowry et al. (1951). The protein in the crude extracts was by precipitated by adding equal volume of 10% (w/v) trichloroacetic acid (Bailey et al., 1967). The mixture was left overnight at 25 °C and then centrifuged at 3,000 x g for 10 min. The precipitate was dissolved in 1 N NaOH and an aliquot was withdrawn for estimating the protein content. A standard curve for protein estimation was prepared by using bovine serum albumin (BSA).

Measurement of heterocyst frequency

Heterocyst frequency was calculated as the number of heterocysts per 100 vegetative cells by using light microscopic observation of the filaments of the cyanobacterium used. Cells were counted using a haemocytometer.

Measurement of electron transport **activity**

Cyanobacterial cells were harvested and washed twice with harvesting buffer containing 20 mM HEPES-NaOH (pH 7.5), 1 mM $CaCl_2$ and 7.5% (w/v) polyethylene glycol (PEG-4000) and resuspended in the same buffer. The cell suspension was centrifuged at 10,000 x *g* for 10 min at 25 °C. Then the cells were suspended in the reaction buffer consisting of 25 mM HEPES-NaOH (pH 7.5) and 20 mM NaCl. The cells were used for measuring PS II activity.

The photochemical activities (PS I and PS II) were measured by following the method of Robinson (1982). Assays of elctron transport activities were carried out using thermostated (28 °C) glass cuvette, fitted within a Clark-type oxygen electrode (Gilson Medical Electronics, Model 5/6 Oxygraph, USA). The cells were illuminated with saturated light (450 W m^{-2}) obtained from a projector at the surface of vessel. Thermostated water bath was used to maintain a constant temperature (28 °C) during the illumination. The changes in the oxygen concentration were recorded.

Photosystem II assay

p-benzoquinon (pBQ) mediated electron tranpsort activity of PS II (H_2O ——→ pBQ) was used for intact cells. Being a lipophilic compound benzoquinone enters into the intact cells and accepts electron at the plastoquinone position (Trebst, 1974). The reaction mixture (3 ml) contanined reaction buffer, 0.5 mM pBQ and the intact cells (equivalent to 12-15 µg Chl a). The sampler were illuminated with the help of a 300 W projector lamp which provided a saturated incandescent light (450 W m) on the surface of the sample cuvette. The changes in O_2 concentration were recorded. Photosynthetic rate was estimated by using intact filaments.

PS II activity of the sonicated cells was measured spectrophotometrically by following the reduction of DCPIP (Mannar and Bose, 1985). Cyanobacterial cells were harvested and washed twice with harvesting buffer (pH 7.8) consisting of 0.3 M sucrose, 10 mM tricine and 0.1% (w/v) BSA and resuspended in the same buffer.

Cells were broken by ultrasonication. The extract was centrifuged at 10,000 x *g* for 30 min at 25 °C. The pellet was used for measruing the PS II activity. The reaction mixture (1.6 ml) for assay of Hill reaction consisted of 5 mM $MgCl_2$, 10 mM NaCl, 5 mM NH_4Cl and 25 μM freshly prepared DCPIP. The thylakoid membranes were added to the above reaction mixture at a concentration of 12 μg. The samples were illuminated for 2 min with saturating white light. The reduction of DCPIP was measured at 600 nm.

$$\mu\text{mol DCPIP reduced mg}^{-1}\ \text{Chl a h}^{-1} = \frac{A_{600} \times 60 \times 1000}{19 \times \mu\text{g Chl a}}$$

Preparation of spheroplasts

Spheroplasts for measurement of PS I activity were prepared by using the method of Peter and Sherman (1978). The cyanobacterial cells in exponential growth phase were harvested and washed with 20 mM Tricine-KOH buffer (pH 7.5) containing 0.4 M sucrose, 10 mM KCl and 10 mM EDTA. The cells were suspended in the same buffer and incubated with 0.5 mg ml^{-1} of lysozyme for 20 min at 37 °C. After the lysozyme treatment, the cyanobacterial cells were centrifuged at 5,000 x *g* for 15 min at 4 °C and used for the measurement of PS I activity.

Photosystem I assay :

PS I mediated electron transport activity was estimated as a measure of oxygen uptake due to methyl viologen (MV) photoreduction

and its subsequent autooxidation with 2,6-dichlorophenol indophenol (DCPIP) and sodium ascorbate donor coupled. The experimental set up was essentially similar to the one described for PS II assay. The reaction mixture (1.6 ml) consisted of 25 mM HEPES-NaOH (pH 7.5), 10 μM DCPIP, 2 mM sodium ascorbate, 0.5 mM MV, 1 mM sodium azide and 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). Spheroplasts equivalent to 15-20 μg Chl a was used for each estimation (Robinson et al., 1982). Photochemical activity estimated in terms of O_2 exchange has been expressed as μmol O_2 evolved/consumed mg^{-1} Chl a h^{-1}.

Radiolabelled transport assays

The method of Rai et al. (1984) was used for the measurement of uptake of amino acids. Exponentially growning cyanobacterial cells were harvested by centrifugation and washed twice with 10 mM HEPES-NaOH buffer (pH 7.0) and resuspended in the same, equilibrated for 30 min at 25 °C under photoautotrophic growth conditions. [U-^{14}C] labelled substrate was then added to the cell suspension at a required final concentration. Four hundred microliters were withdrawn at different time intervals and separated from the bathing medium by silicon-oil microcentrifugation technique (Scott and Nicholls, 1980) and counted for cellular level of radio labelled compound by using Beckman LS 1800 Liquid Scintillation Counter. Non-specific binding of radioactivity was determined by measuring its incorporation in toluene treated cells (Rai et al., 1984). The value was always substracted from the value obtained tolune-untreated samples.

Enzyme assays

Nitrogenase (EC 1.8.6.1)

Nitrogenase activity was measured as descrbed by Stewart and Singh (1975). Assay was done in test tubes containing cyanobacterial cells (10-15 μg Chl a) by evacuation and replaced by the desired gas phase which contained 10% (v/v) acetylene. The tubes were incubated with intermittent shaking at 28 °C and a photon fluence rate of 50 μmol m^{-2} s^{-1}. After 1 h of incubation ethylene production was assayed gas chromatographically using CIC Gas Chromatograph. The nitrogenase activity was expressed in terms of μmol C_2H_4 formed mg^{-1} Chl a h^{-1}.

Glutamine synthetase (EC 6.3.1.2)

Glutamine synthetase was assayed under *in vitro* conditions. Cultures were harvested by centrifugation at 2,000 RPM and washed with buffer A (Tris-HCl, pH 7.5) followed by washing with buffer B (buffer A + 5 mM $MnCl_2$, 10 mM sodium glutamate, 5 mM 2-mercaptoethanol and 1 mM EDTA, pH 7.5). The pellet was resuspended in buffer B and cells were broken in liquid nitrogen and the cell extract thus obtained was centrifuged at 15,000 RPM (Remi C24 model Cooling Centrifuge) for 30 min.

Glutamine synthetase (transferase)(EC 6.3.1.2);

The enzyme was assayed using the method of Sampio et al. (1979). The reaction mixture (1.0 ml) in addition to enzyme extract contained 40 mM Tris-HCl (pH 7.0), 3 mM $MnCl_2$, 20 mM potasium arsenate, 0.4 mM sodium-EDTA, 60 mM hydroxalamine (neutralized with 2

N NaOH before use) and 30 mM glutamine. The reaction was allowed to proceed for 10 rain at 37 °C in dark and terminated by adding 2 ml of stop mixture (4 : 1 : 0.5 (v/v) of $FeCl_3$, 24% TCA (w/v) and 6 N HC1). The γ-glutamyl hydroaxamate formed was estimated by measuring the absorbance at 540 nm. The amount of γ-glutamyl hydroxamate formed was determined from the standard curve. The transferase activity was espressed as nmol γ-glutamyl hydroxamate formed mg^{-1} protein min^{-1}.

Glutamine synthetase (biosynthetic) (EC 6.3.1.2)

Glutamine synthetase (biosynthetic) activity was measured by the method of Kingdon et al. (1968) using the following reagents: 60 μmol sodium glutamate, 3 μmol ATP, 150 μmol Tris-HCl (pH 7.5), 200 μmol NH_4Cl, 150 μmol KCl, 150 μmol $MgCl_2$, 0.45 μmol NADH, 0.5 μmol phosphoenolpyruvate (PEP), 20 Units lactate dehydrogenase (LDH) and 8 Units pyruvate kinase (PK).

To 1 ml of the enzyme extract 200 μl each of the reagents were added. The absorbance was measured at 340 nm in Hitachi Spectrophotometer. The GS (biosynthetic) activity was expressed in terms of nmol NADH oxidized mg^{-1} protein min^{-1}.

Chemicals

MSX, EDA, Tris-HCl, potassium arsenate, Na-EDTA, hydroxalamine, sodium glutamate, Na-ATP, NADH, PEP, LDH, PK, PBQ and lysozyme were procured from Sigma Chemical Co., USA; PPT from HOECHST, Germany. All other chemicals were of analytical grade from BDH or Fluka Chemie AG.

The radiolabelled compounds were procured **from BRIT, Bombay, India.**

Statistical Analysis

Standard Error *(SE)*

The standard error about mean of the data on specific observations was calculated as :

$$\text{Standard Error (SE)} = \frac{\text{Standard deviation (SD)}}{\sqrt{n}}$$

where n = number of variants

Hence, the value was represented as, mean of the value ± SEM.

CHAPTER 3

ENGINEERING MULTIPLE HERBICIDE RESISTANT AMMONIA EXCRETING PHENOTYPES IN *NOSTOC MUSCORUM* THROUGH THE TECHNIQUES OF GENETIC TRANSFORMATION AND MUTATIONAL MANIPULATION

Introduction

Contribution of diazotrophic cyanobacteria to the nitrogen fertility of paddy fields and thereby to the productivity and yields of rice crop result from mineralization of their biomass and not from immediate availability of fixed nitrogen (Singh, 1961; Roger and Kulasooriya, 1980; Tirol et al., 1982). The cyanobiont *Azolla* in symbiotic state lacks effective ammonia assimilating system and therefore it can supply the host plant 'ith ammonia derived from N_2 fixation (Rai et al., 1984). Strains of free living diazotrophic cyanobacteria defective in ammonia assimilation leading to extracellular liberation of N_2-derived ammonia, would be ideal for use as biofertilizer in paddy fields.

The glutamine synthetase-glutamate synthase (GS-GOGAT) is the main primary route of ammonia assimilation in free-living aerobic, N_2-fixing, heterocystous cyanobacteria (Dharmwardene et al., 1973; Stewart and Rowell, 1975, Wolk et al., 1976; Thomas et al., 1977; Rowell et al., 1977). The inhibitor of glutamine synthetase (GS), L-Methionine-DL-sulfoximine (MSX) has been observed to cause excretion of ammonia resulting from N_2 fixation and the release of heterocyst and nitrogenase production from ammonia

inhibition by a mechanism involving inhibition of glutamine synthetase activity (Stewart and Rowell, 1975).

The glutamine synthetase catalyzes glutamine formation from ammonia and glutamate and the MSX inhibition of GS activity in turn results in the inhibition of glutamine formation (Thomas et al., 1977; Stewart, 1980).

MSX resistance involves production of an MSX-resistant GS severely altered in its γ-glutamyl-transferase activity and this mutant excretes a considerable amount of ammonia during photoautotrophic N_2-fixation (Singh et al., 1983). It was also shown that the production of MSX-resistant strains as a useful technique for isolating ammonia excreting mutants of N_2-fixing cyanobacteria with potential for biological production of ammonia under complete autotrophic conditions. Such ammonia excreting diazotrophic cyanobacterial strains have been shown to supply N_2-derived ammonia to rice and wheat plants under laboratory conditions (Lattore et al., 1986; Spiller and Gunasekharan, 1990).

However, successful exploitation of ammonia excreting diazotrophic cyanobacteria as a source of biofertilizer in agriculture require them to have rice field herbicide resistant phenotypes as well. These strains must have inherent ability to grow and fix nitrogen in the presence of commonly used herbicides.

Cyanobacteria in general are similar to the higher plants in respect of photosynthetic physiology and photoautotrophic

nutrition. Most commonly used herbicides are normally those which are inhibitory to the photosynthetic processes and photoautotrophic mode of growth. It is therefore not surprising to find that rice field herbicides are found to be growth toxic to cyanobacteria. Creation of rice field herbicide resistant strains scientifically strengthen considerably the effectiveness of their meaningful use as biofertilizer in rice ecosystems, regularly subjected to herbicide treatment. This will also serve as a source of herbicide resistant gene pools for the crop plants.

Genetics of herbicide resistance in cyanobacteria is little known. Few reports indicate presence of DCMU- and atrazine-resistant genes in *Plectonema boryanum* (Mallison and Cannon, 1984) and DCMU-resistant gene in *Nostoc muscorum* (Vaishampayan, 1984). If modern agricultural technologies have to be improved for more production in agriculture, there is no alternative to development of effective biotechnology for raising herbicide resistant crops and biofertilizers.

Some studies have dealt with the physiological and growth toxic effects of various rice field herbicides on N_2-fixing and non N_2-fixing cyanobacteria (Singh, 1973, 1974; Das and Singh, 1977; Venkataraman and Rajyalakshmi, 1977). Herbicides such as machete has been reported not only growth inhibitor but also as a strong mutagen in *Nostoc muscorum* (Singh and Vaishampayan, 1978; Singh et al., 1979). Singh and Tiwari (1988) have studied the effect of herbicides machete, basalin and propanil on *Gloeocapsa*, *Nostoc muscorum* and *Anabaena* ATCC 27893 in relation to inoculum size and

suggested that the incolum size is a very important parameter to determination of resistance of cyanobacteria to such herbicides. P.K. Singh group at CRRI, Cuttack (India) has involved in the evaluation of the effect of a number of pesticides on diazotrophic cyanobacteria under laboratory and field conditions (Das and Singh, 1977b, 1979; Kar and Singh, 1978, 1979).

DCMU and atrazine are specific inhibitors of PS II activity and the target of their inhibitory action had been shown to be 32 kDa protein of the PS II reaction centre (Brusslan and Haselkorn, 1989). Propanil is known to be the inhibitor of Hill reaction and believed to inhibit the process by its interaction with cytb553 (Good, 1961; Nishimura and Takarmiya, 1966). Machete has been suggested to be an inhibitor of protein synthesis in susceptible plants. Similarly the mode of action of basalin is not known in higher plant systems. Singh et al. (1985) have isolated a diazotrophic *Gloeocapsa* sp. from rice fields, which shows extreme resistance to the growth inhibition by machete and basalin and have found evidently for the genetic basis for this herbicide resistance. Evidently this does seems to be a great possibility of naturally occurring forms of diazotrophic cyanobacteria with in built genetic resistance to variety of other rice field herbicides. Therefore, identification of such source of herbicide resistance genes and methods if developed for their genetic transfer to appropriate diazotrophic strains is vital to use the diazotrophic cyanobacteria as biofertilizer.

Genetic basis of phenotypes are analyzed basically by two methods, one mutational and other gene transfer system. Cyanobacterial genetics lag far behind and only from mid 1970's serious attempts started to develop methods of gene transfer system. Kumar (1962) found apparent genetic recombination for antibiotic markers in *Anacystis nidulans.* Stewart and Singh (1975) reported transfer of *nif* genes from parent *N. moscorum* to its non N_2-fixing mutant strain. Singh and Singh (1983) analyzed the nature of *het* gene, *nif* gene and their common regulator gene by recombination method and found evidence to conclude distinct classes of genes operating in organization of heterocyst and nitrogenase.

Transformation as a mode of gene transfer system leading to production of genetic recombinants for antibiotic resistance markers was demonstrated in *Anacystis nidulans* 602 by Shestakov and Khyen (1970). Subsequent studies have also showed transformation as a process of gene transfer system taking place in *Aphanocepsa* 674 (Astier and Espardellier, 1976), *Gloeocapsa apicola* (Devilly and Houghton, 1977), *Agmenellum quadruplicatium* PR-6 (Stevens and Porter, 1980) and *Synechocystis* PCC 6803 (Grigorieva and Shestokov, 1982). Such studies used genetic markers which were chromosomally located and thus provided evidence for naturally occurring chromosomal transformation systems in cyanobacteria.

Demonstration of plasmid transformation in cyanobacteria is expected to have plasmid cloning vehicles that can be used to produce cyanobacterial merodiploids as well as to introduce foreign genes in cyanobacterial cells. The development and establishment of

efficient transformation system with chromosomal or plasmid DNA in unicellular cyanobacteria has opened the possibility for wide range of genetic analysis based on the methods of transposon mutagenesis, recombination mutagenesis and site directed mutagenesis. These methods have helped in identifying and characterizing genes specifically involved in organization of PS II in unicellular forms (Williams and Szalay, 1983; Vermaas et al., 1987; Brusslan and Haselkorn, 1989). Transformation as a system of gene transfer process has not been found as successfully working in heterocystous filamentous bacteria as it has been found in unicellular cyanobacteria. Recently Singh et al. (1987 a) demonstrated genetic transformation of azide assimilating phenotype from *Nostoc muscorum* to *Anabaena doliolum*.

In this chapter, we present evidence to show that DNA is the basis of five herbicide resistant phenotypes machete resistance (Mat^r), basalin resistance (Bas^r), DCMU resistance ($DCMU^r$), atrazine resistance (Atr^r) and propanil resistance (Prp^r) in *Gloeocapsa* sp., that Bas^r, $DCMU^r$, Atr^r and Prp^r phenotypes are cotransformable with Mat^r phenotype from *Gloeocapsa* sp. to *N. muscorum*, thus suggesting them to be closely genetically related. Herbicide sensitive/resistance nature of PS II activity is primarily the physiological basis of cyanobacterial growth response to the five herbicides and that L-methionine-DL-sulfoximine (Msx^r), ethylene diamine (Eda^r) or phosphiothricin (Ppt^r) resistant mutant GS strains are defective in GS (transferase) activity and proficient in excretion of N_2-derived ammonia.

Experimental Procedure

Organisms

Parent *Nostoc muscorum* and multiple herbicide resistant aerobic diazotroph *Gloeocapsa* sp. were grown under diazotrophic conditions as described in detail in Chapter 2.

Total genomic DNA isolation and purification

Total genomic DNA was isolated from cyanobacterial strains by the method of Mazur et al., (1980) with appropriate modification. Cyanobacterial cells in their late exponential phase were harvested, washed and resuspended in 50 mM Tris-Cl, 100 mM EDTA, 25% (w/v) sucrose, pH 8.0 (TES) at 1 gm (wet weight) per 10 ml TES. Lysozyme in 250 mM Tris-Cl (pH 8.0) was added to a final concentration of 10 $\mu g\ ml^{-1}$ and incubated at 37 °C for 1 h. Then the cells were gently lysed by adding sodium dodecyl sulphate (SDS) to a final concentration of 1%. Immediately NaCl was added to a final concentration of 1 M and the mixture was extracted once with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and then twice with chloroform : isoamyl alcohol (24 : 1), chilled ethanol (2 vol) was added to the aqueous phase, and the high molecular weight DNA was spooled onto a glass rod and dissolved in an appropriate volume of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE). The DNA was treated with ribonuclease (RNase-A) at 100 $\mu g\ ml^{-1}$ (predigested at 70 °C for 10 min) and then with pronase (at 50 $\mu g\ ml^{-1}$). DNA was again precipitated with ethanol and the aqueous phase was dialysed overnight against TE (pH 8.0) with at least three changes of buffer and dissolved in TE. The purity of DNA was analyzed

spectrophotometrically and integrity was checked by electrophoresis on agarose gel (0.8%) before using it in transformation experiment. Serial glassware and buffer solutions were used throughout.

Agarose gel electrophoresis

This was according to the method described by Maniatis et al., (1982). Agarose gel of 0.8% (w/v) was prepared in electrophoresis buffer (90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA) TBE using type I low endo-osmotic agarose. 15 μl of DNA sample was mixed with 5 μl of loading buffer (0.25% bromophenol blue and 40% (w/v) sucrose in sterile distilled water) and then slowly loaded into the slots of the submerged gel using a P 20 disposable micropipettman (Gilson). The enough electrophoresis buffer containing 0.5 μg ml^{-1} ethidium bromide was added till the gel is immersed to a depth of about. 1 mm. A low voltage of 50 mV was applied to start the electrophoresis. After the run, the gel was directly visualized using trans UV-illuminator to check the integrity of DNA sample.

Sensitivity to DNase and RNase

The DNA isolated from cyanobacterial cells was treated with DNase (100 μg ml^{-1}) for 15 min at 37 °C and checked by gel electrophoresis for its sensitivity. Similarly the DNA was treated with RNase A.

Determination of colony forming units (CFU)

Filamentous cyanobacteria in its late exponential phase of growth contains hundreds of cells per filament. Even though every

vegetative cell has the capacity to form a colony, the whole filament also forms a singly colony, after plating on agar plates. Hence, for the sake of convenience to calculate transformation frequency, such long filaments were broken gently by vortexing (for 30-60 sec) in the presence of sterile glass beads. This process produced filaments with an average of 100 cells per filament. This unit was taken as CFU. The whole process was carried out aseptically and after spreading the CFU on agar plate the percentage survival was found to be greater than 90%.

Permeaplast preparation

Permeaplasts were made of recipient cells according to Daniell and McFadden (1986) with appropriate modifications. Freshly prepared modified Chu No. 10 medium 200 ml was inoculated with 15 ml of exponentially growing cultures to give a final concentration of 2×10^8 cells ml^{-1} (2×10^6 CFU ml^{-1}) and the cells were grown overnight under standard growth conditions. Cells were centrifuged and washed once in fresh medium and then twice with 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) (TE) buffer and resuspended in 50 ml of the same buffer and 2 mg ml^{-1} of lysozyme was added and incubated at 37 °C for 1 h in 150 ml Erlenmyer flask under cool white fluorescent light with intermittent shaking. After 1 h of incubation the cells were washed thoroughly in fresh modified Chu No. 10 medium and resuspended in 200 ml of fresh medium. Permeaplasts of exponentially growing cultures were prepared and used in transformation experiments.

Transformation procedure

The permeaplasts of recipient cell cultures prepared by 2 h treatment were used for transformation experiments. one milliliter of permeaplast at a concentration of 2 x 10^6 CFU ml^{-1} was taken and donor DNA at a final concentration of 1 µg ml^{-1} was added. The transformation mixture was incubated at 28 °C under a light intensity of 50 µmol m^{-2} s^{-1} for 60 min. Then the DNA uptake was stopped by addition of DNase I (100 µg ml^{-1}) in presence of 5 mM $MgCl_2$ and was incubated at 37 °C for 10 min and then spread on nutrient agar plates at a concentration of 2 x 10^5 CFU per plate. The drug was added underneath the nutrient agar after 72 h of seeding of DNA treated permeaplast. This basic procedure was modified in various ways to test different parameters.

Standardization of conditions

(a) Effect of washing on transformation : Exponentially growing cells were harvested by centrifugation and resuspended in fresh diazotrophic medium at a concentration of 2 x 10^6 CFU per ml. Two different sets of cells were taken, washed and resuspended in N_2-medium and TE buffer (pH 7.5) respectively. Permeaplasts were prepared and transformation was carried out as described earlier in this chapter.

(b) DNA contact period and DNA concentration versus transformation frequency : After repeated testing the optimum DNA concentration which gives maximum number of transformants was found to be 1 µg per 2 x 10^6 CFU of

recipient cells and the optimum contact period was found to be 60 min (data not shown). The DNA uptake was stopped by the addition of DNase 1.

(c) Effect of light and dark on transformation efficiency : The permeaplast of recipient cells were treated with 1 $\mu g\ ml^{-1}$ donor DNA and incubated at 28 °C. One set was incubated in presence of photosynthetic light and another set in dark and after 1 h the process of DNA uptake was stopped by the addition of DNase I (100 $\mu g\ a"^{1}$). Samples were taken from both the sets and plated on non selective agar plates and after 72 h the selective drug was added underneath the agar aseptically.

Isolation of ammonia excreting mutant strains of parent and multiple herbicide resistant transformant Nostoc muscorum

Parent *N. muscorum* and its multiple herbicide resistant transformant were grown separately in bulk under diazotrophic growth conditions and then screened for the presence of spontaneous mutants resistant to growth inhibitory concentration of MSX (0.36 $\mu g\ ml^{-1}$) and PPT (0.18 $\mu g\ ml"^{1}$) as described by Singh et al. (1983) and of EDA (0.9 $\mu g\ ml^{-1}$) as described by Stewart et al. (1987). These spontaneous resistant mutants thus isolated from the two strains were then examined for their inhibitor resistant GS (transferase) activity and only such mutants were isolated and grown for studying their ammonia excreting ability during photoautotrophic diazotrophic growth.

Ammonia estimation

Ammonia estimation was done following the method **of Solorzano** (1969).

(a) Reagents :

(1) Phenol-alcohol solution : 10 g of distilled phenol in 100 ml 95% (v/v) Ethanol.

(2) Sodium nitroprusside : 0.5% (w/v) in distilled water.

(3) Alkaline solution : 100 g trisodium citrate + 5 g NaOH in 500 ml of distilled water.

(4) Sodium hypochlorite : Commercial grade solution.

(5) Oxidizing solution : 100 ml (3) + 2.5 ml (4).

(b) Procedure : 10 ml of sample +0.4 ml of phenol solution + 0.4 ml of sodium nitroprusside solution + 1 ml of oxidizing solution. After 1 h, the blue colour of indophenol was read at 640 nm.

Chemicals

The enzymes and chemicals used in DNA isolation and purification were purchased from Sigma Chemical Co., USA. All other chemicals used were of analytical grade from BDH, India.

Herbicides

Machete : Trade name of 2-chloro-2',6'-diethyl-*N*- (butoxy-methyl) acetanilide and commonly known as "Butachlor" was obtained from Monsanto Chemical PVT Ltd., Missourie, USA, having a concentration of 50%.

Basalin : Trade name of *N*-{2'-chloroethyl) 2,6-dinitro-*N*- propyl-4-(trifluoromethyl) aniline and commonly known as "Fluchloradin" was obtained from BAST Aktiengesell Schaff, Germany having a concentration of 48%.

Atrazine : Trade name of 2-chloro-4-(ethylamino)-6-(isopropyl amino) S-triazine was obtained BASF India Ltd. The stock solution of 1 mM was prepared in dimethyl sulfoxide (DMS) taking care to keep the final concentration of DMS in the reaction mixture below 0. 1%.

DCMU : Trade name of 3- (3, 4-dichlrophenyl)-1, 1 -dimethyl urea commonly known as "Diuron" was obtained from Sigma Chemical Co. , USA. DCMU was sparingly soluble in water and a stock of 1 mM was made in isopropanol taking care to keep the final concentration below 0.1%.

Propanil : Trade name of 3',4'-dichloropropionaniline and commonly knwon as "Stam f-34" was obtained from Indofil Chemicals Ltd., India, having a concentration of 36%.

Results

Gloeocapsa sp. was shown to contain two herbicide resistant genes, machete resistant (Mat^r) and basalin resistant (Bas^r) which was successfully cotransformed to *Nostoc muscorum* (Singh et al., 1987b). Now it has been found that *Gloeocapsa* sp. contains resistant genes for machete, basalin, atrazine, DCMU and propanil. The aim of the present investigation was to generate transformants of *N. muscorum* resistant to the above herbicides and then use one of such transformant to select out ammonia excreting MSX-resistant, EDA-resistant or PPT-resistant GS strains.

Normally exponentially growing cells of *N. muscorum* when exposed to exogenous DNA of *Gloeocapsa* sp. gave erratic results with respect to machete resistant transformants under photosynthetic conditions. Transformation experiments involving calcium treatment of recipient cells or with permeaplasts prepared in normal minimal medium proved unsuccessful. Cold-shock treatment of normal population also did not succeed in inducing competence in them for reproducible transformation to machete resistance (data not shown). The most successful system for transformation with *Gloeocapsa* sp. DNA to machete resistance involved use of permeaplast preparation in TE buffer. Table 3.1 summarizes the various conditions used for transformation experiments. Basically there are four parameters regulating a transformation experiments, *viz.*, age of permeaplast, concentration of transforming DNA, contact period of transforming DNA with permeaplast and conditions under which transformation reaction *is* carried out. Some basic conditions for stable

transformation in *Anabaena doliolum* have already been worked out by Singh et al. (1987a).

As shown in Table 3.1, the transformation frequency was nearly 100-fold higher in photosynthetic condition over that where the DNA was treated with DNase under similar conditions. Similarly, transformation frequency in photosynthetic conditions was nearly 200-fold higher over that in non-photosynthetic (dark) conditions. It is therefore concluded that transformation for machete resistance is DNase sensitive and photosynthetic light dependent. The results also suggest that the DNA mediated transformation of machete resistant phenotype from *Gloeocapsa* sp. to *N. muscorum* was obligately dependent on the presence of permeaplast of *N. muscorum* and Tris-EDTA buffer in the transformation reaction mixture. The control cultures for scoring spontaneously occurring machete resistant mutants gave zero results, thus suggesting lack of mutational origin of machete resistance phenotype in this cyanobacterium. *Gloeocapsa* sp., the donor of the DNA, showed resistance to basalin, propanil, atrazine and DCMU. We found DNA is a genetic material for machete resistant phenotype. We checked machete resistant transformant whether it was resistant to some or all herbicides. It was found able to grow in growth inhibitory concentrations of the remaining four herbicides as well. We reasoned DNA to be the genetic material for basalin, propanil, atrazine and DCMU resistance as well. On examination, all transformants were found to be basalin resistant, DCMU resistant, atrazine resistant and propanil resistant as well.

Table 3.1

Effect of various treatments on transformation frequency during DNA-mediated transformation of *Nostoc muscorum* to machete resistance with purified donor DNA from machete resistant *Cloeocapsa* sp.

Treatment	Number of transformants per 2×10^6 CFU	Transformation frequency
Whole cell + No DNA (in light)	0.0	0.0
Whole cell + DNA (in light)	0.0	0.0
Permeaplasts prepared in N2-medium + DNA (in light)	0.0	0.0
Permeaplasts prepared in TE buffer + DNA (in light)	760.0 (± 60.3)	3.8×10^{-4}
Permeaplasts prepared in TE buffer + DNA (in dark)	3.0 (± 0.2)	1.5×10^{-6}
Permeaplasts prepared in TE buffer + DNase treated DNA (in light)	8.0 (± 0.3)	4.0×10^{-6}

Transformation frequencies are represented as, number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells were taken as a CFU unit.

Each value is an average (± SE) of three independent experiments.

A comparison of the diazotrophic growth characteristics of the parent and the machete selected transformant in different herbicide media is summarized in Table 3.2a & b. Growth of the transformant in normal minimal medium devoid of the herbicide was slightly better, in comparison to its parent under similar conditions. Presence of the herbicide showed very little inhibitory effect on the growth of the transformant while the parent did not showed any growth and was infact growth toxic in media containing the respective herbicide. The transformant showed slightly higher heterocyst frequency and nitrogenase activity as compared to its parent in herbicide free medium. Nitrogenase activity of the parent was found to be severely sensitive to inhibitory action of the herbicides. On the other hand, nitrogenase activity of the transformant was found to be significantly resistant to inhibitory action of each of the herbicide. These results clearly indicate that the transformants selected for machete resistance were also simultaneously resistant to basalin, atrazine, DCMU and propanil. It is therefore concluded that DNA is the genetic basis for resistant phenotypes to all the five phenotypes in *Gloeocapsa* sp. and that this DNA based genetic trait is transformable to *Nostoc muscorum* and that the genetic determinant controlling the herbicide resistant phenotype are all closely linked.

We compared the response of PS II and PS I activity of *Gloeocapsa* sp., parent *N. musorum* and its transformant, in the presence of five herbicides. As shown in Table 3.3a, PS II activity of parent *N. muscorum* was found sensitive to each of the above herbicides, while that of *Gloeocapsa* sp. (3.3b) and the transformant

Table 3.2a

Effect of various herbicides on the growth (in μg Chl a ml^{-1}), heterocyst frequency (No. of heterocysts per 100 vegetative cells) and nitrogenase activity (μmol ethylene formed mg^{-1} Chl a h^{-1}) in parent *Nostoc muscorum*.

Treatment	Growth	Heterocyst frequency	Nitrogenase activity
Control	4.6	5-6	9.8 (± 0.37)
Machete (20 fig ml^{-1})	**0.06**	4.2	0.96 (± 0.05)
Basalin (20 μg ml^{-1})	0.04	4.0	i.6 (± 0.14)
Atrazine (1 μg ml^{-1})	0.20	**4.0**	4.0 (± 0.1C)
DCMU (1 μg ml^{-1})	0.07	4.0	3.4 (± 0.29)
Propanil (1 μg ml^{-1})	0.32	4.0	3.6 (± 0.24)

Exponentially growing cells under N2-fixing conditions were washed and resuspended in fresh N2-medium containing desired concentrations of various herbicides. Ten-day-old exponentially growing cultures were used for estimation of growth (by estimating Chl a) and heterocyst frequency while nitrogenase activity was estimated after 48 h of incubation under photoautotrophic growth conditions.

Each value is an average of (± SE) three independent experiments.

Table 3.2b

Effect of various herbicides on the growth (in $\mu g Chl\ a\ ml^{-1}$), heterocyst frequency (No. of heterocysts per 100 vegetative cells) and nitrogenase activity (μmol ethylene formed mg^{-1} Chl a h^{-1}) in transformant *Nostoc muscorum*.

Treatment	Growth	Heterocyst frequency	Nitrogenase activity
Control	5.0	7-8	13.7 (± 0.60)
Machete (20 μg ml^{-1})	4.0	6.0	10.4 (± 0.28)
Basalin (20 $\mu g\ ml^{-1}$)	4.2	6.1	14.5 (± 0.70)
Atrazine (1 $\mu g\ ml^{-1}$)	4.16	5.3	9.8 (i 0.54)
DCMU (1 $\mu g\ ml^{-1}$)	3.75	5.0	8.8 (i 0.43)
Propanil (1 $\mu g\ ml^{-1}$)	3.60	4.0	3.6 (± 0.22)

Exponentially growing cells under N_2-fixing conditions were washed and resuspended in fresh N_2-medium containing desired concentrations of various herbicides. Ten-day-old exponentially growing cultures were used for estimation of growth (by estimating Chl a) and heterocyst frequency while nitrogenase activity was estimated after 4S **h** of incubation under photoautotrophic growth conditions.

Each value is an average of (± SE) three independent experiments.

Table **3.3a**

Photochemical activities (PS II and PS I) in parent *Nostoc muscorum* **under diazotrophic** growth conditions.

Treatment	ps II activity (H_2O ⟶ pBQ)	PS I activity (DCIP ⟶ MV)
Control	352.0 (± 14.73)	483.0 (± 16.39)
Machete (20 μg ml^{-1})	126.0 (± 8.24)	432.0 (± 19.79)
Basalin (20 μg ml^{-1})	170.0 (± 14.65)	380.0 (± 17.15)
Atrazine (1 μg ml^{-1})	132.0 (± 7.29)	342.0 (± 20.24)
DCMU (1 μg ml^{-1})	305.0 (± 8.96)	363.0 (± 12.76)
Propanil (1 fig ml^{-1})	90.0 (± 3.54)	324.0 (± 14.24)

Exponentially growing cells, under N_2 fixing conditions were washed and resuspended in fresh N_2-medium in response to various herbicides at desired concentrations and PS II and PS I activities were estimated after 4S h incubation, under photoautotrophic growth conditions.

PS II activity (H_2O ⟶ pBQ) expressed as μmol O_2 evolved mg^{-1} Chl *a* h^{-1}.

PS 1 activity (DCIP ⟶ MV) expressed as μmol O_2 consumed mg^{-1} Chl *a* h^{-1}.

Each value is an average of (i SE) three independent experiments.

Table 3.3b

Photochemical activities (PS II and PS I) in *Cloeocapsa* sp. under diazotrophic growth conditions.

Treatment	ps II activity (H_2O ⟶ pBQ)	PS I activity (DCIP ⟶ MV)
Control	**108.8 (± 8.50)**	**261.0 (± 10.62)**
Machete (20 μg ml^{-1})	**101.8 (± 2.16)**	**256.0 (± 9.53)**
Basalin (20 μg ml^{-1})	115.1 (± 2.94)	296.0 (± 3.49)
Atrazine (1 μg ml^{-1})	105.0 (± 3.86)	260.0 **(± 2.49)**
DCMU (1 μg ml^{-1})	98.1 (i 7.72)	248.0 (± 2.56)
Propanil (1 μg ml^{-1})	86.3 (± 5.73)	237.0 (± 9.09)

Exponentially growing cells, under N_2 fixing conditions were washed and resuspended in fresh N_2-medium in response to various herbicides at desired concentrations and PS II and PS I activities were estimated after 48 h incubation, under photoautotrophic growth conditions.

PS II activity (H_2O ⟶ pEQ) expressed as μmol O_2 evolved mg^{-1} Chl a h^{-1}.

PS I activity (DCIP ⟶ MV) expressed as μmol 0? consumed mg^{-1} Chl *a* h^{-1}.

Each value is an average of (± SE) three independent experiments.

Table 3.3c

Photochemical activities (PS II and PS I) in transformant *Nostoc muscorum* under diazotrophic growth conditions.

Treatment	PS II activity ($H_2O \longrightarrow pBQ$)	PS I activity (DCIP $\longrightarrow$ MV)
Control	276.0 (± 10.50)	488.0 (± 10.62)
Machete (20 μg ml^{-1})	256.0 (± 2.16)	455.0 (± 9.53)
Basalin (20 μg ml^{-1})	218.0 (± 2.94)	483.0 (± 5.C2)
Atrazine (1 μg ml^{-1})	193.0 (± 3.86)	402.0 (±2.49)
DCMU (1 μg ml^{-1})	186.0 (± 7.72)	423.0 (± 3.44)
Propanil (1 μg ml^{-1})	156.0 (± 5.74)	350.0 (± 9.09)

Exponentially growing cells, under N_2 fixing conditions were washed and resuspended in fresh N_2-medium in response to various herbicides at desired concentrations and PS II and PS I activities were estimated after 48 h incubation, under photoautotrophic growth conditions.

PS II activity ($H_2O \longrightarrow pBQ$) expressed as μmol O_2 evolved mg^{-1} Chl *a* h^{-1}.

PS I activity (DCIP $\longrightarrow$ MV) expressed as μmol O_2 consumed mg^{-1} Chl *a* h^{-1}.

Each value is an average of (± SE) three independent experiments.

(Table 3.3c) were found relatively herbicide resistant. As expected, none of the herbicides could significantly influence the PS I activity of parent *N. muscorum* and its transformant and *Gloeocapsa* sp. The results indicate the ability of *Gloeocapsa* sp. and *N. muscorum* transformant to grow in the presence of growth inhibitory concentration of the five herbicides because of their herbicide resistant PS II activity.

Next series of experiments involved in isolating strains of parent *N. muscorum* and its transformant, whose GS activity is resistant to inhibitory action of MSX, EDA and PPT and which are ammonia excreting in nature. The spontaneous mutation frequency leading to production of MSX, EDA and PPT resistant GS in parent and transformant was in the range of 0.5-1.5 x 10^{-7}. All these mutants were as stable as the transformant and showed normal heterocyst frequency and ammonium repressible nature of heterocyst formation and nitrogenase activity. The nitrogenase activity of the parent *N. muscorum*, its Msx^r, Eda^r and Ppt^r mutant strains, the transformant *N. muscorum* and its Msx^r, Eda^r and Ppt^r mutant strains varied in the range of 9.2-14.9 µmol C_2H_4 produced mg^{-1} Chl a h^{-1} under diazotrophic photosynthetic conditions. The level of GS activity was almost similar in both parent and its herbicide resistant transformant. Neither strain excreted ammonia during diazotrophic growth under photosynthetic or non-photosynthetic growth conditions. The results indicate an absence of ammonia excretion under the above conditions. However Msx^r, Ppt^r and Eda^r mutant strains of both parent and transformant *N. muscorum* were associated with ammonia excretion under diazotrophic growth

Table 3.4a

Nitrogenase activity (μmol ethylene formed mg^{-1} Chl a h^{-1}), GS (transferase) activity (nmol γ-glutamyl hydroxamate formed min^{-1} mg^{-1} protein) and ammonia excreting activity (μmol ammonia mg^{-1} Chl a $4h^{-1}$) of L-methionine DL-sulfoximine resistant (Msx^r), ethylene diamine resistant (Eda^r) and phosphinothricin resistant (Ppt^r) strains of parent *Nostoc muscorum.*

Activity	Strain			
	Parent	Msx^r	Eda^r	Ppt^r
Nitrogenase	9.8 (± 0.37)	11.0 (± 0.29)	13.56 (± 0.60)	12.73 (± 0.56)
GS (transferase)				
(a) without inhibitor	1415.6 (± 52.22)	405.0 (± 19.79)	224.69 (± 11.65)	105.12 (± 6.82)
(b) with inhibitor	0.0	399.0 (± 17.15)	235.8 (± 10.48)	200.0 (± 9.96)
Ammonia excreting activity	0.0	18.0 (± 0.9)	20.83 (± 1.03)	22.72 (± 1.1)

Cultures were grown in diazotrophic growth medium. Four day old exponentially growing cultures of different strains were treated with the respective inhibitor for 48 h and then used for estimating GS (transferase) activity.

Each value is an average of (± SE) three independent experiments.

Table 3.4b

Nitrogenase activity (μmol ethylene formed mg^{-1} Chl a h^{-1}), GS (transferase) activity (nmol γ-glutamyl hydroxamate formed min^{-1} mg^{-1} protein) and ammonia excreting activity (μmol ammonia mg^{-1} Chl a $4h^{-1}$) of L-methionine-DL-sulfoximine resistant (*Msx*^{r}), ethylene diamine resistant (*Eda*^{r}) and phosphinothricin resistant (*Ppt*^{r}) strains of multiple herbicide- resistant transformant of *Nostoc muscorum*.

Activity	Strain			
	Parent	*Msx*^{r}	*Eda*^{r}	*Ppt*^{r}
Nitrogenase	10.73 (± 0.28)	12.0 (± 0.52)	14.8 (± 0.73)	14.9 (± 0.7)
GS (transferase)				
(a) without inhibitor	1628.0 (± 47.44)	305.0 (± 14.39)	240.0 (± 12.62)	306.0 (± 14.42)
(b) with inhibitor	0.0	298.0 (± 13.99)	115.8 (± 6.08)	302.0 (± 14.01)
Ammonia excreting activity	0.0	19.49 (± 0.98)	26.02 (± 1.53)	19.3 (± 0.78)

Cultures were grown in diazctrophic growth medium. Four day old exponentially growing cultures of different strains were treated with the respective inhibitor for 48 h and then used for estimating GS (transferase) activity.

Each value is an average of (± SE) three independent experiments.

conditions (Table 3.4a & b). It can be concluded from these results that the *Eda*^{r}-GS strain was more defective in its GS activity, which in turn was more proficient in ammonia excretion than the corresponding *Msx*^{r}-GS or *Ppt*^{r}-GS strains. These cyanobacterial strains fixed N_2 only under photosynthetic conditions. The different inhibitor resistant defective strains excretes ammonia only under photosynthetic diazotrophic conditions. Apparently, the level of extracellular ammonia produced from photobiological fixation of N_2 seems to be dependent on the degree of mutational decrease in the cyanobacterial GS activity.

Discussion

Machete (butachlor) belongs to a class of amide herbicides used in the control of weeds in rice fields (French and Gay, 1963; Armstrong et al., 1973) and has been shown to be highly growth toxic and mutagenic in *N. muscorum* (Singh and Vaishampayan, 197S). *N. muscorum* has been repeatedly tried for isolation of machete resistant mutants but the results have been consistently negative. Natural isolate of *Gloeocapsa* sp. has been found to be inherently resistant to growth inhibitory action of machete and this trait is genetically transformable to *N. muscorum* (Singh et al., 19S7b). When further examined it was also found to be highly resistant to growth inhibitory action of basalin, DCMU, atrazine and propanil. We carried out transformation experiments to find out the genetic basis of such multiple herbicide resistant phenotypes in *Gloeocapsa* sp. and also to examine possibility of genetic linkage among the five herbicides.

Crop plants like other plants are oxygenic phototrophs and most of the herbicides are well known inhibitors of oxygenic photosynthesis. The most commonly used photosynthetic herbicides **are** DCMU and atrazine both of which inhibit the activity of reaction centre D1 protein of PS II. Recent comparative studies on the molecular organisation and functional aspects of photosynthesis in chloroplasts and cyanobacteria suggest them to be very similar. This formed the background for engineering and modifying genes in chloroplasts by manipulating transformable cyanobacteria (Golden et al., 1985). The cyanobacterium *Synechocystis* PCC 6803, a facultative heterotrophic strain, is increasingly being used as a model system for the genetic analysis of oxygenic photosynthesis in view of its ability to produce viable photosynthetic mutants under heterotrophic conditions (Astier et al., 1984). Vermaas et al. (19S8) have developed techniques to generate mutated photosynthetic genes under *in vitro* conditions which have been used for substitution of their wild type alleles.

Many commercial herbicides inhibit electron transport by intercepting flow at the reducing end (Ashton and Crafts, 1973; Wright and Corbett, 1979). There are several evidences to indicate that this inhibition occurs at the level of protein bound plastoquinone called "B" (Pfister and Arntzen, 1979). The product of *psb* A gene is the 32 kDa protein called D_1 which binds quinone "B" and thus serves as the second stable electron acceptor of PS **II. This** Q_B protein, the product of *psb* A gene is the site of DCMU and atrazine binding and mutational alteration of this protein has been shown to be the cause of resistance of PS II activity to DCMU and

atrazine (Kirilovsky et al., 1989; Golden and Haselkorn, 1985). Photoautotrophic growth, nitrogenase activity and PS II activity of *Gloeocapsa* sp. was found resistant to DCMU, atrazine and propanil, **the** well known inhibitors of PS II activity in oxygenic photosynthetic organisms. In view of the known involvement of Q_b protein of PS II system in controlling sensitivity or resistance of oxygenic photosynthesis in cyanobacteria, one is tempted to speculate that this herbicide binding protein is the cause of *Gloeocapsa* sp. resistance to DCMU and atrazine. However, this cyanobacterium is also resistant to propanil which is known to cause inhibition of PS II activity by its interaction with cytb553 component of the PS II complex (Nishimura and Takarmiya, 1966). Accordingly one may conclude that *Gloeocapsa* sp. possesses DCMU and atrazine resistant Q^b protein as well as propanil resistant cyt553 system. There is no previous report of basalin and machete inhibiting PS II activity in oxygenic photosynthetic organisms. Since these two herbicides have shown to inhibit PS II activity of parent *N. muscorum* which is also found extremely sensitive to DCMU, atrazine and propanil. It is suggested that there are certain components of PS II activity in *N. muscorum* sensitive to machete and basalin. Whether these components are Q_B binding protein or cytbSS3 or some other is difficult to establish at this moment. Occurrence of three copies of *psb* A gene namely *psb* Al, *psb* A2 and *psb* A3 occurring at different locations in the cyanobacterial genome has been revealed using the technique of heterologous probing (Curtis and Haselkorn, 1984).

The heterocyst is the site of synthesis and activity of nitrogenase and it lacks Rubisco and PS II activity (Haselkorn, 1978; Stewart, 1980) as well as nitrate reductase (Kumar et al., 1985). Nitrogenase activity requires ATP and reductant. ATP requirement is met by PS I activity of heterocyst but reductant supply requires transport of photosynthetically fixed carbon from vegetative cell to heterocyst (Stewart, 1980). All the five herbicides are strong inhibitors to the nitrogenase activity of *N. muscorum* by inactivating photosynthetic carbon assimilation in vegetative cells through inactivation PS II activity. Hence it can be concluded that the nitrogenase of the *Gloeocapsa* sp. is resistant to all the five herbicides. Such cyanobacteriall multiple herbicide resistant FS II activity in *Cloeocapsa* sp. is the reason for the resistance cf nitrogenase activity in that cyanobacterium.

Mutational production of higher plant cell lines resistant to herbicides glyphosate and atrazine have been successful (Sato et al., 1988). Transgenic plants resistant to inhibitory action of glyphosate have also been generated (Shah et al., 1966). Mutants resistant to 2,4-D have been isolated in *Arobidopsis thaliana* (Esteiie and Somervi1 la, 19S7). The mechanism of detoxification of 2,4-D in *Alcaligenes eutrophus* JMP 134 has been elucidated (Sterber et al., 19S7). This bacterial genes has been engineered into tobacco rendering the plant resistant to the herbicide 2,4-D (Sterber and Willmitzer, 1989). Since the diazotrophic cyanobacteria with potential for use as nitrogen biofertilizer in rice fields have plant like photoautotrophic metabolism, it is essential to develop methods for introduction of herbicide resistant

genes in them, in order to make them effective source of nitrogen in modern agricultural rice field conditons. The source of herbicide resistant genes would be either mutationally created or isolated from naturally occurring microbial forms including cyanobacteria, which contain herbicide resistnant genes. In such cases there has to be a suitable gene transfer method to introduce them into the desired strains. One such method is evidently the method of genetic transformation.

Transformation as a mode of gene transfer has been well demonstrated in unicellular cyanobacteria (Shestakov and Khyan, 1970; Devilly and Hughton, 1977; Astier and Espardeller, 1976; Stevens and Porter, 1980; Grigorieva and Shestokov, 1982; Golden and Scherman, 19S3; Kuhlmier et al., 19S4). Transformation as a gene transfer process has not been found to be successful in heterocystous filamentous cyanobacteria. Wolk et al. (19S4) developed conjugation as ? method of gene transfer system in filamentous cyanobacteria using triparental method of mating. McFarlane et al. (19S7) improved the method by using biparental recombination. Recently Singh et ai. (1987a & b) have shown transformation as a method of gene transfer to be successful in heterocystous cyanobacterial forms as well. The factors that play role in the transformation in such cyanobacteria include purified DNA vs RNA complexes, competence, DNA concentration, DNA contact period light and dark condition and homo and hetero specific nature of transformation process. Unlike the transformation in unicellular cyanobacteria where it is found to be a natural process, heterocystous forms require artificial induction through the

preparation of permeaplasts. The reason for success of transformation in such preparations could be due to unhindered and undamaged uptake of transforming DNA.

Sensitivity of transformation process to DNase has been considered a very strong evidence for DNA to be the cause of transformation process (Avery et al., 1944). DNA binding and uptake has been shown to be an energy requiring process in heterotrophic transormable bacteria (Lacks, 1977). Chauvat et al. (1983) found uptake of transferring DNA to be more or less similar in photosynthetic light and dark. Golden and Sherman (1984) found more efficient process of transformation taking place in dark than in photosynthetic light. Singh et al. (1987a & b) showed requirement of photos., thetic light for effective transformation. Transformation of *N. muscorum* with the purified donor DNA from *Gloeocapsa* sp. which contains five herbicide resistant genes vas found to be sensitive to DNase, dependent on photosynthetic light. The DNA mediated transfer of herbicide resistant phenotype from *Gloeocapsa* sp. to *N. muscorum* is a case of heterospecific transformation. The stable nature of transformant *N. muscorum* with purified *Gloeocapsa* sp. DNA clearly suggests that the herbicide resistant gene of *Gloeocapsa* sp. can be stored, expressed and maintained in *N. muscorum* in a stable and unmodified form. There is report for plasmid born nature of machete and basal in resistance gene in *Gloeocapsa* sp. (Singh et al., 1986). This conclusion was based on the observation that the *Gloeocapsa* sp. naturally resistant to machete and basal in lose their phenotype following treatment with ethidium bromide, a drug reported to eliminate plasmids from cells.

Since transformants in the present study selected for machete resistance were found to be also resistant to basalin, DCMU, atrazine and propanil, a common genetic determinant controlling resistance to all the five herbicides is implied. Transformants selected in the presence of five herbicides arose with a frequency of almost similar to those selected in the presence of machete alone. This finding also suggests that the genes involved in conferring resistance against the five herbicides are closely linked on the transforming DNA. Our results do not suggest anything whether the herbicide resistant genetic determinant or genes are plasmide borne or chromosomally located and this aspect needs furthr study.

Most of the procaryotes including cyanobacteria possesses ammonium transport systems, i.e., proteins which specifically serve for the energy requiring permeation of NH_4^+, thus accumulating it intracellularly. Ammonia on the other hand, diffuses rapidly through biomembranes. Part of the accumulated NH_4^+ leaks out through the membrane in the form of ammonia. This ammonia after getting protonated is recaptured by the ammonium carrier and transported into the cells. Thus a futile cycle (cyclic retention) is operating in the microbes possessing ammonium transport systems. Strains or mutants defective in ammonium carrier called *[Amt*$^-$) would be constantly excrete ammonia (Kleiner, 19S5) in constrast to ammonia excreting mutants described previously (Shanmugam and Valentine, 1975). The mutants of N_2-fixing cyanobacteria defective in ammonium transport system *[Amt*$^-$ strains) are prototrophs i.e., they do not require additional nutrients since *Amt*$^-$ strains because of NH_3 loss

do not accumulate high intracellular ammonium levels and therefore N_2-fixation is much repressed by ammonium than in tie wild type (Kleiner, 1985).

Glutamine synthetase (GS) plays a key role in ammonia incorporation in cyanobacteria. The most common feature of mutants of nitrogen strains that release ammonia continuously have been, the reduced level of GS activity together with increased nitrogenase activity (Polukhina et al., 1982; Singh et al., 1983b; Lattore et al., 1986; Spiller et al., 1986; Boussiba and Gibson, 1991). However, these ammonia excreti;.g mutant cyanobacterial strains, invariably possess normal ammonium transport system, thereby implying that ammonia excretion does not depend on ammonium transport system.

The inhibition of nitrogenase activity and heterocyst production which occurs on adding a mm c ni um to N_2-fixing cultures can be prevented by adding MSX and that the MSX allows nitrogenase synthesis and heterocyst production in ammonium grown cultures. A large number of studies have been done on the metabolic aspects of ammonia excretion in diazotrophic and non-diazotrophic cyanobacteria. Musgrave et al. (1982) demonstrated sustained ammonia production by immobilized filaments of nitrogen fixing heterocystous cyanobacterium treated with MSX, the inhibitor of GS activity. Nitrate assimilating non-diazotrophic cyanobactera also excrete nitrate derived ammonia during photoautotrophic condition in the presence of GS inhibitor (Guerrero et al., 1982; Ramos et al., 1982).

Ammonium transport system does not control ammonium excretion but control its uptake. It would be interesting to find out the fate of ammonium excretion process and the degree of extracellular production of ammonia by *Amt*~ strain of cyanobacteria. All the Msx^r-GS strains excrete ammonia during diazotrophlc growth and the degree of extracellular ammonia production was a function of decreased GS activity. Selection of mutant strains of heterocystous cyanobacteria for MSX resistance as a result of muta ion in *gln* A gene have been shown to result in generation of ammonia excreting strains (Singh et el., 1983b; Thomas et al., 1990). Similarly selection of mutant strains of *A. variabilis* resistant to growth inhibition by EDA at pH 9.0 has also led to production of ammonia excreting strains (Kerby et al.. 1986, 19S7). In the present investigation we found that the EDA resistance is associated with excretion of ammonia and all such Eda^r strains when examined were found to have defective GS (biosynthetic) activity. In cyanobacteria, the *gin* A gene codes ft r cyanobacterial GS enzyme and a mutation in this gene at a particular site results in a mutant GS phenotype. Molecular mechariisms are to be understood in detail controlling *gln* A gene mutation specifically at hose sites leading to the production of mutant GS, which is resistant to inhibition by EDA. A definite role of GS activity in regulation of extracellullar liberation of ammonia produced by photobiological fixation of N_2 is hereby thus suggested. Our results also show for the first time the successful application of techniques of genetic transformation and mutation in generating multiple herbicide resistant ammonia excreting strains in the cyanobacterium *N. muscorum*. Free living diazotrophic cyanobacteria has been suggested to function as a

source of biofertilizer in rice fields of tropical countries (De, 1939; Watanabe, 1956; Singh, 1961; Venkatraman, 1972; Stewart et al., 1979, 1980). It is important to mention here that the ammonia excreting strains resulting from mutational manipulation have been used in laboratory conditions as very effective source of photobiologically produced ammonia for nitrogen nutrition of rice (Lattore et al., 1986) and wheat (Spiller and Gunasekaran, 1990). Cyanobacterial strains with competent biofertilizer potential should have the necessary attributes mentioned in the Chapter 1. Accordingly our aim has been to generate a transformant of *N. muscorum* resitant to herbicides and then use one such transformant to select out a potent ammonia excreting strain. The results of the present study thus imply to have a long bearing for ful 1 meaningful realisation in constructing potent biof ert ilizer strains.

CHAPTER 4

MUTATIONAL ANALYSIS OF GLUTAMINE SYNTHETASE RESPONSE TO THE AMMONIUM ANALOGUE ETHYLENE DIAMINE

Introduction

Nostoc muscorum is a heterocystous, diazotrophic cyanobacterium growing well with N_2, nitrate (NO_3^-) or ammonium (NH_4^+) as nitrogen source. Molecular nitrogen (N_2) is reduced to ammonia by nitrogenase within heterocyst where it is converted to glutamine nitrogen by the ammonia assimilatory enzyme glutamine synthetase (GS). Glutamine thus produced is translocated to adjacent vegetative cells where the glutamate producing enzyme, glutamate synthase (GOGAT) produces it from glutamine and α-ketoglutarate. Nitrate assimilating system is ammonium repressible in *N. muscorum* (Bagchi et al., 1985) and *Anabaena doliolum* (Rai et al., 19SS). In heterocystous nitrogen fixing filaments, nitrogenase remains functional within the heterocysts and nitrate reductase within the vegetative cell which also contains active nitrite reductase (Kumar et al., 1985). Genetic and biochemical evidences in *N. muscorum* suggest that molybdenum cofactor synthesis of nitrate reductase is constitutive and its apoprotein component is ammonium repressible (Bagchi et al., 1985). N_2 -derived, NO_3^- -derived or exogenously supplied ammonia is assimilated into glutamine by glutamine synthetase.

Ammonium assimilation involves two basic steps, one its uptake and the other its incorporation into amino acids. At pH 7-8 NH_4^+ is the predominant form and experiments have shown operation of an efficient biphasic ammonium transport system (Amt) in diazotrophic and non-diazotrophic cyanobacteria. At pH above 8.0, the predominant form is ammonia which freely diffuses into the cell without the participation of Amt system (Rai et al., 1984; Singh et al., 1985; Boussiba et al., 1984; Kleiner, 1985). The incorporation of cellular ammonia into glutamine is then carried out by glutamine synthatase. Any genetic approach aiming at construction of diazotrophic cyanobacterial strains capable of excreting much of the fixed nitrogen (photofixation of N_2) in the form of ammonia would have to take into account the relative role of Amt system and glutamine synthase activity in the process.

There are two ways to induce the cyanobacteria to liberate ammonium extracellularly by inhibiting ammonia assimilation via GS, one is inactivating GS using MSX (Stewart and Rowell, 1975; Musgrave et al., 1982a & b; Kerby et al., 1983) and the second way is, by the selection of mutant strains partially deficient in ammonium assimilation (Polukhina et al., 1982; Sakhurieva et al., 1982). A succesful method of mutant selection for resistance to ethylene diamine was proposed by Polukhina et al. (1982).

GS and GOGAT constitute the primary pathway of ammonia assimilation resulting from N_2-fixation, nitrate reduction or exogenous supply in heterocystous cyanobacteria. On the basis of

enzymatic studies (Dharmawardene et al., 1973; Rowell et al., 1977), usage of analogues (Stewart and Rowell, 1975; Ladha et. al., 1978) and ^{15}N tracer studies (Stewart et al., 1975), it has been demonstrated that glutamine synthetase is a major enzyme involved in ammonium assimilation in nitrogen fixing cyanobacteria. This conclusion is also supported by other workers (Wolk, 1967; Meeks et al., 1975; Thomas et al., 1977) and now there is substantial evidence (Brown et al., 1974) to say that glutamate analogue L-methionine-DL-sulfoximine (MSX) inhibits glutamine synthetase activity and as a result of this, the nitrogenase synthesis and heterocyst production are uninfluenced even in the presence of exogenous ammonium (Stewart and Rowell, 1975), in other words MSX alleviates the inhibitory effect of exogenous ammonium on nitrogenase synthesis and heterocyst production. The addition of exogenous MSX to N_2-fixing cultures leads to extra cellular liberation of ammonia (Gordon and Brill, 1974; Kumar and Kumar, 1980; Lea et al., 1984; Orr and Haselkorn, 1982; Shanmugam *et* al., 1978). Mutant strains that are blocked at the level of ammonium assimilation were first described in *Klebsiella pneumoniae* (Shanmugam and Valentine, 1980). Such mutant strains not only derepressed nitrogenase synthesis but also excrete ammonia produced by the enzyme into the surrounding medium (Shanmugam and Valentine, 1980). Cyanobacteria use light and water as sources of energy and reductant for nitrogen fixation (Haselkorn, 1978; Wolk, 1982). Their photosynthetic ability inherently empowers them to be never energy limited for production and liberation of ammonia and because

of this physiological property nitrogenase-derepressed mutant strains of the heterocystous, ubiquitous cyanobacteria can protentially be used as suppliers of fertilizer nitrogen. Spiller et al. (1986) have isolated nitrogenase-derepressed mutant strains and analyzed their physiological and biochemical properties.

The ammonium analogue ethylene diamine (EDA), like the glutamine analogue MSX, is also a potent inhibitor of cyanobacterial GS activity *in vivo*. *Eda*^{r}-GS strain has been shown to be more efficient producer of extracellular ammonia than the corresponding *Msx*^{r}-GS strains (Polukhina et al., 1982; Kerby et al., 1986; Chapter 3 of this thesis). Methylammonium, another analogue of ammonia has been shown to be assimilated like a fixed nitrogen source, by a class of *Msx*^{r}-GS strains of *N. muscorum* (Bagchi and Singh, 1984). As a sequel to the studies in Chapter 3 we thus proceeded to analyze the role of GS activity in regulation of *Eda*^{r} phenotype, ammonium excretion and EDA assimilation. This was achieved by isolating and characterizing a number of *Eda*^{r} mutant clones derived from the parent strain, *Msx*^{r} strain and *Het*$^-$ *Nif*$^-$ strains of *N. masorum* described below.

Experimental Procedure

Axenic clonal cultures of parent *N. masorum* were grown in modified Chu No. 10 medium as described in Chapter II. *Het*$^-$ *Nif*~ mutant strain of *N. muscorum* was grown in modified Chu No. 10 growth

medium supplemented with 5 mM KNO_3 or 1 mM NH_4Cl. The pH of the ammonium medium was adjusted between 7.2 and 8.0 with HEPES-NaOH. Exponentially grown cultures were used to isolate the desired mutants.

Isolation of mutant strains

L-Methionine-DL-sulfoximine (MSX) resistant *N. musorum* :MSX is a glutamate analogue and an irreversible competitive inhibitor of ammonium assimilating enzyme glutamine synthetase (GS) activity. 2 μM MSX was found to be completely growth toxic. MSX resistant clones were isolated on combined nitrogen free solid medium designated as N_2-medium containing 100 μM MSX. 2 x 10 CFU were spread on solid nutrient plates and incubated for two weeks under photoautotrophic growth conditions. The surviving colonies were isolated and transferred to fresh liquid medium for further experimental analysis.

Ethylene diamine (EDA) resistant *Nostoc muscorum* : EDA is an ammonium analogue which enters inside the cyanobacterial cells at pH 8.5 or above, freely by difusion where it is metabolized by GS to produce aminoethyl glutamine which is not further assimilated, thus causing growth inhibition of the cyanobacterium (Kerby et al., 1985). EDA resistant (*Eda*^{r}) mutants of parent *N. muscorum*, its *Het*$^-$ *Nif*$^-$ and *Msx*^{r}-GS strains were isolated. EDA survival studies of the three strains suggested that they were uniformly sensitive to the inhibitor and could not survive at inhibitor concentration of

30 μM at pH 9.0. Spontaneously occurring *Eda*^{r} clones were selected from 200 μM EDA containing N_2-agar medium (pH 9.0) in the case of parent and *Msx*^{r}-GS strain. For *Het*$^-$ *Nif*$^-$ strain N_2-medium was supplemented with 5 mM KNO_3. *Eda*^{r} clones thus selected were tested for their stability before storing them on inhibitor containing nutrient egar slants.

GS (biosynthetic) activity was measured by following the method of Kingdon et al. (1968). Protein and Chl a were estimated PV using the methods of Lowry et al., (1951) and Mackinney (1941), respectively. These methods are des ribed in Chapter 2.

Results

Cyanobacterial cell lacks transport system for uptake of ethylene dianine (EDA) and this ammonium analogue enters cyanobacterial cell freely by diffusion at or above pH 8.5, where it is netabolized by GS into aminoethyl glutamine leading to cyanobacterial growth inhibition (Kerby et al., 19S5; Stewart et al., 1987). EDA inhibits growth of *N. muscorum* and a dose of 200 μM causes complete inhibition of *in vivo* GS activity. Parent, *Het*$^-$ *Nif*$^-$ and *Msx*^{r}-GS strains were used to isolate *Eda*^{r} clones, growing on N_2/NO_3^--medium, containing 200 μM EDA. Tha nature of the *Msx*^{r}-GS strain used here was that it was not ammonia excreting and is different from the one described in Chapter 3. *Eda*^{r} clones arose at a frequency of nearly 3.5×10^{-7} from parent strain, 4.5×10^{-7}

from *Het⁻ Nif⁻* strain and 3.2 x 10^{-5} from *Msx*^{r}-GS strain. Thus, **it appears that *Msx*^{r}**-GS strain is inherently more prone to spontaneous **mutation leading to *Eda*^{r} phenotype. A careful examination of the size and growth of** the colonies on inhibitor-free control medium **and on inhibitor** containing experimental medium showed that **mainly EDA resistant** colonies (*Eda*^{r}) comprised two types: one small in size and the other large in size. This large and small size colonies were designed as *Eda*$^{r}_{s}$ and Eda$^{r}_{L}$. *Eda*$^{r}_{s}$ colonies were slightly **yellowish-blue-green** in colour and *Eda*$^{r}_{L}$ colonies were deep blue-green colour. The proportion of *Eda*$^{r}_{L}$ colonies among *Eda*^{r} propulation derived from parent or *Het⁻ Nif⁻* strain was nearly 0.1% and those from *Msx*^{r}-GS strain was about 97%. Various *Eda*^{r} strains were designated as : parent-*Eda*$^{r}_{s}$ or *Eda*$^{r}_{L}$ or *Het⁻ Nif⁻*-*Eda*$^{r}_{s}$ or *Eda*$^{r}_{L}$ or *Msx*^{r}-*Eda*$^{r}_{L}$ or *Eda*$^{r}_{s}$ depending upon their source and size.

As shown in Table 4.1, 43 h EDA treatment of culture caused complete inhibition of GS (biosynthetic) activity from parent strain, *Het⁻ Nif⁻* and *Msx*^{r} strains. This suggested that there is no cross-resistant relationship between *Msx*^{r} phenotype and *Eda*^{r} phenotype. Also GS from the *Eda*^{r} strain was found to be sensitive to inhibitory action of MSX. It is important and interesting **to** mention **here, that** preliminary studies in our laboratory **indicate that the *in vivo* GS** (biosynthetic) activity of *Msx*^{r}-GS strain **has been found to** be resistant to inhibitory action of PPT and **that from the *Ppt*^{r}-GS strain to be resistant to the inhibitory action of MSX, thus showing a cross-resistance relationship (data not shown). This**

Table 4.1

In vivo GS (biosynthetic) activity (nmol NADH **oxidized mg^{-1} protein min^{-1}**) of various cyanobacterial strains of *Nostoc muscorum* **with** or without 200 μM ethylene diamine (EDA).

Strain	GS (biosynthetic) activity	
	(- EDA)	(+ EDA)
Parent	65.5 (± 1.63)	0.0
Het$^-$ *Nif*$^-$	68.4 (± 1.87)	0.0
Msx^r-GS	60.8 (± 1.51)	0.0
Parent-*Eda*^r_L	53.1 (± 1.46)	51.4 (± 1.21)
Parent-*Eda*^r_S	14.5 (± 0.38)	12.4 (± 0.44)
Het$^-$ *Nif*$^-$ *Eda*^r_L	53.9 (± 2.38)	54.5 (± 1.21)
Het$^-$ *Nif*$^-$ *Eda*^r_S	13.8 (± 0.61)	15.2 (± 0.29)
Msx^r-GS-*Eda*^r_L	47.6 (± 0.96)	49.2 (± 1.12)
Msx^r-GS-*Eda*^r_S	12.5 (±0.26)	11.8 (± 0.33)

Cyanobacterial cultures were pretreated with EDA for 48 h and then examined for extractable GS (biosynthetic) activity in cell-free preparation.

Each reading is an average (± SE) of three independent experiments.

suggests different mutations within the *gln* A gene responsible for conferring *Msx*^{r}-GS phenotype or *Eda*^{r}-GS phenotype. Another **important finding** is that the spontaneous mutation to *Eda*^{r} **phenotype occurred** at nearly hundred-fold higher frequency in *Msx*^{r} **strain** than **in parent** or *Het*$^-$ *Nif*$^-$ strain. Evidently, spontaneous mutation frequency to *Eda*^{r} phenotype appears to be the function of cyanobacterial *gln* A allele. On microscopic examination, *Eda*^{r} clones were found to produce heterocystous filanents on N_2 medium and non-heterocystous filaments on 200 μM EDA containing N_2-medium. In comparison *Eda*^{r} clones remained neterocystous under both growth conditions.

An shown in Table 4.1 *in vivo* GS (biosynthetic) activity decreased by a factor of nearly one-fifth following mutation to *Eda*^r_L phenotype and nearly by 4-fold following mutation to *Eda*^r_S phenotype. EDA treatment did not change *in vivo* GS activity cf *Eda*^r_L or *Eda*^r_S clones. As shown in Tables 4.2a, b & c, *Eda*^r_L strains in general and their respective parents nave more or less doubling time in N_2 or ammonium growth medium, but *Eda*^r_S strain on the other hand showed a doubling time which was nearly five-fold higher under similar conditions. However, *Eda*^r_L strain, *Eda*^r_S strain and their respective parents all grew with more or less similar doubling time in glutamine medium. Since availability of glutamire as nitrogen source is found to restore the growth rate of *Eda*$^-$ **strain to their parental** level, in N_2^- or ammonium medium glutamine production from N_2 **or** ammonium in *Eda*$^-$ strain seems to limit their phenotypic

Table 4.2a

Doubling time (in hours), nitrogenase activity (nmol C_2H_4 formed μg^{-1} **Chl a** h^{-1}), heterocyst frequency (number of heterocysts per **100** vegetative cells), GS_{-1} (biosynthetic) activity (nmol **NADH oxidized mg** $^{-1}$ **protein** min $^{-1}$ and the level of photobiologically produced extracellular ammonia (nmol NH_3 μg^{-1} **Chl** a 2 h^{-1} **in parent** *Nostoc muscorum* **and its** EDA-resistant (*Eda*^{r}) strains.

Characteristics	Nitrogen source	Parent	*Eda*^{r}	*Eda*^{r}
Doubling time	N_2	30	24	115
	NH_4Cl (1 mM)	28	20	120
	Glutamine (1 mM)	24	18	26
	EDA (0.2 mM)	NG	22	130
Nitrogenase activity				
	N_2	14.5 (± 0.31)	12.6 (± 0.32)	18.6 (± 0.19)
Heterocyst frequency				
	N_2	5-6	5-6	5-6
GS (biosynthetic) activity	N_2	65.5 (± 2.07)	63.1 (± 2.73)	11.5 (± 0.26)
Photobiologically produced extracellular ammonia	N_2	0.0	0.0	8.3 (± 0.26)

NG = No growth
Each value is an average of (± SE) three independent experiments.

Table 4.2b

Doubling time (in hours), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a* h^{-1}), heterocyst frequency (number of heterocysts per 100 vegetative cells), GS (biosynthetic) activity (nmol NADH oxidized mg^{-1} protein min^{-1} and the level of photobiologically produced extracellular ammonia (nmol NH_3 μg^{-1} Chl a 2 h^{-1} in *Msx*^{r}GS strain of *Nostoc muscorum* and its EDA-resistant (*Eda*^{r}) strains.

Characteristics	Nitrogen source	Parent	*Eda*^{r}	*Eda*^{r}
Doubling time	N_2	32	25	130
	NH_4Cl (1 mM)	30	24	135
	Glutamine (1 mM)	28	23	35
	EDA (0.2 mM)	NG	26	132
Nitrogenase activity				
	N_2	12.8 (± 0.32)	14.2 (± 0.38)	18.8 (± 0.35)
Heterocyst frequency				
	N_2	5-6	5-6	5-6
GS (biosynthetic) activity	N_2	60.8 (± 0.29)	47.6 (± 1.43)	12.6 (± 0.52)
Photobiologically produced extracellular ammonia	N_2	0.0	0.0	7.6 (± 0.31)

NG = No growth
Each value is an average of (± SE) three independent experiments.

Table 4.2c

Doubling time (in hours), nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a* h^{-1}), heterocyst frequency (number of heterocysts per 100 vegetative cells), GS (biosynthetic) activity (nmol NADH oxidized mg^{-1} protein min^{-1} and the level of photobiologically produced extracellular ammonia (nmol NH_3 μg^{-1} Chl a 2 h^{-1} in *Het Nif*⁻ strain of *Nostoc muscorum* and its EDA-resistant (*Eda*^{r}) strains.

Characteristics	Nitrogen source	Parent	*Eda*^{r}	*Eda*^{r}
Doubling time	N_2	NG	NG	NG
	NH_4Cl (1 mM)	26	20	105
	Glutamine (1 mM)	24	22	26
	EDA (0.2 mM)	NG	24	106
Nitrogenase activity				
	N_2	0.0	0.0	0.0
Heterocyst frequency				
	N_2	0.0	0.0	0.0
GS (biosynthetic) activity	N_2	68.4 (± 3.51)	53.8 (± 2.01)	13.8 (± 0.66)
Photobiologically produced extracellular ammonia	N_2	0.0	0.0	0.02

NG = No growth
Each value is an average of (± SE) three independent experiments.

growth. **The** observed four-fold decrease **in** GS **(biosynthetic)** **activity** of various *Eda*$^{r}_{s}$ strains appears to be the enzymatic cause **of limited** glutamine **production.** **All such strains had much higher** nitrogenase **activity than their respective parents.** **Since only** ***Eda***^{r} **strain with substantially higher nitrogenase activity was found substantially ammonia excreting during their photosynthetic diazotrophic growth,** their GS activity alone, appears **to** be **the apparent** cause of both extracellular production of ammonia **and intracellular** generation of glutamine. *Het*$^{-}$ *Nif*$^{-}$-*Eda*$^{r}_{L}$ strain **unlike its** ***Met***$^{-}$ *Nif*$^{-}$-*Eda*^{r} strain grew in N_2-medium containing EDA, **thus indicating** its ability to metabolize the ammonium analogue like a fixed nitrogen source. Accordingly various *Eda*^{r} strains of parent and *Msx*^{r} were grown in various nitrogen media to s udy and compare **their** ability to assimilate EDA like a nitrogen source.

As shown in Tables 4.3a & b *Eda*$^{r}_{L}$ strain like parent, *Msx*^{r}-GS and *Eda*^{r} strain differentiated heterocyst with nitrogenase activity in N_2-medium but not in ammonium or glutamine medium. ***Eda***^{r} **strain** also behaved similarly. However, *Eda*$^{r}_{L}$ strain differed from ***Eda***$^{r}_{s}$ strain, parent strain, *Het*$^{-}$ *Nif*$^{-}$ strain and *Msx*^{r}-GS strain **in showing EDA** repression of heterocyst and nitrogenase activity during **their growth in the EDA** medium. **Evidently, mutation has conferred on** ***Eda***^{r} **strain and not on** ***Eda***^{r} **strain to assimilate EDA like ammonium nitrogen source.**

Table 4.3a

Heterocyst frequency (number of heterocysts per 100 vegetative cells), HF and Nitrogenase (N2ase) activity ((nmol C_2H_4 formed μg^{-1} Chl *a* h^{-1} of parent *Nostoc muscorum* and its EDA **resistant (*Eda*^{r}) strains.**

Media	Parent		Eda^r_L		Eda^r_S	
	HF	N2-ase activity	HF	N2-ase activity	HF	N2-ase activity
N_2	5-6	15.2 (± 0.52)	5-6	13.4 (± 0.41)	5-6	19.2 (± 0.54)
NH_4Cl (1 mM)	0.0	0.0	0.0	0.0	0.0	0.0
Glutamine (1 mM)	0.0	0.0	0.0	0.0	0.0	0.0
EDA (0.2 mM)	0.0	0.0	0.0	0.0	4-5	14.5 (± 0.36)

All the strains were first grown in glutamine medium for 6 days, washed and transferred to desired media and then incubated for growth for 72 h before estimating heterocyst frequency and nitrogenase activity.

Each value is an average of (± SE) three independent experiments.

Table 4.3b

Heterocyst frequency (number of heterocysts per 100 vegetative cells), HF and Nitrogenase (N2ase) activity ((nmol C2H4 formed μg^{-1} Chl a h^{-1} of *Msx*^{r}-GS strain of *Nostoc muscorum* and its EDA resistant (*Eda*^{r}) strains.

Media	*Msx*^{r}-GS		*Msx*^{r}-*Eda*^r_L		*Msx*^{r}-*Eda*^r_S	
	HF	N2-ase activity	HF	N2-ase activity	HF	N2-ase activity
N_2	5-6	12.6 (± 0.32)	5-6	13.6 (± 0.39)	5-6	20.1 (± 0.44)
NH_4Cl (1 mM)	0.0	0.0	0.0	0.0	0.0	0.0
Glutamine (1 mM)	0.0	0.0	0.0	0.0	0.0	0.0
EDA (0.2 mM)	0.0	0.0	0.0	0.0	5-6	16.8 (± 0.49)

All the strains were first grown in glutamine medium for 6 days, washed and transferred to desired media and then incubated for growth for 72 h before estimating heterocyst frequency and nitrogenase activity.

Each value is an average of (± SE) three independent experiments.

Discussion

In heterocystous diazotrophic cyanobacterial forms, one **always finds a pattern** distribution of heterocysts where a group of vegetative cells, are found regularly associated with any one of the many heterocysts, in one dimensional array. The heterocyst and its nearby vegetative cell represent a kind of symbiotic association. The continued efficient maintainance of this delicate interdependence of two cell types would need metabolisms that can ensure distribution of heterocysts in a spaced pattern. In a fundamental work on genetics of heterocyst formation in *N. muscorum* and *N. linckia,* Singh et al. (1977) found evidences to conclude, that heterocyst formation and nitrogenase activity is under the positive control of a regulatory gene and that active nitrogenase is not involved in regulation of heterocyst spacing. This heterocyst spacing pattern is under the genetic control and the nature of the mechanism of the heterocyst inhibition by proheterocyst is different from that of heterocyst inhibition by nitrate or ammonium. Mutants defective in heterocyst formation (Het^-) and/or nitrogen fixation *(Nif~)* have been described by many workers in other heterocystous cyanobacteria (Currier et al., 1977; Padhy and Singh, 1978; Wilcox et al., 1975). Development of vegetative cell into heterocyst has been shown to accompanied by *Nif* gene arrangement in *Anabaena* PCC7120 by Golden et al. (1985), Haselkorn et al. (1987). Fogg (1949) suggested that either ammonia or a close derivative of it is the regulator of heterocyst differentiation and heterocyst

pattern formation. Wolk (1967), found evidence to conclude that heterocyst controls new heterocyst formation in a negative way, by exerting heterocyst inhibitory influence, involved in the regulation of heterocyst pattern formation and comes into operation at a much early stage of heterocyst development called proheterocyst. Stewart and Rowell (1975) presented evidence resulting from the action of MSX on GS activity in ammonium assimilating non-heterocystous filament that ammonia itself is not the regulator and that glutamine or some other metabolic product of ammonia was likely to be the regulator molecule of heterocyst differentiation and nitrogenase activity. Singh et al. (1983a) have isolated a GS deficient glutamine axotrophic mutant of *Anabaenae cycadae*, where ammonia was found inhibiting heterocyst formation and nitrogenase activity. They found ammonium inhibition of heterocyst formation and nitrogenase activity to get derepressed in the presence of MSX and it was shown that the derepression was a result of MSX inhibition of ammonium uptake. This led them to propose that ammonium *per se* is the initial repressor signal of heterocyst formation and nitrogenase activity in the cyanobacteria. Turpin et al. (1984) and Mackerras and Smith (1986) concluded that indeed ammonia *per se* is the regulator of heterocyst formation and nitrogenase activity.

Cyanobacterial diazotrophic and non-diazotrophic strains grow at pH 7.5-8.0 with ammonia as nitrogen source and under such conditions ammonia is expected to exist predominantly in the form of ammonium ion which can enter cyanobacterial cells only through the

involvement of the active ammonium transport system (Amt). Active transport of NH_4^+ has been investigated in a variety of bacterial species (Kleiner, 1985) using [^{14}C]methylammonium as a model substrate. Studies of the nitrogen regulation of Amt system in *E. coli* has demonstrated the involvement of a *ntr* system (Jayakumar et al., 1986). Operation of biphasic Amt system was demonstrated for the first time by Rai et al. (1984). Boussiba et al. (1984, 1989), Singh et al. (1985), Rai et al. (1986) have confirmed the existence of Amt system in cyanobacteria as well. This Amt system can be repressed by ammonium. A role of Amt system has been suggested to be a mechanism for conservation of internally generated ammonia from N_2-fixation, nitrate reduction or deamination (Boussiba et al., 1984; Kleiner, 1985). According to this view, absence of Amt activity from cells fixing N_2 or assimilating NO_3^- is expected to result in extracellular liberation of N_2-derived or NO_3^--derived ammonia. Both parent and *Het*$^-$ *Nif*$^-$ mutant strain of *N. muscorum* used in the present study were Amt$^+$, did not show any Amt activity during growth with nitrate as nitrogen source and also did not generate extracellular ammonia during such growth. It supports the view, that in cyanobacteria Amt system does not function as a mechanism for cellular conservation of ammonia, derived from N_2-fixation or NO_3^- reduction.

A large number of studies have been done on the metabolic aspects of ammonia excretion in diazotrophic and non-diazotrophic cyanobacteria. The mutants that excrete ammonia will have certain

common features like reduced GS actvity and increased nitrogenase activity (Polukhina et al., 1982; Singh et al., 1983b; Lattore et al., 1986; Spiller et al., 1986; Boussiba and Gibson, **1991).** **Ammonia excreting** mutants of the cyanobacteria invariably possess normal **Amt** system, thereby implying NH_4^+ excretion does not depend on **Amt** system. Selection of mutant strains of heterocystous cyanobacteria for MSX resistance as a result of mutation in *gln* A gene have been shown to result in generation of ammonia excreting strains (Singh et al., 1983b; Thomas et al., 1990). Similarly selection of mutants of *Anabaena variabilis* resistant to growth inhibition by EDA at pH 9.0 has also led to production of ammonia excreting strains (Kerby et al., 1986, 1987). In the present study, EDA resistance was als found to be associated with excretion of ammonia and all such Eda^r strains when examined were found to have defective GS by biosynthetic activity. Interestingly, one class of Eda^r mutants arose as a result of their ability to assimilate EDA like fixed nitrogen source. The *gln* A gene codes for cyanobacterial GS enzyme. Since mutant GS activities comprise two distinct groups, one associated with utilization of EDA as nitrogen source and the other without acquiring the ability to utilize the ammonium analagoue as nitrogen source. Such mutant GS phenotypes are **obviously** due to the result of mutations at different sites in the *gln* A gene that are regulating in GS activity. These findings clearly suggest us, to understand in detail, the molecular mechanism controlling *gln* A gene mutation specifically at those sites, that lead **to** the production of mutant GS which is resistant to inhibition

by EDA, but which is incapable of assimilating it like nitrogen source. Our results suggest that the mutant *Msx*^{r}-GS strain and mutant *Eda*^{r}-GS strains Jack a cross-resistance relationship, that **the frequency** of spontaneous mutation to *Eda*^{r} phenotype is apparently a function of *gln* A allele and that the mutant *Eda*^{r}-GS strain comprises two distinct physiological groups. Such areas although little studied have a great bearing on the biofertilizer technology of cyanobacteria. Verma et al. (1990) have isolated glutamine auxotrophic (*gln*$^-$) mutants of *N. muscorum* specifically deficient in GS activity, such mutants of heterocystous forms with transformable systems are expected to provide ideal systems for :

(a) determining the organization, regulation and functioning of cyanobacterial *gln* A, and

(b) cloning of *gln* A gene of cyanobacteria and bacteria in the background of the present findings.

CHAPTER 5

CHARACTERISTICS OF METHIONINE TRANSPORT SYSTEM IN SPONTANEOUS MUTANTS OF *NOSTOC MUSCORUM* RESISTANT TO METHIONINE ANALOGUES : ETHIONINE AND NORLEUCINE, GS INHIBITOR : PHOSPHINOTHRICIN AND GROWTH INHIBITOR : A2IDE

Introduction

In enterobacteria amino acids can serve as a source of nitrogen and carbon through the *ntr* system of genetic control (Merrick, 1988). There are no detailed studies about the role of amino acids in nitrogen or carbon nutrition in cyanobacteria. Glutamine, arginine and proline can serve as nitrogen source in *Nostoc muscorum*, in which glutamate is found to be growth toxic (Singh et al., 1991, 1992). Spence and Stewart have demonstrated a role of proline oxidase in assimilation of proline as nitrogen source In *Anabaena* sp. PCC7120 (Spence and Stewart, 1976). Several other cyanobacteria have also been examined, with respect to amino acid nutrition and growth studies have suggested, some of them to function, as very effective nitrogen source like ammonium (Nielson and Larsson, 1980; Vaishampayan, 1982; Rawson, 1985). There are very few studies dealing with the mechanism of amino acid transport in heterocystous cyanobacteria. There are reports for the occurrence of high and low affinity transport system for glutamate and glutamine (Chapman and Meeks, 1983) and single

transport for leucine (Thiel, 1988) in *A. variabilis* ATCC29413, glutamine transport system in *A. cylindrica* (Rowell et al., 1977), a glutamate/aspartate in *Nostoc* sp. (Strausser and Falkner, 1986) and a glutamine/glutamate transport system in *Anabaena* sp. PCC7120 (Flores and Muro-Pastor, 1988). Occurrence of proline transport system, has also been demonstrated in *N. muscorum* (Singh et al., 1991) and *Anabaena* sp. PCC7120 (Spence and Stewart, 1986). The results of amino acid transport system indicate the occurrence of limited number of amino acid transport systems, one specific for basic amino acids, the other for neutral amino acids and a third one specific for aromatic amino acids.

The activities of amino acid transport systems are expected to determine the intracellular accumulation of exogenous amino acids in cyanobacteria. In addition, one would also like to know the role of amino acid transport systems in regulating intracellular and extracellular pool of amino acids and the role of intracellular amino acids in nitrogen nutrition as well as protein synthesis.

Mutational studies in *N. muscorum* have indicated a definite role of GS activity in the assimilation of arginine or proline as nitrogen source (Singh et al., 1991). Methionine analogues such as ethionine and norleucine have been used to isolate methionine analogue resistant mutants of *N. muscorum.* Phosphinothricin, a potent glutamine synthetase (GS) inhibitor, has been used to isolate

its resistant mutant. Azide, a well-known inhibitor of microbial growth, has been used to isolate its resistant mutant strain. Earlier studies suggest that azide inhibits growth by inhibiting ATPase activity (Rosin and Kepes, 1972), nitrate reductase activity (Solmonson and Vennesland, 1972), nitrogenase (Lockshin and Burris, 1965), cytochrome oxidase activity and respiratory electron flow (Peschek, 1981). Resistance to azide in cyanobacteria may occur in three different ways :

(i) by derepressing heterocyst and nitrogenase in combined nitrogen media (Singh and Singh, 1978),

(ii) by utilization as substrate for nitrogenase (Schollhorn and Burris, 1977), and

(iii) by being defective in azide uptake (transport mutants).

Those found defective in the methionine transport system, were used to find out the role of transported methionine as a source of nitrogen in cyanobacteria. The results suggest that these mutant strains, are all nearly fifty per cent defective in glutamine and methionine transport and such transport defective mutants, lack repression control of methionine or glutamine on heterocyst-nitrogenase system. Such amino acid transport defective mutants would be useful for use as biofertilizer since they would keep fixing nitrogen while assimilating exogenous amino acids available from the growth medium.

Materials and methods

Axenic cultures of *Nostoc muscorum* were routinely grown in modified Chu No. 10 medium as described in Chapter 2, free of combined nitrogen source or containing 1 mM glutamine or methionine as nitrogen source where necessary. Log phase cultures were used for the isolation of various mutant strains after determining the lethal dose to the parent culture.

Isolation of spontaneous mutants resistant to various inhibitors

(i) Isolation of L-ethionine resistant strain :

L-Ethionine at a concentration of 2 $\mu g\ ml^{-1}$ was found to be completely growth toxic to parent strain under diazotrophic growth conditions. L-Ethionine concentration of 50 $\mu g\ ml^{-1}$ was used for selecting out spontaneous mutants resistant to L-ethionine. Diazotrophically grown log phase cultures were harvested, washed, fragmented and approximately 2 x 10^6 CFU were spread on agar plates containing N2-medium with 50 $\mu g\ ml^{-1}$ L-ethionine. The inoculated plates were incubated at photoautotrophic growth conditions at a temperature of 28 ± 2 °C and photon fluence rate of 50 μmol m s for fortnight.

(ii) Isolation of L-norleucine resistant strain :

L-Norleucine at a concentration of 1 mM (131.17 $\mu g\ ml^{-1}$) was found

to be completely growth toxic to parent strain under diazotrophic growth conditions. L-Norleucine concentration of 4 mM was used for isolating spontaneous mutants resistant to L-norleucine. Diazotrophically grown log phase cultures were harvested, washed, fragmented and approximately 2 x 10^6 CFU were spread on agar plates containing N2-medium with 4 mM L-norleucine. The inoculated plates were incubated at photoautotrophic growth conditions at a temperature of 28 ± 2 °C and photon fluence rate of 50 μmol m^{-2} s^{-1} for fortnight.

(iii) Isolation of phosphinothricin resistant strain :

Phosphinothricin at a concentration of 0.1 μg ml^{-1} was found to be completely growth toxic to parent strain under diazotrophic growth conditions. Phosphinothricin concentration of 1 μg ml^{-1} was used for selecting out spontaneous mutants resistant to phosphinothricin. Diazotrophically grown log phase cultures were harvested, washed, fragmented and approximately 2 x 10^6 CFU were spread on agar plates containing N2-medium with 1 μg ml^{-1} phosphinothricin. The inoculated plates were incubated at photoautotrophic growth conditions at a temperature of 28 ± 2 °C and photon fluence rate of 50 μmol m^{-2} s^{-1} for fortnight.

(iv) Isolation of azide resis :ant strain :

Sodium azide at a concentration of 20 μg ml^{-1} was found to be completely growth toxic to parent strain under diazotrophic growth

conditions. Azide concentration of 100 μg ml^{-1} was used for selecting out spontaneous mutants resistant to phosphinothricin. Diazotrophically grown log phase cultures were harvested, washed, fragmented and approximately 2 x 10^6 CFU were spread on agar plates containing N2-medium with 100 μg ml^{-1} sodium azide. The inoculated plates were incubated at photoautotrophic growth conditions at a temperature of 28 ± 2 °C and photon fluence rate of 50 μmol m^{-2} s^{-1} for fortnight.

In all cases, the surviving colonies were repeatedly restreaked on fresh nutrient plates containing the respective inhibitor, tested for their stability and then isolated and stored on agar slants for further experimental use. The mutant strain resistant to L-ethionine was designated as *Etn*^{r}, to L-norleucine as *Norleu*^{r}, to phosphinothricin as *Ppt*^{r} and to azide as *Azi*^{r}. Exponentially growing parent *N. muscorum* and its mutant strains were grown either diazotrophically or in N2-medium containing 1 mM methionine was used to determine the heterocyst frequency and nitrogenase activity as described in Chapter 2.

[^{35}S]*Methionine transport assay*

Parent and its mutant strains were grown in N2-medium, 1 mM glutamine containing medium and 1 mM methionine medium. Exponentially growing cultures were harvested, washed and suspended in 10 mM HEPES-NaOH buffer (pH 7.5) and transport activity was

assayed as described in Chapter 2. [^{35}S]Methionine was suitably diluted with cold methionine stock solution. Aliquots were then drawn from it and added to the cell suspension at a final concentration of 100 μM. [^{35}S]Methionine (Specific activity 5 Cl mol^{-1}) was purchased from BRIT, Bombay, India.

Results

The mutant strains were checked for methionine transport activity, in relation to their wild type parent, and those that showed deficient methionine transport under diazotrophic growth conditions, were selected for further study. The frequency of such inhibitor resistant mutants, were normally in the range of 1-5 x 10^{-6}. The methionine transport was measured by making use of [^{35}S] methionine and the results of such studies are shown in Table 5.1. Parent *N. muscorum* showed almost similar activity of methionine transport system in diazotrophic grown, glutamine grown or methionine grown cultures. It is important to mention here that glutamine or methionine grown cultures of the parent strains never produced heterocysts and nitrogenase activity, thus indicating their utilization like a fixed nitrogen source in cyanobacterial growth. While one can infer that glutamine or methionine functions as co-repressor of heterocyst-nitrogenase system in the cyanobacterium, no such methionine or glutamine repression of methionine transport

Table 5.1

L-Methionine transport (nmol ^{35}S-methionine mg^{-1} Chl a min^{-1}) in the parent *Nostoc muscorum* and its various mutant strains.

Strains	Nature of the growth media		
	N_2-grown	Glutamine grown	Methionine grown
Parent	40.13 (± 2.17)	46.06 (± 2.82)	40.0 (± 2.93)
Etn^r	15.65 (± 0.87)	30.13 (± 2.13)	36.0 (± 1.78)
Norleu^r	20.67 (± 1.13)	24.15 (± 1.89)	22.33 (± 1.77)
Ppt^r	8.07 (± 0.65)	19.20 (± 1.08)	35.71 (± 2.67)
Azi^r	7.82 (± 0.49)	10.0 (± 0.58)	10.13 (± 0.64)

Six day old drowing cultures were harvested, washed and suspended in 10 mM HEPES-NaOH buffer (pH 7.5) before the uptake was measured.

All values are average (± SE) of three independent experiments.

activity, is evident in the parent strain. The *Etn*^{r} mutant suffered more than two-fold decrease in methionine transport activity, under diazotrophic growth conditions. However, methionine transport activity in this mutant increased in methionine grown cultures or glutamine grown cultures. The increase of methionine transport activity was almost similar to similarly grown cultures of the parent strain. One can therefore infer that methionine transport system in *Etn*^{r} mutant has become glutamine/methionine activable. The *Norleu*^{r} mutant like *Etn*^{r} mutant also suffered decrease in methionine transport activity under diazotrophic growth condition and such decreased transport activity was not significantly influenced by glutamine or methionine. The *Ppt*^{r} strain and *Azi*^{r} strain were more deficient in methionine transport activity than *Etn*^{r} or *Norleu*^{r} mutant strains under diazotrophic growth conditions. However, both glutamine to a lesser extent and methionine to a much greater extent, activated the methionine transport activity in the *Ppt*^{r} strain. In comparison, the *Azi*^{r} mutant strain did not show glutamine/methionine activation of methionine transport system. *Azi*^{r} mutant strain and *Norleu*^{r} mutant strains thus seem similar in respect of their response to glutamine/methionine activation of their methionine transport system. Similarly *Etn*^{r} and *Ppt*^{r} mutant strains belong to a different category in terms of their methionine transport activity being glutamine/methionine activable. Not much can be said at the moment, about nitrogen regulation of methionine

transport activity except that, it is not glutamine/methionine repressible.

Since the various mutant strains were defective in methionine transport system at different degree, we examined methionine repression control of heterocyst and nitrogenase activity in them. Results of such studies were given in Table 5.2. The parent *N. muscorum* neither showed heterocyst nor nitrogenase activity, in the growth medium containing 1 mM L-methionine. In comparison to its diazotrophic culture, *Etn*^{r} strain produced heterocyst and nitrogenase activity, under diazotrophic growth as well as in the growth medium containing 1 mM methionine. However, the frequency of heterocyst and the level of nitrogenase activity were slightly lower in methionine media than in diazotrophic growth medium. It thus appears, that mutational decrease in methionine transport is the reason for apparent lack of methionine repression on heterocyst formation and nitrogenase activity in the mutant strain. *Nor leu*^{r} mutant strain behaved more or less like the Etn^r strain in respect of lack of methionine repression control of aerobic diazotrophy. *Azi*^{r} and *Ppt*^{r} mutant strains were also similar qualitatively in respect of loss of methionine repression control on heterocyst system in the cyanobacterium. Quantitatively speaking *Azi*^{r} and *Ppt*^{r} mutant strains were more efficient than *Etn*^{r} or *Norleu*^{r} mutant strains in showing lack of methionine repression particularly on the nitrogenase system. When one compares the

Table 5.2

Heterocyst frequeny (number of heterocysts per 100 vegetative **cells),** HF and nitrogenase (N2ase) activity of the parent *Nostoc* ***muscorum*** **and its** various mutant strains grown in **diazotrophic and methionine medium.**

Strains	Growth media			
	N_2-medium		Methionine medium	
	HF	N2ase activity	HF	N2ase activity
Parent	5-6	1.63 (± 0.09)	0.0	0.0
Etn^r	5-6	2.49 (± 0.13)	3-4	1.014 (± 0.05)
Norleu^r	6.0	2.12 (± 0.11)	5-6	1.75 (± 0.09)
Ppt^r	5.0	1.21 (± 0.08)	5-6	2.1 (± 0.11)
Azi^r	5.0	1.65 (± 0.13)	5-6	2.25 (± 0.09)

The values are average (± SE) of three independent experiments.

methionine transport data from various amino acid analogue resistant mutants with the data on the degree of lack of methionine repression control on heterocyst-nitrogenase system in the cyanobacterium, one finds a correlation between the loss in methionine transport activity and lack of methionine repression control on heterocyst-nitrogenase system.

Discussion

Transport of amino acids in bacteria often involves multiple stereospecific transport systems. Transport of branched-chain amino acids *viz.*, L-leucine, L-isoleucine and L-valine is one of the best studied of these and probably involves several transport systems (Yamato et al., 1979). A series of studies have also been conducted to study the genetics of nitrogen regulation of amino acid catabolism in *E. coli* and S. *typhimurium* and the results suggest the operation of a nitrogen starvation signal in activation of operons for metabolism of amino acids like histidine and arginine (Merrick, 1988).

Cyanobacteria can provide an ideal system for examining the reasons, about the multiplicity of specific amino acid transport systems in enterobacteria. The results of such studies will also throw light on, why some amino acids are toxic to cyanobacteria and why others are not (Thiel, 1988). Cyanobacteria would also provide

a simple system, to study how metabolite uptake is energized in photosynthetic organisms. It has been suggested that obligate photoautotrophic cyanobacteria do not control their intermediary metabolism by repression/derepression of enzyme synthesis and that this lack of transcription regulation is a characteristic and causative feature of obligate autotroph physiology (Carr, 1973). Amino acids such as methionine or glutamine are known to block the inhibitory effect of MSX on *Chlorella* GS activity by inhibiting its transport (Meins and Abrams, 1972). The inclusion of amino acids in the growth medium and demonstration of their assimilation into cell material is not accompanied by reduction in levels of amino acid biosynthetic enzymes. This has been demonstrated for arginine and branched-chain amino acids in *A. variabilis* (Hood and Carr, 1972) and for 3-deoxy-D-arabino- heptalosonic-7-phosphate (Waber and Bock, 1968). Exogenous glutamine within the cell is known to be assimilated like a nitrogen source causing repression of its biosynthetic enzyme GS (Singh et al., 1993; Verma et al., 1990). Proline has also been shown to be utilized like a fixed nitrogen source in *Anabaena* sp. PCC7120 through the enzymatic mechanism involving the participation of cyanobacterial proline oxidase enzyme (Spence and Stewart, 1986). A definite role of GS in assimilation of amino acids like arginine and proline have been shown in *N. muscorum* where the cyanobacterial amino acid transport system for proline and arginine seem to function normally in the absence of GS enzyme activity. This also suggests while the GS enzyme activity is

essential for amino acid assimilation like a fixed nitrogen source it is not essential for regulating their transport (Singh et al., 1991).

Hole of amino acid transport systems in amino acid accumulation, excretion and utilization as nitrogen source needs to be understood at molecular level for construction of strains for extracellular production of amino acids, as well as for construction of strains that lack amino acid repression-control of heterocyst and nitrogenase activity in diazotrophic cyanobacteria. Few studies have been carried out on this aspect of amino acid transport in some cyanobacterial strains and all these studies suggest mainly operation of three amino acid transport systems in the metabolism of amino acids (Herrero and Flores, 1990; Labarre et al., 1987; Thiel, 1988; Chapman and Meeks, 1983). Mutation to resistance to amino acid analogues in cyanobacteria could be at the transport level, at the biosynthetic enzyme level or at their catabolic enzyme level. It is known that mutations to MSX resistance in cyanobacterium *A. doliolum* occurs both at the transport level and at the GS activity level. Such MSX transport defective mutants produce the MSX-sensitive GS enzyme (Singh et al., 1987c). The resistant mutant strains of *N. muscorum* described here include, resitance to methionine analogues ethionine (*Etn*^{r}) and norleucine (*Norleu*^{r}), to the GS inhibitor : phosphinothricin - (*Ppt*^{r}) and a potent growth inhibitor : sodium azide (*Azi*^{r}). All these mutants are defective

in methionine transport. These results do support the view for a common occurrence of transport system for the inhibitor as well as for methionine. The parent strain neither differentiated heterocysts nor showed nitrogenase activity during growth with methionine. This clearly suggests the ability of the cyanobacterium to utilize methionine as a nitrogen source. Earlier studies have shown the ability of *Anacystis nidulans* (Delaney et al., 1973)and *N. muscorum* (Vaishampayan, 1982) to utilize methionine like a nitrogen source.

The lack of methionine repression on heterocyst and nitrogenase activity in ethionine *[Etn^r)*, norleucine(*Norleu^r)*, phosphinothricin (*Ppt^r*) or azide *(Azi^r*) resistant cyanobacterlal mutant strains coupled with the observation for the operation of methionine defective transport system in them, suggests a direct role of the amino acid transport system in regulation of the amino acid catabolism, like a fixed nitrogen source.

The present findings on the behavior of *Azi^r* mutant strain corroborates with earlier observations of derepressed heterocyst and nitrogenase activity in combined nitrogen media (Singh and Singh, 1978) and lack of amino acid repression on heterocyst and nitrogenase activity may be useful in studying the property of the mutated gene products in *nif$^-$* or *NR$^-$* mutants of heterocystous diazotrophic cyanobacteria. In addition such mutants are useful

from the point of view of their utilization as biofertilizer strains in view of their ability to scavenge amino acids from the external medium in rice fields without suffering any adverse effect on their nitrogen fixing ability. It is therefore suggested, in any scheme or plan for construction of cyanobacterial biofertilizer strains, introduction of amino acid transport defective trait must be included.

CHAPTER 6

MUTATIONAL STUDY OF SALINITY/OSMOTIC REGULATION OF GROWTH, NITROGEN FIXATION, OXYGENIC PHOTOSYNTHESIS, AMMONIA ASSIMILATION AND AMMONIUM TRANSPORT SYSTEM

Introduction

Maintenance of structural parameters such as turgor and cell volume needs water. Biological membranes are permeable to water. Cells have mechanisms for retaining solutes (electrolytes or non-electrolytes) within a semi-permeable membrane against a concentration gradient but water is not retained and tends to move towards low thennodynamic water activity i.e., when a cell is water stressed either due to dessication or due to external solute concentrations, let water movement out of the cell is favoured. High solute concentrations in the medium can cause extensive influence on a cell in the following ways :

(i) Either by osmotic effects (water stress),
(ii) Ion specific effects (ion imbalance) or
(iii) Toxic effects (excessive accumulation of solutes or ions)

Depending on the type and concentration of the solutes surrounding the cell one of these factors may dominate. The osmotic effect may be counter balanced because higher solute concentration in the nutrient medium leads to increased rate of ion/solute uptake. This

lowers the water potential and stimulates the water uptake by the cells which raises the cell turgor. This change in the osmotic potential as a means of maintaining positive water balance, is known as osmotic adjustment or osmoadaptation or osmoregulation (Turner and Jones, 1980). It is a well-known fact that osmolarity like temperature is a physical parameter of great relevance to biological systems which have evolved various strategies to overcome this problem. Osmolarity of a natural habitat is subjected to great fluctuations and knowledge of the osmoregulatory mechanism of organisms from such habitats provide methods to artifically construct osmotolerant forms which can grow and multiply under fluctuating constrains of environmental osmolarity. Ionic and non-ionic environments of varying osmolarities are known to limit the survival and growth of organisms. Knowledge about the genetic and physiological mechanism of biological adaptation to salinity and osmotic stress is expected to be of great fundamental relevance to agriculture, food microbiology, plant-microbe interactions and medical microbiology (Csonka and Hanson, 1991). Problems of salinity and water stress in agriculture is now an ever-growing problem and future success of agriculture would depend greatly on the availability of crop plants resistant to salinity and water stress.

The genetics and physiology of osmoregulation has been studied in greater detail in bacteria, where accumulation of a variety of compatible solutes such K^+ ions, amino acids : glutamate, glutamine, proline, γ-aminobutyrate and alanine, quaternary amines

like glycinebetaine and sugars like trehalose and sucrose have been demonstrated to be the physiological mechanism of their adaptation to osmotic stress (Flowers et al., 1977; Imhoff, 1986).

The genetic basis of osmotic adaptation in enteric bacteria has been studied in detail which includes *kdp* A to E genes required for inducible K^+ uptake (Walderbaug et al., 1987), *pro* U and P genes required for the transport of proline and glycinebetaine (Cairney et al., 1985) and *pro* A, B and C genes required for synthesis of proline (Jakowec et al., 1985; Mahan and Csonka, 1983), *ots* A and B genes required for synthesis of trehalose (Giaver et al., 1988) and *bet* A, B and C genes required for transport of choline and synthesis of glycinebetaine from choline (Styrvold et al., 1986). *omp* genetic system has also been shown to contribute osmotic adaptation through control of synthesis of porin proteins of bacterial plasma membrane (Csonka, 1989).

N_2-fixation process in *Klebsiella pneumoniae* has been found to be an extremely osmosensitive process and a role of glycinebetaine in its protection has been implicated (Le Rudulier and Valentine, 1982). Bacterial osmotolerant mutants are available and over production of proline in these bacteria is found to be the biochemical basis of osmotolerance (Strom et al., 1983). Recent studies also advocate role of polyamines as compatible osmolytes in higher plants under osmotic stress (Flores and Galston, 1984).

Accumulation of glycinebetaine as a compatible osmolyte has been found to be widespread in higher plants (Jones and Storey, 1981) and the enzyme activities of its synthetic pathway have been shown to be osmoinducile (Broukisse et al., 1989). Genetic engineering of glycinebetaine pathway into important crops has been suggested to be one of the possible solutions to osmotic stress problem afflicting them (McCue and Hanson, 1990).

Studies on adaptation of cyanobacteria to osmotic/ salinity stress was started by Borowitzka, 1980; MacKay et al., 1983). Subsequently, a series of other studies were conducted to find out the physiological mechanism of osmotic stress resistance in various cyanobacteria characteristic of fresh water, marine and brackish water habitats (Reed and Stewart, 1988). These studies showed involvement of disacharides sucrose and trehalose or a heteroside such as glucosyl-glycerol or quaternary ammonium compounds such as glycinebetaine as compatible solutes produced under osmotic stress. The extreme osmotolerant cyanobacterial forms were found to accumulate glycinebetaine, the moderately osmotolerant forms glucosyl-glycerol and the least osmotolerant forms sucrose or trehalose. Very few studies have touched the aspects of nitrogen fixation and GS activity in relation to osmotic stress (Warr et al., 1988; Reed and Stewart, 1988). No studies have so far been made with regard to response of aerobic nitrogen fixation, ammonia/amino acid transport to naturally occurring osmotic stress situations. While hyperosmotic shock has been found to generate various types of organic osmotica within cyanobacterial cells there has been little

study with regards to the consequences of the hypoosmotic shock in such cyanobacteria. Few available studies indicate extracellular liberation of various organic carbon and fixed nitrogen compounds following hypoosmotic shock in *Synechocystis* sp. PCC6714 and *Calothrix scopulorum* (Reed and Stewart, 1988).

Diazotrophic cyanbacteria are natural biological systems of nitrogen input in wet land rice agriculture in tropical countries (Singh, 1961) and serious efforts are being made to increase their potential as biofertilizers in such ecosystems (Modi et al., 1991). The scientific merit of the present practice of using them as biofertilizer suffers from lack of knowledge about their salt tolerance, osmotic tolerance, herbicide tolerance and their ability to compete successfully against the native strains. It therefore, becomes imperative to generate technology to construct cyanobacterial biofertilizer strains which are resistant to salinity/osmotic stress. Chapter 3 of this thesis reports for the first time the successful application of techniques of genetic transformation and mutation in production of multiple herbicide resistant ammonia excreting strains of diazotrophic cyanobacterium and one of the desirable characters to be present in cyanobacterial biofertilizer strains relates to their resistance against salinity/osmotic stress as well. It therefore becomes essential to study the role of osmotic stress in regulating the production, assimilation and liberation of ammonia resulting from N_2-fixation. This has formed the basis for the origin of the present problem for study. No information exists about impact of osmotic stress on

biofertilzer quality of cyanobacteria. The biofertilzer quality of caynobacterial strains under field condition is a function of its survival, competetive ability, N_2-fixation and excretion of fixed nitrogen. Osmotic modulation of nitrogenase activity, N_2-fixation, ammonium transport and assimilation and amino acid transport needs detailed physiological and genetic analysis for developing appropriate osmotolerant cyanobacterial strains for use as biofertilizer. We have begun a systematic study of these aspects in the cyanobacterium *Nostoc muscorum* and our results do suggest a possible practical application of mutational technology in construction of genetic traits offering competent protection against salinity/osmotic stresses.

Materials and methods

Axenic clonal cultures of parent *N. muscorum* and various mutant strains (as described below) were grown and maintained photoautotrophically in modified Chu No. 10 diazotrophic medium.

Isolation of salinity (NaCl) resistant ($NaCl^r$) mutant strain

Sodium chloride at 150 mM was found completely lethal to cyanobacterial growth under diazotrophic growth conditions. Spontaneous mutants resistant to NaCl were selected out at this concentration. Such colonies were isolated and repeatedly restreaked and tested for their stability on fresh NaCl containing medium. This strain was designated as $NaCl^r$ strain.

Isolation of Osmotic (sucrose) resistant ($Sucrose^r$) *mutant strain*

Sucrose at 300 mM was found completely lethal to **cyanobacterial** growth under diazotrophic growth conditions. Spontaneous mutants resistant to sucrose were selected out **at this concentration.** Such colonies were isolated and repeatedly restreaked and tested for their stability on fresh sucrose containing medium. This strain was designated as $Sucrose^r$ strain.

Both the strains were maintained on agar slants and grown as and when required.

Sodium (Na^+) *transport*

Exponentially growing diazotrophic cultures of parent *N. muscorum* and its $NaCl^r$ and $Sucrose^r$ strains were harvested, washed twice with deionized water and resuspended in the same and equilibrated for 30 min in light at a photon fluence rate of 50 μmol m s . Na uptake was measured as depletion of Na from the bathing medium containing 100 μmol ml^{-1} NaCl using a flame photometer (Digisun Electronics, India). 100 ppm NaCl solution prepared in deionized water was used here as standard.

[^{14}C]Sucrose transport

[^{14}C]Sucrose (Sp. activity 300 Ci mol^{-1}) transport was assayed by following the method of Singh et al. (1985) with **appropriate** modifications, at 300 μM final sucrose concentration in

the reaction mixture following silicon-oil/ microcentrifugation technique (Scott and Nicholls, 1980), as described in Chapter 2.

[^{14}C]Methyl ammonium transport

[^{14}C]Methylammonium (Sp. activity 7.5 kBq ml^{-1}) transport **was** assayed by following the method of Singh et al. (1985) with appropriate modifications, at 50 μM final methyl ammonium concentration in the reaction mixture following silicon-oil/ microcentrifugation technique (Scott and Nicholls, 1980), as described in Chapter 2.

PS II mediated oxygen evolution was measured by using a Clark type Oxygen electrode fitted in a Gilson Oxygraph as described in Chapter 2. Nitrogenase activity and Chl a content were also estimated as described earlier (Chapter 2).

Results

As shown in Fig. 6.1a & b, parent *N. muscorum* did not survive a growth medium salinity of 150 mM NaCl or osmolority of 300 mM sucrose. The spontaneously occurring mutant clones of the cyanobacterium resistant to NaCl lethality or sucrose lethality arose with a frequency of 0.8 - 1.5 x 10^{-7} thus suggesting the $NaCl^r$ or $Sucrose^r$ phenotype to be the result of a single mutational event. The $NaCl^r$ strain was found to be equally resistant to sucrose **lethality** and *vice versa* showing a cross-resistant **nature (Fig. 6.1a & b).**

Legend to Fig. 6.1a

Survival curves of the various strains of *Nostoc muscorum* to different concentrations of NaCl.

Parent strain (●—•)

NaCl^{r} strain (■—•)

Sucrose^{r} strain (▲—▲)

Survival was estimated by counting the number of colonies seen on each nutrient plate after a week of incubation, under photoautotrophic diazotrophic growth conditions. Survival values thus obtained from the control plates, were treated as hundred percent and those obtained from the hypersaline nutrient plates, were expressed in percentage with respect to the control. Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols.

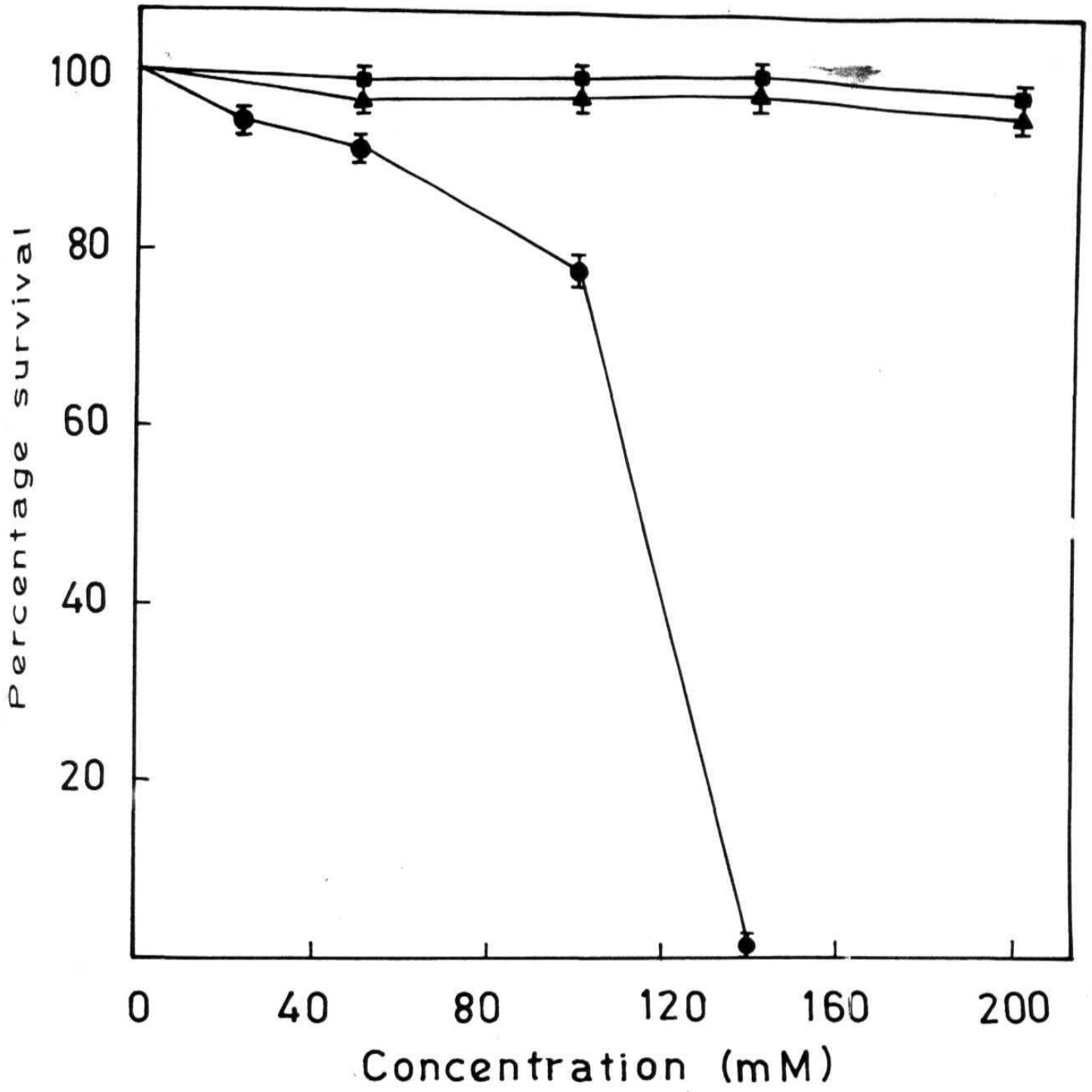
100
80
60
40
20
Percentage survival
0
40
80
120
160
200
Concentration (mM)

Fig. 6.1a

Legend to Fig. 6.1b

Survival curves of the various strains of *Nostoc muscorum* to different concentrations of sucrose.

Parent strain (●—•)

NaCl^{r} strain (■—•)

Sucrose^{r} strain (▲—A)

Survival was estimated as detailed in the legend to Fig. 6.1a. Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols.

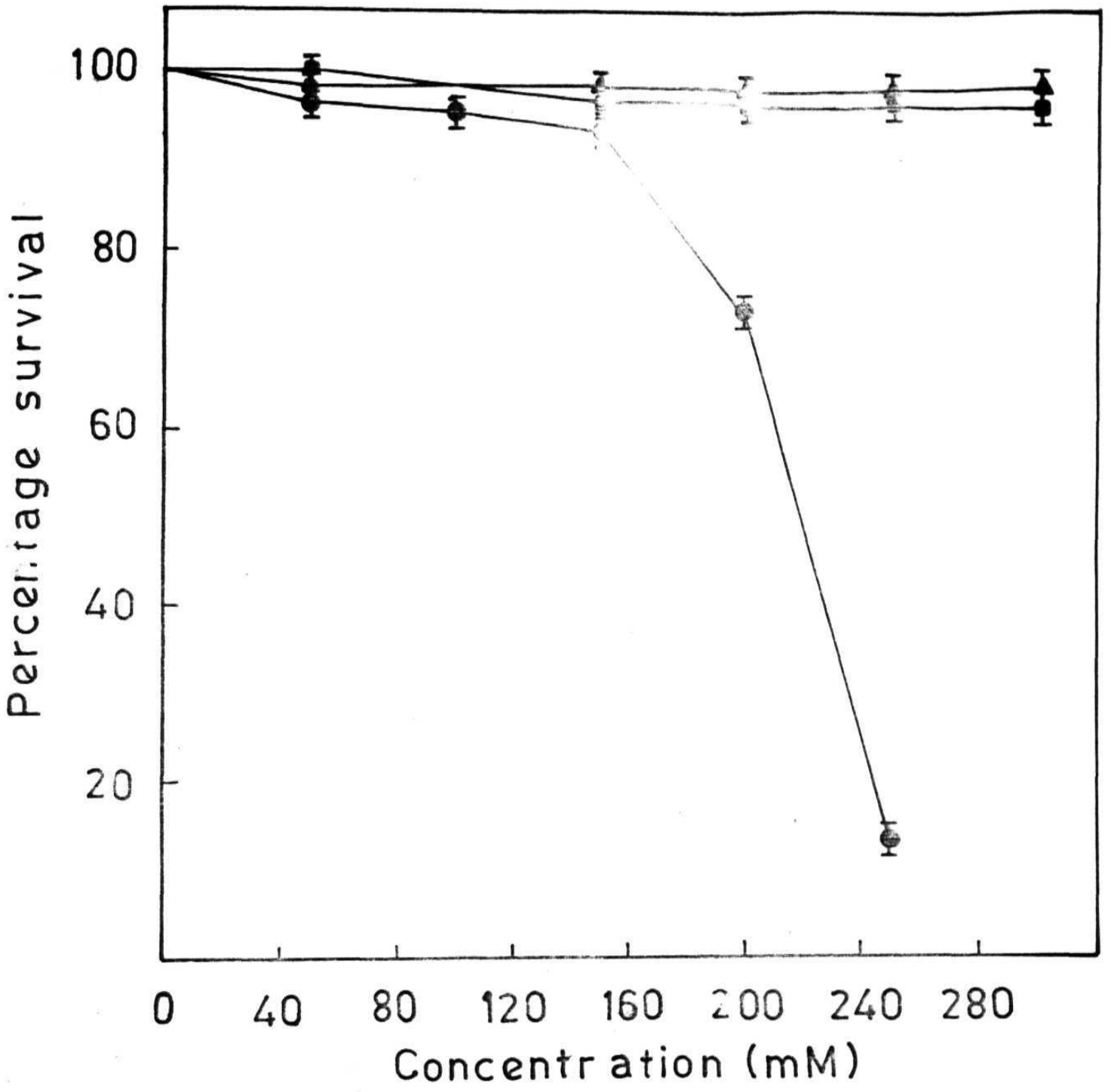
100
80
60
40
20
Percentage survival
0
40
80
120
160
200
240
280
Concentration (mM)

Fig. 6.1b

Inorganic ions like Na^+ at high cellular level are known perturbants of the structure and function of the enzymes and other cellular proteins (Yancey et al., 1982). Thus, any osmoregulatory cellular strategy must not only be osmoprotective and osmobalancing in nature but at the same time must also be functioning in effective curtailment of influx of perturbant ions like Na^+. This aspect of Na^+ transport was examined in the three strains of the cyanobacterium. As shown in Fig. 6.2, the mutant strains showed severe decrease in uptake of Na^+ with respect to the parent strain.

^{14}[C]Sucrose transport was also assayed in all the three strains where in all of them showed a similar pattern of transport (Fig. 6.3). Studies on time course influence of salinity/osmotic stress on nitrogenase of the three cyanobacterial strains has been presented in Fig. 6.4 a & b. Mutation to salinity/osmotic resistance was accompanied by a significant decrease in cyanobacterial nitrogenase activity. Nitrogenase activity of the parent strain was comparatively a little more sensitive to salinity stress than to osmotic stress. In comparison, the nitrogenase activity of the two mutant strains showed significant stimulation with sucrose treatment than with NaCl treatment. This might reflect a positive role of osmolarity in regulation of nitrogenase activity in osmotolerant cyanobacterial strains.

The parent and its mutant strains were also compared for photosynthetic oxygen evolving activity following treatment for 14 h with 150 mM NaCl or 300 mM sucrose. The salt or osmotic stresed cultures of the parent strain showed nearly five-fold decline in its

Legend to Fig. 6.2

Na^+ uptake in the three strains of *Nostoc muscorum*.

Parent strain (●—•)

NaCl^r strain (■—•)

Sucrose^r strain (▲—▲)

Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols. (For other details see text)

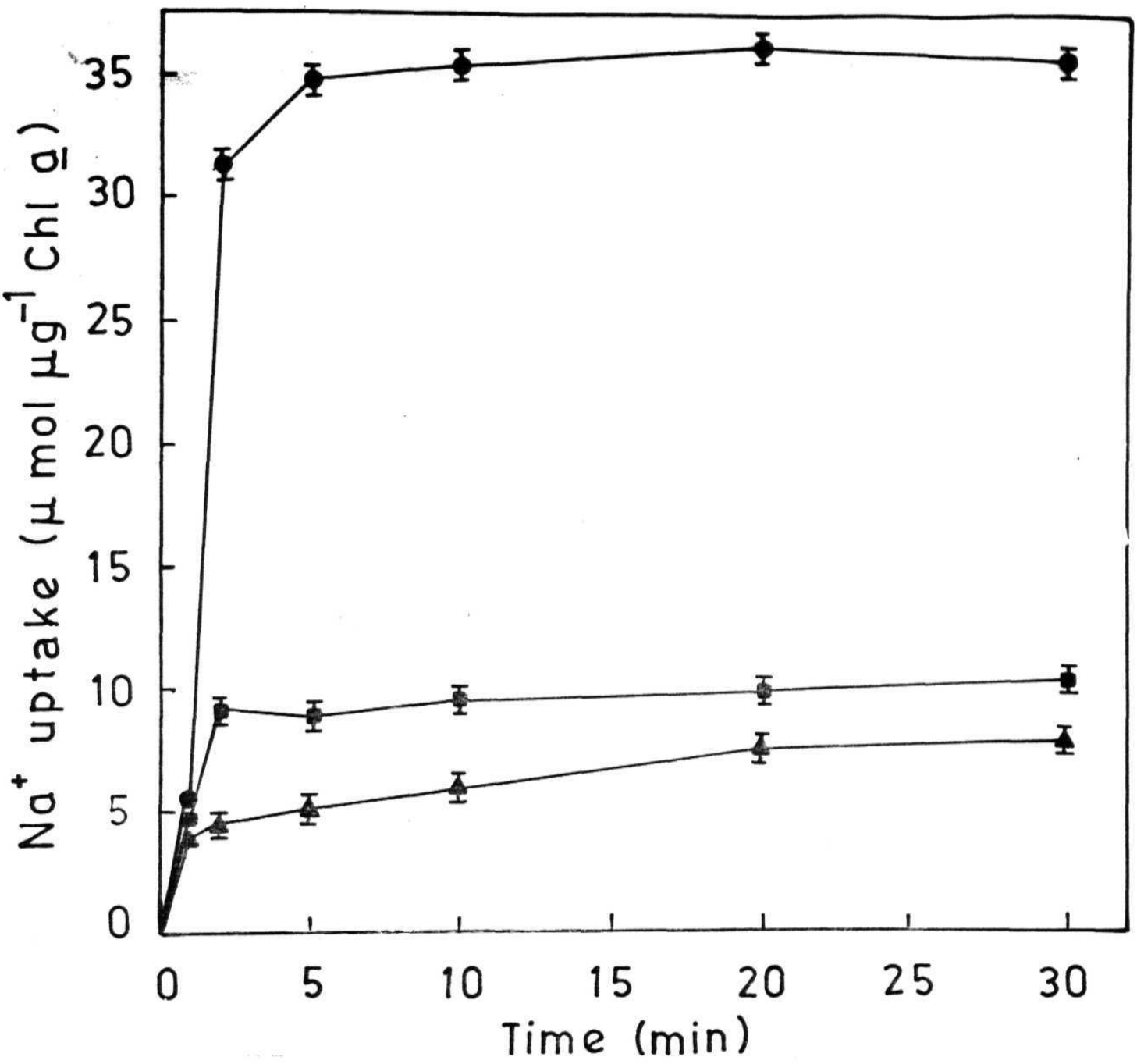
Na+ uptake (μ mol μg-1 Chl a)
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Time (min)

Fig. 6.2

Legend to Fig. 6.3

[^{14}C]Sucrose uptake in the three strains of *Nostoc muscorum*.

Parent strain (●—•)

NaCl^{r} strain (■—•)

Sucrose^{r} strain (▲—•)

Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols. (For other details see text)

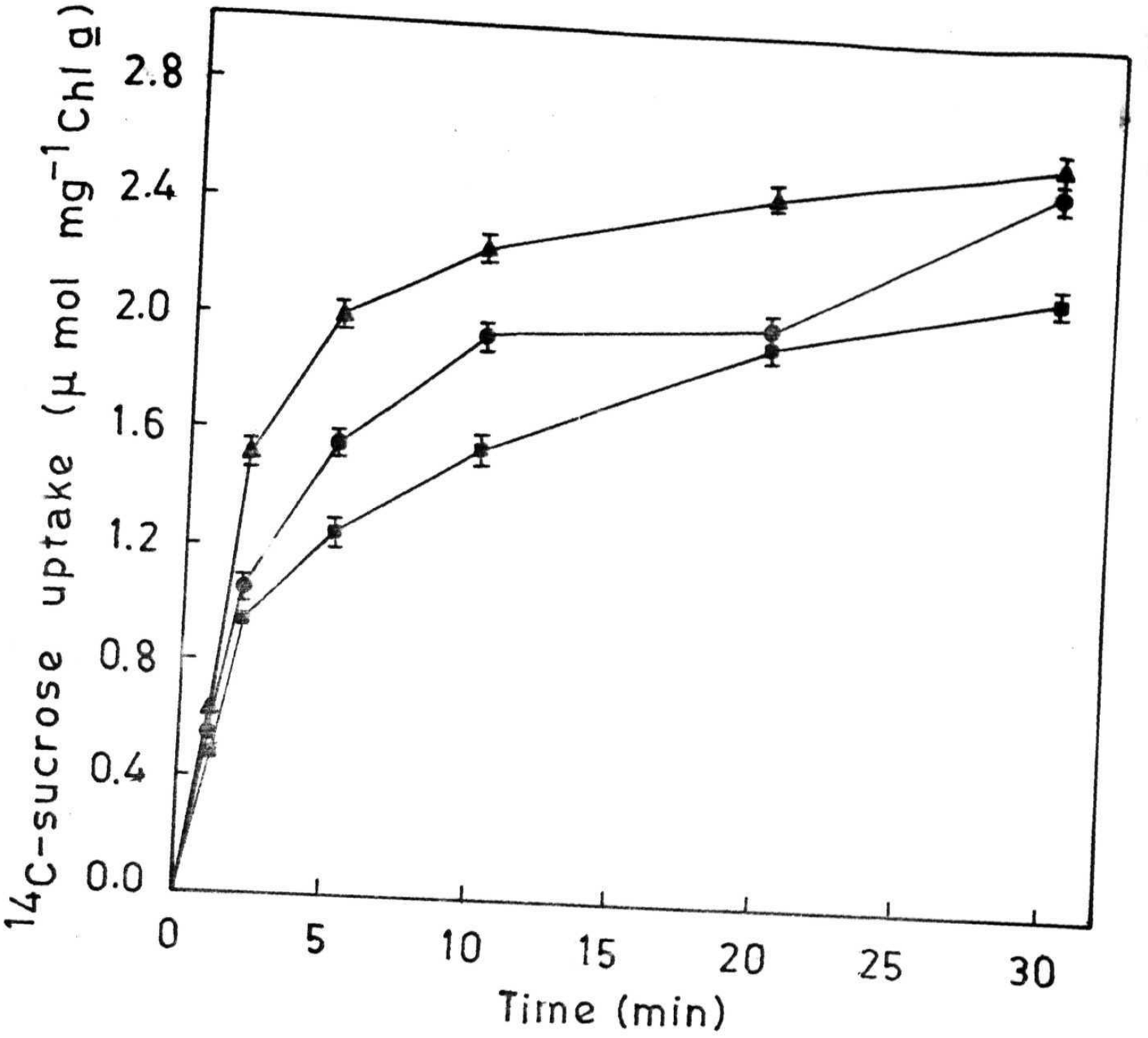
^{14}C-sucrose uptake (μ mol mg^{-1}Chl a)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0
5
10
15
20
25
30
Time (min)

Fig. 6.3

Legend to Fig. 6.4a

Effect of 150 mM NaCl (hypersalinity stress) on the nitrogenase activity of the three strains of *Nostoc muscorum*.

Parent strain (●—•)

NaCl^{r} strain (■—•)

Sucrose^{r} strain (▲—▲)

Six-day old diazotrophially growing cultures were harvested, washed and incubated in fresh N2-medium in the absence and presence of 150 mM NaCl at different time intervals. They were then harvested, washed, resuspended in fresh N2-medium and assayed for nitrogenase activity as described in Chapter 2.

Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols. (For other details see text)

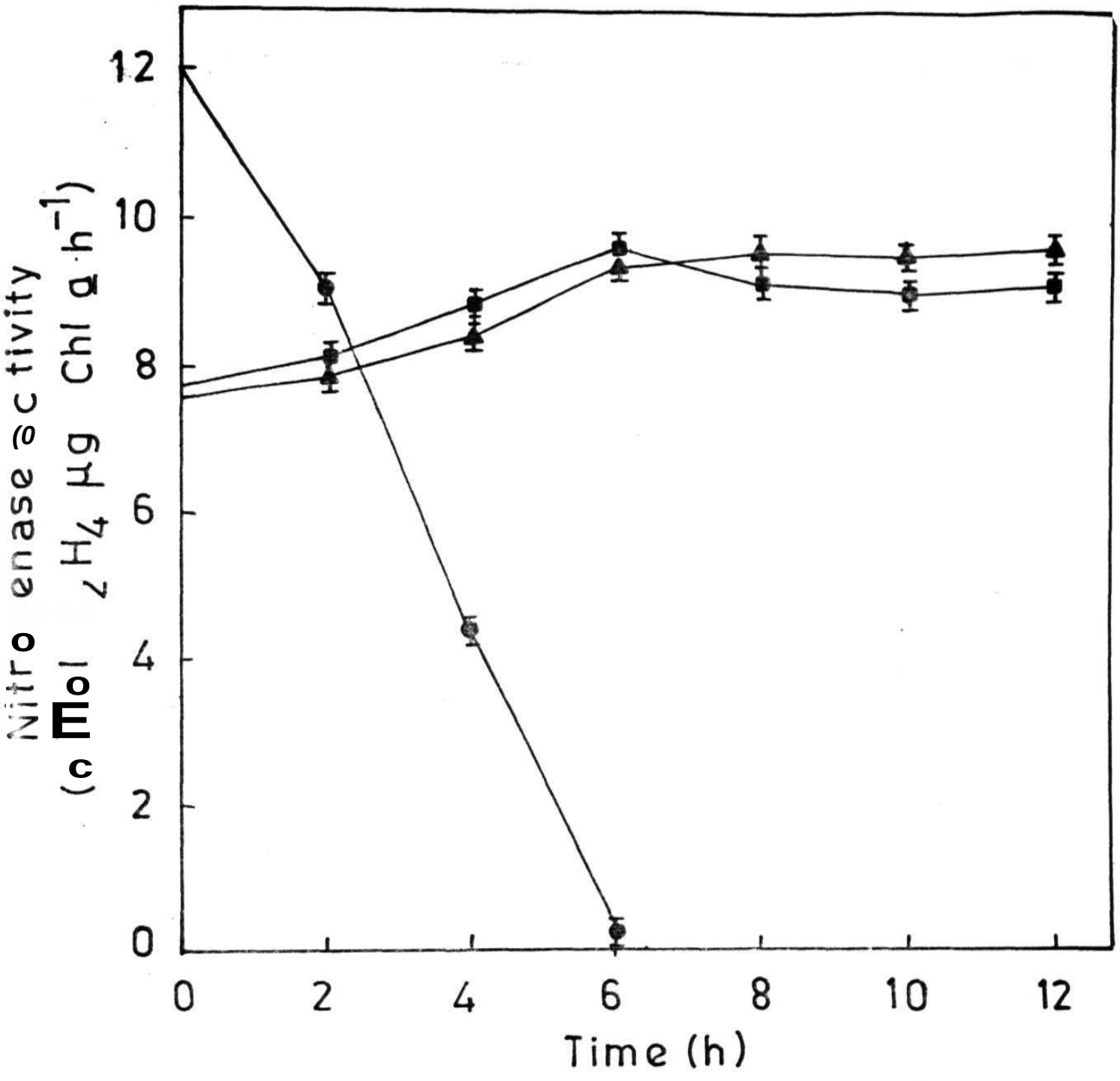

Fig. 6.4 a

Legend to Fig. 6.4b

Effect of 300 mM sucrose (hyperosmotic stress) on the nitrogenase activity of the three strains of *Nostoc muscorum*.

Parent strain (●—•)

NaCl^{r} strain (■——•)

Sucrose^{r} strain (▲——▲)

Six-day old diazotrophically growing cultures were harvested, washed and incubated in fresh N2-medium in the absence and presence of 300 mM sucrose at different time intervals. They were then harvested, washed, resuspended in fresh N2-medium and assayed for nitrogenase activity as described in Chapter 2.

Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols. (For other details see text)

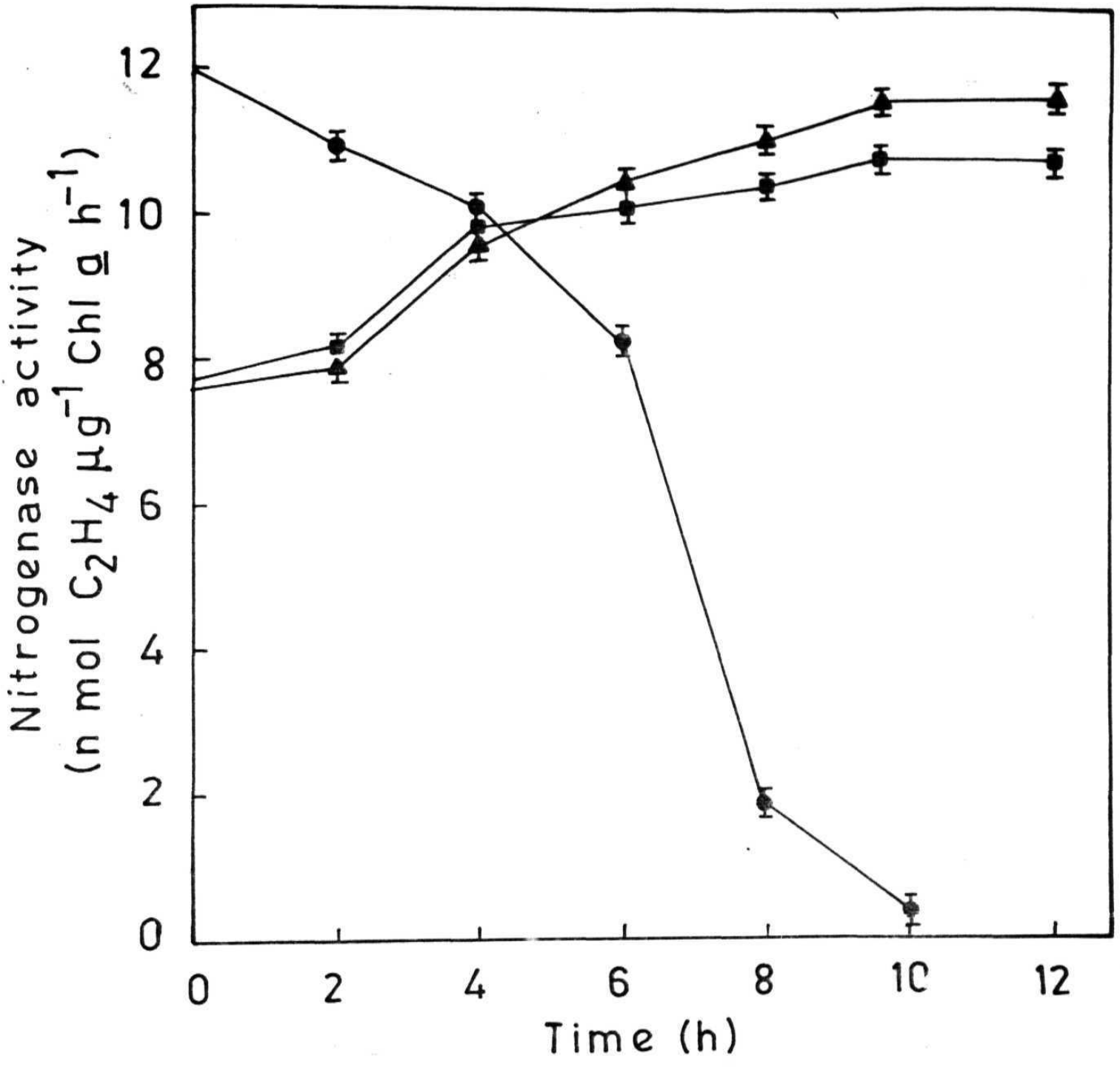
Nitrogenase activity
(n mol C_2H_4 μg^{-1} Chl a h^{-1})
0
2
4
6
8
10
12
Time (h)

Fig. 6.4 b

oxygen evolving activity. In comparison similarly treated cultures of *NaCl*^{r} or *Sucrose*^{r} strains showed no significant variation in photosynthetic O_2 evolution activity (Table 6.1). However, oxygenic photosynthetic process was found to be relatively more osmotolerant than N_2-fixation process.

Studies on ammonium (methylammonium) transport activity (Amt) in diazotrophically grown cultures showed that the activity decreased by almost fifty per cent in the parent strain (Fig. 6.5a) under stressed conditions, while it remained almost uninfluenced in the two mutant strains (Fig. 6.5b & c), under unstressed and stressed conditions.

Saline or osmotic sensitivity of *in vivo* GS activity of the parental strain was also examined in diazotrophic cultures stressed with 150 mM NaCl or 300 mM sucrose for 4 h. While extractable GS (biosynthetic) activity was 78.4 ±3.6 SEM μmol NADH oxidized mg^{-1} protein min^{-1} for stressed cultures and 85.5 ±4.2 SEM μmol NADH oxidized mg^{-1} protein min^{-1} for unstressed cultures, the activity of GS (transferase) was 1985 ± 37 nmol γ-glutamyl hydroxamate formed mg^{-1} protein min^{-1} for stressed cultures and 2012 ± 77 nmol γ-glutamyl hydroxamate formed mg^{-1} protein min^{-1} for unstressed cultures. Clearly cyanobacterial GS is little influenced by osmotic stress.

Table **6.1**

Oxygen evolution (PS **II)** activity **(nmol** O_2 evolved h^{-1} μg^{-1} **Chl** a) **of parent** *Nostoc muscorum* and **its NaCl-resistant** (*$NaCl^r$*) **and sucrose-resistant** *[$Sucrose^r$*) **mutant strains.**

Treatment	Parent	*$NaCl^r$*	*$Sucrose^r$*
Control	225.0 (± 14.55)	183.0 (± 11.15)	168.0 (± 9.4)
NaCl (150 mM)	46.5 (± 2.83)	188.2 (± 10.41)	172.5 (± 10.62)
Sucrose (300 mM)	43.1 (± 2.65)	184.5 (± 9.52)	166.5 (± 8.46)

Cultures of the strains were grown diazotrophically for 6 days, treated with ionic osmoticum (NaCl) or non-ionic osmoticum (sucrose) for 14 h and then examined for oxygen evolving activity.

Each value is an average (± SE) of three independent experiments.

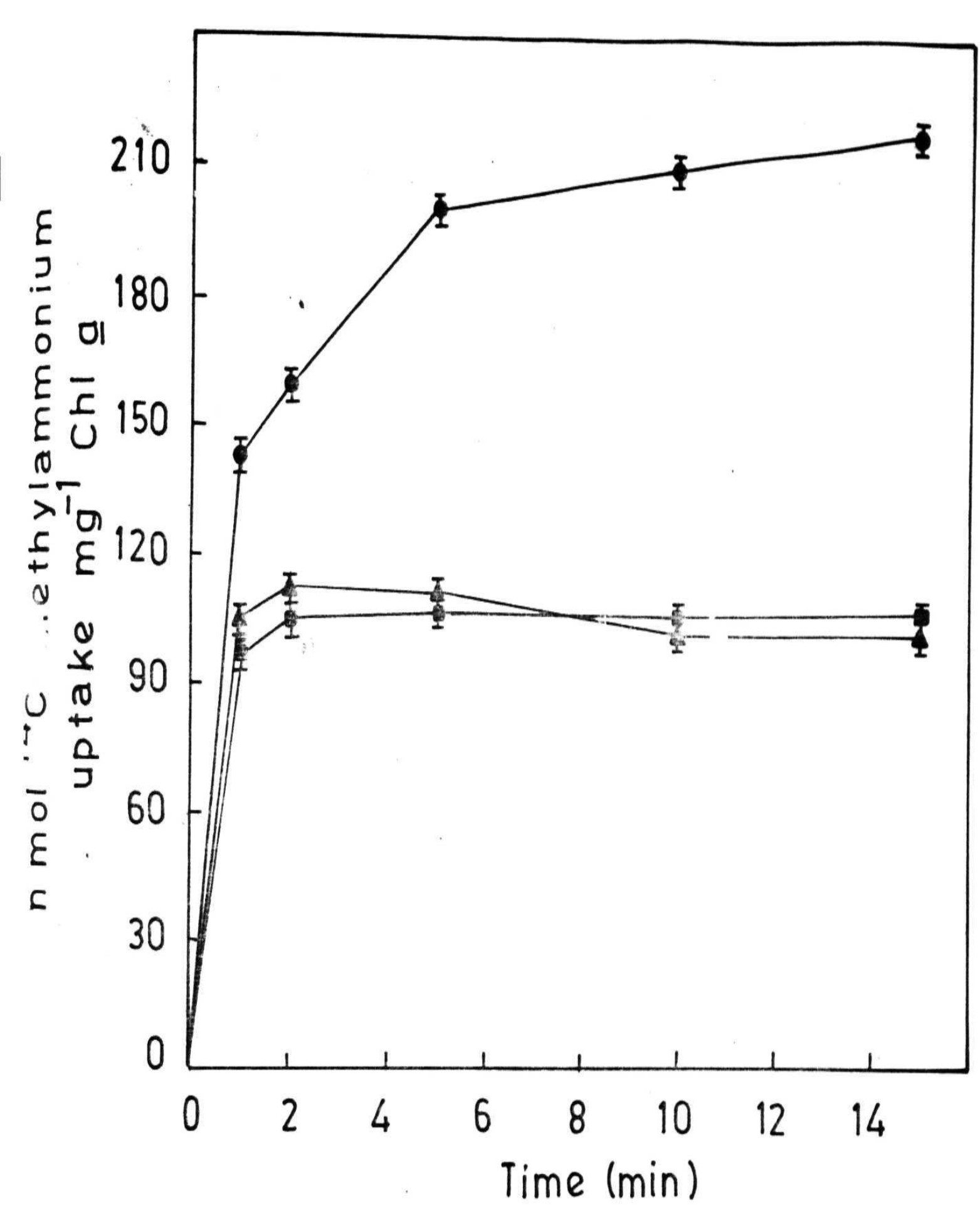
nmol ¹⁴C ..ethylammonium uptake mg⁻¹ Chl a
210
180
150
120
90
60
30
0
0
2
4
6
8
10
12
14
Time (min)

Fig. 6.5 a

Legend to Fig. 6.5a

Uptake of [^{14}C]methylammonium in the parent *Nostoc muscorum* under diazotrophic growth condition.

Six day old culture was harvested, washed and incubated in 10 mM HEPES-NaOH (pH 7.5) buffer (•——•) or in buffer containing 150 mM NaCl (•——•) or in buffer containing 300 mM sucrose (▲——▲), for 2 h. They were then washed and resuspended in 10 mM HEPES-NaOH buffer (pH 7.5) and uptake was assayed as described in the text.

Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols.

Legend to Fig. 6.5b

Uptake of [^{14}C]methylammonium in the *NaCl*^{r} mutant strain of *Nostoc muscorum* under diazotrophic growth condition. The sample was prepared for assay as described in legend to Fig. 6.5a.

10 mM HEPES-NaOH buffer, pH 7.5 (●——•)

10 mM HEPES-NaOH buffer, pH 7.5 + 150 mM NaCl (■——•)

10 mM HEPES-NaOH buffer, pH 7.5 + 300 mM sucrose (▲——▲)

Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols.

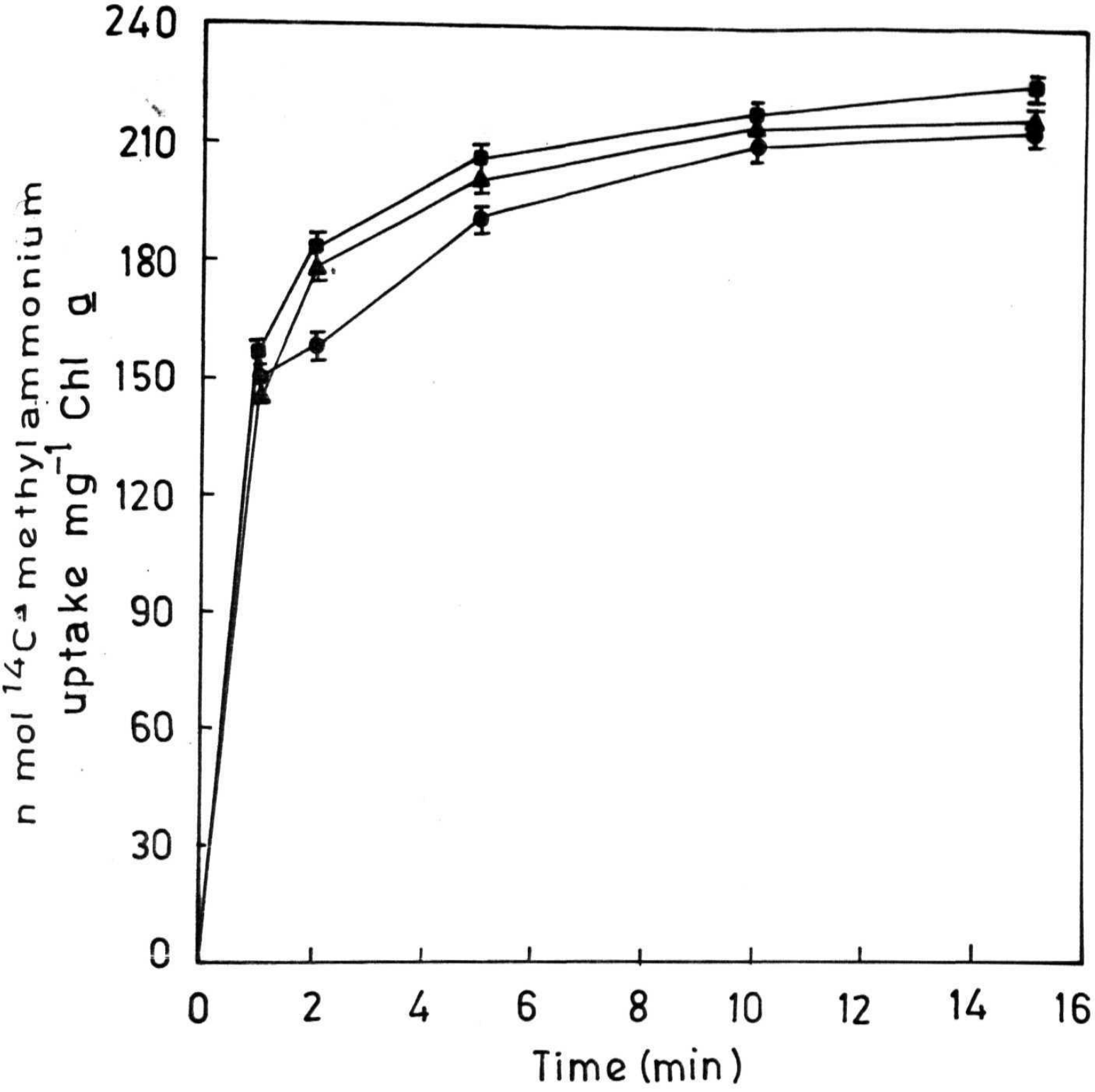
240
210
180
150
120
90
60
30
0
n mol ^{14}C-methylammonium uptake mg^{-1} Chl a
0
2
4
6
8
10
12
14
16
Time (min)

Fig. 6.5 b

Legend to Fig. 6.5c

Uptake of [^{14}C]methylammonium in the *Sucrose* mutant strain of *Nostoc muscorum* under diazotrophic growth condition. The sample was prepared for assay as described in legend to Fig. 6.5a.

10 mM HEPES-NaOH buffer, pH 7.5 (●——•)

10 mM HEPES-NaOH buffer, pH 7.5 + 150 mM NaCl (■——•)

10 mM HEPES-NaOH buffer, pH 7.5 + 300 mM sucrose (▲——▲)

Mean values from three experimental determinations are shown ± SEM values where these exceed the dimensions of the symbols.

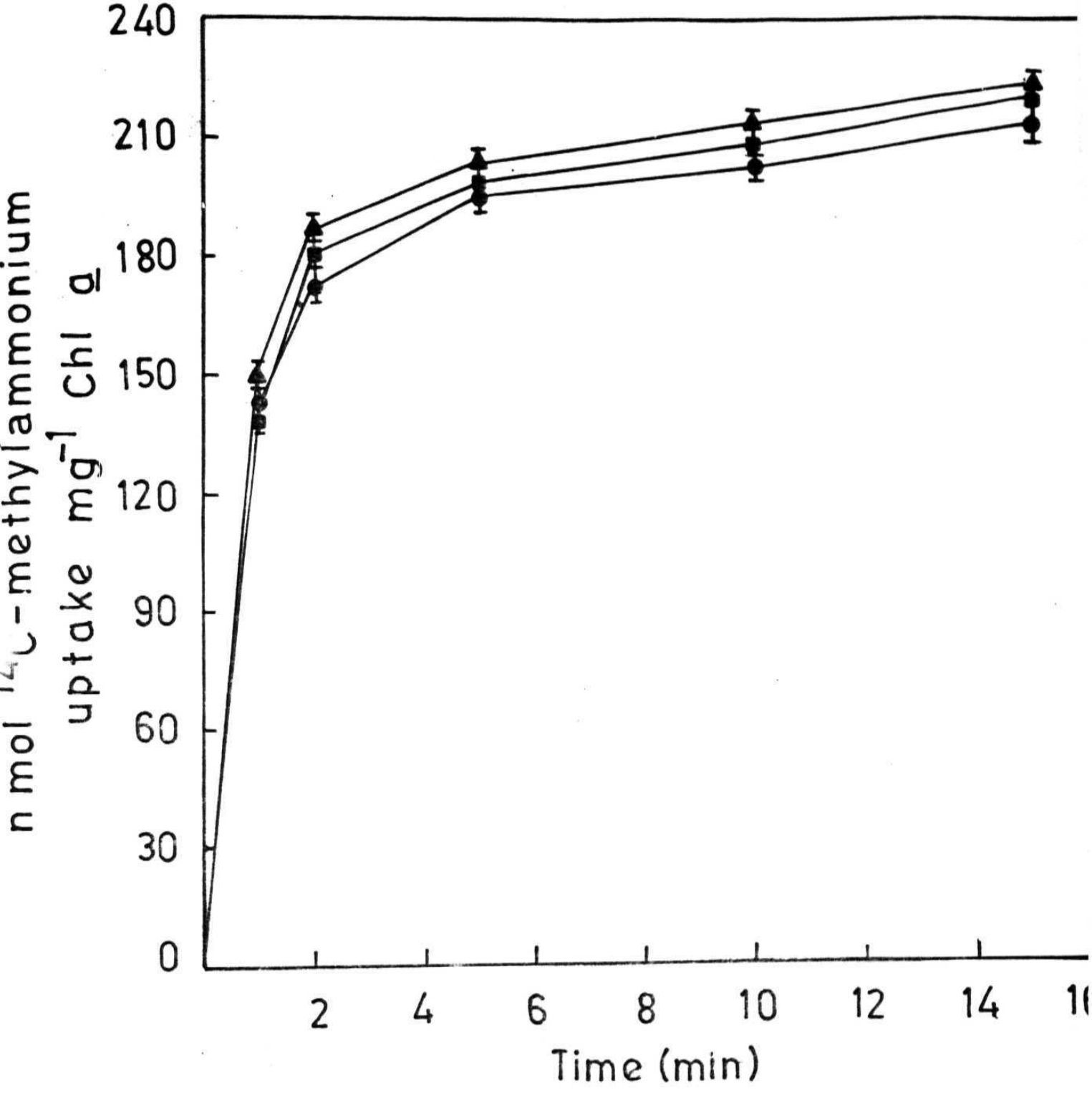
240
210
180
150
120
90
60
30
0
n mol ^{14}C-methylammonium uptake mg^{-1} Chl a
2
4
6
8
10
12
14
Time (min)

Fig.6.5c

The next studies involved on the role of heterocyst-nitrogenase repressible nature of nitrogen sources like ammonium and proline (Singh et al., 1991) in cyanobacterial osmotic adaptation. The main reason for choosing proline in the present study was because of its well-characterized osmoprotective role in enterobacteriaceae (Csonka and Hanson, 1991). As shown in Table 6.2, NaCl lethality did not change with the change in nature of nitrogen source from N_2 to ammonium, thus ruling out any special role for ammonium nitrogen in osmoregulation of the cyanobacteirium. However, the most interesting results came from proline cultures which under unstressed conditions metabolized proline like a fixed nitrogen source causing repression of cyanobacterial heterocyst-nitrogenase system and under osmotic stress conditions used it as a most effective osmoprotectant permitting the cultures to differentiate heterocysts and fix N_2. This led us to examine the uptake of proline in diazotrophically grown cultures. The unstressed cultures showed a proline uptake of 0.5 ± 0.02 SEM μmol proline mg^{-1} Chl a min^{-1}, while cultures stressed with 150 mM NaCl for 30 min showed a value of 4.5 ± 0.18 SEM μmol proline mg^{-1} Chl *a* min^{-1}.

Discussion

High external osmolarity inhibits growth and development of organisms mainly by reducing the turgor pressure. The ease with which various organisms sense the osmotic or salt stress signal and respond to it by metabolic readjustment to maintain their cellular

Table 6.2

Evaluation of the role of NH_4^+ and proline on survival (X), heterocyst frequency (HF, '/.) and nitrogenase (N2ase) activity (nmol C_2H_4 μg^{-1} Chl a h^{-1}) of the parent *Nostoc muscorum* under hyper-saline (150 mM NaCl) growth condition. Saline treated cultures served as control.

Nitrogen source	Parent strain					
	Control culture			Hypersaline culture		
	Survival	HF	N2ase activity	Survival	HF	N2ase activity
N_2	100	5-6	10.4 (± 0.72)	0.0	0.0	0.0
NH_4^+ (1mM NH_4Cl)	100	0.0	0.0	0.0	0.0	0.0
Proline (1mM)	100	0.0	0.0	86.7	4-5	8.5 (± 0.53)

Source of inocula for control and hypersaline cultures was N2-fixing heterocystous cultures. Survival was estimated by counting the number of colonies formed on each nutrient plate after a week of incubation under growth condition. Survival values thus obtained from control colonies were treated as hundred percent and those obtained from hypersaline cultures were expressed as percentage of control. Similarly heterocyst frequency and nitrogenase activity were determined in a week old cultures.

Each value is an average (± SE) of three independent experiments.

turgor is a measure of their osmotic or salt adaptability. Turgor sensitive accumulation of compatible molecules by either transport or fresh synthesis or both in response to salinity or osmotic stress is the physiological mechanism of osmotic adaptation in cyanobacteria (Reed and Stewart, 1985) and bacteria (Csonka, 1989). In general salinity and osmotic stresses have been considered to be similar by some workers in bacteria (Csonka, 1989; Weretilnyk and Hanson, 1990) and dissimilar by others in cyanobacteria (Blumwald and Tel-or, 1982; Fernandes et al., 1993). Similarity or dissimilarity could be analyzed unambiguously by isolating mutant strains resistant against salinity or osmotic stress and then examining their cross-resistant relationship. The results presented in this Chapter show a spontaneous mutation frequency characteristic of a single mutational event thus suggesting the *NaCl*^{r} or *Sucrose*^{r} phenotype to be under the control *of* a single genetic determinant. Since 150 mM NaCl (150 mM NaCl aqueous solution gives about 278 mOsmol equivalent to - 0.72 MPa) and 300 mM (300 mM sucrose in aqueous solution gives about 333 mOsmol equivalent to - 0.84 MPa) are approximately of similar osmotic strength (Wolf et al., 1979) one may conclude that NaCl or sucrose induced lethality is primarily due to osmotic and not ionic cause. Further support to osmotic stress as a cause for NaCl or Sucrose lethality comes from the finding that *NaCl*^{r} mutant strain was also found resistant to sucrose induced lethality and *vice versa.* Such cross-resistant results at genetic level suggests that a single genetic determinant functions in the genetic regulation of both salt resistance and osmotic resistance and that salinity/osmotic induced stress signal generates

physiologically similar response in the cyanobacterium. The present finding, assumes significant in view of earlier reports that found no evidence for the involvement of any single gene product in the regulation of various osmogenes in bacteria (Higgins et al., 1987) or salt resistant genes in the cyanobacterium *Anabaena torulosa* (Apte and Haselkorn, 1990). In addition, present mutational results also indicate a powerful role of mutational approach in producing genetically-engineered cyanobacterial strains capable of growth and multiplication in hypersaline or hyperosmotic media and opens out the possibility of identifying specific cyanobacterial genes and their products in conferring osmotic adaptation. NaCl inducible transcription of about a hundred genes in the salt-tolerant cyanobacterium *A. torulosa* has been reported (Apte and Haselkorn, 1990) but their specificity in salt adaptation depends upon demonstrating the essentiality of their products in such process. In this context it is worth mentioning that all osmoinducible transcripts in enterobacteriaceae are not essential for acquisition of osmotolerance (Csonka and Hanson, 1991; Csonka, 1989). Hyperosmotic treatment of *A. variabilis* with NaCl, sorbitol or sucrose have also been found to generate physiologically similar response in the cyanobacterium (Reed and Stewart, 1985). Fernandes et al. (1993) have forwarded that cyanobacterial responses to salt and osmotic stresses are physiologically distinct phenomena. However, as already stated our results do not support this view.

Reduction in Na^+ influx has been reported to be the major mechanism of salt tolerance in diazotrophic cyanobacteria (Apte

et al., 1987). Our results also show a severe curtailment of Na^+ influx in the mutant strains while showing similar pattern of sucrose transport. Mutational curtailment of Na^+ influx associated with simultaneous acquisition of osmotic resistance does suggest a definite role of curtailed Na^+ influx in osmoadaptation of the cyanobacterium. Nitrogenase activity was more sensitive to NaCl or sucrose stress than its photosynthetic oxygen evolving activity, a finding in agreement with previous report on salt inhibition of nitrogen fixation in cyanobacteria (Tel-or, 1980). Mutation of the cyanobacterium to *NaCl*^{r} or *Sucrose*^{r} phenotypes resulted in significant decrease in its nitrogenase activity under unstressed conditions. However, under stressed conditions nitrogenase activity in the mutant strains showed a significant raise approaching almost to the parent level with passage of time. While the ammonium assimilation (through GS) seems to be little influenced by salinity/osmotic stress, the transport system of ammonium is very much inhibited under the same conditions. These results impliacate that cyanobacterial ammonium metabolism is regulated not at the level of ammonium assimilation but at the transport level.

Utilization of proline as a nitrogen source in cyanobacteria and proline repression of heterocyst and nitrogenase is already well-known (Singh et al., 1991). Utilization of proline as osmoprotectant seems to be osmoregulated through osmotic inhibition control of its catabolism. Prolinebetaine function as nitrogen source or as an osmoprotectant in *Rhizobium meliloti* has been shown to exhibit such osmotic control (Gloux and Le Rudulier,

1989). In enterobacteriaceae exogenous proline uptake has been shown to be osmoinducible (Measures, 1975). Our results show an increased proline uptake by a factor of almost nine-fold under osmotic stress conditions, thereby showing that the proline uptake like proline catabolism is osmoregulated in the cyanobacterium.

The single mutational acquisition of osmoresistance by the cyanobacterium suggests the involvement of a compatible osmolyte mechanism. Though proline is found to function as a compatible solute of osmoprotective nature, the nature of the compatible osmolyte produced in the two osmoresistant mutants is proline or some other osmolyte is difficult to infer at the moment and needs further studies.

To construct an osmotolerant biofertilize; strain we must have a knowledge of their physiology and genetics that regulate their osmotolerance. Thus the following objectives should be achieved in a systematic way in the construction of competent osmotolerant strains :

(i) A range of osmotolerant characteristics of oxygenic photosynthesis, N2-fixation, ammonia and amino acid transport and their assimilation in the parent and various mutant strain should be determined,

(ii) The nature of the inorganic and organic osmolytes accumulated intracellularly during osmotic stress should be identified,

(iii) Transport systems specific to these accumulated osmolytes should be analyzed with respect to their function in csmoadaptation,

(iv) Mutational acquisition of osmoresistant/osmosensitive genetic traits must be identified with respect to their biofertilizer potential.

(v) Mutational and transformational identification of genes specific to osmotolerance should also be looked into.

In this context, the results presented here provide a beginning for genetic construction of a competent osmotolerant strain using the techniques of mutational analysis and genetic transformation for use as a biofertilizer.

CONCLUSIONS

CONCLUSIONS

From the studies presented in the foregoing chapters, the following conclusions have been arrived at :

(i) DNA is the genetic basis for machete resistance phenotype, as well as for all the other herbicide resistant phenotypes (Basalin, DCMU, Atrazine and Propanil) in *Gloeocapsa* sp. This is the first report on the presence of multiple herbicide resistance in a unicellular diazotrophic cyanobacterium, and that the genetic determinant controlling the herbicide resistant phenotypes are all closely linked and that this genetic trait is transformable to *Nostoc muscorum*.

(ii) The transformation process was found to be obligately dependent, on photosynthetic light, on the presence of permeaplasts of recipient *N. muscorum* and on TRIS-EDTA buffer in the transformation mixture, but was found to be DNase sensitive.

(iii) Sensitivity of *N.muscorum* by all the herbicides may be because of their inhibition on nitrogenase activity, due to inhibitory carbon dioxide fixation through their inhibition of PS II activity, for supply of ATP and

reductant, while resistance to them by *Gloeocapsa* sp. and transformant *N.muscorum* is due to their herbicide resistant PS II activity. PS I activity in all the strains remained uninfluenced by the herbicides.

(iv) Genetic acquisition of resistance by GS to MSX, PPT or EDA is invariably found associated with the development of ammonia excreting ability during diazotrophic growth of the cyanobacterium.

(v) *Eda*^{r} -GS strains were more deficient in GS activity and proficient in ammonium excretion, than their corresponding *Msx*^{r}-GS strains or *Ppt*^{r}-GS strains. Apparently, the level of extracellular ammonia liberated seems to be a function of mutational decrease in cyanobacterial GS activity.

(vi) For the first time, strains have been constructed which are multiple herbicide resistant and ammonia excreting, under diazotrophic growth conditions, using the techniques of genetic transformation and mutation.

(vii) *Msx*^{r}-GS strain and *Eda*^{r}-GS strain did not show a cross-resistance relationship.

(viii) Spontaneous mutation frequency to the *Eda*^{r}-GS phenotype appears to be a function of the cyanobacterial *gln* A allele.

(ix) *Eda*^{r} clones comprised of two physiologically distinct types, one assimilating EDA like a nitrogen source and the other non-EDA assimilating, but ammonia excreting.

(x) Glutamine or methionine functions as a co-repressor of heterocyst-nitrogenase system, but not of the methionine transport system in the cyanobacterium.

(xi) Loss of methionine transport activity, leads to a lack of methionine repression control, on the heterocyst-nitrogenase system.

(xii) An evidence is shown for a role of amino acid transport system, in the regulation amino acid catabolism like a nitrogen source.

(xiii) A single genetic determinant, functions in genetic regulation of salinity resistance and osmotic resistance.

(xiv) Mutational curtailment of influx of Na^+ ions associated with simultaneous acquisition of osmotic resistance suggests, a definite role of curtailed Na^+ influx, in osmoadaptation of the cyanobacterium.

(xv) Mutation to salinity/osmotic resistance was accompanied by a reduced level of nitrogenase activity under unstressed conditions. However, a positive role of osmolarity in regulation of nitrogenase activity is evident in the osmotolerant strains.

(xvi) Photosynthetic oxygen evolving activity is sensitive to salinity/osmotic stress in the parent strain, but tolerant in the mutant strains. However, photo-synthetic oxygen evolving activity, was found more tolerant than nitrogen fixation process.

(xvii) While cyanobacterial ammonium assimilation (through GS) is little influenced in both parent and mutant strains, its ammonium transport activity is inhibited in the parent strain, while remaining unaltered in the mutant strains, under salinity/osmotic stress conditions.

(xviii) Nature of nitrogen source has no special role in osmoregulation of the cyanobacterium.

(xix) Role of proline as an osmoprotectant under osmotic stress and that exogenous proline uptake is osmoinducible has been shown.

(xx) Mutational acquisition to osmoresistance by the cyanobacterium suggests, involvement of a compatible osmolyte mechanism.

REFERENCES

REFERENCES

Allen, J.F. (1992) *Biochim. Biophys. Acta* **1098:** 275.

Apte, S.K. and Haselkorn, R. (1990) *Plant Mol. Biol.* 15: 723.

Apte, S.K., Reddy, B.R. and Thomas, J. (1987) *Appl. Environ. Microbiol.* 53: 1934.

Armstrong, T.F., William, F.M. and Donald, P. (1973) *Weed Sci.* 21: 354.

Ashton, F.M. and Crafts, A.S. (1973) In: *Mode of Action of Herbicides,* (John Wiley, ed) ppl26. A Wiley Interscience Publication, New York.

Astier, C., Boussac, A. and Etienne, A.L. (1984) *FEBS Lett.* **167:** 321

Astier, C. and Espardellier, F. (1976) *C.R. Acad. Sci. Paris.* 282: 795.

Avery, O.T., MacLeod, C.M., and McCarthy, M. (1944) J. *Exp. Medicine* 89: 137.

Bagchi, S.N., Rai, A.N. and Singh, H.N. (1985) *Biochim. Biophys. Act a* 838: 370.

Bagchi, S.N. and Singh, H.N. (1984) *Mol. Gen. Genet.* **193:** 82.

Bailey, J.L. (1967) *Techniques in Protein Chemistry.* pp340. Elsevier/North-Holland Biomedical Press, Amsterdam.

Bishop, P.E., Hawkins, M.E. and Eady, R.R. (1986) *Biochem. J.* **238:** 437.

Blumwald, E. and Tel-Or, E. (1982) *Arch. Microbiol.* **132;** 163.

Borowitzka, L.J., Demerle, S. , Mackay, M.A. and Norton, R.S. (1980) *Science* **210:** 650.

Bothe, H. (1982) In: *The Biology of Cyanobacteria* (Carr, N.G. and Whitton, B.A. eds.), pp87. Blackwell Scientific Publications, Oxford.

Boussiba, S. (1989) *Plant Cell Physiol.* 30: 303.

Boussiba, S. and Gibson, J. (1991) *FEMS Microbiol. Rev.* 88: 1.

Boussiba, S. , Resch, CM. and Gibson, J. (1984) *Arch. Microbiol.* **139:** 287.

Brill, W.J. (1977) *Sci. Amer.* 236: 68.

Broukisse, R. Weigel, P. Rhodes, D., Yocum, C.F. and Hanson, A.D. (1989) *Plant Physiol.* 90: 322.

Brown. CM., MacDonald-Brown, D.S. and Meers. J.L. (1974) *Adv. Microbiol. Physiol.* 11: 1.

Brusslan, J. and Haselkorn, R. (1989) *EMBO J.* 8: 1237.

Bryant, D.A. and de Marsac, N.T. (1988) *Method. Enzymol.* **167:** 755.

Buikema, W.J. and Haselkorn, R. (1991) *Genes and Development* Cold Spring Harbor Press, 5: 321.

Buikema, W.J. and Haselkorn, R. (1993) *Annu. Rev. Pl. Physiol.* 44: 33.

Burger-Wiersma, T., Veenhuis, M., Korthals, H.J., von de Wiel, C.C.M. and Mur, L.R. (1986) Nature 320: 262.

Cairney, J., Booth, I.R. and Higgins, C.F. (1985) J. *Bacterlol.* **164:** 1218.

Carr, N.G. (1973) J. *Gen. Microbiol.* 75: 5.

Chapman, J.S. and Meeks, J.C. (1983) *J. Bacteriol.* **156:** 122.

Chauvat, F., Astier, C., Vedel, F. and Espardellier, F. (1983) *Mol. Gen. Genet.* **191:** 39.

Codd, G.A., Okabe, K. and Stewart, W.D.P. (1980) *Arch. Microbiol.* 124: 149.

Cohen-Bazire, G. and Bryant, D.A. (1982) In: *The Biology of Cyanobacteria* (Car, N.G. and Whitton, B.A., eds) p. 143. Blackwell Scientific Publications, Oxford.

Csonka, L.N. (1989) *Microb. Rev.* 53: 121.

Csonka, L.N. and Hanson, A.D. (1991) *Annu. Rev. Microbiol.* 45: 569.

Currier, T.C., Haury, J.F. and Wolk, C.P. (1977) *J. Bacteriol.* 129: 1556.

Curtis, S.E. and Haselkorn, R. (1984) *Plant Mol. Biol.* 3: 249.

Daniell, H. and McFadden, B.A. (1986) *Mol. Gen. Genet.* **204:** 243.

Das, B. and Singh, P.K. (1977a) *Arch. Environ. Contamin. Toxicol* 5: 437.

Das, B. and Singh, P.K. (1977b) *Hicrob. Lett.* 4: 99.

Das, B. and Singh, P.K. (1979) *Nov. Hedwigia* 63: 161.

De, P.K. (1939) *Proc. R. Soc. Lond* **B127:** 121.

Delaney, S.F., Dickson, A. and Carr, N.G. (1973) *J. Gen. Microbiol.* 79: 89.

Dharmawardene, M.W.N., Haystead, A. and Stewart, W.D.P. (1973) *Arch. Microbiol.* 90: 281.

Devilly, C.I. and Houghton, J.A. (1977) *J. Gen. Microbiol.* 98: 277.

Eisbrenner, G. and Bothe, H. (1979) *Arch. Microbiol.* **123:** 37.

Elhai, J. and Wolk, C.P. (1990) *EMBO. J.* 9: 3379.

Estelle, M.A. and Somervilla, C. (1987) *Mol. Gen. Genet.* 206: 200.

Evans, H.J., Harker, A.R., Papen, H., Sterling, A.R., Hanus, F.J. and Zuber, M. (1987) *Anna. Rev. Microbiol.* 41: 335.

Fay, P. (1981) In: *Nitrogen fixation,* Ecology, (Wibroughton, W.J. ed.) I: 1, Clarendon Press, Oxford,

Fay, P. (1992) *Microbiol. Rev.* 56: 340.

Fernandes, T.A., Iyer, V. and Apte, S.K. (1993) *Appl. Environ. Microbiol.* 59: 899.

Fleming, S. and Haselkorn, R. (1973) *Proc. Natl. Acad. Sci. USA* 70: 2727.

Fleming, S. and Haselkorn, R. (1974) *Cell* 3: 159.

Flores, H.E. and Galston, A.W. (1984) *Plant Physiol.* 75: 102.

Flores, E. and Muro-Pastor, M.I. (1988) *FEMS Microbiol. Lett.* 56: 127.

Flowers, T.J., Troke, P.R. and Yeo, A.R. (1977) *Anna. Rev. Plant Physiol.* 28: 89.

Fogg, G.E. (1949) *Ann. Bot.* **13:** 241.

French E.W. and Gay, W.B. (1963) *World Crops* 15: 196.

Gallon, J.R. and Chaplin, A.E. (1988) In: *Biochemistry of the Algae and Cyanobacteria* (Rogers, L.J. and Gallon, J.R., eds.) ppl46. Oxford Science Publications, Oxford.

Gallon, J.R. (1992) *New Phytol.* 122: 571.

Gerloff, G.L., Fitzgerald, G.P. and Skoog, F. (1950) *Am. J. Bot.* 37: 216.

Giaever, H.M., Styrvold, O.B., Kaasen, I. and Strom, A.R. (1988) J. *Bacteriol.* **170:** 2841.

Gloux, K. and Le Rudulier, D. (1989) *Arch. Microbiol.* 151: 143.

Golden, S.S. and Sherman, L.A. (1983) *J. Bacteriol.* 155: 966.

Golden, S.S. and Sherman, L.A. (1984) *J. Bacteriol.* **158:** 36.

Golden, S.S. and Haselkorn, R. (1985) *Science* 229: 1104.

Golden, J.W., Robinson, S.J. and Haselkorn, R. (1985) *Nature* **314:** 419.

Golden, S.S., Brusslan, J. and Haselkorn, R. (1986) *EMBO J.* 5: 2789.

Golden, J.W., Carrasco, CD., Mulligan, M.E., Schneider, G.J. and Haselkorn, R. (1988) J. *Bacteriol.* **170:** 5034.

Good, N.E. (1961) *Plant Physiol.* 36: 788.

Gordon, J.K. and Brill, W.J. (1974) *Biochem. Biophys. Res. Commun.* 59: 967.

Grigorleva, G.A. and Shestakov, S.V. (1982) *FENS Microbiol. Lett.* 13: 367.

Guerrero, M.G., Ramos, J.L. and Losada, M. (1982) *Experientia* 38: 53.

Haselkorn, R. (1978) *Annu. Rev. Plant Physiol.* 29: 319.

Haselkorn, R., Lammers, P.W. and Mulligan, M.E. (1987) In: *A Century of Nitrogen Fixation Research: Present Status and Future Prospects* (Bergerston, E.J. and Postgate, J.R, eds) pp. 173-181. The Royal Society, London.

Helber, J.T., Johnson, R.T., Yarbrough, L.R. and Hirchberg, R. (1988) J. *Bacteriol.* 170: 532.

Herrero, A and Flores, E. (1990) *J. Biol. Chem.* 265: 3931.

Higgins, C.F., Cairney, J., Stirling, D.A., Sutherland, L. and Booth, I.R. (1987) *TIBS* 12: 339.

Hood, W. and Carr, N.G. (1972) J. Gen. *Microbiol.* 73: 470.

Hughes, E.D., Gorham, T.R. and Zehuder, A. (1958) *Can. J. Microbiol.* 4: 255.

Imhoff, J.F. (1986) *FENS Microbiol. Rev.* 39: 57.

Jakowec, M.W., Smith, L.T. and Dandekar, A.M. (1985) *Appl. Environ. Nicrobiol.* 50: 441.

Jayakumar, A., Schulman, T.I., MacNeil, D. amd Barness, E.M. (1986) *J. Bacteriol.* 166: 1281.

Jones, R.G. and Storey, R. (1981) In : *The Physiology and Biochemistry of Drought-Resistance in Plants* (Paleg, L.G. and Asplnall, D. , eds.) ppl71. Academic Press, New York.

Kar, S. and Singh, P.K. (1978) *Bull. Environ. Cont. Toxicol.* 20: 707.

Kar, S. and Singh, P.K. (1979) *Microb. Lett.* 10: 111.

Kentemich, T. , Dannesburg, G. , Hunderhagen, B. and Bothe, H. (1988) *FEMS Microb. Lett.* 51: 40.

Kerby, N.W., Musgrave, S.C., Codd, G.A., Rowell, P., Stewart, W.D.P. (1983) *Proc. Biotech 83.* Online Publications Ltd., Northwood, U.K. pp1029.

Kerby, N.W., Rowell, P. and Stewart, W.D.P. (1985) *Arch. Microbiol.* 141: 244.

Kerby, N.W., Musgrave, S.C., Rowell, P., Shestakov, S.V. and Stewart, W.D.P. (1986) *Appl. Microbiol. Biotech.* 24: 42.

Kerby, N.W., Niven, G.W., Rowell, P. and Stewart, W.D.P. (1987) In: *Algal Biotechnology* (Stadler, J.M., Verdus, M.C., Karamanos, Y., Morvan, H. and Christian, D. , eds.) pp324. Applied Sciences, London.

Kerby, N.W. and Stewart, W.D.P. (1988) In: *Biochemistry of the Algae and Cyanobacteria* (Roger, L. and Gallon, J.R. eds.) pp319. Oxford Science Publications, Oxford.

Kingdon, H.S., Hubbard, J. and Sutman, E.R. (1968) *Biochemistry* 7: 2136.

Kirilovsky, D.L., Ajlani, C, Picaud, M. and Etienne, L.A. (1989) *Plant mol. Biol.* 13: 355.

Kleiner, D. (1985) *FEMS Microbiol. Rev.* 32: 87.

Kuhlmier, C.J., Tweywsen, V.J.P., Janssen, M.J.T. and Van Arkel, G.A. (1984) *Gene* 31: 109.

Kulasooriya, S.A., Lang, N.J. and Fay, P. (1972) *Proc. R. Soc. Lond. Ser* **B181:** 199.

Kumar, A and Kumar, H.D. (1980) *Curr. Microbiol.* 3: 213.

Kumar, A.P., Rai, A.N. and Singh, H.N. (1985) *FEBS Lett.* **179:** 125.

Kumar, H.D. (1962) *Nature* 96: 1121.

Labarre, J. Thuriax, P. and Chauvat, F. (1987) J. *Bacteriol.* **169:** 4668.

Lacks, S. (1977) In: *Microbial Interactions* (Reissig, J. ed.) pp179, Chapman and Hall, London.

Ladha, J.K., Rowell, P. and Stewart, W.D.P. (1978) *Biochem. Biophys. Res. Commun.* 83: 688.

Lang, N.J. and Fay P. (1971) *Proc. R. Soc. Lond. Ser.* B178: 193.

Lattore, C., Lee, J.H., Spiller, H. and Shanmugum, K.T. (1986) *Biotech. Lett.* 8: 507.

Lea, P.J., Joy, K.W., Ramos, J.L. and Guerrero, M.G. (1984) *Phytochemistry* 23: 1.

Le Rudulier, D. and Valentine. R.C. (1982) *Trends in Biochem. Sci.* 7: 431.

Levin. R.A. (1976) *Nature* **261:** 697.

Liang, J., Scappino, L. and Haselkorn, R. (1992) *Proc. Natl. Acad. Sci. USA* 89: 5655.

Lockshin, A. and Burris, R.H. (1965) *Biochim. Biophys. Acta* **111**: 10.

Lowry, O.H., Rosebrough, N.J., Farr, A. and Randall, R.J. (1951). *J. Biol. Chem.* 193: 265.

Lucht, J.M. and Bremer, E. (1994) *FEMS Microbiol. Rev.* 14: 3.

Mackay, M.A., Norton, R.S. and Borowitzka, L.J. (1983) War. *Biol.* 73: 301.

Mackerras, A.H. and Smith, G.D. (1986) *Biochem. Biophys, Res, Commun.* 134: 835.

Mackinney, G. (1941) *J. Biol. Chem.* 140: 315.

Mahan, M.J. and Csonka, L.N. (1983) *J. Bacteriol.* 156: 1248.

Mallison III, S.M. and Cannon, R.E. (1984) *Appl. Environ. Microbiol.* 47: 910.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning : A Laboratory Manual.* Cold-Spring Harbor Laboraory, Cold-Spring Harbor, New York.

Mannar, M.R. and Bose, S. (1985) *Indian J. Biochem. Biophys.* 22: 179.

Mazur, B.J., Rue, D. and Haselkorn, R. (1980) *Proc. Natl. Acad. Sci. USA.* 77: 186.

McCue, K.F. and Hanson, A.D. (1990) *Trends in Biotech.* 8: 358.

McFarlane, G.J.B., Machray, G.C., and Stewart W.D.P. (1987) In: *A Century of Nitrogen Fixation Research : Present Perspectives and Future Prospects,* Bergerson, E.J. and

Postgate, J.R. eds.) pp245, Royal Soc, London.

Measures, J.C. (1975) Nature 257: 398.

Meeks, J.C, Wolk, C.P., Lokan, W., Schilling, N., Shaffer, P.W. and Chien, W.S. (1975) *J. Bacteriol.* **134:** 125.

Meins, F. and Abrams, M.L. (1972) *Biochim. Biophys. Acta* **266:** 307.

Merrick, M.J. (1988) In: *The Nitrogen and Sulphur Cycle* (Cole, J.A. and Ferguson, S.J., eds.) pp331, Cambridge University Press, Cambridge.

Merrick, M. and Dixon, R. (1984) *Trends in Biotech.* 2(6): 162.

Modi, D.R., Singh, A.K., Rao, K.S., Chakravarty, D. and Singh, H.N. (1991) *Biotech. Lett.* 13: 793.

Musgrave, S.C., Kerby, N.W., Codd, G.A. and Stewart, W.D.P. (1982a) *Biotech. Lett. 4:* 647.

Musgrave, S.C., Kerby, N.W., Codd, G.A. and Stewart, W.D.P. (1982b) *Eur. J. Applied. Microbiol. Biotechnol.* 17: 133.

Neilson, A.H. and Larsson, T. (1980) *Physiol. Plant.* 48: 542.

Nishimura, M. and Takarmiya, A. (1966) *Biochem. Biophys. Res. Commun.* **120:** 45.

Orr, J. and Haselkorn, R. (1982) J. *Bacteriol.* 152: 626.

Ortego-Calvo, T.J. and Stal, L.J. (1991) *J. Gen. Micrbiol.* **137:** 1789.

Padhy, R.N. and Singh, P.K. (1978) *Mol. Gen. Genet.* **162:** 203.

Patterson, G.M. amd Withers, N.W. (1982) *Science* 217: 1934.

Peschek, **G.A. (1981)** *Biochem.* ***Biophys Res.*** *Commun.* **98: 72.**

Peter, J.N. and Sherman, L.A. (1978) ***Biochem. Biophys.*** *Res. Commun.* **503: 343.**

Peterson, R.B., Dolan, **E. , Calvert, H.E. and** Ke, **B. (1981)** *Biochim.* ***Biophys. Acta*** **634: 237.**

Pfister, K. **and Arntzen, C.J.** (1979) **Z.** *Naturforsch.* 34C: 996.

Polukhina, L.E., Sakhurieva, G.N. and Shestakov, S.V. (1982) *Microbiology* 51: 90.

Rai, A.N. and Singh, S. (1982) *FEMS Microbiol. Lett.* **14:** 303.

Rai, A.N., Rowel 1, P. and Stewart, W.D.P. (1984) *Arch. Microbiol.* 137: **241.**

Rai, A.N., Singh, D.T. and Singh. H.N. (i936) *Physiol. Plant.* **68:** 1320.

Rai, A.N., Rao, V.V. and Singh, H.N. (1988) *New Phyti* . **109:** 133.

Ramos, J.L., Guerrero, M.G. and Losada, M. (1982) *Biochim. Biophys. Acta* **679:** 323.

Rawson, D.M. (1985) J. *Gen. Microbiol.* **131:** 2549.

Reed, R.H. and Stewart, W.D.P. (1985) *Blochim. Biophys.* ***Acta*** **812: 115.**

Reed, R.H. and Stewart, W.D.P. (1988) In: ***Biochemistry of the Algae and Cyanobacteria*** **(Rogers, L.J. and Gallon, J.R., eds.) pp217, Oxford University Press, Oxford.**

Robinson, S.J., De Roo, C.S. and Yocum, C.F. (1982) *Plant Physiol.* 70: 154.

Roger, P.A. and Kulasooriya, S.A. (1980) In: *Blue-Green Algae and Rice,* pp112, *IRRI*, Los Banos.

Rosin, M.P. and Kepes, A. (1972) *Biochim. Biphys. Acta* 275: 333.

Rowell, P., Enticott, S. and Stewart, W.D.P. (1977) *New Phytol.* 79: 41.

Sakhurieva, G.N., Polukhina, L.E. and Shestakov, S.V. (1982) *Microbiol.* 51: 258.

Sampaio, M.J.A.M., Rowell, P. and Stewart, W.D.P. (1979) J. *Gen. Microbiol.* 85: 26.

Sato, F., Shegamatsu, Y. and Yamada, Y. (1988) *Mol. Gen. Genet.* 214: 358.

Schollhorn, R. and Burris, R.H. (1967) *Proc. Natl Acad. Sci. USA* 57: 1317.

Scott, I.D. and Nicholls, D.G. (1980) *Biochem. J.* 186: 21.

Saville, B., Straus, N. and Coleman, J.R. (1987) *Plant Physiol.* 85: 26.

Shah, D.M., Harsch, R.B., Klee, H.J., Kishore, G.M., Winter, J.A., Turner, N.E., Hironaka, C.M., Sanders, P.R., Gasser, C.S., Aykent, S., Siegel, N.R., Rogers, S.G. and Fraley, R.T. (1986) *Science* 233: 478.

Shanmugum, K.T., Gara, F., Andersen, K. and Valentine, R.C. (1978) *Annu. Rev. Plant Physiol.* 29: 263.

Shanmugum, K.T. and Valentine, R.C. (1975) *Proc. Natl. Acad. Sci. USA* 72: 136.

Shanmugum, K.T. and Valentine, R.C. (1980) *Method. Enzymol.* **69:** 47.

Shestakov, S.V. and Khyen, N.T. (1970) *Mol. Gen. Genet.* **107:** 372.

Singh, A.K., Sailaja, M.V. and Singh, H.N. (1989) *FEMS Microbiol. Lett.* **60:** 187.

Singh, A.K., Singh, H.N. and Rai, A.N. (1991) *Biochem. Intl.* 25: 887.

Singh, A.K., Verma, S.K., Singh, H.N. and Rai, A.N. (1992) *Indian J. Exptl. Biol.* 30: 384.

Singh, D.T., Rai, A.N. and Singh, H.N. (1985) *FEBS Lett.* **186:** 51.

Singh, D.T., Modi, D.R. and Singh, H.N. (1986) *FEMS Microbiol. Lett.* 37: 95.

Singh, D.T., Bagchi, S.N., Modi, D.R. and Singh, H.N. (1987a) *New Phytol.* **107:** 347.

Singh, D.T., Nirmala, K., Modi, D.R., Katiyar, S. and Singh, H.N. (1987b) *Mol. Gen. Genet.* 208: 436.

Singh, H.N., Ladha, J.K. and Kumar, H.D. (1977) *Arch. Microbiol.* **114:** 155.

Singh, H.N. and Singh, H.N. (1978) *Arch. Microbiol.* **119:** 197.

Singh, H.N., and Vaishampayan, A. (1978) Environ. *Exp. Bot.* **18:** 87.

Singh, H.N., Vaishampayan, A. and Singh, R.K. (1978) *Biochem. Biophys. Res. Commun.* 81: 67.

Singh, H.N., Singh, H.R. and Vaishampayan, A. (1979) Environ. *Exp. Bot.* 19: 5.

Singh, H.N. and Singh, H.N. (1983) *Plant Cell Reports* 2: 310.

Singh, H.N., Rai, A.N., Rao, V.V. and Bagchi, S.N. (1983a) *Biochem. Biophys. Res. Commun.* **111**: 180.

Singh, H.N., Singh, R.K. and Sharma, R (1983b) *FEBS Lett.* **154:** 10.

Singh, H.N., Chakravarty, D., Srinivasa Rao, K. and Singh A.K. (1993a) J. Basic. *Microbiol.* 33: 201.

Singh, L.J. and Tiwari, D.W. (1988) J. *Appl. Bacteriol.* 64: 25.

Singh, P.K. (1973) *Arch. Microbiol.* 92: 56.

Singh, P.K. (1974) *Arch. Microbiol.* 97: 69.

Singh, R.K. and Stevens, S.E. (1992) *FEMS Microbiol. Lett.* 94: 227.

Singh, R.N. (1961) *The Role of Blue-green Algae in Nitrogen Economy of Indian Agriculture.* Indican Council of Agricultural Research, New Delhi.

Singh, S., Chakravarty, D. and Singh, H.N. (1993b) *Biochem. Mol. Biol. Intl.* 29: 1083.

Singh, S., Hasija, S.K., Negi, S. and Singh, H.N. (1994) *Biochem. Mol. Biol. Intl.* 33: 359.

Smith, A. J. (1982) In: *The Biology of Cyanobacteria* (Carr, N.G. and Whitton, B.A., eds.) pp47, Blackwell Scientific Publications, Oxford.

Solmonson, L.P. and Vennesland, B. (1972) *Biochim. Biphys.* **267:** 544.

Solorzano, L. (1969) *Limnol. Oceanogr.* 14: 799.

Spence, D.W. and Stewart, W.D.P. (1986) *Biochem. Biophys. Res. Commun.* **139:** 940.

Spiller, H. and Gunasekaran, R. (1990) *Appl. Microbiol. Biotech.* 33: 77.77.

Spiller, H., Lattore, C., Hassan, M.E. and Shanmugum, K.T. (1986) J. *Bacteriol.* 165: 412.

Stanier, R.Y. and Cohen-Bazire, G. (1977) *Annu. Rev. Microbiol.* 34: 497.

Sterber, W.R. and Willimitzer, L. (1989) *Biotech. Lett.* 7: 811.

Sterber, W.S., Timmis, K.L. and Zenk, M.H. (1987) J. *Bacteriol.* 169: 2950.

Stevens, S.E., and Porter, R.D. (1980) *Proc. Natl. Acad. Acad. Sci. USA* 77: 6052.

Stewart, W.D.P. (1973) *Annu. Rev. Microbiol.* 27: 283.

Stewart, W.D.P., Haystead, A. and Dharmawardene, M.W.N. (1975) In: *Nitrogen Fixation by Free living Microorganisms* (Stewart, W.D.P., ed.) ppl29, Cambridge University Press, Cambridge.

Stewart, W.D.P. and Rowell, P. (1975) *Biochem. Biophys. Res. Commun.* 65: 846.

Stewart, W.D.P. and Singh, H.N. (1975) Biochem. Biophys. Res. Commun.**62**: 62.

Stewart, W.D.P., Rowell, P., Ladha, J.K. and Sampaio, M.J.A.M. (1979) In: Proc. *Nitrogen and Rice Symposium IRRI, Mimilla,* pp263.

Stewart, W.D.P. (1980) *Annu. Rev. Microbiol.* 34: 497.

Stewart, W.D.P., Rowell, P. and Rai, A.N. (1980) In: *Proc. Intl. Symposium on Nitrogen fixation* (Stewart, W.D.P. and Gallon, J.R. eds.) 451: 263.

Stewart, W.D.P., Rowell, P., Kerby, N.W. and Machray, G.C. (1987) *Phi* J. *Trans. R. Soc. London* B317: 245.

Strasser, P. and Falkner, G. (1986) *Planta* 168: 385.

Strom, A.R., Le Rudulier, D. Jakowec, M., Bunnell, R.C. and Valentine, R.C. (1983) In: *Genetic Engineering of Plants : An Agricultural Perspective.* (Kosuge, T. Meredith, L.P. and Hollaender, A. eds.), NewYork, Plenum Press.

Styrvold, D.B., Falkenberg, P., Landfald, B., Eshoo, M.W., Bjornsen, T. and Strom, A.R. (1986) *J. Bacteriol.* 165: 856.

Tel-Or, E. (1980) *Appl. Environ. Microbiol.* 40: 689.

Thiel, T. (1988) *Arch. Microbiol.* 149: 466.

Thomas, J., Meeks, J.C., Wolk., C.P., Saffer, P.W., Austin, S.M. and Chien, W.S. (1977) J. *Bacteriol.* 129: 1545.

Thomas, S.P., Zaritsky, A. and Boussiba, S. (1990) *Appl. Environ. Microbiol.* 56: 3499.

Trebst, A. (1974) *Anna. Rev. Plant Physiol.* 25: 423.

Tirol, A.C., Roger, P.A. and Watanabe, I. (1982) *Soil Sci. Plant Nutr.* 28: 559.

Tsinoremas, N.F., Castets, A.M., Harrison, M.S., Allen, J.F. and Marsac, T.D. (1991) *Proc. Natl. Acad. Sci . USA.* 88: 4565.

Turner, N.E., Robinson, S.J.and Haselkorn, R. (1983) *Nature* 306: 337.

Turner, N.C. and Jones, M.N. (1980) In: *Adaptation of plants to water and high temperature stress* (Turner, N.C. and Krant, P.J. eds.) pp87, John Wiley and Sons, NewYork.

urpin, D.H., Eddie, S.A. and Canvin, D.T. (1984) *Plant Physiol.* 74: 701.

Vaishampayan, A. (1982)*New Phytol.* 90: 545.

Vaishampayan, A. (1984) New *Phytol.* 96: 7.

Vega-Palas, M.A., Madueno, F., Herrero, A. and Flores, E. (1990) J. *Bacteriol.* 172: 643.

Venkataraman, G.S. (1972) In: *Algal Biofertilizers and Rice Cultivation,* Today and Tommorrow's Puiblications, New Delhi

Venkataraman, G.S. (1975) In: Nitrogen fixation by Free-living Microorganisms, (Stewart, W.D.P. ed.) pp207, Cambridge University press, NewYork.

Venkataraman, G.S. and Rajyalakshmi, B. (1977) *Ind. J. Agric. Sci.* 42: 119.

Verma, S.K., Singh, A.K., Katiyar, S. and Singh, H.N. (1990) Arch. *Microbiol.* **154:** 414.

Vermaas, W.E.J., Williams, J.G.K. and Arntzen, C.J. (1987) *Plant Mol. Biol.* 8: 317.

Vermaas, W.E.J., Ikeuchi, M. and Inoue, Y. (1988) *Photosynthesis Res.* 17: 97.

Walderhaug, M.O., Dosch, D.C. and Epstein, W. (1987) In: *Ion Transport in Prokaryotes* (Rosen, B.P. and Silver, S., eds.) pp85, Academic Press, New York.

Warr, S.R.C., Reed, R.H. and Stewart, W.D.P. (1988) *Plant Cell Env.* 11: 137.

Watana[illegible], A. (1956) *Bot. Mag.* 69: 530.

Weber, H.L. and Bock, A. (1968) *Arch. Microbiol.* 61: 159.

Weretilnyk, E.A. and Hanson, A.D. (1990) *Proc. Natl Acad. Sci. USA* 87: 2745.

Wilcox, M., Mitchison, G.J. and Smith, R.J. (1975) *Arch. Microbiol.* 103: 219.

Williams, J.G.K. (1988) *Method. Enzymol.* 167: 766.

Williams, J.G.K. and Szalay, A.A. (1983) *Gene* 24: 37.

Wolf, A.V., Brown, M.G. and Prentiss, P.G. (1979) In: *Conversation Tables. CRC Handbook of Chemistry and Physics* (Weast, R.C. and Astle, M.J., eds.) pp59, CRC Press Inc., Bocaraton, FLD. 265.

Wolk, C.P. (1967) *Proc. Natl. Acad. Sci. USA.* 57: 1246.

Wolk, C.P. (1982) In: *The Biology of Cyanobacteria* (Carr, N.G. and Whitton, B.A., eds.) pp359, Blackwell Scientific Publications, Oxford.

Wolk, C.P., Thomas, J., Schaffer, P.W., Austin, S. and Galonsky, A. (1976) *J. Biol. Chem.* **251:** 5027.

Wolk, C.P., Vonshak, A., Kehie, P. and ELhai, J. (1984) Proc. *Nat* J. *Acad. Sci. USA* 81: 1561.

Wright, K. and Corbett, J.K. (1979) Z. *Naturforsch. Teil.* **C34:** 996.

Yamato, J., Okki, M. and Anraku, Y. (1979) J. *Bacteriol.* 138: 24.

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R. and Somero, G.M. (1982) *Science* **217:** 1214.