STUDIES ON DIAZOTROPHY AND ITS REGULATION BY SALINITY STRESS AND OSMOTIC STRESS IN THE CYANOBACTERIUM *NOSTOC MUSCORUM*

A THESIS SUBMITTED FOR THE DEGREE OF

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Prayer

Guru madhye Sthitham Vishwam
Vishwa madhye shitho Guruhu;
Gurur- Vishwam Nacha-anyosthi
Thasmai Shri Gurave namh.

The universe has its being within the Guru (dispeller of darkness); the Guru abides within the Universe (as its essence); the Guru is the Universe; nothing exists besides him. Salutations to such a Guru.

(Shri Guru Gita)

With blessings from:

My parents,

Sree Sampath Vinayagar Swamy (Visakhapatnam),

Sree Jnana Saraswathi Devi (Basar),

Samartha Sadguru Sree Sainath *Maliaraj* ***(Shirdi) and***

Bhagavan Sree Sathya Sai Baba (Puttaparthi)

This thesis is dedicated with most reverence to

Sree Venkateswara Swamy - Lord of the Seven Hills
(Tirumala)

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 500 046, **India** and that this thesis has **not** been submitted for any degree or diploma **of** any **other** University or Institution.

Prof. H.N. Singh
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CERTIFICATE

This is to certify that the thesis entitled **Studies on Diazotrophy and its regulation by Salinity stress and Osmotic stress in the cyano-bacterium Nostoc muscorum** is based on the results of the work done by Mr. D. Chakravarthy 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.

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As apparent from my work, it would be a great injustice on my part if I do not acknowledge my petite and pragmatic cyanobacterial friend-Nostoc *muscorum,* into whose diaphanous physiology I had interloped.

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limitations in understanding and interpreting the available information and partly to the fact that a few of the publications could not be consulted in original.

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Thank you all, I hope this work reflects your best hopes for me and the future of mankind.

Chakravarthy Devulapalli

ABBREVIATIONS USED

ADP	Adenosine diphosphate
AMPSO	3-[(1,1-dimethyl-2-hydroxyethyl) amino]-2-hydroxy propane sulfonic acid
ATP	Adenosine triphosphate
CAPS	3-(cyclohexyl amino)-1-propane sulfonic acid
CAPSO	3-(cyclohexyl amino)-2-hydroxy propane sulfonic acid
CCCP	Carbonyl cyanide-m-chlorophenyl hydrazine
DCCD	Dicyclohexyl carbodiimide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
EDTA	Ethylene diamine tetraacetic acid
× g	multiples of gravitational constant
GOGAT	Glutamine-oxo-glutamate amino transferase (glutamate synthase)
GS	Glutamine synthetase
HEPES	N-2-hydroxyethyl piperazine-N'-ethane sulfonic acid
het	Genes coding for heterocyst formation
MSX	L-Methionine-DL-Sulfoximine
NADH	Nicotinamide adenine dinucleotide (reduced)
nia	Genes coding for nitrate assimilation
nif	Genes coding for the conventional nitrogen fixation
ntr	Genes coding for overall nitrogen regulatory system
PS-I	Photosystem I
PS-II	Photosystem II
Rubisco	Ribulose-1,6-bis-phosphate carboxyalse
Tris	Tris-(hydroxymethyl) amino methane
v/v	Per cent 'volume in volume' number of cm of a constituent of 100 cm
w/v	Per cent 'weight in volume' number of grams of constituent in 100 cm of solution

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Chapter-1

GENERAL INTRODUCTION

Cyanobacteria, popularly called blue-green algae are Gram negative, oxygenic photosynthetic prokaryotes (Fay, 1983). They are capable of growth and multiplication in a wide range of ecological habits ranging from extreme environments such as deserts (de Chazal et *al*. 1992; Palmer & Friedmann 1990), hot springs (Ward et *al*. 1989), hot brines (Dor & Paz 1989), frigid lakes (Orcutt et *al*. 1986), soda lakes (Ciferri 1983) and open oceans poor in nutrients (Fogg 1982), to symbiotic association with plants, animals and fungi (Rai 1990). A majority of them are diazotrophic, capable of growth at the simple expense of light, water and air. Since 1970s, they are being used as model systems for studies on photosynthetic N -fixation, H -production, NH -production as well as for their use as biofertilizers in nitrogen economy of natural ecosystems. In addition, they are also being used as model systems for understanding the molecular mechanisms of vegetative cell differentiation into heterocysts, hormogonia, akinetes, buoyancy regulation, secondary metabolism (Fay & Van Baalen 1980) and for studies on role of light, ions and temperature in regulation of gene expression for components of the photosynthetic apparatus and in photosynthetic state transitions (Debus 1992).

Their application potentials as agents in biotechnology is enormous (Stewart et *al*. 1987; Elhai 1994, Hall et *al*. 1995) and include application in desert reclamation (Painter 1993), waste water treatment (Wilde et *al*. 1991), bioremediation (Kuritz & Wolk 1994), as biofertilizer in rice agriculture (Venkataraman 1975) and in reclamation of *usar* lands (Singh 1961). Several laboratories have proposed that cyanobacteria can be used as bioinsecticides to deliver toxins for grazing mosquito larvae (Murphy & Stevens 1992;

Soltes-Rak et *al.* 1993) and in biological control of plant pathogenic fungi and bacteria (Kulik 1995). In addition, they are also considered as potential resources of pharmaceuticals (Metting & Pyne 1986). The successful exploitation of these cyanobacterial potentials would greatly depend on our understanding of the molecular biology of these oxygenic photosynthetic prokaryotes, which at the moment is under intense investigation. The techniques that have developed during the last two decades for genetic analysis of cyanobacteria at molecular level include; introduction of foreign DNA into cyanobacteria by various means (Porter 1986; Elhai & Wolk 1988; Thiel & Poo 1989; Thiel 1994), to make mutations tagged with transposons (Wolk et *al.* 1991) and to identify genes by complementation of mutations induced by classical means (Wolk et *al.* 1988; Golden 1988; Haselkorn 1991). It is also possible to fuse the regulatory region of one gene with an open reading frame of another called a reporter gene to monitor the regulatory aspect of gene expression during heterocyst differentiation, *nif* gene expression etc. (Elhai & Wolk 1990). Most of these tools have found their use in genetic analysis and in physical genetic mapping of cyanobacterial genomes (Thiel 1994). Recent advances in bioengineering has also made it possible to artificially create a stable cyanobacterium-crop association (Ganter et *al.* 1991).

In the following pages, an attempt has been made to delineate areas of current interest and discuss only few of the fundamental aspects while giving due respect (whereever necessary) to other important milestones in the field of cyanobacteriology. The first definite evidence for heterocystous forms fixing N was provided by Drewes (1928) and Fogg (1942).

Fay et *al.* (1968) proposed a role of heterocyst in N -fixation in heterocystous cyanobacteria while Stewart et *al.* (1969) and Fleming & Haselkorn (197 3) showed occurence and synthesis of nitrogenase in it. Wyatt & Silvey (1969) provided the first conclusive evidence for aerobic N -fixation in laboratory cultures of a non-heterocystous cyanobacterium. Subsequently microaerobic N -fixation in a non-heterocystous filamentous cyanobacterium was reported by Stewart & Lex (1970). Wolk & Woljciuch (1971) provided conclusive evidence for localization of nitrogenase activity in heterocysts in isolated population of cyanobacteria. Stewart & Singh (1975) reported *nif* gene transfer in *Nostoc muscorum*. Mazur et *al.* (1980) identified *nif* genes at molecular level in cyanobacteria. Mullineaux et *al.* (1981) demonstrated temporal separation of oxygenic photosynthesis and N -fixation in a non-heterocystous cyanobacterium as a mechanism of protection of nitrogenase against oxygen toxicity. Golden et *al.* (1985) discovered the classical feature of cyanobacterial *nif* gene rearrangement during heterocyst differentiation and Wolk et *al.* (1984) carried out gene transfer between *E. coli* and cyanobacteria. It has now been established beyond doubt that heterocyst is the oxygen protection mechanism of nitrogenase activity in filamentous cyanobacteria (Fay 1992; Gallon 1992). An exception to this rule are the two filamentous strains *Trichodesmium* (Bergman et *al.* 1994) and *Lyngbya aestuarii* (Paerl et *al.* 1991). Recent techniques of immunofluorescence and TEM have conclusively demonstrated that nitrogenase in *Trichodesmium* is confined only to a limited number of cells of unknown cell type (Bergman & Carpenter 1991) and the quantity of nitrogenase in such cells has been shown to vary diurnally (Fredriksson & Bergman 1995). The oxygen protection mechanisms of N -fixation in unicellular forms are under intense investigation and forms which exhibit N -fixation under photosynthetic condition are suggested to develop oxygen protection mechanisms under such conditions (Fay 1992;

Gallon 1992). Further detailed studies showed that mature heterocysts lack ribulose 1,6-*bis*-phosphate carboxylase (Rubisco) activity (Cossar et *al*. 1985), PS-II activity (Fay et *al*. 1968), nitrate reductase (NR) activity (Kumar et *al*. 1985), glutamate synthase (GOGAT) activity (Thomas et *al*. 1977) and contains an uptake hydrogenase (Peterson & Wolk 1978), functional *nif* genes and nitrogenase enzyme involved in production of ammonia from N_2 (Haselkorn 1978). The activity of PS-I of heterocyst and its active respiration at the expense of organic carbon received from adjacent vegetative cells together constitute the source of ATP and reductant for the oxygen labile nitrogenase activity (Haselkorn 1986).

Strains of *Anabaena* or *Nostoc* are photosynthetic prokaryotes capable of aerobic N_2-fixation in morphologically and biochemically distinct cells called heterocysts. Heterocysts and their adjacent vegetative cells live in a kind of nutritionally symbiotic relation - the heterocyst supplying fixed nitrogen to the vegetative cells and the latter in turn providing photosynthetically fixed carbon to the former (as a source of reductant energy) that is required for N -fixation. The differentiation of a photosynthetic vegetative cell to a N -fixing heterocyst requires the co-ordinated regulation of many sets of genes. The genes controlling novel envelopes of heterocysts are induced or activated. The developing heterocyst turns-off PS-II, stops fixing CO_2, breaks down phycobilisomes, turns-on the system for generation of ATP and reductant for nitrogenase and induces the synthesis for nitrogenase, peptides, polypeptides and co-factors (Buikema & Haselkorn 1993). In addition, heterocysts also lack NR activity (Kumar et *al*. 1985). It has been shown in *N. muscorum* that heterocysts are the

exclusive sites of nitrogenase activity and vegetative cells the exclusive sites of NR activity (Bagchi et *al*. 1985a). Such heterocystous filaments, on growth with NO_3^- as nitrogen source, develop into non-heterocystous, non-N_2-fixing filaments composed of exclusively vegetative cells with an active NO_3^- -assimilating enzyme system. The repressor signal for NO_3^- -repression of heterocyst and N_2 -fixation seems to be a metabolic product of NO_3^- -assimilation in *N. muscorum* (Bagchi & Singh 1984). NH_4^+-assimilating cultures of *N. muscorum* show complete repression of N_2 -fixing heterocyst, NO_3^- -assimilating enzyme systems and NH_4^+ -transport system (Bagchi & Singh 1984; Verma et *al*. 1990; Singh et *al*. 1994).

Vegetative cells of NH_4^+ -grown cultures of *Nostoc* or *Anabaena* on transfer to combined nitrogen-free medium (N_2), undergo nitrogen starvation leading to activation of proteases catalyzing degradation of phycobilisomes in certain cells evenly spaced along the filament. These cells are the first visible symptoms of a differentiating vegetative cell leading to an intermediate stage called proheterocyst. Heterocyst spacing pattern comes into operation at the stage of proheterocyst formation by which time the proheterocysts, while lacking oxygenic photosynthesis still do not develop the ability to fix N_2. In *Anabaena* sp. PCC 7120, a major rearrangement of *nif* gene region occurs during the heterocyst differentiation. During this rearrangement, two regions of DNA (11 and 55 kb) are excised from the chromosome thus producing a functional *nif* regulon (Haselkorn et *al*. 1987, 1991; Adams 1992). However, no such *nif* gene rearrangements have been reported in any non-heterocystous cyanobacterium wherein the nifHDK is already clustered (Kallas et *al*. 1983; Zehr 1992). This characteristic process has been found in other heterocystous cyanobacteria of *Nostoc* type, but not in *Fischeriella* spp. (Saville et *al*. 1987) or *Mastigocladus* spp. (Singh & Stevens 1992). Still the

physiological significance of *nif* gene rearrangements during heterocyst development remains contentious (Gallon 1992).

Heterocyst formation and nitrogenase activity are well-established processes that exhibit NH -repression control. Transcription of *glnA* gene as a function of inorganic nitrogen source has been demonstrated in *Anabaena* sp. PCC 7120 (Turner et *al*. 1983). Mutational and recombinational evidences suggest involvement of a regulatory gene in positive control of heterocyst formation and nitrogenase activitiy (Singh et *al*. 1977; Singh & Singh 1981). A nitrogen control gene called *ntck* has been discovered to function, in controlling the expression of all the genes that are subjected to repression by NH in non-diazotrophic and diazotrophic cyanobacteria (Frias et *al*. 1993). The product of ntcA gene has been found to be a sequence specific DNA-binding protein interacting with upstream region of several genes including *hetR*, *glnA*, *gisk* or *rbcL* and *nifL*. *ntck* mutant strains of *Anabaena* sp. PCC 7120 failed to produce N -fixing heterocysts and to assimilate N_2 as sole nitrogen source (Wel et *al*. 1994). NH -repressible *hetR* along with patP (Singh et *al*. 1994) or *patk* (Buikema & Haselkorn 1993) or *hetN* (Black & Wolk 1994) have been implicated in the regulation of heterocyst pattern formation. Mutants of *N. muscorum* selected for glyphosate resistance are known to lack NH transport system, heterocyst formation and nitrogenase activity (Singh et *al*. 1989). The identity of such ntr like gene in positive control of NH transport activity, heterocyst formation and nitrogenase activity is still unknown. In the present study on the role of V in nitrogen nutrition, use has been made of $Het^{+}Nif^{+}Nia^{+}$, $Het^{+}Nif^{-}Nia^{+}$, $Het^{+}Nif^{+}Nia^{-}$ and $Het^{-}Nif^{-}Nia^{+}$ strains of *N. muscorum.* The molecular nature of the

mutational defect in these mutants is yet to be identified and characterized. We believe that the *Het⁻Nif⁻Nia⁺* mutant might be a class of mutant defective in hetR or other NH -repressible nitrogen regulatory genes. However, the complexity of developmentally regulated genes in heterocyst formation and nitrogenase activity is an ever growing area of exciting research and nothing definite can be proposed as a molecular model for heterocyst formation and N -fixation at the present juncture.

The differentiation of a vegetative cell to a N -fixing heterocyst exhibits nitrogen control similar to nitrogen control on NH -transport system, NO - and NO - assimilation (Haselkorn 1978; Singh et *al.* 1989; Bagchi et *al.* 1985b). Mutants lacking the ability to produce functional NH -transport system and nitrogenase activity suggested the occurrence of a regulatory gene mediating nitrogen control of N₂-fixation and NH -transport activity (Singh et *al.* 1989). The real existence of such a nitrogen control gene (ntcA) was discovered recently in *Synechococcus* sp. PCC 7942 (Vega-Palas et *al.* 1990). It was shown that mutational loss of ntcA gene activity was associated with loss in full expression of NR, nitrite reductase (NiR) and GS activities. Subsequent studies established the positive regulatory role of the product of ntcA gene in regulating the expression of all the genes known to be under the repression control of NH in the cyanobacterium. A ntcA mutant failed to show heterocyst development and induction of *hetR* (Frias et *al.* 1994). Recent genetic evidences indicate an essential role for ntcA gene in assimilation of NO₃ or N₂ as nitrogen source and in differentiation of heterocyst and *nif* gene rearrangement (Wel et *al.* 1994). A role of ntcA gene in positive regulation of genes under the repression control of NH is now beyond doubt (Luque et *al.* 1994). The ntcA gene has been characterised by sequencing analysis and its product has been shown to belong to a family of bacterial transcriptional

activators that include CRP (cAMP receptor protein), Fnr, FixK etc., (Vega-Palas et *al.* 1992). The mechanism mediating the response of NtcA (=BifA) to the nitrogen status of the cell is little understood. It is believed that the NtcA protein (factor VF1) recognizes a sequence designated as VF1-binding site (Wel et *al.* 1993) present upstream to *rbcL*, *glnA*, *nifH* genes and xisA, thus acting as a positive transcriptional activator of *glnA*, nirA (first gene of the NO -assimilation operon nirA nrtABCD narB) and ntcA itself and as a negative effector of *xisA* (Wel et *al.* 1994). Further, two nitrogen-regulated genes nirB and ntcB have been recently identified in *Synechococcus* sp. PCC 7942 and appear to constitute an operon (nirB ntcB) and transcribed divergently from the nirA (Suzuki et *al.* 1995). The transcription of nirB ntcB increases under nitrogen limited condition and requires NtcA protein. The role of NtcB protein still needs to be ascertained.

One of the most exciting developments in the molecular biology of nitrogen control of nitrogen nutrition in cyanobacteria has been the discovery of P_{II} protein, which seems to respond primarily to cellular nitrogen status and imbalanced electron transport (Tsinoremas et *al.* 1991). The activity of this regulatory protein in control of nitrogen nutrition is regulated by phosphorylation/dephosphorylation carried out by a cyanobacterial protein kinase/phosphatase enzymes. Such kinase and phosphatase activities which modify the state of P_{II} might be the sensor of primary nitrogen signal or merely a step in a more complex nitrogen signal transduction pathway (Forchhammer & de Marsac 1994). Although an involvement of P_{II} interacting with NtcA has been postulated (Vega-Palas et *al.* 1992), the phenotype of the ginB mutant is

quite distinct from that of the ntcA mutant (Forchhammer & de Marsac 1995). If P_{II} was required for activation of NtcA then the phenotypes should have been similar indicating that P_{II} is not required for NtcA activity. The P_{II} in *Synechococcus* strains seems to be involved in mediating the co-ordination of nitrogen and carbon assimilation and it is yet to be shown which sensory systems monitor the phosphorylation state of P_{II} to adjust the cellular functions in response to nitrogen supply. The enterobacterial system of nitrogen control of nitrogen nutrition is different from the cyanobacterial system, in that uridylylation/deuridylylation of P_{II} is known to modulate the activity of GS enzyme via the classical *ntr* mediated control of nitrogen nutrition in the former (Merrick 1995). The NtcA in cyanobacteria appears to be analogous to the NtrC of enterobacteria. There is no evidence of a *ntr* mediated system in gram positive bacteria nor is there any evidence for alternative global nitrogen regulatory systems in them. However, novel mechanisms of nitrogen regulation have been found in *Streptomyces* spp., *Bacillus subtilis* and *Clostridium* spp. (Merrick 1995). It must also be mentioned here that cyanobacterial GS activity has been shown to be inactivated by NH_4^+ and/or dark treatments and found to be fully reversible on exposure to photosynthetic light, thus suggesting a role of photosynthetic electron transport via the P_{II} mediated cascade, in control of GS activity (Merida et *al.* 1991; Marques et *al.* 1992). In *Phormidium laminosum* (a non-N-fixing cyanobacterium), GS activity itself is required for *in vivo* inhibition of NO - or NO_3^--assimilation, by NH_4^+/glutamine (Tapia et *al.* 1995). Studies on glutamine auxotrophic mutants of *A. cycadeae* have shown operation of a common genetic regulation between GS, NO -uptake and NR (Singh et *al.* 1985) and it is interesting to note that Bagchi et *al.* (1985b) had then predicted a direct role for GS in regulation of NO_3^--uptake and NR activity in it. It is also worth mentioning here that in *Bacillus subtilis* GS itself together with

the *glnB* product seems to regulate its own transcription (Schreier 1993). However, the molecular mechanism of regulatory interaction involving NtcA protein and *P* protein in cyanobacterial inorganic nitrogen nutrition such as diazotrophy, NO^-_3 -assimilation and NH^+_4-assimilation remains to be analyzed in detail.

Nitrogen control of photosynthesis is comparatively a little investigated area. Few available reports show a role of nitrogen source in regulation of photosynthetic state transitions in algae and cyanobacteria (Mullineaux & Holzwarth 1990; Weger & Turpin 1989). The discovery of the role of P_I protein in control of oxygenic photosynthesis as a function of nitrogen source has opened up an entirely new field of research on the molecular mechanisms coupling oxygenic photosynthesis to the mode of nitrogen nutrition (Tsinoremas et *al.* 1991). Such a molecular signal cascade operating via the *P* protein coupling both nitrogen and carbon nutrition is already known in enterobacteria (Merrick 1988). The *P* protein and its gene *glnB* have now been shown to be present in a wide range of cyanobacteria where it is shown to undergo phosphorylation in the absence of NH and/or under PS-II light, i.e. when functioning of PS-II is favoured over PS-I (Tsinoremas et *al.* 1991; Allen 1992). This directly implicates a definite role of *P* regulatory protein in co-ordinated regulation of oxygenic photosynthesis and inorganic nitrogen metabolism in cyanobacteria. In *Calothrix* spp., photosynthetic electron transport is a major factor controlling phycobilisome gene expression and regulating differentiation of vegetative cells into heterocysts or hormogonia (Campbell et *al.* 1993).

The next question is how the product of ntcA gene interacts with *hetR* gene (essential for terminal heterocyst differentiation and in control of the process of heterocyst differentiation and pattern formation) in view of the well-known NH_4^+-repressible nature of *het* genes. A role of *hetR* gene along with the genes of heterocyst pattern formation *patA* and patB, in controlling terminal heterocyst differentiation and pattern distribution of intercalary heterocyst has been recently shown (Buikema & Haselkorn 1993). The product of *hetN* gene has been suggested to generate a secondary metabolite that regulates heterocyst spacing pattern by functioning as an inhibitor of heterocyst differentiation (Black & Wolk 1994). Recently, Bauer et *al.* (1995) have generated a fragmentation mutant from *Anabaena* sp. PCC 7120 by insertional inactivation of fraC and have demonstrated the essentiality of FraC in maintaining the integrity of cell junctions, though not directly involved in normal differentiation and N_2-fixation. In order to avoid any ambiguity in understanding the molecular mechanisms of heterocyst pattern formation, the relational identity between ntcA, hetN and *patA* and patB genes has to be clarified by future studies.

While the genes of heterocyst differentiation and heterocyst pattern formation are expressed in response to nitrogen starvation signal in evenly-spaced cells of the filament, it is not clearly known whether their expression is developmentally regulated or environmentally regulated. According to the environment control model, DNA rearrangements and transcription of *nif* genes occur in response to a combination of nitrogen deprivation and anaerobiosis (conditions that are present in a mature heterocyst), but differentiation itself is unnecessary (Helber et *al.* 1988). According to the developmental control model, a certain degree of differentiation must precede rearrangement and/or transcription of *nif* related genes (Elhai & Wolk 1990). Since heterocyst is the site of N_2-fixation

and since heterocyst differentiation is nitrogen regulated, there has been a general thinking that N -fixation in heterocystous cyanobacteria is nutritionally regulated as NH does in regulating the expression of *nif* genes in *Klebsiella pneumoniae* (Merrick *et al.* 1988). This problem was approached by the use of *lux* reporter gene fused to the promoter of nifHDK cyanobacterial gene in *Anabaena* sp. PCC 6942 (Elhai & Wolk 1990). Such reporter gene containing NH -grown filaments of the cyanobacterium on transfer to combined nitrogen-free medium, exhibited reported gene activity only in those evenly-spaced cells already committed to heterocyst differentiation. This powerful technique developed for monitoring the problem of N -fixing heterocyst differentiation (whether developmentally or nutritionally regulated), has led to the conclusion that heterocyst differentiation and nif gene expression is developmentally and not environmentally regulated in *Anabaena* sp. PCC 7120 (Elhai & Wolk 1990). However, mutants of *N. muscorum* are known which produce heterocysts in NH -medium (without showing N -fixation) while continuing to fix N under diazotrophic growth conditions (Kumar et *al.* 1988). Clearly, there is a strong evidence in favour of NH-nitrogen regulated expression of *nif* genes within the heterocyst and not developmentally regulated as suggested by Elhai & Wolk (1990) and Ernst et *al*. (1992).

In a novel study involving the use of *in situ* hybridization technique in heterocystous cyanobacteria, Anuradha & Nierzwicki-Bauer (1993) have detected *rbcL* and *rbcS* (coding for large and small subunits of Rubisco) in heterocysts, while also detecting *nif* transcripts in all the vegetative cells of a filament. This clearly appears to be in direct conflict with earlier observations reporting absence of Rubisco in

heterocysts (Cosser et *al.* 1985) while detecting *nif* gene expression that is developmentally regulated (Elhai & Wolk 1990). Only further studies on this aspect can rule out such a paradox. The importance of the knowledge about the mechanism controlling heterocyst differentiation and N -fixation lies in the fact that once techniques are available, it will be easy to manipulate and construct appropriate diazotrophic cyanobacterial strains for use as biofertilizer in agriculture. In addition, heterocystous cyanobacteria hold great promise as a potential source of photosynthetically generated ammonia under diazotrophic growth conditions (Modi et *al.* 1994). Thus, a complete knowledge on the biology of N -fixing heterocysts might enable scientists to photoproduce ammonia at commercial scale from heterocystous cyanobacterial forms in the near future.

Recent studies discovered a gene called *glnN* coding for a new type of GS in *Synechocystis* sp. PCC 6803 which also contains *glnA*, coding for the conventional cyanobacterial GS (Reyes & Florencio 1994). This has opened up an entirely new field on the physiology of NH -assimilation and its regulation by different environmental conditions. Rhizobial strains in symbiotic association with legumes are known to produce two different GSs, one characteristic of prokaryotic system and another characteristic of eukaryotic system with distinct •etabolic roles under different environmental conditions (Carlson & Chelm 1986). How wide spread the distribution of *glnN* gene encoding for the novel cyanobacterial GS remains to be investigated. But in *N. muscorum*, there does not seem to be any reason to suspect the presence of such ginN gene encoded GS as spontaneously occurring *GS* mutants are easily obtainable from this cyanobacterium (Verma et *al.* 1990; Singh et *al.* 1994). This inference is based on the known fact that ginA gene encoded and *glnN* gene encoded cyanobacterial GSs are found functionally equivalent as primary enzymes of NH -assimilation. However, *GS*

glutamine auxotrophic mutants are possible to arise in cyanobacterial strains containing both the *glnA* and *glnN* genes if the two genes form part of a common operon. But, such information at the moment is totally lacking.

Two types of glutamate synthases have been described in higher plants and algae. One uses pyridine nucleotides (NAD[P]H-GOGAT) and the other ferredoxin (Fd-GOGAT) as electron donors (Suzuki & Gadal 1984). Bacteria are known to exhibit only NADPH-GOGAT (Vanoni et *al.* 1991), while cyanobacteria contain only the Fd-GOGAT (Marques et *al.* 1992). In bacteria, the NADPH-GOGAT comprises two subunits, a smaller subunit encoded by *gltD* and a larger subunit encoded by *gltB* (Oliver et *al.* 1987; Pelenda et *al.* 1993). However, no information is available about the Fd-GOGAT in prokaryotes to trace the origins of plant GOGATs (Gregerson et *al.* 1993; Valentin et *al.* 1993). Recently, studies have shown the existence of two different genes *gltB* and *gltS* coding for Fd-GOGAT in the cyanobacterium *Synechocystis* sp. PCC 6803. These genes are actively expressed under normal growth conditions and can be inactivated independent of the other without affecting the growth of the cyanobacterium. Further, it was shown that the *gltB* gene is more related to the gene encoding for the NADH-GOGAT of higher plants than to its Fd-GOGAT counterpart. A common origin for various GOGATs has thus been hypothesized (Navarro et *al.* 1995).

A finding of importance in recent years has been the obligate requirement of functional phosphoenolpyruvate carboxylase for growth of cyanobacteria and that this requirement cannot be eliminated even with the addition of exogenous TCA cycle intermediates and amino acids (Luinenberg & Coleman 1990). Rapid strides have also been made in understanding the physical,

biochemical and molecular genetic basis of CO -concentrating mechanisms (CCMs) in cyanobacteria with the isolation of high CO_2-requiring mutants and characterisation of *ccm* genes (Ogawa et *al*. 1994a & b). Inactivation of genes coding for NAD(P)H dehydroogenase complex leads to a high CO. requirement. Evidence is now shown for the involvement of NAD(P)H dehydrogenase in cyclic electron flow and it is the ATP generated cyclic electron flow of PS-I that transports the inorganic carbon into the cell (Kaplan et *al*. 1994). Evidences also suggest that this is accomplished by packaging Rubisco and carbonic anhydrase (CA) into discrete structures called carboxysomes analogous to pyrenoids of microalgae (Badger & Price 1992). Photosynthetic flux density (PPFD) is an important parameter for growth of cyanobacteria and the molecular mechanisms by which it is sensed still needs to be clarified. The importance of PPFD in regulating the expression of many genes related to photosynthesis have been recently reviewed (de Marsac & Houmard 1993).

Modern techniques of molecular genetics have succeeded in identifying a large number of genes controlling sulfate transport and metabolism in cyanobacteria (Laudenbach & Grossman 1991). A comparative study on the sulfate metabolizing systems in chloroplast and cyanobacteria have shown them to be identical. Similar techniques have also led to the discovery of multi-gene families (unusual for prokaryotes) coding for D_1 and D_2 proteins of PS-II (Curtis & Haselkorn 1984; Golden 1994). Some photosynthetic genes are expressed differentially as a function of light intensity. Howver the relevance of this regulation is little understood at the moment (Golden 1994). In *Anabaena* sp. PCC 7937, plastocyanin and cytochrome *c* are alternative electron carriers of the electron transport chain connecting the two photosystems and their synthesis is governed by a copper regulated switch. Plastocyanin is made only in cells grown in the presence of copper while

cytochrome c_{55} is made in its absence. Such a copper regulated switch operates at the transcriptional level where copper-dependent increase in plastocyanin mRNA and decrease in cytochrome c_{55} mRNA has been shown (Bovy et *al.* 1992).

The discovery of biological clocks in prokaryotes has been a surprise finding in recent years. It is widely believed that circadian rhythms are a eukaryotic characteristic (Kippert 1991). However, the cyanobacterium *Synechococcus* RF-1 displays endogenous rhythms for nitrogenase activity (Grobbelar et *al.* 1986). Recent studies have also shown that circadian regulation of nitrogenase activity in *Synechococcus* RF-1 is controlled at the level of transcription (Huang & Chow 1990; Huang et *al.* 1994). Thus, cyanobacteria form useful model systems for studying the control mechanism of circadian rhythms at molecular and genetic level. Recently, Kondo et *al.* (1993) have used luciferase reporter gene and continuous automated monitoring of bioluminescence to demonstrate unequivocally that cyanobacteria exhibit circadian behaviour that are fundamentally the same as circadian rhythms of eukaryotes thus overturning the non-prokaryote dogma.

Azotobacter vinelandii is known to contain three genetically distinct nitrogenases (Bishop & Premkumar 1992). The molybdenum (Mo) nitrogenase, the vanadium (V) nitrogenase and a third nitrogenase containing only iron (Fe). Multiple enzymes systems, like the three nitrogenases carrying out a single function, inherently imply a role of distinct environmental factors controlling N_2-fixation. Mo is a known repressor of V- or Fe-nitrogenase, while V is an inhibitor of Fe-nitrogenase as well (Jacobitz & Bishop 1992; Premakumar et *al.* 1992). Such

hierarchial regulation of diazotrophy by Mo and V, suggests a specific role of these nutrients governing the distribution of alternative N -fixing systems in nature. Physiological studies indicate presence of a V-nitrogenase in the cyanobacterium *Anabaena variabilis* as well (Kentemich et *al*. 1988). V-dependant nitrogenase and NR activities have also been evidenced in N. *muscorum* (Singh et *al*. 1993a; Singh et *al*. 1993b) and **the** results are discussed in detail in Chapter 3 of this thesis. Studies at molecular level involving heterologous probes to identify genes for V-nitrogenase have also been recently successful in *A. variabilis* (Thiel 1993). The physiological significance of the three nitrogenases is still obscure.

It is encouraging to note that there has been an upsurge of interest in understanding the molecular mechanisms of biological production of ammonia, NH assimilation, NH transport and amino acid metabolism. The concept of diazotrophic cyanobacteria as photobiological sources of ammonia arose from the fact that *A. azollae* (living in symbiotic association with *Azolla)* provides to its host N_2-derived ammonia through a mechanism involving repression of the GS-GOGAT pathway (Stewart et *al*. 1987; Rai et *al*. 1984). Ammonium transport system in cyanobacteria is characteristically biphasic and its regulation by nitrogen source and GS activity has been studied in detail (Rai et *al* . 1984; Singh et *al*. 1985, 1987, 1989, 1990). Strains of free-living diazotrophic cyanobacteria defective in ammonia assimilation result in extracellular liberation of N_2-derived ammonia (Stewart et *al*. 1987; Singh et *al*. 1983, 1992) and **thus would be** ideal for use as biofertilizer in paddy fields. Methods are now available to produce and select out ammonia excreting strains of heterocystous diazotrophic cyanobacteria (Stewart et *al*. 1987; Singh et *al*. 1983) and such ammonia excreting diazotrophic cyanobacterial strains have been shown to supply N -derived ammonia to rice and wheat plants under

laboratory conditions (Lattore et *al*. 1986; Spiller et *al*. 1993). The physiological and genetic linkage between NH_4^+-transport system, NH_4^+-excretion and NH -assimilation still remains to be explored at molecular level for succesful construction of strains with biofertilizer potential. An ideal cyanobacterial biofertilizer strain needs to have the following attributes:

(i) Photosynthetic N -fixing strains derepressed for combined nitrogen,
(ii) Continuous excretion of ammonia generating from N -fixation,
(iii) Resistance to field herbicides and pesticides,
(iv) Resistance to salinity and/or osmotic, temperature and pH stresses, and
(v) Ability to compete succesfully against native strains.

Although N -fixing cyanobacterial strains derepressed for combined nitrogen are known (Kerby & Stewart 1988; Kumar et *al*. 1988), the molecular biology of repression-control of N_2-fixation is still unclear. Rice-field herbicides have been found to be extremely growth toxic and mutagenic to cyanobacteria (Singh & Vaishampayan 1978; Singh et *al*. 1979). Evidently, herbicide studies do indicate the need for constructing diazotrophic cyanobacterial biofertiliser strains resistant to them. Recently, multiple herbicide resistant ammonia excreting strains have been generated using the techniques of genetic transformation and mutation (Modi et *al*. 1991). However, inoculation of non-native strains is found to be a failure owing to interspecific competition and environmental constrains (Grant et *al*. 1985). An ammonia excreting strain from a

rice-field isolate *A. siamensis* has been generated which shows faster growth rate and adapts to wide temperature fluctuations as well as to salinity stress conditions (Thomas et *al.* 1991). *Spirulina platensis* is an alkalophilic cyanobacterium and is known to withstand and utilise ammonia even at high environmental pH. A high internal pH inhibits intracellular accumulation of ammonia in it, thus preventing uncoupling of photosynthesis and that this high internal pH of the cell is the result of an increase in the intrathylakoid pH (Belkin & Boussiba 1991) . Such studies are useful in that, strains competitive for growth of the cyanobacteria under field conditions can be selectively removed. A recent study in a yet another alkalophilic cyanobacterium *Hapalosiphon welwitschii*, demonstrated the operation of a pH regulated copper and zinc efflux system in it, as a strategy to grow and multiply in an alkalophilic environment rich in copper and/or zinc at concentrations inhibitory to its photosynthetic activity (Dwivedi et *al.* 1992). A copper efflux system of similar physiological significance has been found to operate in the neutrophilic cyanobacterium *N. muscorum* (Verma & Singh 1991).

Organisms are known to grow and multiply in environments of fluctuating temperature, dessication, salinity and osmotic stresses. A knowledge about the molecular nature of such stress tolerance would be extremely useful in production of strains for use in agriculture, food industry and in medical microbiology (Bartels & Nelson 1994). The cyanobacterium *N. commune* has been shown to be relatively more dessication tolerant and has been studied as model system for analysis of dehydration induced stresses in photosynthetic cells (Potts 1994). Special proteins called dehydrins are known to confer tolerance against dessication stress (Bartels et *al.* 1993). Genetic evidences suggest that acclimation-induced cold tolerance is like dessication tolerance a quantitative characteristic controlled by a number of additive

genes (Guy 1990). Recent biochemical and genetic engineering experiments have demonstrated a definite role of fatty acid desaturases in acquistion of cold tolerance in higher plants (Murata et *al*. 1992) and in cyanobacteria (Wada et *al*. 1994). It is thus possible to manipulate photosynthetic organisms for cold tolerance by the technique of genetic engineering so common in biotechnological researches at the moment.

Growing problem of hyperosmolarity in agricultural ecosystems is now making it obligatory to generate genetically stable osmotolerant crops and microbes. It is therefore necessary to identify a range of genes that can be engineered into plants and microbes of agricultural importance. This requires a knowledge of genetic make-up of osmotolerance or salt tolerance that are characteristic of different biological groups growing in non-saline/saline habitats. Diazotrophic cyanobacteria are important natural components of various ecosystems from the view point of nitrogen supply. It has now become necessary to identify cyanobacterial genes which confer salinity/osmotolerance and which can be introduced easily for generation of diazotrophic strains tolerant to salinity/osmotic stress for use as biofertilizer in agriculture. In this thesis, studies have been conducted in *N. muscorum* to understand the nature of osmotic and salinity stresses in it and generate mutant strains resistant to them. Chapters 4, 5, 6, & 7 constitute a small contribution in this direction.

On the whole, results presented in this thesis attempts to address questions from a basic and a fundamental perspective and explores avenues for furtherance of basic and applied cyanobacterial research.

Chapter-2

2.1 Organism used

Nostoc muscorum is a fresh water 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 N_2-medium and when it is supplemented with a final concentration of 5 mol m^{-3} KNO_3, 5 mol m^{-3} $NaNO_2$ and 1 mol m^{-3} NH_4Cl (buffered with 50 mol m^{-3} 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 & Singh, 1975).

Axenic batch cultures of this strain were grown in liguid medium in air-conditioned illuminated culture room at a photon fluence rate of 50 $\mu mol\ m^{-2}\ s^{-1}$ and temperature of 28+2 °C. Clonal cultures were maintained on agar slants which were prepared by adding 1.2% (w/v) agar-agar to the liquid medium prior to autoclaving.

2.2 Culture media

5 cm^3 each of the macronutrients (Table 2.1) and 1 cm^3 of the micronutrient mixture solution (Table 2.2) were taken in a final volume of 1000 cm^3 (1 L) double distilled water and its pH 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.

2.3 Sterilization

Culture media were sterilized prior to inoculation under aeseptic conditions by autoclaving at 15 lbs sq. inch pressure for 15 min at 121 °C.

Macronutrient stock

Macronutrient	Concentration (gm per 100 cm)
1) $CaCl_2.2H_2O$	**1.1**
2) $MgSO_4.7H_2O$	0.5
3) K_2HPO_4	0.2
4) Na_2SiO_3	0.88
5) Na_2CO_3	0.44
6) (a) Citric acid	0.06
(b) Ferric citrate	**0.06**

Table 2.2

Micronutrient stock

Micronutrient	Concentration (gm per 1000 cm)
1) $MnCl_2$	1.81
2) H_3BO_3	2.86
3) $ZnSO_4.7H_2O$	0.222
4) $CuSO_4..5H_2O$	0.079
5) $Na_2MoO_4.2H_2O$	0.0177
6) $Co(NO_3)_2.6H_2O$	0.05

2.4 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 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.

2.5 Estimation of chlorophyll a

The chlorophyll a content was estimated by using the method of Mackinney (1941) by extracting into 100% methanol. 5 cm of the culture is withdrawn and centrifuged at 2,000 x g for 15 min. The supernatant is discarded and the pellet is dissolved in equal volume of 100% methanol in a test tube. It is then kept in hot water bath maintained at 60 °C for 10 min. The solution is cyclomixed and once again centrifuged at 2,000 x g for 10 min. The absorbance of the supernatant (chlorophyll a extract) was read at 663 nm. The chlorophyll a content is calculated by using the following formula:

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

2.6 Estimation of cellular protein

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

2.7 Measurement of heterocyst frequency

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

2.8 Estimation of percent survival

Nutrient agar plates at different graded concentrations of the drug/inhibitor are prepared. A known number of colony forming units (CFUs) per nutrient plate are inoculated on to each plate. A control plate containing no drug/inhibitor is also inoculated. After 6-days of growth, the number of CFUs appearing on each plate is counted. The number of CFUs appearing on each plate is compared with respect to the control and expressed as percent of the control. The total number of CFUs survival on the control plate is presumed to show 100% survival.

2.9 Assay of nitrogenase (EC 1.8.6.1) activity

Nitrogenase activity was measured as described by Stewart & Singh (1975). Assay was done in rubber-stoppered 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 s After 1 h of incubation ethylene production was assayed gas chromatographically using a CIC Gas Chromatograph. The nitrogenase activity was expressed in terms of mmol C H formed g^{-1} Chl a^{-1} h^{-1}.

2.11 Chemicals used

Unless otherwise stated, all chemicals used in the present study were of high purity (analytical grade) and were obtained from M/s Sigma Chemical Co., USA and from British Drug House (BDH), India. Radiochemicals were procured from Board of Radiation and Isotope Technology (BRIT), India.

2.12 Statistical analysis

2.12.1 Standard deviation (SD) and standard error about mean (SEM)

The standard deviation (SD) of the data on specific observations was calculated as:

$$\text{Standard deviation (SD)} = \sqrt{\frac{1}{n} \sum (x_i - \bar{x})^2}$$

The standard error of the data was calculated as:

$$\text{Standard error (SEM)} = \frac{\text{Standard deviation (SD)}}{\sqrt{n}}$$

where n = number of variants (experimental determinations).

Chapter-3

AN EVIDENCE FOR A ROLE OF VANADIUM IN DIAZOTROPHIC GROWTH OF THE CYANOBACTERIUM *NOSTOC MUSCORUM*

3.1 INTRODUCTION

The ability to fix atmospheric nitrogen and convert it into a usable form (diazotrophy) by N -fixing microorganisms is catalyzed by the enzyme nitrogenase. The genes required specifically for nitrogen fixation are called *nif* genes. The essentiality of molybdenum (Mo) in N_2-fixation became apparent when Bortels (1930) showed that Mo was required by *Azotobacter vinelandii* for growth on N_2 but not for growth on NH_4. This observation was given a biochemical meaning first by Bulen & Lecomte (1966) who showed that purified nitrogenase contained Mo and subsequently by (Miller 1991) who isolated Mo-Fe protein of nitrogenase from a variety of diazotrophic bacteria. Early studies also indicated a role for vanadium (V) in N -fixation in *Azotobacter* (Burk & Horner 1935; Bortels 1936). The first indirect evidence for the occurrence of Mo-independent nitrogenase enzyme system came from the observation that a class of tungstate resistant (*W-R*) mutants of *Nostoc muscorum* did not fix N nor grew diazotrophically with Mo in the growth media (Singh et *al.* 1978). However, the most serious challenge to the dogma of the unique role of Mo came with genetic studies on *nif* mutants of *A. vinelandii*, which showed that some of these mutants could fix N in growth medium devoid of Mo (Bishop et *al.* 1980). Eventually, unequivocal confirmation for the occurrence of a non-Mo nitrogenase system came with the construction of strains of both *A. chroococcum* (Robson et *al.* 1986) and *A. vinelandii* (Chisnell et *al.* 1988; Hales et al 1986) where the structural genes for Mo-nitrogenase *nif*HDK were specifically deleted. Further studies revealed operation of genetically three distinct nitrogenase systems for N -fixation in *A. chroococcum* and *A. vinelandii* (reviewed in Joerger & Bishop 1990; Pau 1991). Nitrogenase-1 is the conventional Mo-nitrogenase, expressed only

when Mo is present in the nutrient medium. The Mo-nitrogenase is encoded by the three structural genes nifHDK. Nitrogenase-2 is the V-nitrogenase, expressed only under conditions of complete Mo-starvation but containing V in the nutrient medium and encoded by the structural genes *vnf***HDGK.** Nitrogenase-3 is the Fe-nitrogenase, expressed only under conditions of both Mo- as well as V-starvation and encoded by the structural genes anfHDGK (Bishop & Premakumar 1992).

The conventional nitrogenase is coded by the *nif* cluster *nif***FMZWVSUXNEYTKDH** and is similar to that in *Klebsiella pneumoniae* (Eady et *al*. 1988; Evans et *al*. 1988; Jacobson et *al*. 1989) but without the flanking genes *nif***QBA** or the *nif***J.** The *nif***QB** genes have been recently sequenced and analysed (Joerger & Bishop 1988). The genes encoding the V-nitrogenase include *vnf***H** (in *A. chroococcum)* which is next to a gene encoding the ferredoxin-like with vnfDGK located 2.5 Kb away from the *vnf***H** (Eady 1994). A similar nifH2 and a gene encoding a ferredoxin-like protein were also found in *A. vinelandii* (Raina et *al*. 1988). Other genes specific for the V-nitrogenase have not yet been identified. The third nitrogenase (Fe-nitrogenase) has been discovered in *A. vinelandii*, but apparently absent in *A. chroococcum* (Chisnell et *al*. 1988). Its synthesis is repressed by addition of either Mo or V or both (Bishop et *al*. 1982; Premakumar et *al*. 1989). Joerger et *al*. (1989) have sequenced the structural genes of this nitrogenase (anfHDGK) from *A. vinelandii*. Although the structural genes coding for the three systems are distinct, few *nif* genes are required for activity of all the three enzymes in *A. vinelandii*. These are nifM (Kennedy et *al*. 1986), nifB (Joerger et *al*. 1986; Joerger & Bishop 1988) and nifU, S & V (Kennedy & Dean 1992). Mutations in these genes abolish N -fixation process under all conditions (in presence of Mo, V, both Mo and V or neither metal). The

requirement of nifB and *nifV* suggests similarities in the structure of FeMoco, FeVco and FeFeco. Specifically the requirement of *nifV* implies that homocitrate is a likely component (Eady & Leigh 1994). The H-gene encoding the small sub-unit (nitrogenase reductase) shows a high degree of sequence homology in all the three nitrogenases (Kentemich et *al.* 1990).

The V-nitrogenase (nitrogenase-2) in *Azotobacter spp.*, has a structure similar to the two components of the molybdoenzyme (nitrogenase-1). The V-Fe protein however differs from its counterpart (Mo-Fe) in that it has three polypepetides (two in the Mo-Fe protein) encoded by the KGD genes (Eady & Leigh 1994). The V-nitrogenase reduces more H_2 and reduces acetylene (C_2H_2) with lower rates, than the conventional Mo-nitrogenase and forms low, but significant amounts of ethane (C_2H_6). The nitrogenase-3 probably contains solely Fe-S centres at the prosthetic group (Chisnell et *al.* 1988) and reduces N_2 and particularly acetylene with lower rates and produces H_2 with even higher rates than the V-enzyme and also forms small amounts of ethane. Ethane production is the characteristic feature of non-Mo-nitrogenases (Dilworth et *al.* 1987).

The role of Mo and V in regulation of the three genetically distinct nitrogenases in *A. vinelandii* have been studied in detail (Premakumar et *al.* 1992). Mo is a requirement for the expression of genes for Mo-nitrogenase but functions as inhibitor for expression of V-nitrogenase and Fe-nitrogenase enzyme systems. The implication is that V-nitrogenase specific genes would be expressed only under conditions of Mo-deficiency. It has also been found that in bacterial systems V-nitrogenase expression is V-dependant. V like Mo has been found to inhibit Fe-nitrogenase in bacteria. The organization of V-nitrogenase genes in *Anabaena variabilis* was found similar to that of *A. vinelandii* and it was further shown that V-nitrogenase is not expressed in the presence of Mo in cyanobacteria (Thiel 1993).

Bortels (1940) also proposed a role for V in cyanobacterial N_2-fixation. Subsequently, neither Allen & Arnon (1955) nor Holm-Hanson found any evidence of V requirement for cyanobacterial diazotrophic growth. These findings were extended by Fay & de Vasconcelos (1974) who found V-inhibiting N -fixation in *Anabaena variabilis.* The V-nitrogenase is known to reduce acetylene to ethylene and ethane, while Mo-nitrogenase catalyzes the reduction of acetylene to ethylene alone. This has led to estimation of ethane production as a measure of V-nitrogenase in bacterial systems. *A. variabilis* grows and produces ethane from acetylene when grown with V in a Mo-deficient medium (Kentemich et *al.* 1988; Yakunin et *al.* 1991) suggesting that this organism may have an alternative N -fixing enzyme system. Following this approach in *A. variabilis*, the presence of a functional V-nitrogenase has been shown (Kentemich et *al.* 1988; 1990). Thiel (1993) has characterized a cluster of genes from *A. variabilis* ATCC 29413 that appear to encode a V-dependant nitrogenase system. Evidence for the expression of nitrogenase-3 (Fe-nitrogenase) in *Anabaena variabilis* has also been recently shown (Kentemich et *al.* 1990).

An earlier observation (Singh et *al.* 1978) showing Mo-independent diazotrophic growth in *N. muscorum* was further investigated during the present study, to find out whether such Mo-independent diazotrophic system is V-dependent. It has also been earlier observed that Mo is indispensable not only for diazotrophy in *N. muscorum* but also for its nitrate assimilation (Singh et *al.* 1978). This led us to examine the role of V in nitrate nutrition as well. In the present study on the investigation for a role of V in nitrogen nutrition, use has been made of $Het^{+}Nif^{+}Nia^{+}$, $Het^{+}Nif^{-}Nia^{+}$, $Het^{+}Nif^{+}Nia^{-}$, $Het^{-}Nif^{-}Nia^{+}$ strains of *N. muscorum* (Singh et *al.* 1989).

W being an analogue of Mo, resistance to it has been used as a more successful method for demonstration of V-nitrogenase in *A. chroococcum* (Robson et *al.* 1986). In the present study, tungstate resistant (*W-R*) mutants of heterocystous, N -fixing, nitrate assimilating (Het *Nif Nia*) strain; heterocystous, non-N_2-fixing, nitrate assimilating *(Het Nif Nia*) strain; heterocystous, N -fixing, non-nitrate assimilating (Het *Nif Nia*) strain; non-heterocystous, non-N_2-fixing, nitrate assimilating (Het *Nif Nia*) strains of *N. muscorum* have been isolated and examined for their N_2 and NO nutritional characteristics with respect to Mo/V requirement. Evidence is provided here to show replacement of Mo by V in activity control of nitrogenase and nitrate reductase essential for growth on N or NO as nitrogen source in the *W-R* mutant strains the of cyanobacterium *N. muscorum.* The present report of V-dependent nitrate reductase activity and nitrate assimilation is a new finding in its entirety and there is no previous examples published.

3.2 MATERIALS AND METHODS

Axenic clonal cultures of parent *N. muscorum (Het Nif Nia* strain) was routinely grown in Chu No. 10 medium as described in Chapter 2 (Section 2.1) in combined nitrogen-free (called diazotrophic or N -medium) alone. The *Het Nif Nia* and the *Het Nif Nia* strains were grown in media containing 5 mol m KNO_3, while the $Het^+Nif^+Nia^-$ strain was grown in N -medium. 6-day old exponentially growing cultures were used for isolation of the desired mutants.

3.2.1 Isolation of tungstate resistant (*W-R*) mutant strains

W is antagonistic to synthesis of active nitrogenase and nitrate reductase in *N. muscorum* whose N -fixing cultures are nearly 10-fold more sensitive to W than its NO -assimilating cultures (Singh et *al.* 1978). This observation formed the basis

for isolating spontaneously occurring mutants capable of surviving/growing in W-containing diazotrophic growth medium. A dose of 0.3 mol m sodium tungstate was found completely lethal to N_2-fixing cultures. *W-R* mutants of N -fixing cultures were isolated by plating cultures on N -medium containing 2 mol m^{-3} sodium tungstate. The *W-R* mutants of non-N -fixing NO -assimilating cultures were isolated in a similar way, but in the presence of 5 mol m sodium tungstate on NO -medium. In both cases the growth medium contained Mo at its normal concentration as given in Table *2.%* (Chapter-2). 2 x 10 CFUs each of the cyanobacterial cultures were seeded on solid nutrient plates containing the inhibitor for the isolation of sponatneously occurring mutants. Such cultures were incubated for two weeks under photoautotrophic growth conditions. Few pin-head sized colonies appeared on W-containing respective nitrogen media, which on subsequent testing on fresh Mo-containing N - or NO -medium lacking W failed to grow, thus suggesting them to be defective in Mo-nutrition and metabolism. Addition of V as vanadyl sulphate at 0.01 mol m to W-containing selective or W-free selective N_2- or NO -media, made all such pin-head tungstate resistant (*W-R*) colonies to respond and grow very efficiently. Such *W-R* mutant clones growing with V were isolated and maintained on respective nitrogen media containing 2 mol m or 5 mol m sodium tungstate and 0.01 mol m vanadyl sulphate. 1 mol m ammonium chloride containing Mo/V-free medium was used to grow the two strains separately for about 6-successive sub-cultures, in order to reduce to the minimum possible contaminating Mo or V in the cultures which were subsequently used as inocula in the experiments detailed as below.

3.2.2 Estimation of ^{99}Mo-transport activity

The method of Elliott & Mortenson (1975) was used for the measurement of uptake of ^{99}Mo (as molybdate) into the cells. NH_4^+-growing exponential cultures of the various parent and mutant strains of *N. muscorum* were used and incubated in respective N - or NO$_3^-$-media for 6-days. They were then harvested by centrifugation, washed twice with 10 mol m^{-3} HEPES-NaOH buffer (pH 7.0), resuspended in the same and equilibrated for 30 min at 25 °C under photoautotrophic growth conditions. ^{99}Mo (as sodium molybdate) was added to the cell suspensions at a final concentration of 0.05 mol m^{-3} (specific activity 20.4 mCi g^{-1}). 400 µl samples were drawn at specific time intervals and separated from the bathing medium by the silicon oil microcentrifugation technique of Scott & Nicholls (1980) and counted for cellular level of the radiolabel in a Beckman LS 1800 liquid scintillation counter. Non-specific binding of the radiolabel was determined by measuring its incorporation in toluene-treated cells (Rai *et al.* 1984). This value was always subtracted from the value obtained of untreated samples before analysing the results.

3.2.3a Nitrate reductase (EC 1.6.6.3) assay

Cyanobacterial cells in their exponential phase of growth were harvested by low-speed centrifugation, washed twice with 50 mol m^{-3} Tris buffer (pH 7.5) containing 100 mol m^{-3} NaCl, 300 mol m^{-3} sucrose and 1 mol m^{-3} EDTA and resuspended in the same. This suspension was sonicated at 4 °C. The broken material was centrifuged at 30,000 x g for 30 min and the resulting supernatant was used as a crude enzyme extract.

The method of Manzano *et al.* (1976) as described by Bagchi & Singh (1984) was followed for estimating nitrate reductase activity. The enzyme activity was measured colorimetrically following the appearence of nitrite using methyl

donor. The reaction mixture contained in a final volume of 10 ml, glycine-KOH buffer (pH 10.5 100 μmol) ; KNO (20 μmol) ; methyl viologen (4 μmol) and sodium dithionite (10 μmol in 0.1 ml of 250 mol m sodium bicarbonbate) and an appropriate amount of enzyme. This was incubated at 30 °C for 10 min and the reaction •ixture was terminated by the addition of 0.2 ml of 1000 mol m " zinc acetate. The mixture is then pelleted by low-speed centrifugation. The nitrite thus formed in the supernatant was estimated by following the method of Snell & Snell (1966).

3. 2.3b Determination of nitrite

To 1 cm of the supernatant 1 cm of 1% sulphanilamide (in 25:75 HCl:Water) and 1 cm^3 of 0.02% NED (N-1-naphthylethylene diamine dihydrochloride) is added, mixed and incubated for 10 min. The absorbance is read at 540 nm. A standard plot for NO is also made using 1 mol m aqueous solution of potassium nitrite (KNO) .

Growth, chlorophyll a, protein, heterocyst frequency, percent survival and nitrogenase activity were determined as described in Chapter-2 (Sections 2.4, 2.5, 2.6, 2.7, 2.8 & 2.9 respectively) .

3. 2. 4 Chemicals used

Vanadyl sulfate was procured from M/s Aldrich Chemical Co., USA while all other chemicals used in the present study were from M/s Sigma Chemical Co. , USA. ^{99}Mo was purchased from Board of Radiation and Isotope Technology (BRIT), India.

3.3 RESULTS

Heterocystous, N -fixing, NO_3^--assimilating (*Het* $^+$ *Nif* $^+$ *Nia* $^+$) strain, heterocystous, non-N -fixing, NO -assimilating (Het $^+$ *Nif* $^-$ *Nia* $^+$) strain, heterocystous, N -fixing, non-NO -assimilating (*Het* $^+$ *Nif* $^+$ *Nia* $^-$) strain and non-heterocystous, non-N -fixing, NO -assimilating (Het *Nif Nia*) strains of *N. muscorum* used in the present study were those that have been already described (Singh et *al.* 1989). A brief description of their phenotypes are shown in Table 3.1. The first three strains were similar with respect to heterocyst formation, heterocyst frequency and nitrogenase activity under diazotrophic growth condition and their repression under NH_4^+-assimilating condition. Also while the *Nia* strain contains active nitrate reductase and shows NO - repression of heterocyst formation and nitrogenase activity, the *Nia* strain lacks active nitrate reductase as well as such NO -repression control. This behaviour is consistant with the reported role of nitrate reductase activity in NO -repression of heterocyst formation and nitrogenase activity in this cyanobacterium (Bagchi & Singh 1984).

The spontaneous mutation frequency with which the *W-R* mutant clones of the four strains arose was in the range 0.8 - 1.2 $\times$ 10^- . All such *W-R* clones on preliminary examination were found to survive in the form of pin-head colonies on both W-containing selective and W-free non-selective N - or NO - growth media. The lack of growth on Mo-containing W-free diazotrophic/NO - growth medium suggested that the mutant strains were impaired in Mo-metabolism. This led us to check for Mo-transport activity in the parent as well as in the mutant strains. As shown in Fig. 3.1a, b, c & d, the *W-R* mutant strains were severely defective in Mo-uptake by about 20-fold with respect to their parent strains. These results clearly show, that the *W-R* mutant strains are deficient in the Mo-transport system.

Table 3.1

Strains of *Nostoc muscorum* used in the present study with their phenotypes and growth characteristics.

Strains	Phenotypes	Growth characteristics
parent strain	*Het*$^+$*Nif*$^+$*Nia*$^+$, produces heterocyst, Mo-nitrogenase and Mo-nitrate reductase.	Grows with N_2, NO_3^- or NH_4^+ as nitrogen source and shows NO_3^-- or NH_4^+-repression of heterocyst and nitrogenase activity.
non-N_2-fixing Strain	*Het Nif Nia* , produces heterocyst and Mo-nitrate reductase but showing no nitrogenase activity due to defect in processing of Mo-cofactor into active nitrogenase.	Grows with NO_3^- or NH_4^+ but not with N_2 as nitrogen source and shows NO_3^- or NH_4^+-repression of heterocyst.
non-NO$_2^-$-assimilating strain	*Het Nif Nia* , produces heterocyst and Mo-nitrogenase but shows no nitrate reductase activity due to lack of its apoprotein component.	Grows with N_2 or NH_4^+ but not with NO_2^- as nitrogen source and deficient in NO_3^- but not in NH_4^+-repression of heterocyst and nitrogenase activity.
non-heterocystous, non-N_2-fixing strain	*Het Nif Nia* , shows no heterocyst and nitrogenase activity but producing Mo-nitrate reductase.	Grows with NO_3^- or NH_4^+ but not with N_2 as nitrogen source.

Fig. 3.1a

Mo transport activity in the ***Het Nif Nia*** (parent) strain of *N. muscorum* (0) and in its ***W-R*** mutant (•).
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

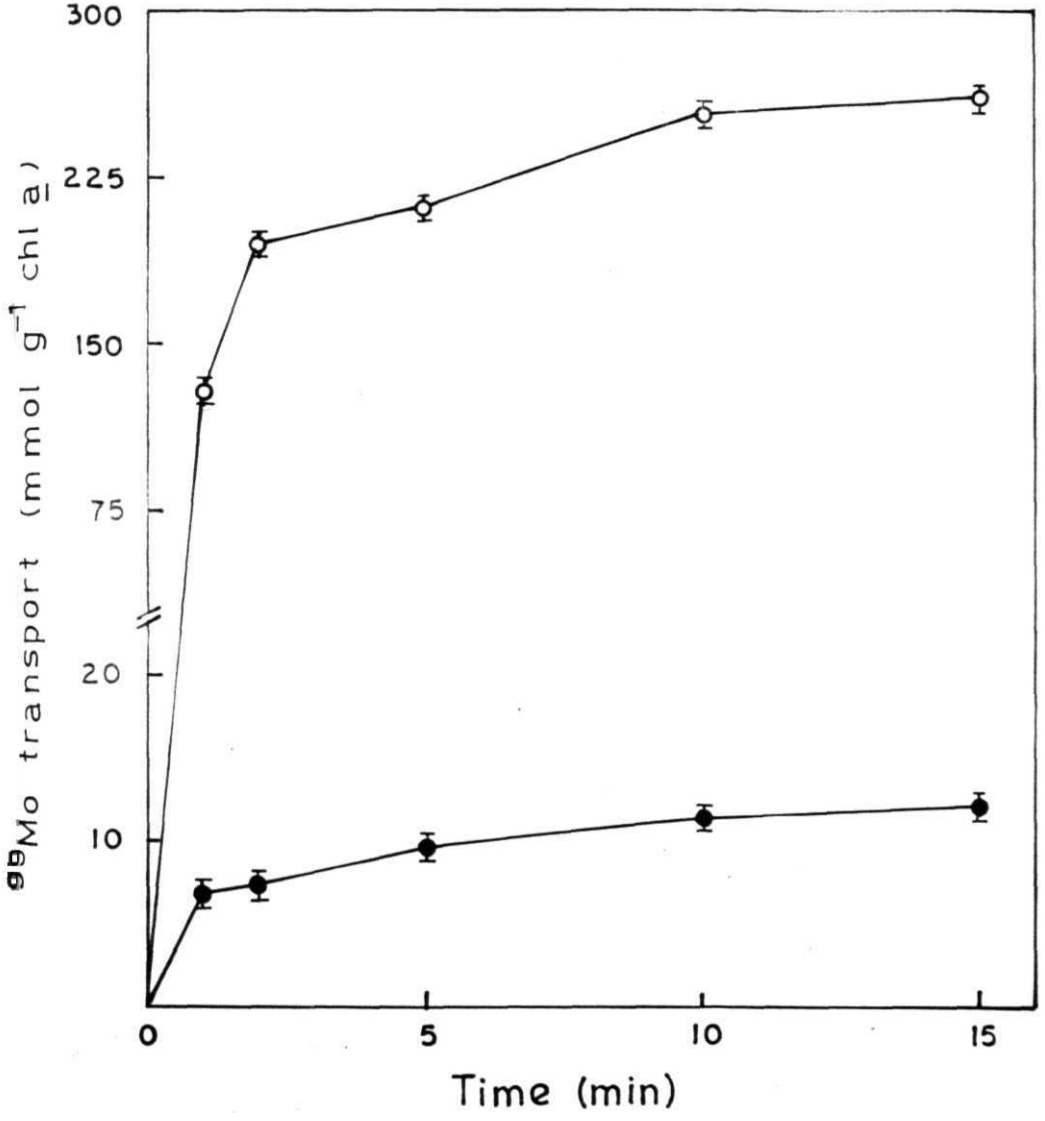
300
225
150
75
20
10
^{99}Mo transport (m mol g^{-1} chl a)
0
5
10
15
Time (min)

Fig. 3.1a

Fig. 3.1b

99 + - +

Mo transport activity in the *Het Nif Nia* strain of *N. muscorum* (•) and in its *W-R* mutant (•).

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

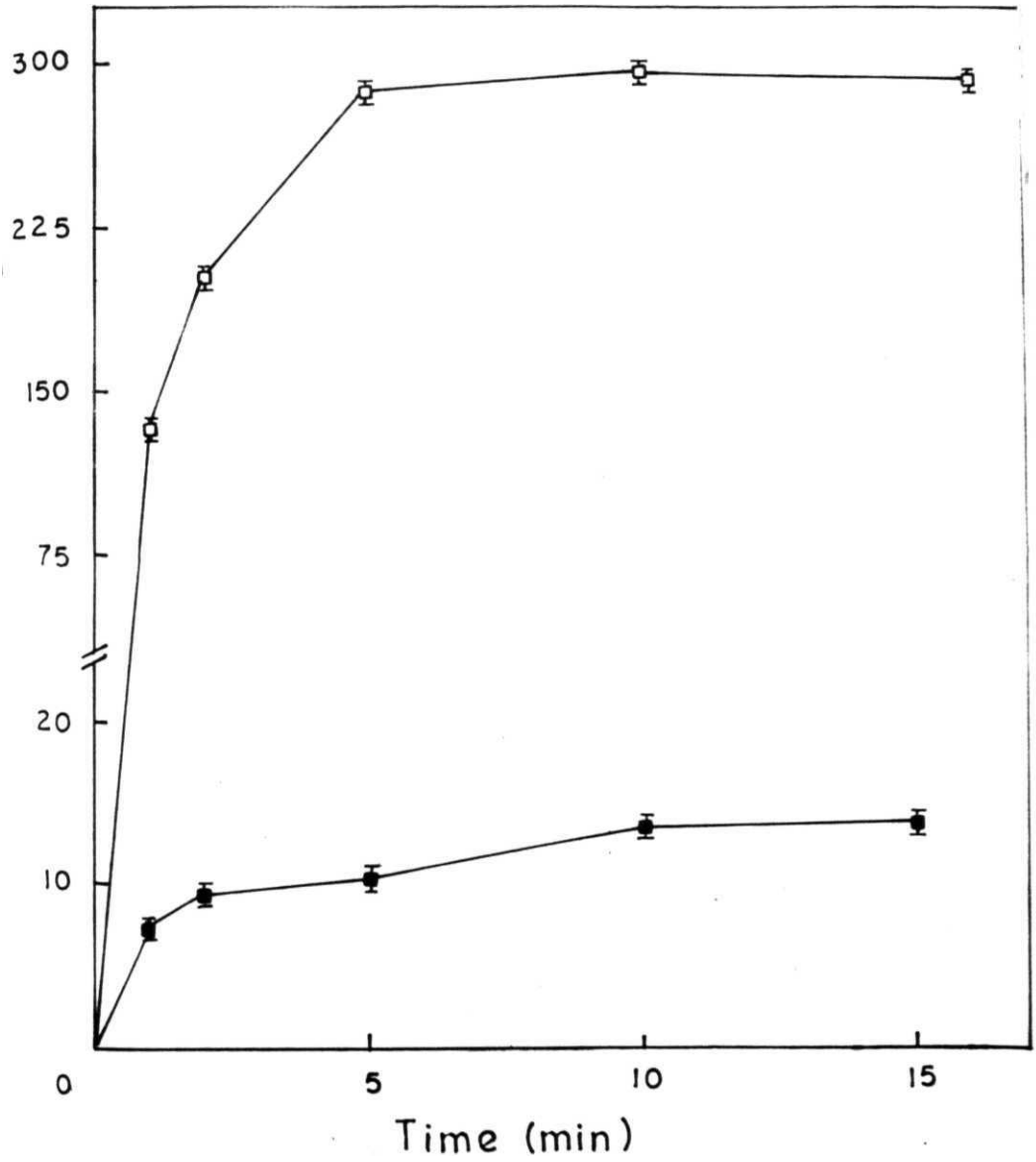

Fig. 3.1b

Fig. 3.1c

99 + + —

Mo transport activity in the *Het Nif Nia* strain of *N. muscorum* (▲) and in its *W-R* mutant (▲).

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

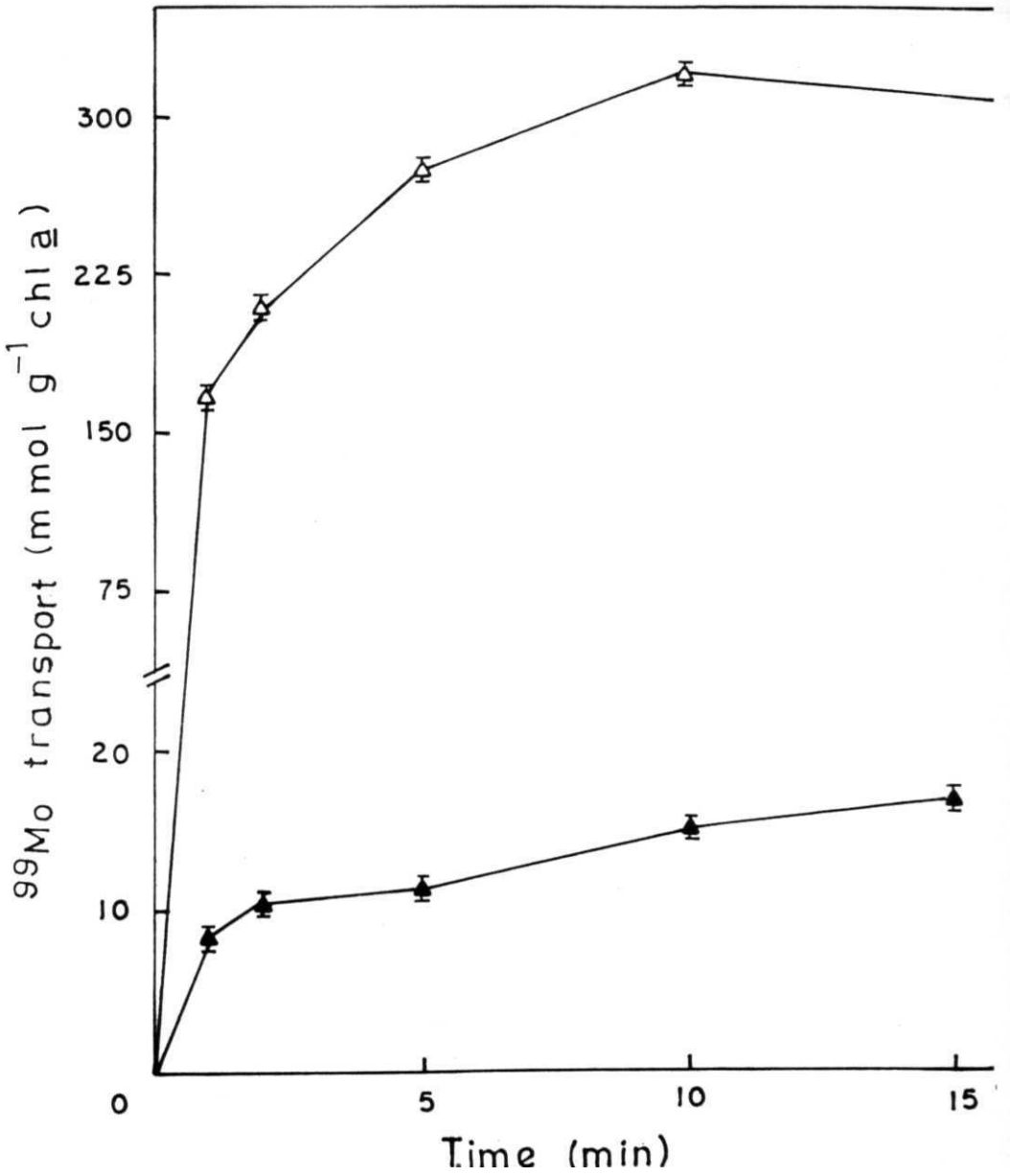
300
225
150
75
20
10
0
5
10
15
Time (min)
^{99}Mo transport (m mol g^{-1} chl a)

Fig.3.1c

Fig. **3.1d**

99 — — +

Mo transport activity in the *Het Nif Nia* strain of *N. muscorum* (O——0) and in its *W-R* mutant (O——0).

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

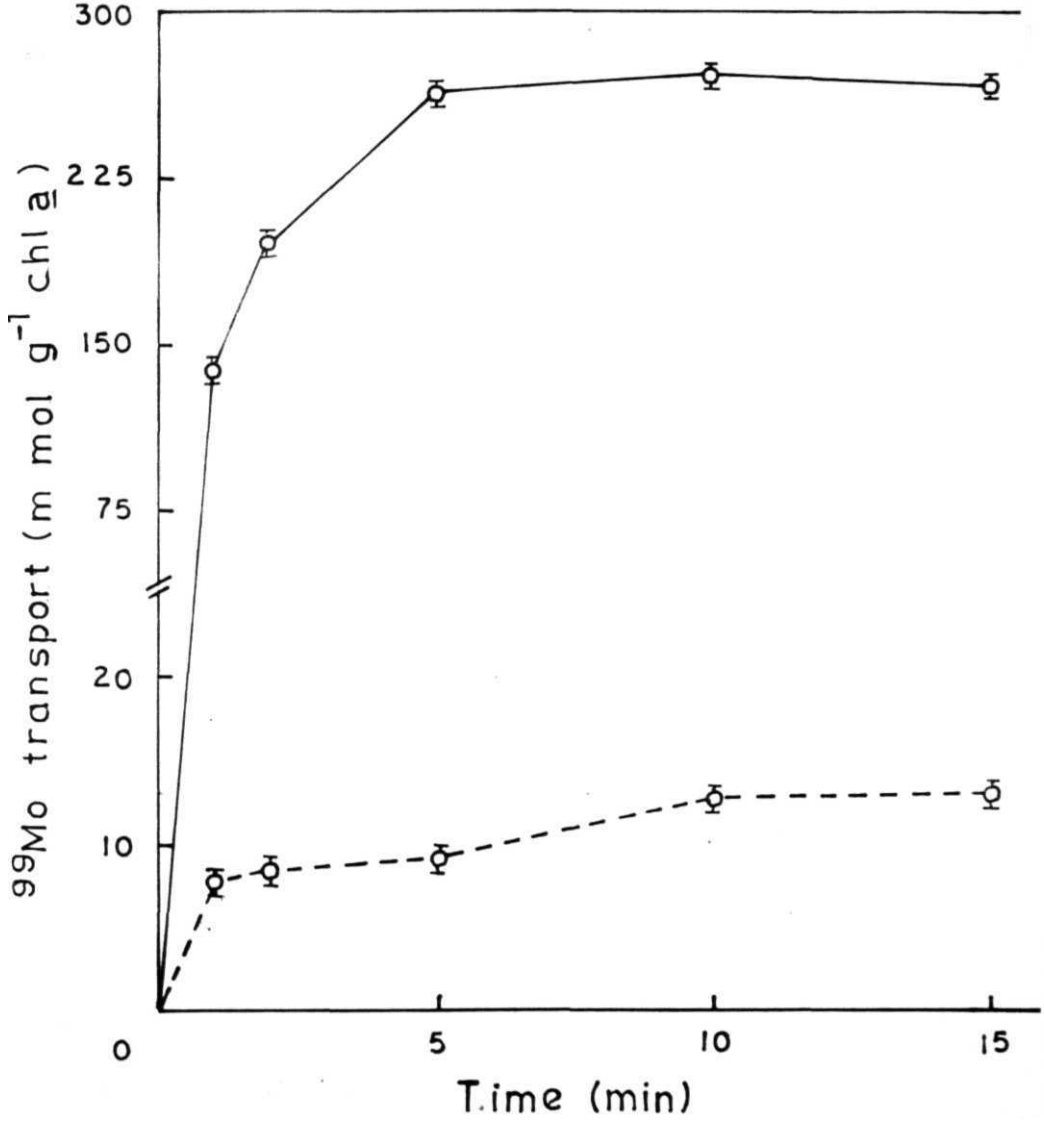
300
225
150
75
20
10
0
5
10
15
T.ime (min)
99Mo transport (m mol g-1 chl a)

Fig.3.1d

As shown in Table 3.2b, growth, heterocyst frequency, nitrogenase activity and nitrate reductase activity of the *W-R-Het Nif Nia* mutant strain in diazotrophic growth medium occured in the presence of V but not in medium with or without Mo and increased with increasing concentration of V. The presence of Mo in the V-medium did not influence the V-dependent growth and activities. In comparison, the parent *Het Nif Nia* strain showed such a nature with an increased dependence for Mo in the growth medium (Table 3.2a). Growth, heterocyst frquency, nitrogenase activity and nitrate reductase of the parent $Het^{+}Nif^{+}Nia^{+}$ or its $W\text{-}R\text{-}Het^{+}Nif^{+}Nia^{+}$ strain responded to increasing concentration of Mo + V combination in a way the parent $Het^{+}Nif^{+}Nia^{+}$ strain did to Mo or the $W\text{-}R\text{-}Het^{+}Nif^{+}Nia^{+}$ strain did to V.

Growth, heterocyst frequency, nitrogenase activity and nitrate reductase activity of the parent *Het Nif Nia* strain and its *W-R* strain in NO_3^--growth medium were also studied (Table 3.2c & d). While the parent strain required Mo for growth on NO_3^- and NO_3^--repression of heterocyst formation and nitrogenase activity, the mutant strain required V. The parent strain produced heterocysts in NO_3^--growth medium lacking Mo, or in presence of V alone, while the mutant strain produced heterocysts in NO_3^--growth medium lacking V or in medium containing Mo alone. Both the strains showed similar heterocyst frequencies in cultures grown with or without Mo or V and showed NH_4^+ repression/derepression of heterocyst formation, nitrogenase activity and nitrate reductase activity. Both the strains, also showed significantly lower level of nitrate reductase activity under N_2-assimilating condition than under NO_3^--assimilating condition. Growth, nitrogenase activity and nitrate reductase activity of the parent $Het^{+}Nif^{+}Nia$ strain was visible in medium containing V and could have resulted from contaminating Mo in trace amounts.

Table 3.2a

Growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells), nitrogenase (N_2ase) activity (m mol C_2H_4 formed g^{-} Chl a h^{-1}) and nitrate reductase (NR) activity (m mol NO_2^- formed g protein min^{-1}) of *Het*$^{+}$*Nif Nia* strain of *N. muscorum* (parent) in N_2-medium containing graded concentrations of sodium molybdate (Mo), vanadyl sulphate (V) or sodium molybdate + vanadyl sulphate (Mo + V).

Concentration ($\times 10^{-3}$ mol m^{-3})	Growth	HF%	N_2ase activity	NR activity
Mo-addition				
0	0.08 ± 0.006	5 - 6	1.3 ± 0.08	0.48 ± 0.03
15	0.34 ± 0.022	5 - 6	3.8 ± 0.21	2.1 ± 0.12
30	0.66 ± 0.04	5 - 6	9.5 ± 0.74	3.5 ± 0.20
60	0.62 ± 0.045	5 - 6	9.3 ± 0.81	3.4 1 0.18
V-addition				
0	0.08 ± 0.004	5 - 6	1.2 ± 0.06	0.52 ± 0.026
15	0.16 ± 0.008	5 - 6	1.4 ± 0.075	0.78 ± 0.04
30	0.21 ± 0.012	5 - 6	1.5 ± 0.08	0.84 ± 0.043
60	0.18 ± 0.009	5 - 6	1.1 ± 0.09	0.81 ± 0.041
Mo + V-addition				
0	0.08 ± 0.004	5 - 6	1.1 ± 0.044	0.52 ± 0.025
15	0.29 ± 0.014	5 - 6	2.8 ± 0.3	2.2 ± 0.16
30	0.58 ± 0.03	5 - 6	8.5 ± 0.88	3.1 ± 0.31
60	0.54 ± 0.045	5 - 6	8.2 i 0.55	2.9 ± 0.22

1 tool m^{-} NH -grown culture of the parent strain sub-cultured for 5-successive generations (devoid of Mo or V) was used as inoculum for the experiments.

Each reading is an average (± SEM) of three independent experimental determinations.

Table 3.2b

Growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells), nitrogenase (N_2ase) activity (m mol C_2H_4 formed g^{-} Chl a h^{-}) and nitrate reductase (NR) activity (m mol NO_2^- formed g^{-1} protein min^{-1}) of the *W-R-Het*$^{+}$ *Nif*$^{+}$ *Nia*$^{+}$ mutant strain of *N. muscorum* in N_2-medium containing graded concentrations of sodium molybdate (Mo), vanadyl sulphate (V) or sodium molybdate + vanadyl sulphate (Mo + V).

Concentration (x 10^{-3} mol m^{-3})	Growth	HF%	N ase activity	NR activity
Mo-addition				
0	0.02 ± 0.002	5 - 6	0.23 ± 0.02	0.12 ± 0.03
15	0.02 ± 0.002	5 - 6	0.28 ± 0.02	**0.16 ± 0.031**
30	0.02 ± 0.002	5 - 6	0.24 ± 0.019	**0.14 ± 0.015**
60	0.02 ± 0.002	5 - 6	0.29 ± 0.022	0.18 ± 0.022
V-addition				
0	0.02 ± 0.001	5 - 6	0.25 ± 0.024	0.16 ± **0.04**
15	0.32 ± 0.019	5 - 6	3.2 ± 0.16	1.96 ± **0.06**
30	0.58 ± 0.037	5 - 6	7.6 ± 0.31	3.2 ± **0.14**
60	0.54 ± 0.033	5 - 6	7.8 ± 0.55	3.1 ± **0.03**
Mo + V-addition				
0	0.02 ± 0.002	5 - 6	0.26 ± 0.025	0.18 ± 0.012
15	0.29 ± 0.015	5 - 6	2.9 ± 0.26	1.8 ± 0.012
30	0.51 ± 0.031	5 - 6	7.6 ± 0.45	2.9 ± **0.27**
60	0.50 ± 0.033	5 - 6	8.2 ± 0.75	3.2 ± **0.32**

1 mol m^{-} NH_4^+-grown culture of the *W-R* mutant strain sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

Growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells), nitrogenase (N_2ase) activity (m mol C_2H_4 formed g^{-1} Chl a h^{-1}) and nitrate reductase (NR) activity (m mol NO_2^- formed g^{-1} protein min^{-1}) of the *Het*$^+$*Nif*$^+$*Nia*$^+$ strain of *N. muscorum* (parent) in growth medium containing 5 mol m^{-3} KNO_3 and graded concentrations of sodium molybdate (Mo), vanadyl sulphate (V) or sodium molybdate + vanadyl sulphate (Mo + V).

Concentration (x 10 mol m)	Growth	HF%	N ase activity	NR activity
Mo-addition				
0	0.09 ± 0.005	5 - 6	1.2 ± 0.06	0.6 ± 0.03
15	0.44 ± 0.022	0.0	0.0	3.1 ± 0.17
30	0.85 ± 0.043	0.0	0.0	5.9 ± 0.41
60	0.82 ± 0.041	0.0	0.0	5.2 ± 0.12
V-addition				
0	0.09 ± 0.004	5 - 6	1.3 ± 0.06	0.6 ± 0.03
15	0.18 ± 0.008	4 - 5	1.4 ± 0.07	0.6 ± 0.08
30	0.22 t 0.011	4 - 5	1.8 ± 0.09	0.7 ± 0.03
60	0.16 ± 0.01	4 - 5	2.1 ± 0.01	0.6 ± 0.09
Mo + v-addition				
0	0.08 ± 0.004	5 - 6	1.3 ± 0.06	0.7 ± 0.039
15	0.39 ± 0.017	0.0	0.0	2.9 ± 0.1
30	0.79 ± 0.045	0.0	0.0	5.6 ± 0.56
60	0.79 ± 0.05	0.0	0.0	5.4 ± 0.05

l mol m NH -grown culture of the parent strain sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

Growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells), nitrogenase (N_2ase) activity (m mol C_2H_4 formed g^{-1} Chl a h^{-1}) and nitrate reductase (NR) activity (m mol NO_2^- formed g protein min^{-1}) of the *W-R-Het*$^+$ *Nif*$^+$ *Nia*$^+$ mutant strain of *N. muscorum* in growth medium containing 5 mol m^{-3} KNO_3 and graded concentrations of sodium molybdate (Mo), vanadyl sulphate (V) or sodium molybdate + vanadyl sulphate (Mo + V).

Concentration (x 10^{-3} mol m^{-3})	Growth	HF%	N ase activity	NR activity
Mo-addition				
0	0.02 ± 0.001	5 - 6	0.24 ± 0.02	0.5 ± 0.015
15	0.02 ± 0.002	5 - 6	0.18 ± 0.012	0.6 ± 0.021
30	0.02 ± 0.002	5 - 6	0.21 ± 0.02	0.6 ± 0.027
60	0.02 ± 0.001	5 - 6	0.21 ± 0.021	0.5 ± 0.023
V-addition				
0	0.02 ± 0.009	5 - 6	0.21 ± 0.016	0.5 ± 0.04
15	0.38 ± 0.034	0.0	0.0	2.6 ± 0.12
30	0.77 ± 0.037	0.0	0.0	5.1 ± 0.39
60	0.79 ± 0.07	0.0	0.0	4.9 ± 0.25
Mo + v-addition				
0	0.02 ± 0.002	5 - 6	0.23 ± 0.01	0.5 ± 0.043
15	0.34 ± 0.034	0.0	0.0	2.9 ± 0.21
30	0.71 1 0.033	0.0	0.0	4.1 * 0.3
60	0.68 ± 0.034	0.0	0.0	4.5 i 0.22

1 mol m^{-3} NH_4^+-grown culture of the *W-R* mutant strain sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

It has been shown here that V can effectively substitute for Mo in nitrogenase activity and in nitrate reductase activity of the *W-R-Het Nif Nia*$^{+}$ strain. *N. muscorum* thus shows both Mo-dependent nitrogenase activity and nitrate reductase activity as well as V-dependent nitrogenase activity and nitrate reductase activity. Can *N. muscorum* show a mutually exclusive V-dependant nitrogenase activity or V-dependant nitrate reductase activity? This problem was approached by isolating *W-R* mutants from *Het Nif Nia* strain, *Het Nif Nia* strain and *Het Mif Nia* strains of *N. muscorum.* Growth and nitrate reductase activity of the parent *Het Nif~Nia* strain was found to be Mo-concentration dependent (Table 3.3a) while its *W-R* mutant V-concentration dependent (Table 3.3b). Similarly, N -dependent growth and nitrogenase activity of the *Het Nif Nia* strain was found to be Mo-concentration dependent (Table 3.4a) and of its *W-R* mutant V-concentration dependent (Table 3.4b). Parent *Het Mif Nia* also showed such Mo-concentration dependent nature while its *W-R* mutant strain showed a V-concentration dependence (Table 3.5).

The V-dependent and Mo-dependent diazotrophy in the cyanobacterium *N. muscorum* might not exhibit a similar response to environmental stresses like salinity and osmotic stresses and since further experiments in the present study involved understanding the role of alkali metals as an agent of cyanobacterial nutrients/stresses, it was thought proper to examine, on a preliminary basis, the response of the diazotrophic cyanobacterial strains to NaCl stress. As shown in Fig. 3.2, both the parent *Het Nif Nia* strain and its *W-R* mutant showed progressive decrease in percent survival with increase in salinity stress and a concentration of 100 mol m NaCl was found almost completely lethal. It was therefore concluded that the two cyanobacterial strains while differing in respect of the nature of metal requirement for their diazotrophy, exhibited no significant difference in their response to salinity stress.

Table 3.3a

Influence of graded concentrations of sodium molybdate (Mo) and vanadyl sulphate (V) on growth (optical density at 663 nm), nitrate reductase (NR) activity (m mol NO_2^- formed g protein min) and NO_3-repression of heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) of the *Het*$^+$ *Nif*$^-$ *Nia*$^+$ strain of *N. muscorum* in growth medium containing 5 mol m KNO_3.

Concentration (× 10 mol m~)	Growth	HF	NR activity
Mo-addition			
0	0.08 *t* 0.004	5 - 6	0.6 ± 0.033
15	0.48 ± 0.017	0.5 - 1	3.4 *t* 0.25
30	0.86 ± 0.04	0.0	6.1 ± 0.57
60	0.88 *t* 0.04	0.0	5.8 ± 0.4
V-addition			
0	0.07 1 0.074	5 - 6	0.6 i 0.033
15	0.12 *t* 0.012	5 - 6	0.8 ± 0.04
30	0. 14 4 0.014	5 - 6	0.8 ± 0.04
60	0.18 i 0.009	5 - 6	0.8 ± 0.039

l mol m NH -grown culture of the *Het Nif Nia* strain sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

; 2.3b

Influence of graded concentrations of sodium molybdate (Mo) and vanadyl sulphate (V) on growth (optical density at 663 run), nitrate reductase (NR) activity (m mol NO_2^- formed g~ protein min^{-1}) and NO_3^--repression of heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) of the *W-R-Het*$^+$*Nif*$^-$*Nia*$^+$ mutant strain of *N. muscorum* in growth medium containing 5 mol m^{-3} KNO_3.

Concentration (× 10 mol m~)	Growth	HF%	NR activity
Mo-addition			
0	0.02 + 0.002	5 - 6	0.5 ± 0.013
15	0.02 i 0.002	5 - 6	0.5 *t* 0.025
30	0.02 ± 0.002	5 - 6	0.6 ± 0.05
eo	0.02 ± 0.001	5 - 6	0.6 ± 0.053
V-addition			
0	0.02 ± 0.002	5 - 6	0.6 ± 0.06
15	0.42 ± 0.034	0.0	3.1 ± 0.06
30	0.78 i 0.03	0.0	5.8 *t* 0.56
60	0.84 *t* 0.043	0.0	5.2 ± 0.51

1 mol m NH_4^+-grown culture of the *W-R-Het Nif~Nia* strain sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 4a

Influence of graded concentrations of sodium molybdate (Mo) and vanadyl sulphate (V) on growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) and nitrogenase activity (m mol C_2H_4 formed g Chl *a* h^{-1}) of the *Het*$^+$*Nif*$^+$*Nia*$^-$ strain of *N. muscorum* in diazotrophic growth medium.

Concentration. (x 10" mol m^{-})	Growth	HF%	N_2ase activity
Mo-addition			
0	0.06 + 0.006	5 - 6	1.1 ± 0.03
15	0.28 ± 0.002	5 - 6	4.1 ± 0.21
30	0.56 *i* 0.044	5 - 6	9.6 ± 0.97
60	0.54 ± 0.031	5 - 6	8.5 ± 0.35
V-addition			
0	0.06 *t* 0.004	5 - 6	1.1 *t* 0.036
15	0.11 *t* 0.009	5 - 6	1. 5 ± 0.05
30	0. 14 *t* 0.01	5 - 6	1.3 ± 0.05
60	0.12 *t* 0.023	5 - 6	1.5 ± 0.09

1 mol m^{-} NH -grown culture of the *Het Nif Nia* strain sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 3.4b

Influence of graded concentrations of sodium molybdate (Mo) and vanadyl sulphate (V) on growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) and nitrogenase activity (m mol C_2H_4 formed g Chl a h~) of the *W-R-Het*$^{+}$*Nif*$^{+}$*Nia*$^{-}$ mutant strain of *N. muscorum* in diazotrophic growth medium.

Concentration (x 10 mol m)	Growth	HF%	N ase activity
Mo-addition			
0	0.02 ± 0.002	5 - 6	0.2 ± 0.01
15	0.02 ± 0.002	5 - 6	0.2 *t* 0.01
30	0.02 ± 0.001	5 - 6	0.2 ± 0.006
60	0.02 • 0.001	5 - 6	0.2 ± 0.006
V-addition			
0	0.02 * 0.002	5 - 6	0.2 4 0.01
15	0.24 *t* 0.004	5 - 6	3.9 ± 0.04
30	0.55 i 0.055	5 - 6	7.8 4 0.45
60	0.57 4 0.027	5 - 6	7.9 4 0.42

1 mol m NH -grown culture of the *W-R-Het*$^{+}$*Nif Nia*$^{+}$ strain sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 5

Influence of graded concentrations of sodium molybdate (Mo) and vanadyl sulphate (V) on growth (optical density at 663 nm) and nitrate reductase (NR) activity (m mol NO_2^- formed g protein min^{-1}) of the *Het⁻Nif⁻Nia⁺* and *W-R-Het⁻Nif⁻Nia⁺* strains of *N. muscorum* in growth medium containing 5 mol m KNO_3.

Concentration (x 10" mol m~)	Strains			
	Het⁻Nif⁻Nia⁺		*W-R-Het⁻Nif⁻Nia⁺*	
	Growth	NR activity	Growth	NR activity
Mo-addition				
0	0.083 (0.003)	0.54 (0.021)	0.023 (0.004)	0.35 (0.03)
15	0.45 (0.012)	3.1 (0.25)	0.023 (0.001)	**0.53 (0.03)**
30	0.72 (0.05)	5.6 **(0.28)**	0.026 (0.001)	**0.67 (0.055)**
60	0.77 (0.06)	5.6 (0.32)	0.022 (0.001)	**0.69 (0.02)**
V-addition				
0	0.07 **(0.006)**	0.53 **(0.021)**	0.022 (0.003)	**0.55 (0.011)**
15	**0.11** (0.05)	0.61 (0.027)	0.44 (0.013)	**2.7 (0.26)**
30	0.11 (0.06)	0.65 **(0.024)**	0.63 (0.06)	**4.7 (0.43)**
60	**0.14 (0.01)**	0.63 **(0.024)**	0.67 (0.063)	**5.1 (0.45)**

1 mol m^{-3} NH_4^+-grown cultures of the *Het Nif⁻Nia* and *W-R-Het Nif⁻Nia* strains of *N. muscorum* sub-cultured for 6-successive generations (devoid of Mo or V) was used as inoculum for the experiments.
values in parentheses are ± SEM values of three independent experimental determinations.

Fig. 3.2
Effect of increasing concentrations of NaCl on **the** per cent survival of **the** *Het*$^{+}$*Nif*$^{-}$*Nia*$^{+}$ strain of *N. muscorum* (parent) (0) and in **its** *W-R* mutant (•).
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

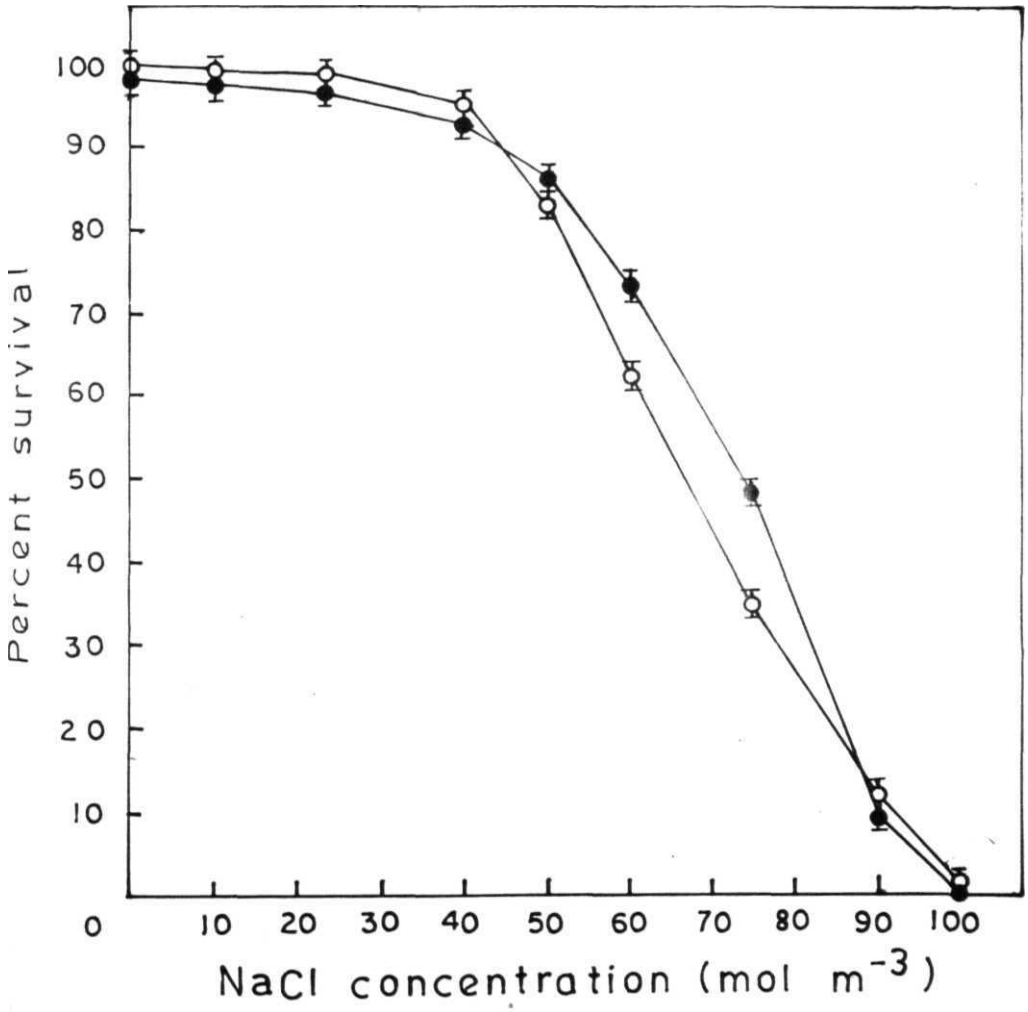
Percent survival
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
NaCl concentration (mol m^{-3})

Fig. 3.2

Also, both the parent and the *W-R* mutant strain did not make heterocysts during growth with 1 mol m^{-} NH Cl. Non-heterocystous NH -grown cultures on transfer to fresh N -medium required 24 h for producing N -fixing heterocysts. When such cultures were treated for 8 h with different concentrations of NaCl (salinity stress) and then transfered to normal growth medium, both the strains developed heterocysts after a progressive lag and showed a progressive decrease in the number of heterocysts and nitrogenase activity with increase in salinity stress. A salinity stress of 90 mol m NaCl for 8 h resulted in complete inhibition of the formation of heterocysts as well as inhibition of nitrogenase activity (Tables 3.6a & b) . Evidently, the N -fixing appartus in the two strains of the cyanobacterium is salinity sensitive.

3.4 DISCUSSION

The role of Mo and V in regulation of diazotrophic mode of nutrition or in NO -nutrition in the various strains of *N. muscorum* described here were investigated by examining the ability of their respective *W-R* derivatives for growth under diazotrophic or NO -assimilating conditions. Since, all the *W-R* mutants failed to exhibit significant growth in N -medium it was concluded that *W-R* mutants have resulted due to impairment of Mo-metabolism, causing defect in the activity of Mo-dependant nitrogenase or nitrate reductase activity. Since, all such *W-R* mutants resumed growth when they were provided with exogenous V in their respective growth media, it was evident that they were V-dependant for their growth. Since, the parents showed considerably more rise in N or NO_3, and in nitrogenase or nitrate reductase activities with rise in external concentration of Mo than with rise in external concentration of V, it is concluded that the parents predominantly produced Mo-dependant nitrogenase activity and/or nitrate reductase activity in

Table 1 (cont.)

Effect of increasing concentrations of NaCl on time for heterocyst differentiation (t_{Het}; in hours), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) and nitrogenase (N_2ase) activity (m mol C_2H_4 g^{-1} Chl a h^{-1}) of *Het Nif Nia* strain of *N. muscorum* (parent).

Treatment	t_{Het}	HF%	N_2ase activity
Control	24-30	5-6	9.8 ± 0.45
+ 30 mol m^{-3} NaCl	48	5-6	7.2 ± 0.66
+ 60 mol m^{-3} NaCl	70	3-4	3.8 ± 0.36
+ 90 mol m^{-3} NaCl	a	0.0	0.9 ± 0.06

Non-heterocystous NH -grown cultures were stressed with NaCl at different concentrations for 8 h, later washed and used as inoculum for incubation on diazotrophic growth medium containing Mb They were periodically examined for their respective characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 3.6b

Effect of increasing concentrations of NaCl on time for heterocyst differentiation (t_{Het}; in hours), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) and nitrogenase (N_2ase) activity (m mol C_2H_4. g^{-1}Chl a h^{-1}) of *W-R-Het*$^{+}$ *Nif*$^{+}$ *Nia*$^{+}$ mutant strain of *N. muscorum*.

Treatment	t_{Het}	HF%	N_2ase activity
ontrol	24	5 - 6	8.1+0.11
+ 30 mol m^{-3} NaCl	48	5 - 6	5.3 ± 0.43
+ 60 mol m^{-3} NaCl	72	3 - 4	3.4 ± 0.2
+ 90 mol m^{-3} NaCl	a	0.0	0.4 ± 0.016

Non-heterocystous NH_4-grown cultures were stressed with NaCl at different concentrations for 8 h, later washed and used as inoculum for incubation on diazotrophic growth medium containing V. They were periodically examined for their respective characteristics.

Each reading is an average (± SEM) of three independent experimental determinations.

Mo-containing growth medium or in growth medium containing both Mo and V. The observed lack of significant increase in growth of the parent strains in Mo-free V-medium might be the result of the presence of Mo as a contaminant under such condition which would then cause inhibition of the activity of V-dependant nitrogenase or nitrate reductase system. Such Mo induced complications in analysis of V nutrition could be avoided if the cyanobacterial strains are defective in Mo-transport. Since all the cyanobacterial *W-R* mutants showing dependence of growth on V with N_2 or NO_3^- as nitrogen source lacked Mo-transport activity and since they all exhibited rise in growth with rise in external V concentration, it is obvious that presence of Mo interferes with V-dependant process of N -fixation and NO -assimilation in the cyanobacterium. Lack of Mo in cyanobacterial cells is a pre-requisite for them to express their V-dependant nitrogenase or V-dependant nitrate reductase systems. This also explains why V fails to influence N_2-fixation or NO -assimilation in the parent strains which have a normal Mo-transport system.

The observed significantly lower level of Mo-dependant nitrate reductase activity under diazotrophic growth condition than under NO -assimilating condition is because of the limited availability of Mo-co-factor for nitrate reductase activity under the former condition as shown earlier (Bagchi et *al.* 1985). Presumably, the same explanation applies to why V-dependant nitrate reductase activity under diazotrophic growth condition is less than under NO -assimilating condition. In other words, V-co-factor in place of Mo-co-factor level is diazotrophically regulated in the cyanobacterium. These results simply suggest that Mo-transport defective *W-R-Het Nif Nia* strain apparently active in V-transport is the producer of V-dependent nitrogenase or V-dependent nitrate reductase leading to V-dependent growth in N_2 or in NO_3^--medium. Accordingly, the parent strain with normal Mo-transport activity is predominantly a producer of Mo-nitrogenase or Mo-nitrate reductase under the given growth conditions. The ability of the parent strain not to respond as

significantly to exogenous V as to exogenous Mo, suggests either it is not as efficient as its *W-R* mutant in V-transport or that it is equally efficient in transport of Mo and V, but presence of Mo in it inhibits its V-dependent functions as has been reported for *Azotobacter spp.* (Robson et *al.* 1986; Hales et *al.* 1987). However, information about the status of V-transport system in the parent strain is essential, in order to analyze the precise role of Mo-transport activity in regulation of V-nutrition and metabolism in the cyanobacterium. There is no apparent role of Mo/V in heterocyst differentiation and frequency as both the parent and the mutant had similar heterocyst frequency in cultures growing with or without Mo/V. Both the parent and the mutant strain showed NH_4^+-repression of heterocyst formation, nitrogenase activity and nitrate reductase activity and was neither Mo-dependant nor V-dependant. In comparison NO_3^--repression of heterocyst formation and nitrogenase activity was found Mo-dependant in the parent strains and V-dependant in the mutant strains. These results further emphasize a role of V in NO_3^--repression control of heterocyst formation and nitrogenase activity in the mutant strain. The nutritional and regulatory role of NO_3^--nitrogen is a functiona of Mo-dependant nitrate reductase in the parent and V-dependant nitrate reductase in the mutant strain.

W-R phenotype is a result of mutational inactivation of Mo-transport process which blocks Mo entry in the cyanobacterial cells and thereby blocking synthesis of Mo-nitrogenase and Mo-nitrate reductase. In a recent study, two strains of *A. vinelandii* (FL2 & FL4) with *Tn5* insertion mutations showed alternative nitrogenase dependent diazotrophic growth in the presence of Mo. The mutations were in a region which contained 4 open reading frames (ORFs 1-4) whose products showed strong homology to bacterial periplasmic transport proteins. The products of ORF4 and ORF3 were implied in Mo-transport and share

homology with £. *coli* *chlD* and *chlJ* genes respectively (Luque et *al*. 1993). Evidently, the genetic determinant controlling Mo-transport activity is one of the common genetic determinants involved in the processing of Mo for active Mo-nitrogenase or Mo-nitrate reductase, a finding confirming earlier reports of such common genetic determinant in *N. muscorum* (Bagchi et *al*. 1985; Bagchi & Singh 1984). Since V-dependant nitrogenase activity and V-dependent nitrate reductase activity operate simultaneously only under conditions of Mo-deficiency, it is suggested that like the cyanobacterial Mo-nitrogenase and Mo-nitrate reductase its V-dependent nitrogenase and V-dependent nitrate reductase also share some initial steps of V-metabolism.

While the parent *N. muscorum* produces both Mo-dependent nitrogenase and Mo-dependent nitrate reductase simultaneously, its *W-R* mutant produces V-dependent nitrogenase and V-dependent nitrate reductase simultaneously. Can *N. muscorum* exclusively produce either V-nitrogenase or V-nitrate reductase independent of each other? This aspect was examined in the *W-R* mutant clones isolated from the heterocystous, non-N -fixing, nitrate assimilating strain (Het *Nif Nia*), from heterocystous, N -fixing, non-nitrate assimilating strain (Het *Nif Nia)* and from the non-heterocystous, non-N -fixing, nitrate assimilating strain (Het *Nif~Nia*) of *N. muscorum.* *W-R* derivatives of N -fixing nitrate reductase deficient mutant showed absolute dependence on V for diazotrophic growth. Clearly, V-dependent nitrogenase production is possible in the cyanobacterium in the absence of its nitrate reductase activity. Similarly, since the *V-R* derivatives of the $Het^{+}Nif^{-}Nia^{+}$ strain produce V-dependent nitrate reductase activity and showed V-dependent NO -repression heterocyst formation, it is again concluded that the cyanobacterium *N. muscorum* can produce V-nitrate reductase in the absence of its V-nitrogenase. The ability of the *W-R* derivatives of the $Het^{-}Nif^{-}Nia^{+}$ strain to grow with NO_3^- and to produce active nitrate reductase activity only in the presence of V, indicate that V-nitrate reductase can occur in the cyanobacterium even

without the presence of heterocyst and V-nitrogenase activity. Evidently, *N. muscorum* can make or regulate V-dependent nitrogenase in the absence of active V-dependent nitrate reductase and vice versa, as it makes or regulates its Mo-nitrogenase and Mo-nitrate reductase independently. An obvious implication of this finding, would be the occurrence of V-nitrogenase and Mo-nitrate reductase in some cyanobacteria as is implied for *Anabaena variabilis* (Kentemich et *al.* 1988). Similarly, one can expect cyanobacterial strains producing Mo-nitrogenase and V-nitrate reductase or simply V-nitrate reductase alsone as is evidenced in the *W-R-Het Nif~Nia* and *W-R-Het^{-} Nif^{-} Nia^{+}* strains.

Mo is known to cause repression of V-nitrogenase in *A. vinelandii* (Jacobitz & Bishop 1992). Since W is an inhibitor of Mo-requiring enzymatic processes, it is also expected to inhibit Mo-dependent repressor signal not only for V-nitrogenase, but also for V-nitrate reductase. One possible explanation for the observed requirement of W for cyanobacterial growth on N - or NO -nitrogen as reported earlier (Singh et *al.* 1978), is that W is required for the inactivation of Mo-generated inhibitor signal for cyanobacterial V-dependent nitrogenase and V-dependent nitrate reductase in the mutant strain, and that presence of contaminating V in the tungsten salt enables such mutant strains to grow well with either nitrogen source. This explanation however, does not exclude the possibility of an alternative mechanism of still unknown nature underlying such W-requirement for cyanobacterial growth on N - or NO -nitrogen.

The known mechanisms of salinity tolerance in microbes include the accumulation of compatible organic osmolytes sucrose, glucosyl-glycerol, trehalose, glycine betaine or proline (Csonka 1989) and the activity of Na^{+}/H^{+} antiporter (Padan & Schuldiner 1994). Since response of V-dependant diazotrophy in

the ***W-R*** strain to salinity induced stress remained similar to the Mo-dependent diazotrophy in the parent strain further detailed studies on salinity and osmotic stress tolerance have been confined to the parent strain alone in the subsequent chapters of the thesis.

Chapter-4

DIAZOTROPHIC RESPONSE OF THE CYANOBACTERIUM *NOSTOC MUSCORUM* TO SALINITY (NaCl) STRESS AND OSMOTIC (SUCROSE) STRESS

4.1 INTRODUCTION

Microbes grow in a variety of habitats of differing water potentials. Such variations in water potentials of the habitats could arise from the presence of varying levels of inorganic/organic osmotica. Naturally, the success of microorganisms for growth and survival in such habitats, would greatly depend on their ability to adjust their osmotic potential according to the nature of the habitats. When a microbial cell increases in volume, part of the increase is brought about by increasing volume of water within the outer membrane. The rate of flow of water into the cell must be such that the pressure generated does not cause the membrane to burst. When the cell has a wall, the pressure with which the outer covering can withstand is much greater. The driving force for the movement of water into a cell is the difference in its chemical potential across the outer membrane. At one extreme are the microorganisms capable of growth in a habitat of very low water potential and at another extreme are the organisms which grow in habitats of high water potential and the mechanisms governing their adaptation to such osmotically differing habitats is being studied in detail for practical purposes.

Osmotic studies are apparently of great economic importance in food industry, fermentation industry, medical microbiology and in agriculture. Agriculturally useful microbes can be genetically engineered for tolerance to salinity or osmotic stress and then used in saline agriculture for augmenting the productivity of crops on an industrial scale. In recent years, there has been a tremendous increase in studies on molecular biology of salt/osmotolerance in microbes and plants (Csonka & Hanson 1991; Rhodes & Hanson 1993; Bartels & Nelson

1994). Strains of *Escherichia coli* or *Salmonella typhimurium* when subjected to salinity or osmotic stresses are known to undergo loss of turgidity, associated with an active accumulation of K leading to prevention of further water loss and restoration of cell turgor. The cytoplasmically associated K ions in turn are subsequently replaced by accumulation of compatible organic solutes such as trehalose, glucosyl-glycerol, glycinebetaine, proline etc. These compatible osmolytes function as salinity/osmotic protectants in bacteria as well as in plants growing under salinity/osmotically stressed habitats (Higgins et *al*. 1987; Warr et *al*. 1988; Csonka 1989). As a rule most bacteria prefer the accumulation of exogenous osmoprotectants to endogenous biosynthesis. Much of the genetic information on osmoregulation is available mainly from studies on members of enterobacteriaceae where the products of *kdp***A-E** genetic system have been shown to function in the transduction of osmotic signal leading to the activation of proP or proU genes that transport and accumulate proline/glycinebetaine from the medium or tre and *bet* genes that are necessary for synthesis of high cellular levels of trehalose and glycinebetaine (betaine) respectively as alternative forms osmotic adaptation in them (Csonka 1989).

Cyanobacteria are prokaryotic photoautotrophs distributed in a wide variety of habitats (Chapter 1). Free living forms are often abundant in habitats where there is substantial fluctuation in a range of environmental conditions of salinity/water status. Studies on adaptation of cyanobacteria to osmotic stress and salinity stress was started by Borowitzka (1980). Subsequently, a series of further studies were conducted to find out the physiological mechanism of osmotic and salinity stress resistance in various cyanobacteria characteristic of fresh water, marine and brackish water habitats (Mackay et *al*. 1984; Blumwald & Tel-or 1982; Reed et *al*. 1986a; Warr et *al*. 1988). Some correlation has been found between the nature of habitat and the osmoregulatory solute present in

cyanobacteria adapted to growth under such habitats. The fresh-water strains (least osmotolerant) have been shown to accumulate predominantly disaccharides such as sucrose and trehalose, the marine isolates (moderately osmotolerant) a heteroside like glucosyl-glycerol and the hypersaline forms (extremely osmotolerant) quaternary ammonium compounds like glycine betaine (Warr et *al.* 1988) when challenged with salinity/osmotic stress. The involvement of single metabolites in controlling resistance to osmotic stress in cyanbacteria is a very welcome information as it raises the possibility of easy isolation cyanobacterial mutants showing upper limit of osmotic tolerance. Most of these studies concentrated only on growth and viability of cyanobacteria under osmotically stress conditions. Very few studies have touched the process of N -fixation and GS activity in relation to osmotic stress. Warr et *al.* (1988) found activity of GS to be sensitive to osmotic stress under *in vitro* conditions. Reed et *al.* (1986a) have shown compatiblity of organic osmolytes like glycinebetaine, glucosyl-glycerol and sucrose with *in vitro* GS activity at their physiological concentration. While hyperosmotic stress has been found to generate various types of organic osmolytes within the cyanobacterial cell, there has been little study with regards to hypoosmotic shock in such cyanobacteria. Few available studies show extracellular liberation of various organic carbon and fixed nitrogen compounds following hypoosmotic shock (Reed & Stewart 1988; Fulda et *al.* 1990). In none of these studies, experimental attempts have been made to generate genetically stable salinity resistant or osmotic resistant strains of fresh-water cyanobacteria and to examine the physiological basis of such salinity-/osmo-resistance in them.

Diazotrophic cyanobacteria are natural components of water-logged rice ecosystems where they grow under fluctuating constrains of environmental osmolarity and constitute biologically the most stable source of fixed nitrogen year after yaer (Singh 1961). They are being studied for developing a viable biofertilizer technology that can generate appropriate biofertilizer strains for use as supplement along with chemical nitrogen fertilizers. In this respect one of the required attributes include genetic acquisition of salinity and osmotic tolerance (Venkatraman 1980; Stewart et *al.* 1987). Few physiological studies have shown cyanobcterial N -fixation process to be extremely sensitive to salinity and osmotic streses (Tel-Or 1980; Blumwald & Tel-Or 1982; Fernandes et *al.* 1993). In general, salinity and osmotic stresses have been considered to be similar in bacteria (Csonka 1989; Weretilnyk & Hanson 1990) and dissimilar in cyanobacteria (Blumwald & Tel-Or 1982; Fernandes et *al* . 1993). Similarity or dissimilarity of biological response of cyanobacteria to the two stresses could be analysed unambiguously by isolating cyanobacterial mutant strains resistant to salinity and osmotic stresses and then examining for their cross-resistant relationship. The present study is an attempt towards examining the possibility of generating salinity resistant and osmotic resistant mutant strains of **the** cyanobacterium *Nostoc muscorum* and then examining them physiologically for their ability to tolerate such salinity or osmotic stresses. The results presented here significantly show that over-accumulation of proline is the cause for increased tolerance of **the** cyanobacterium to salinity and osmotic stress.

4.2 MATERIALS AND METHODS

Axenic clonal cultures of parent *N. muscorum* was grown and maintained photoautotrophically as described in Chapter-2.

4.2.1 Isolation of salinity (NaCl) stress and osmotic (sucrose) stress tolerant strains

NaCl at a concentration of 100 mol m and sucrose at a concentration of 250 mol m were found completely lethal to the cyanobacterial growth. NaCl resistant (*NaCl-R*) and sucrose resistant (*Sucrose-R*) mutants of the cyanobacterium were isolated by plating 3.0 - 3.5 > 10 CFUs on diazotrophic growth medium containing 100 mol m NaCl or 300 mol m sucrose.

Further, the upper NaCl tolerance limit of such mutants was also examined. The mutant strain resistant to lethal action of 100 mol m NaCl grew with 100% survival at 100 or 150 mol m^{-3} NaCl but its survival dropped to zero percent at 200 mol m^{-3} NaCl. The 100 mol m NaCl resistant clone was designated as *NaCl-R* and the second clone of mutants derived from the *NaCl-R* strain and capable of survival at 200 mol m NaCl were designated as *NaCl-R* . The *NaCl-R* mutant clones could survive and grow normally upto 230 mol m NaCl. The third step mutants capable of normal growth and survival in 250 mol m NaCl were selected from the second step $NaCl\text{-}R_{200}$ mutant clones by plating them in bulk on diazotrophic medium containing 250 mol m^{-3} NaCl. The third step mutants were designated as

The *Sucrose-R* strain did not survive a concentration beyond 300 mol m^{-3} sucrose. The various spontaneously occuring

mutant clones of the cyanobacterium resistant to NaCl lethality or sucrose lethality arose with a frequency between 0.8 - **1.5** × 10^{-7}.

4.2.2 Estimation of photosynthetic 0 -evolution

Photosynthetic 0 -evolution was measured with a Clark-type O_2 electrode. Exponentially growing cyanobacterial cells were deposited on a flat platinum cathode that was polarized at 0.6V with reference to a large Ag/AgCl electrode. **The** electrodes were immersed in an electrolyte (consisting of 0.05 **M** phosphate buffer, pH 7.8; 0.1 M KC1). The electrode was separated from the magnetically stirred assay medium by a teflon membrane. The difference between the output of the electrode in water in equilibrium with air and water in equilibrium with pure N was considered to represent 0.235 mmol m in the assay medium. After injection of the same into the assay medium, the medium was illuminated from opposite side with projector lamps. The rate of 0 -evolution was determined from the initial slope of electrode output as a function of time.

4.2.3 Estimation of intracellular proline

The intracellular levels of proline was measured by the method of Bates et *al*. (1973) with slight modification. Growing cultures of cyanobacteria at a strength of 1.5 > 10 CFUs were harvested by centrifugation (4000 × g), washed twice with HEPES-NaOH buffer (pH 7.5), pelleted and broken in liquid N_2, and then again centrifuged. To 2 cm^3 of of the resulting supernatant, 2 cm^3 of acid ninhydrin reagent (1.25 g ninhydrin, 30 cm^3 glacial acetic acid, 8 cm^3 orthophosphoric acid 12 cm^3 water and stored in an amber coloured bottle) and 2 cm^3 of glacial acetic acid were added and the mixture was boiled for 1 h in a water bath. The reaction was terminated by dipping the test tubes in an ice bath. 4 cm^3 of toluene is then added and the reaction mixture vigourously mixed on a cyclomixer for 30 seconds. The reactant chromophore extracted into the toluene

phase is then aspirated from the aqueous phase and its absorbance measured at 520 nm. A standard plot is also simultaneously calibrated using L-proline.

4.2.4 Estimation of ^{14}C-proline transport activity

The method of Rai et *al*. (1984) was used for measurement of ^{14}C-proline uptake. The cyanobacterial cells were centrifuged, washed and resuspended in 10 mol m HEPES-NaOH buffer (pH 7.5) and equilibrated for 30 min at 25 °C at a photon fluence rate of 50 μmol m S . C-proline was then added to the incubation mixture to a final concentration of 0.05 mol m (specific activity 7.5 KBq mol). Aliquots of 400 μl were withdrawn at regular time intervals and quickly separated from the bathing medium by microcentrifugation through silicone oil/dinonyl phthalate (40:60, v/v) into perchloric acid/water (15:85, v/v) as described by Scott & Nicholls (1980), and then counted in a LS 1800 liquid scintillation counter. Non-specific binding of the radiolabel was determined by measuring its incorporation in toluene-treated cells (Rai et *al*. 1984). This value was always subtracted from the value obtained from the untreated samples.

4.2.5 Estimation of ^{22}Na^{+} transport activity

The protocol for determination of ^{22}Na^{+} transport activity was essentially the same as described for estimation of ^{4}C-proline transport activity (Section 4.2.4) except that Na (as NaCl, carrier free, specific activity 7.4 MBq mol) at a concentration of 0.5 mol m was added to the incubation medium.

4.2.6 Estimation of ^{14}C-sucrose transport activity

The protocol for determination of C-sucrose transport

activity was essentially the same as described for estimation of ^{14}C-proline transport activity (Section 4.2.4) except that ^{14}C-sucrose at a final concentration of 0.3 mol m^{-3} (specific **activity** 9.4 KBq mol) was added to the incubation medium.

Estimation of growth, chlorophyll a, protein, heterocyst frequency, percent survival and nitrogenase activity were done as described in Chapter-2 (Sections 2.4, 2.5, 2.6, 2.7, 2.8 & 2.9 respectively).

4.2.7 Chemicals used

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C-proline was obtained from Board of Radiation and Isotope Technology (BRIT), India while Na was procured from M/s Amersham plc, UK. All other chemicals used in the present study were either from M/s Sigma Chemical Co. , USA or British Drug House (BDH), India.

4.3 RESULTS

Parent *N. muscorum* did not make N -fixing heterocysts during growth with 1 mol m NH Cl. Such cultures always remained non-heterocystous. The first series of experiments involved examining the effect of salinity and osmotic stresses on differentiation of N -fixing heterocysts. The doses for inducing increasing salinity stress were 30, 60 and 90 mol m^{-3} NaCl and 160, 200 and 240 mol m sucrose for inducing osmotic stresss in the growth medium. The treatments were given for 8 hours to non-heterocystous NH -grown cultures of the cyanobacterium. Such treated cultures along with their respective controls were incubated under diazotrophic growth conditions and then periodically examined microscopically for heterocyst formation and assayed for nitrogenase activity by the acetylene reduction technique. The results are presented in Tables 4.1a & b. Non-heterocystous NH_4^+-grown cultures on transfer to fresh N_2-medium required 24 - 30 hours for producing N_2-fixing

Table 4.1a

Effect of increasing concentrations of NaCl on time for heterocyst differentiation (t_{Het}; in hours), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) and nitrogenase (N_2ase) activity (m mol C_2H_4 g~ Chl a h^{-1}) in parent *N. muscorum*.

Treatment	t_{Het}	HF%	N_2ase activity
Control	24-30	5-6	9.3 ± 0.7
+ 30 mol m^{-3} NaCl	48-56	5-6	7.4 ± 0.75
+ 60 mol m^{-3} NaCl	72	3-4	4.1 ± 0.4
+ 90 mol m^{-3} NaCl	a	0.0	1.1 ± 0.033

Non-heterocystous NH -grown cultures were stressed with NaCl at different concentrations for 8 h, later washed and used as inoculum for incubation on diazotrophic growth medium and then periodically examined for their respective characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 4.1b

Effect of increasing concentrations of sucrose on time for heterocyst differentiation (t_{Het}; in hours), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) and nitrogenase (N_2ase) activity (m mol C_2H_4 g^{-1}Chl a h^{-1}) on parent *N. muscorum*.

Treatment	t_{Het}	HF%	N_2ase activity
Control	24-30	5 - 6	10.2 ± 1-22
+ 160 mol m^{-3} Sucrose	48	5 - 6	5.7 ± 0.45
+ 200 mol m^{-3} Sucrose	72	5 - 6	3.2 ± 0.24
+ 240 mol m~ Sucrose	a	0.5	0.5 ± 0.01

Non-heterocystous NH -grown cultures were stressed with sucrose at different concentrations for 8 h, later washed and used as inoculum for incubation on diazotrophic growth medium and then periodically examined for their respective characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

heterocysts. Such cultures treated for 8 hours with increasing concentrations of NaCl or sucrose were subsequently transferred to growth medium. There was a progressive lag in the differentiation of N_2-fixing heterocysts with increase in salinity/osmotic stress and a dose of 90 mol m^{-3} NaCl or 240 mol m^{-3} sucrose for 8 hours resulted in complete prevention of the formation of N_2-fixing heterocysts. Evidently, the differentiation of aerobic N_2-fixing apparatus in the cyanobacterium is salinity/osmosensitive and this might be the reason for nitrogenase of the cyanobacterium for exhibiting greater sensitivity to NaCl stress under diazotrophic growth condition.

Similarly effects of NaCl and sucrose stresses on oxygenic photosynthesis in the parent strain were also made. Tables 4.2a & b contains the results of such a study along with that on N_2-fixing ability in order to bring out clearly the differential sensitivity of oxygenic photosynthesis and nitrogenase activity of the cyanobacterium to NaCl/sucrose stress. N_2-fixation of the cyanobacterium got completely inhibited at a dose of 100 mol m^{-3} NaCl or 250 mol m^{-3} sucrose. Oxygenic photosynthetic activity though reduced was still significant even upto 150 mol m^{-3} NaCl or 300 mol m^{-3} sucrose. It was therefore concluded oxygenic photosynthetic process is also sensitive to NaCl/sucrose stress but its sensitivity is significantly less than that of its N_2-fixation process. It is because of such differential sensitivity of the two cyanobacterial processes it may not be unreasonable to conclude that cyanobacterial sensitivity to salinity and osmotic stresses under diazotrophic growth condition is primarily because of greater sensitivity of its N_2-fixation process to the stress

Table 4.

Comparison of photosynthetic O_2-evolution (m mol 0, evolved g Chl a h^{-1}) and N_2-fixation (N_2ase activity) process (m mol C_2H_4 formed g^{-1} Chl a h^{-1}) of the parent *N. muscorum* to increasing concentrations of NaCl.

Treatment	O_2-evolution	act?vitv
Control	225 *t* 21	10.4 ± **1.1**
+ 50 mol m^{-3} NaCl	179 ± 12	4.7 ± 0.4
+ 75 mol m~^{3} NaCl	112 ±1 1	2.1 1 **0.12**
+ 100 mol m^{-3} NaCl	59 ± 3.7	0.0
+ 150 mol m~^{3} NaCl	46 ± 2.3	0.0

6-day old diazotrophically grown cultures were treated with different concentrations of NaCl respectively for 12 h and then examined for their respective characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 4.1

Comparison of photosynthetic 0,-evolution (m mol O_2 evolved g Chl a h^{-1}) and N_2-fixation (N_2ase activity) process (m mol C$_2$H$_4$ formed g^{-1} Chl *a* h^{-1}) of the parent *N. muscorum* to increasing concentrations of sucrose.

Treatment	O_2-evolution	N_2ase activity
Control	235 ±15.6	10.4 ± 1.0
+ 160 mol m^{-3} sucrose	132 ± 12.2	5.1 ± 0.14
+ 200 mol m^{-3} sucrose	49 ± 2.4	1.9 ± 0.11
+ 250 mol m^{-3} sucrose	34 ± 1.7	0.6 ± 0.045
+300 mol m^{-3} sucrose	21 ± 2.3	0.0

6-day old diazotrophically grown cultures were treated with different concentrations of sucrose respectively for 12 h and then examined for their respective characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

parameter.

The spontaneously occuring mutant clones of the cynaobacterium resistant to NaCl induced lethality or sucrose induced lethality arose with a frequency of 0.8 - 1.5 × 10 **which** is characteristic of single mutational events. A comparison was made on the effect of proline on heterocyst formation and nitrogenase activity in the parent and its various *NaCl-R* mutant clones (Tables 4.3a & b). It can be seen that the parent strain did not produce N -fixing heterocysts in proline medium while its various *NaCl-R* mutants produced normal frequency of heterocysts and showed nitrogenase activity. Evidently, the mutation for resistance against different levels of NaCl stress in the cyanobacterium seems to have rendered N -fixing heterocysts derepressed in proline medium. A comparative study on the percent survival of the three *NaCl-R* mutant strains to NaCl was made under diazotrophic growth condition (Fig. 4.1). Evidently, the three mutant strains did differ in respect of their tolerance characteristic to NaCl stress. Since NaCl stress tolerance characteristic of the three mutant strains remained intact over a period of 5-successive sub-cultures in the absence of NaCl stress in the growth medium, it is concluded that the observed three increasing levels of NaCl stress tolerance in the cyanobacterium are heritable phenotypes. Since proline could not cause repression of heterocyst formation and nitrogenase activity in the three *NaCl-R* mutants, it was thought that they might have undergone alteration in their proline metabolism as a result of mutation exhibiting different levels of resistance to NaCl stress.

The intracellular levels of proline were estimated under NaCl stress (100 mol m~) NaCl condition in N -medium containing or lacking 1 mol m proline. Salinity stress in the diazotrophic (N) medium did not cause any significant rise in intracellular level of proline in any of the mutant strains.

Table 4.3a

Growth (optical density at 663 nm), heterocyst frequency (HP%, number of heterocysts per 100 vegetative cells) and nitrogenase (N_2ase) activity (m mol C_2H_4 formed g~ Chl a h^{-1}) of the parent *N. muscorum* and its various salinity (NaCl) resistant mutant clones under diazotrophic (N_2) growth conditions.

Strains	Growth	HF%	activity
Parent	0.64 ±0.02	5 - 6	9.8 ± 0.83
NaCl-R_{100}	0.61 ± 0.03	5 - 6	9.6 ± 0.88
NaCl-R_{200}	0.58 ±0.03	5 - 6	9.6+1.1
NaCl-R_{250}	0.56 ±0.04	5 - 6	9.2 ± 0.6

Non-heterocystous NH -grown cultures were washed twice and used as inoculum for incuBation in diazotrophic medium for 6-days before examining them for their characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

table 4.3b

Growth (optical density at 663 run), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells) and nitrogenase (N ase) activity (m mol C_H formed g~ Chl a h) of the parent *N. muscorum* and its various salinity (NaCl) resistant mutant clones in growth medium containing 1 mol m⁻ proline.

Strains	Growth	HF%	activity
Parent	0.87+0.05	0.0	0.0
NaCl-R_{100}	0.64 ± 0.03	5 - 6	9.8 ± 0.28
NaCl-R_{200}	0.62 ±0.06	5 - 6	9.1 ± 0.66
NaCl-R_{250}	0.60 ± 0.064	5 - 6	8.7 ± 0.5

Non-heterocystous NH -grown cultures were washed twice and used as inoculum for incubation in growth medium containing 1 mol m proline for 6-days before examining for their characteristics. Each reading is an average (± SEM) of three independent experimental determinations.

Fig. 4.1

Per cent survival of the parent *N. muscorum* (0) and its ***NaCl-R*** mutant (•), ***NaCl-R***$_2$ mutant (A) and ***NaCl-R***$_{50}$ mutant (A) strains to increasing concentrations of **NaCl** under diazotrophic growth conditions.

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

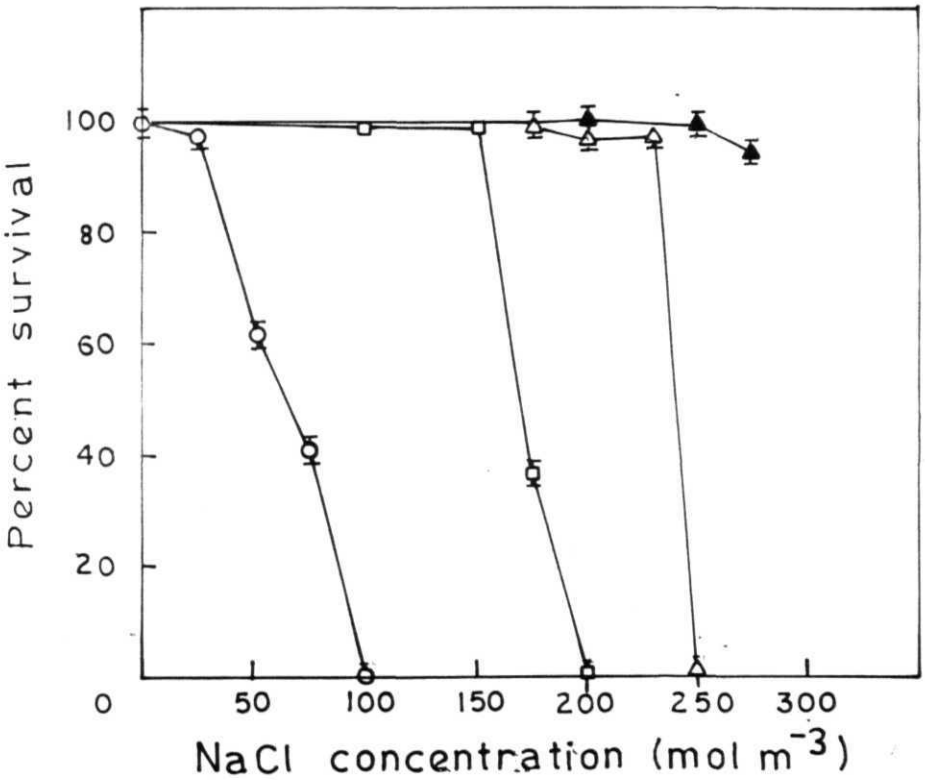

Percent survival
100
80
60
40
20
0
50
100
150
200
250
300
NaCl concentration (mol m^{-3})

Fig.4.1

However, the parent strain showed an increase by 20.6 μ mol proline g protein during its growth under normal in the presence of 1 mol m proline. Similar cultures of the parent when stressed by 100 mol m NaCl caused 3.5-fold rise in the intracellular proline level (Table 4.4). Evidently, the cause of 3.5-fold rise in intracellular proline level in the parent under salinity stress is the result of increased uptake of exogenous proline. In comparison, *NaCl-R* while showing about 2 - 3-fold rise in intracellular proline level under stress condition containing proline did not show any significant increase under similar conditions in the absence of NaCl stress. Such a behaviour was true for the other two *NaCl-R* mutant strains (Table 4.4). These results suggest that the parent strain contains both normal proline uptake process and osmo(salinity)-stimulable proline uptake process and utilizes proline like a nitrogen source and consequently causing repression of heterocyst formation in proline medium. The salinity stress resistant mutants seemed to have only osmo(salinity)-stimulable proline uptake process while showing a loss in normal proline transport activity and thus consequently showing derepression for heterocyst formation and nitrogenase activity in proline medium. It was therefore decided to confirm the operation of normal and salinity-stimulable proline transport activity in the parent and in the *NaCl-R* mutant strain using ^{4}C-proline. As shown in Fig. 4.2a, parent exhibited normal proline transport activity, as well as a osmo(salinity)-stimulable proline transport activity while the mutant strain did not show significant level of normal proline uptake process. The degree of increase in proline uptake following salinity stress in the parent as well as in the mutant strain was almost similar. Thus, mutation to salinity resistance

Table 4.4

Intracellular levels of proline (μ mol proline g^{-1} protein) under unstressed and 3 h NaCl stressed conditions in cultures of the parent *N. muscorum* and its various salinity resistant (*NaCl-R*) mutant clones following their incubation on diazotrophic medium and on growth medium containing proline (1 mol m~).

	Strains			
	Parent	*00*	*NaCl-R*$_{200}$	
N -medium	**10.8** (0.32)	**40.5** (2.45)	**41.3** (0.67)	**48.5** **(2.34)**
+ NaCl	11.6 (0.54)	52.4 (3.11)	**53.7** (3.56)	54.2 **(3.08)**
+ proline	31.4 (1.8)	69.5 (4.97)	**62.4** (3.1)	**61.6** **(3.44)**
+ proline + NaCl	103.5 (3.69)	114.6 (5.05)	118.7 (5.5)	**120.1** **(8.21)**

6-day old diazotrophically grown cultures were used as source of inocula in the present series of experiments. Proline and NaCl were added to the experimental cultures at the start of the experiment.
Values in parentheses are *i* SEM of three independent experimental determinations.

Fig. 4.2a

Comparison of ^{14}C-proline uptake in parent *N. muscorum* (0) and in its *NaCl-R* mutant (D), *NaCl-R*$_{200}$ mutant (A) and *NaCl-R*$_{250}$ mutant (1) strains under unstressed (——) or 75 mol m~ NaCl stressed (——) diazotrophic growth conditions.

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

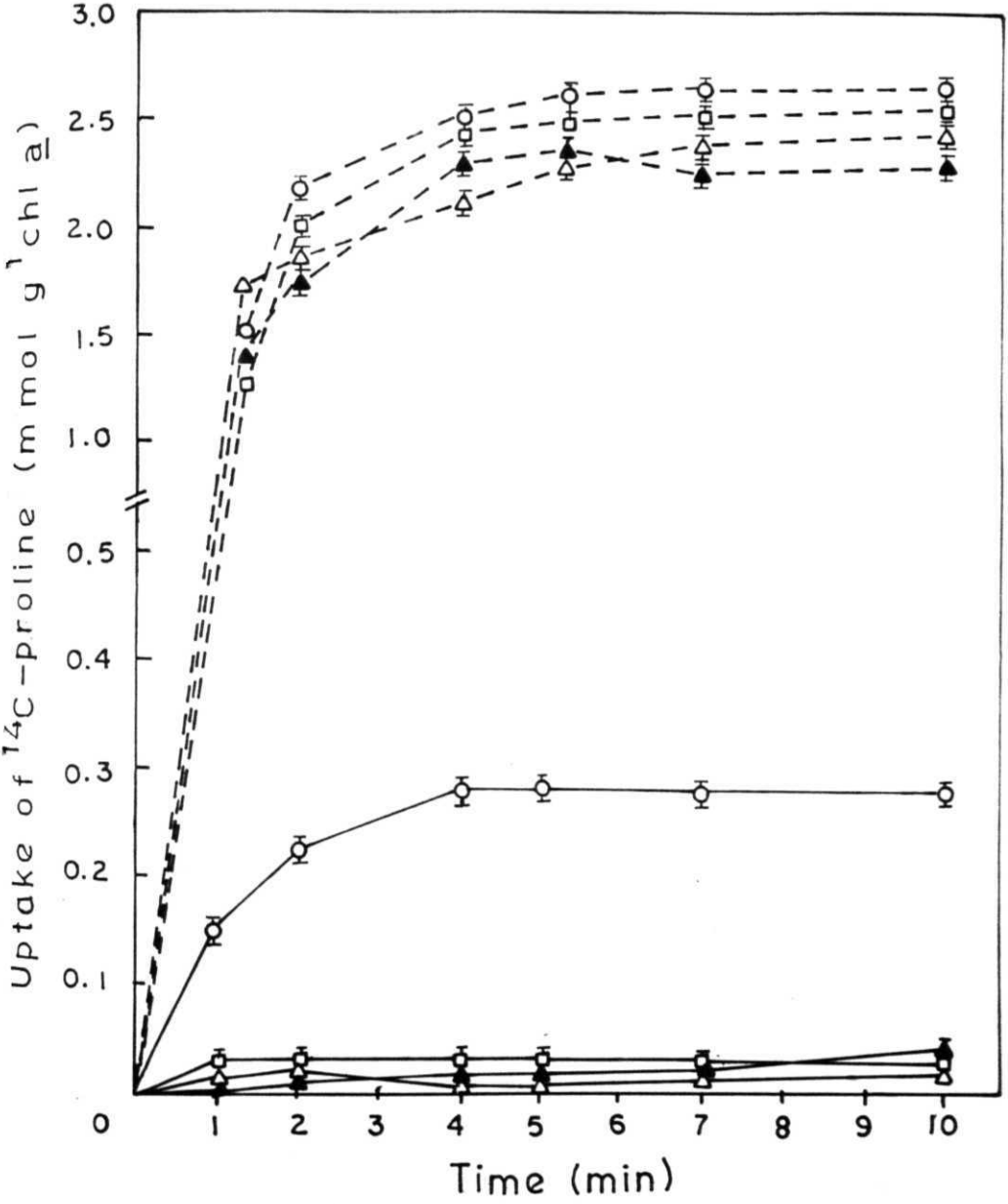

Uptake of ^{14}C-proline (m mol g^{-1} chl a)
3.0
2.5
2.0
1.5
1.0
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
10
Time (min)

Fig.4.2a

seems to have resulted in loss of normal proline uptake process and simultaneous rise in intracellular proline content. However no such loss of normal proline uptake process was visible in the *Sucrose-R* strain (Fig. 4.2b). Intracellular levels of proline in the *Sucrose-R* strain also showed a similar nature to that exhibited by the *NaCl-R* strains (Table 4.5).

The next series of the experiments were done to examine the kinetics of $^{22}Na^{+}$ transport process in the parent and in the mutant strains. All the NaCl resistant mutant strains examined in this respect were found almost similar with respect to the $^{22}Na^{+}$ transport and it was therefore decided to include the result of the parent, *NaCl-R* and *Sucrose-R* strains (Fig. 4.3). The two mutant strains showed about 3-fold less level of $^{22}Na^{+}$ transport activity than the parent strain. Evidently, mutation associated with salinity or osmotic resistance seems to have been resulted in curtailment of $^{22}Na^{+}$ influx in the cyanobacterium.

Experiments were done to examine whether the *NaCl-R* mutants were also resistant to osmotic stress induced by sucrose. Similarly, attempts were made to study the cross-resistance nature of osmotic resistant *(Sucrose-R)* mutants with salinity (NaCl) stress. Subsequently, we also examined the intracellular proline level in the *Sucrose-R* mutant in order to find out the common biochemical basis of cross-resistance relation between *Sucrose-R* and *NaCl-R* phenotypes. The survival characteristics of *Sucrose-R* and *NaCl-R* on diazotrophic media are shown in the Fig. 4.4. *NaCl-R* strain and *Sucrose-R* strain showed almost 100% survival on 100 mol m NaCl or on 300 mol m sucrose. The results of cross-resistance relation between the two mutant strains thus suggest that there is a common mechanism for stress induced lethality as well as mutationally induced resistance in the cyanobacterium. This also means that the cyanobacterium *N. muscorum* is capable of mutating spontaneously to NaCl or sucrose resistant phenotypes. The frequency of spontaneous

Fig. 4.2b
Comparison of C-proline uptake in parent *N. muscorum* (O) and in its *Sucrose-R* mutant (D) under unstressed (——) or 75 mol m NaCl stressed (——) diazotrophic growth conditions.
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

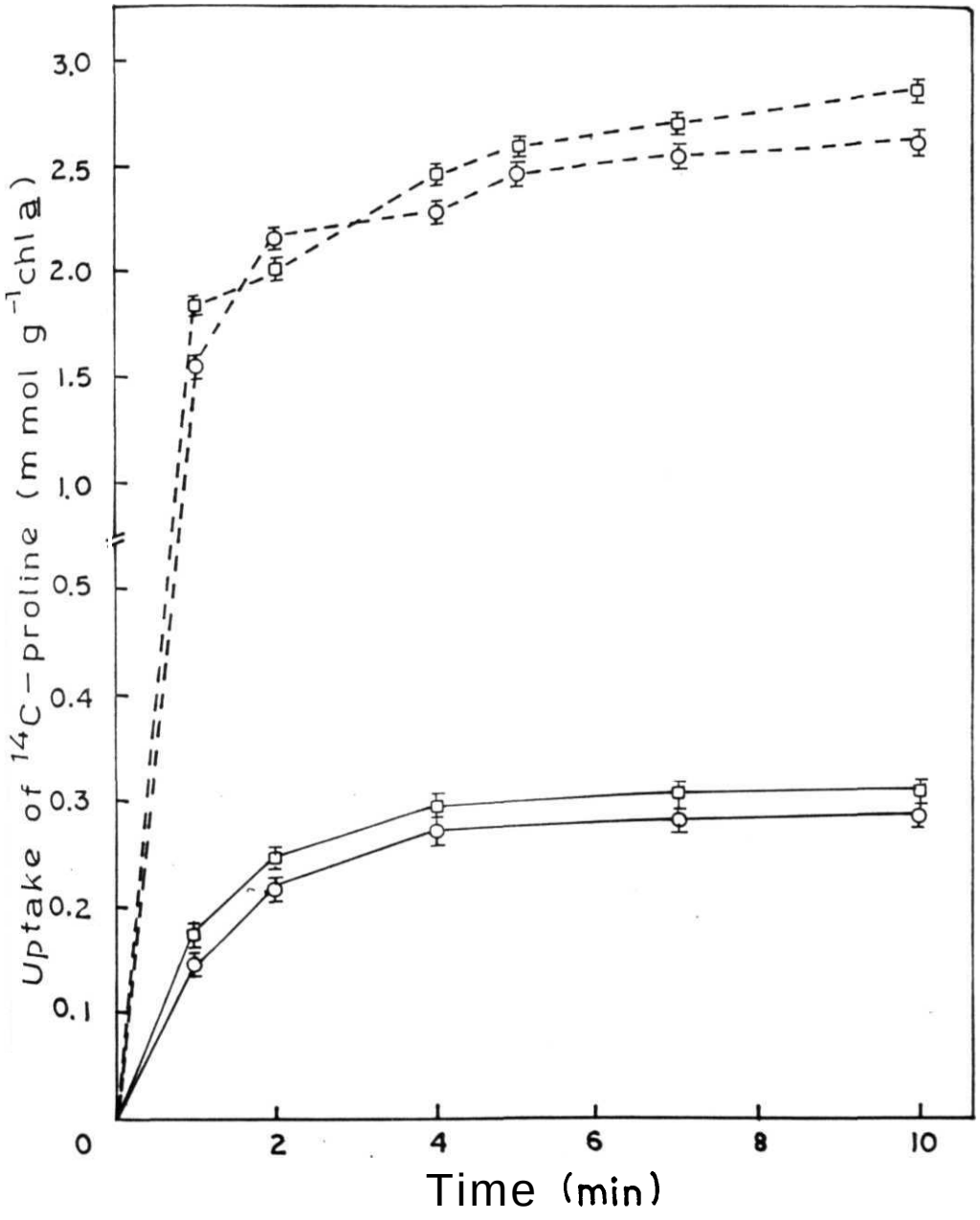

Uptake of ^{14}C-proline (m mol g^{-1} chl a)
3.0
2.5
2.0
1.5
1.0
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
Time (min)

Fig. 4.2 b

Influence of salinity (100 mol m^{-} NaCl) stress and osmotic (250 mol m~ sucrose) stress on the intracellular level of proline (μ mol proline g~ protein) on the parent *N. muscorum* and its salinity stress tolerant (*NaCl-R* $_{OO}$) and osmotic stress tolerant (*Sucrose-R*) mutant strains.

Treatment	Strains		
	Parent	*NaCl-R* $_{O}$	*Sucrose-R*
N_2-medium	11.4 ± 1.1	43.6 ± 2.7	47.3+2.3
+ NaCl	11.5 ± 1.1	50.4 ± 3.9	• 51.1 ± 2.0
+ sucrose	10.4 ± 1.1	50.6 ± 3.45	47.1 ± 2.1

6-day old diazotrophically grown cultures were used as source of inocula. Incubation of the cultures in the medium at respective stresses was for 3 h.
Each reading is an average (± SEM) of three independent experimental determinations.

Fig. 4.3
Uptake of $^{22}Na^{+}$ in the parent *N. muscorum* (0) and in its *NaCl-R_{100}* mutant (D) and *Sucrose-R* mutant (Δ) strains under diazotrophic growth conditions.
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

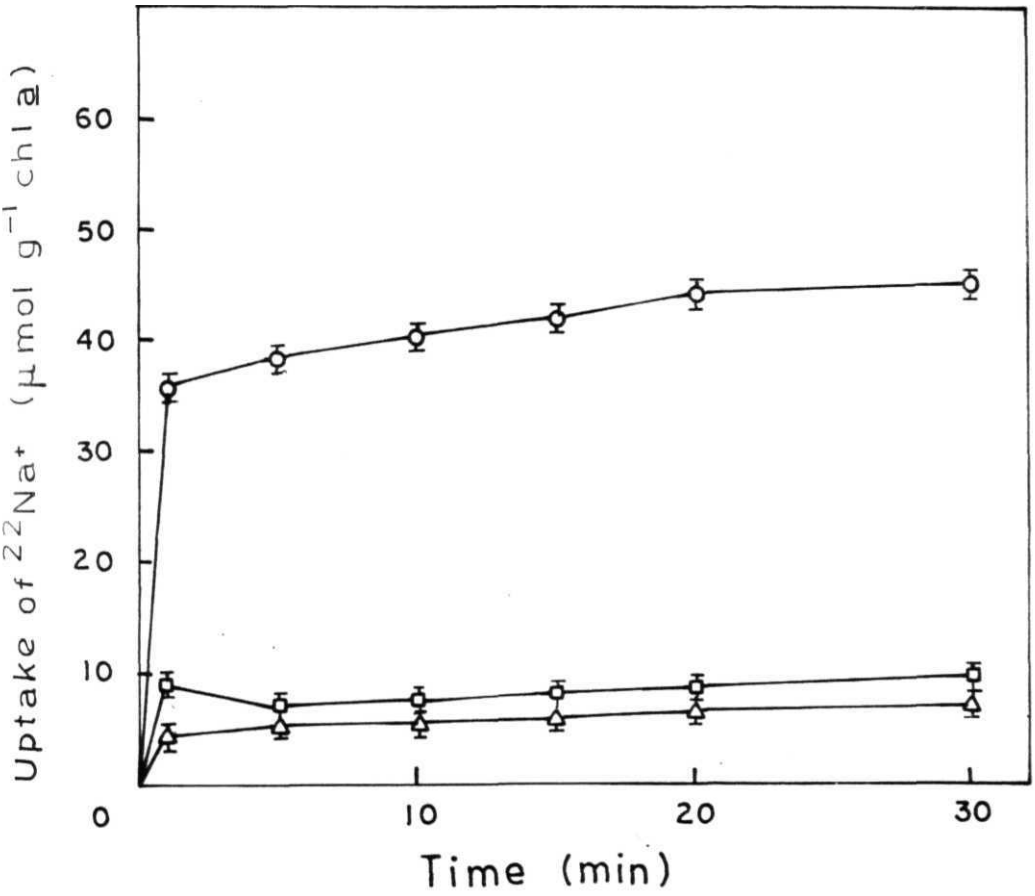


Fig.4.3

Fig. 4.4

Per cent survival of the parent *N. muscorum* (0) and of its ***NaCl-R*** mutant (D) and *Sucrose-R* mutant (A) strains to increasing concentrations of NaCl (——) or sucrose (——) under diazotrophic growth conditions.

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

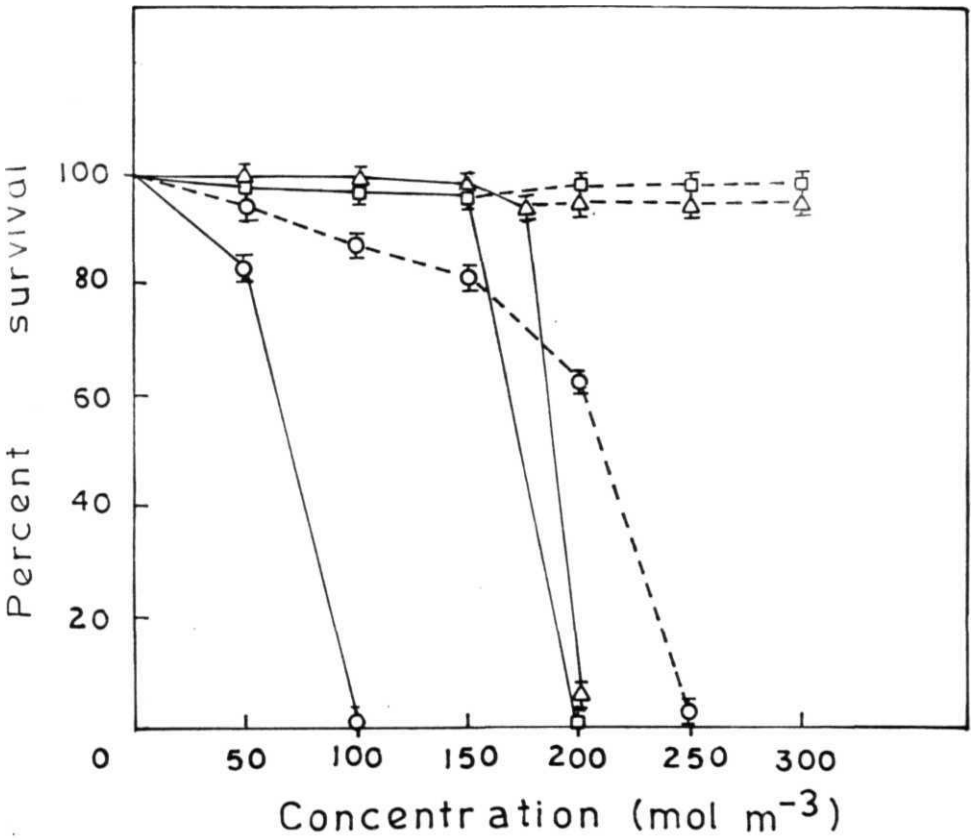

Percent survival
100
80
60
40
20
0
50
100
150
200
250
300
Concentration (mol m^{-3})

Fig. 4.4

mutation of the parent strain to *NaCl-R* or *Sucrose-R* phenotypes was in the range 1.5 x 10 . Such a mutational frequency are reliable measures of a single mutational event in the system. Accordingly, it can be inferred that single mutational events in *N. muscorum* can generate variants resistant to salinity/osmotic stress.

14
C-sucrose was utilized to measure the sucrose-transport characteristics in the parent, *NaCl-R* and *Sucrose-R* mutants of *N. muscorum.* The results are shown in Fig. 4.5. All the three strains in general seemed to be similar in respect of their sucrose transport characteristic. It is therefore concluded that mutation to *NaCl-R* phenotype or *Sucrose-R* phenotype has not affected significantly the sucrose transport while bringing about a simultaneous reduction in $^{22}Na^{+}$ influx (Fig. 4.3). Since the *NaCl-R* strain and *Sucrose-R* strain showed cross-resistance relationship and since *NaCl-R* phenotype seemed to result from mutationally induced accumulation of proline, it was thought proper to examine the intracellular level in *Sucrose-R* strain in relation to its parent and *NaCl-R* strains. As shown in Table 4.5, *Sucrose-R* strain like *NaCl-R* strain had higher level of intracellular proline content than the parent strain. Also, sucrose or NaCl stress did not influence in any significant way the intracellular proline content of parent, *NaCl-R* or *Sucrose-R* strains under diazotrophic growth conditions (in the absence of exogenous proline). Thus, over-accumulation of proline seems to be the reason for protection against NaCl stress or sucrose stress in cyanobacterium.

Studies were also conducted to examine the influence of nitrogen source on survival characteristics of the parent strain.

Fig. 4.5

Uptake of ^{14}C-sucrose in the parent *N. muscorum* (0) and in its *NaCl-R$_{10}$* mutant (D) and *Sucrose-R* mutant (A) strains under diazotrophic growth conditions.

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

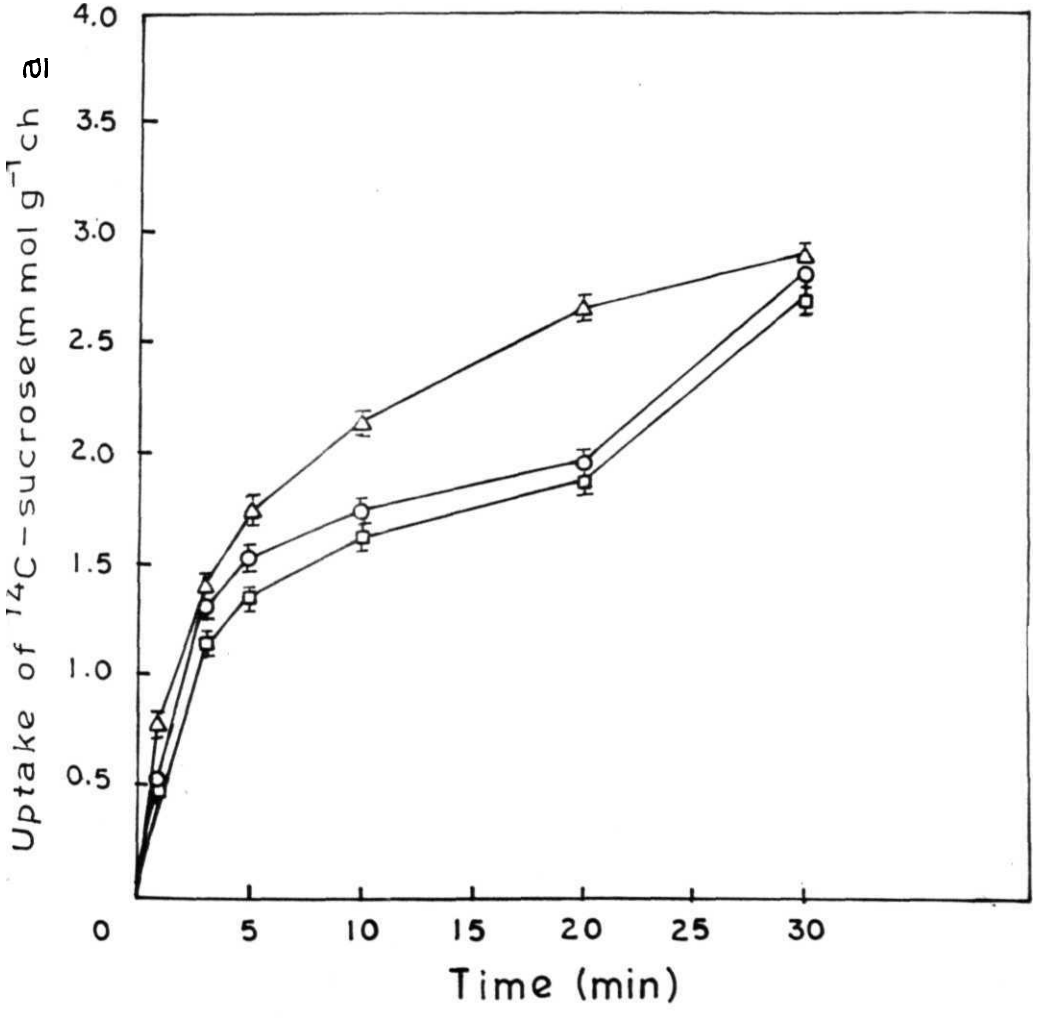

Uptake of ^{14}C-sucrose (m mol g^{-1} ch
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
5
10
15
20
25
30
Time (min)

Fig. 4.5

The results of such studies are shown in Tables 4.6a & b. Diazotrophically grown cultures on N_2 or NH_4^+ media, showed similar degree of lethality on 100 mol m^{-3} NaCl or 250 mol m^{-3} sucrose. This indicates that NH_4^+ as a post-nitrogen source does not confer any protection to diazotrophic cultures of the parent strain. However, when such diazotrophic cultures of the parent strain were plated on N -medium containing 1 mol m^{-3} proline and 100 mol m^{-3} NaCl or 250 mol m^{-3} sucrose, the percent survival of the parent increased by 90%. Clearly, the presence of proline in the stress medium offers near-total protection to the cyanobacterium against NaCl induced lethality. When these experiments were repeated with NH -grown cultures, the survival characteristics of the parent exhibited almost a similar pattern shown by N -grown cultures under a parallel condition. In conclusion, proline among the nitrogen sources is capable of almost fully recovering the lethality induced by NaCl in the cyanobacterium.

Experiments were also done to examine the effect of N_2 or NH_4^+ as nitrogen source on Na transport in the parent and in the two mutant strains. Such experiments were done with N_2-grown cultures for N as nitrogen source and NH -grown cultures for NH_4^+ as nitrogen source. The results of such studies are shown in Fig. 4.6. Na transport was found more in NH -grown cultures than in N_2-grown cultures of the parent strain. In comparison, Na uptake and accumulation process in the *NaCl-R* and *Sucrose-R strains* was very much reduced and the influence of NH -nitrogen on Na uptake and accumulation was not as pronounced in the mutant strains as it was in the parent strain. In conclusion, it can be said that NH -grown cultures are more efficient in uptake and accumulation of Na^+ than its N -fixing cultures. In this respect, such NH -regulation of Na uptake and accumulation is not significantly evident in the two mutant strains.

Table 4.6a

Influence of post-nitrogen source on per cent (%) survival and nitrogenase (N_2ase) activity (mrool C_2H_4 formed g Chl a h) of diazotrophically grown cultures of the parent *N. muscorum* under unstressed, salinity (100 mol m^- NaCl) stressed and osmotic (250 mol m^- sucrose) stressed conditions.

Treatment	% survival	activity
N_2-medium	100	9.4 ± 0.7
+ NaCl	0	0.0
+ Sucrose	0	0.0
NH_4-medium	100	0.0
+ NaCl	2	0.0
+ Sucrose	2 - 3	0.0
Proline medium	100	0.0
+ NaCl	92	8.5 ± 0.5
+ Sucrose	94	8.7 ± 0.8

6-day old diazotrophically grown cultures were used as source of inocula and were incubated on the respective growth media for ten days before examining them for their characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 4.6b

Influence of pre-nitrogen source on per cent (%) survival and nitrogenase **(N_2ase)** activity **(m mol C_2H_4** formed **g^{-1} Chl** a h) of **NH_4-grown** cultures of the parent *N. muscorum* under **unstressed,** salinity (100 mol **m** $^{-3}$ **NaCl)** stressed and osmotic (250 **mol m** $^{-3}$ sucrose) stressed conditions.

Treatment	% survival	activity
N_2-medium	100	8.6 ± 0.5
+ NaCl	0	0.0
+ Sucrose	0	0.0
NH_4-medium	100	0.0
+ NaCl	0	0.0
+ Sucrose	0	0.0
Proline medium	100	0.0
+ NaCl	94	8.1 ± 0.6
+ Sucrose	94	9.2 ± 0.6

6-day old NH_4-grown cultures were used as source of inocula and were incubated on the respective growth media for ten days before examining them for their characteristics.
Each reading is an average (± SEM) of three independent experimental determinations.

Fig. 4.6

Uptake of Na^+ in the parent *N. muscorum* under diazotrophic growth condition (0) and NH_4^+-growth condition (•); in the *NaCl-R* mutant strain under diazotrophic growth condition (D) and NH_4^+-growth condition (•); and in the *Sucrose-R* mutant strain under diazotrophic growth condition (A) and NH_4^+-growth conditions (A).

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

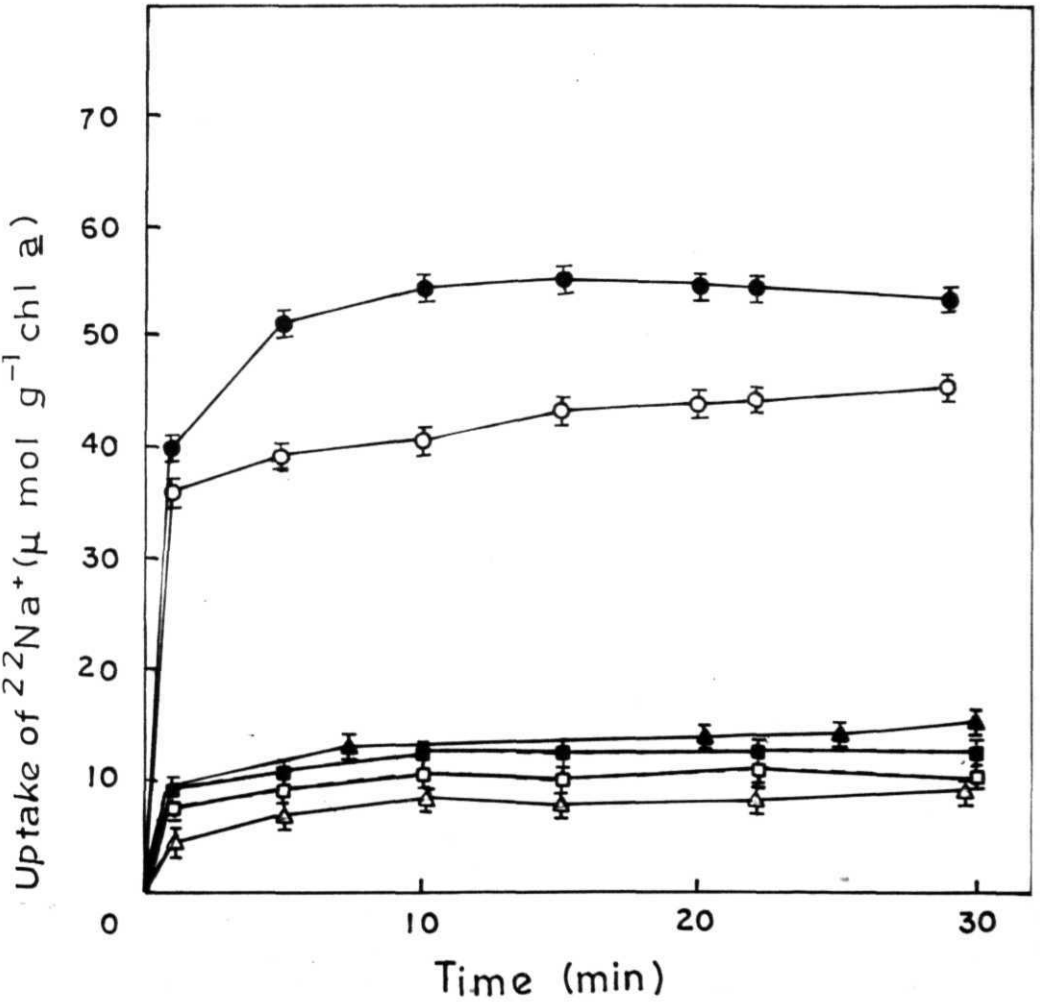

70
60
50
40
30
20
10
0
10
20
30
Time (min)
Uptake of $^{22}Na^{+}$ (μ mol g^{-1} chl a)

Fig.4.6

4.4 DISCUSSION

NaCl is an ionic osmoticum, while sucrose is a non-ionic osmoticum. NaCl stress to the cyanobacterium could result either from its ionic effect or osmotic effect. Since, parent *N. muscorum* failed to survive in medium containing 10^{-3} mol m^{-3} NaCl or 250 mol m^{-3} sucrose and since spontaneously occuring mutant clones resistant to NaCl induced lethality or sucrose induced lethality arose with a frequency characteristic of a single mutational event, it is suggested that *NaCl-R* or *Sucrose-R* phenotypes are the result of single mutational events as a consequence of identical stress conditions imposed by NaCl or sucrose (i.e., primarily an osmotic stress). This is supported further by the cross-resistant relationship of *NaCl-R* and *Sucrose-R* mutant strains of the cyanobacterium. Another point in support of this contention is that the *NaCl-R* and *Sucrose-R* mutant strains show a considerably reduced level of Na uptake and accumulation than in the parent strain. Inorganic ions like Na at high cellular levels are known perturbants of the structure and function of enzymes and other cellular proteins (Yancey *et al.* 1982). Thus, any osmoregulatory cellular strategy should not only be osmoprotective and osmobalancing in nature but also at the same time must function in effective curtailment of influx of perturbant ions like Na . Thus, when the $^{22}Na^{+}$ transport characteristics (Fig. 4.3) are analyzed in conjunction with the ^{14}C-sucrose transport characteristics (Fig. 4.5), it is seen that the parent and its mutant strains while showing a similar pattern of sucrose transport differed in respect of $^{22}Na^{+}$ influx, which was severely curtailed in the mutant strains. Mutational curtailment of Na influx associated with simultaneous acquisition of osmotic or salinity resistance does suggest a definite role of curtailed Na influx in salinity-

or osmoadaptation of the cyanobacterium. Reduction in Na influx has been reported to be the major mechanism of salt tolerance in N_2-fixing cyanobacteria (Apte *et al*. 1987). Evidently, the results also suggest that resistance to the two stresses in this cyanobacterium has a common physiological basis.

When the parent strain was examined for relative sensitivity of its oxygenic photosynthesis and nitrogenase activity to NaCl or sucrose stress, the latter was found more sensitive than the former, there by suggesting that differential inhibition of the two activities is the primary reason for cyanobacterial death under salinity or osmotic stress conditions. This is in agreement with a previous report on osmoregulation of oxygenic photosythesis and N_2-fixation (Tel-Or 1982). But if that be so, an examination of the osmosensitivity of NH_4 -assimilating cultures to NaCl or sucrose is expected to throw light on this aspect. Since the survival characteristics of N_2 -grown and NH_4 -grown cultures of the parent were almost similar, nature of inorganic nitrogen source like N_2 or NH_4 does not seem to be the factor influencing cyanobacterial resistance to NaCl or sucrose stress. The differentiation of a vegetative cell into a N_2 -fixing heterocyst was delayed and completely inhibited with the rise in NaCl or sucrose stress. The structural and organizational fate of different N_2 -fixing heterocysts under sub-lethal stress of NaCl and sucrose still remains to be analyzed.

Turgor sensitive accumulation of compatible molecules either by transport or fresh synthesis or both in response to salinity or osmotic stresses is the physiological mechanism of adaptation in cyanobacteria (Reed & Stewart 1985) and in bacteria (Csonka 1989). No previous study has attempted any mutational approach to examine the possibility of generating genetic variants capable of overcoming salinity or osmotic stresses. It is also not clear why proline, the well-known osmoprotectant in

enterobacteria and in higher plants (Rhodes & Hanson 1991) has not been examined for its osmoprotective role in any cyanobacterium. Proline under normal growth condition is found to be metabolized like a fixed nitrogen source, leading to repression of heterocyst formation and nitrogenase activity in the parent strain. Since exogenous proline in *N. muscorum* is found protecting against NaCl and induced lethality, as it has been reported to do in members of enterobacteriaceae (Csonka 1989), it thus appears that in this cyanobacterium too exogenous proline functions as an effective osmoprotectant. This finding led us to examine the intracellular levels of proline in parent and its various mutants exhibiting different levels of resistance to NaCl stress under various growth conditions.

Before discussing this aspect it is important to point out here that *N. muscorum* can initially mutate spontaneously to NaCl resistance at a level of 100 mol m NaCl and not beyond. Such *NaCl-R* mutant clones have been found to increase their resistance level upto 200 mol m NaCl by undergoing a second step of spontaneous mutation. Similarly, spontaneous mutant derivatives of the *NaCl-R* strain exhibited a NaCl resistance level of 250 mol m . These results imply the involvement three independent sequential spontaneous mutations to increase the salinity resistance level of the cyanobacterium to 250 mol m The three mutant strains on examination for their intracellular proline level in relation to the intracellular proline level in the parent strain, showed a correlation between their intracellular proline levels and the degree of resistance to NaCl resistance. The mutant strains like the parent strain also showed a salinity-stimulable proline uptake process. All these results suggest clearly over accumulation of proline resulting

either from salinity-stimulable uptake of exogenous proline or from endogenous biosynthetic pathway as the cause of increased salinity or osmotic tolerance in the cyanobacterium. Since exogenous proline did not cause rise in the intracellular level of proline in any of the mutant strains as it did in the parent strain, it is concluded that mutation to salinity resistance in the cyanobacteria has inactivated the activity of normal proline uptake process.

Since proline oxidase enzyme activity has been shown essential for assimilation of exogenous proline as nitrogen source in *Anabaena* PCC 7120 (Spence & Stewart 1986) and in *N. muscorum* (Singh et *al*. 1991) and since all the three salinity resistant mutant strains of *N. muscorum* produced N -fixing heterocyst in proline medium, lack of normal proline uptake process or proline oxidase activity or both, could be the reason why exogenous proline could not be assimilated like a fixed nitrogen source in the three salinity resistant cyanobacterial mutant strains. Since all the three strains are proline over-producers and since increased proline levels is know to repress proline oxidase activity and normal proline transport activity in bacteria, the observed lack of proline uptake and proline accumulation in the mutant strains could be result of repression of proline oxidase enzyme activity and proline uptake activity by the over-accumulation of intracellular proline resulting from increased biosynthesis. Such conclusions are consistent with similar findings reported earlier in bacterial systems (Dendinger & Brill 1970). However, in the absence of complete knowledge on proline metabolism in cyanobacteria under stress and unstressed conditions, nothing definite can be said about the primary cause leading to loss of the ability to

assimilate proline like a fixed nitrogen source in the mutant strains that seem to over-accumulate proline.

The *Sucrose-R* differed from the *NaCl-R* in showing repression for heterocyst formation in proline medium and in showing normal proline transporta activity under unstressed conditions. It is thus apparent that different mechanisms operate both for uptake of proline under unstressed conditions as well as for its utilization as a nitrogen source under such conditions in the two mutant strains. However, *Sucrose-R* strain showed over-accumulation of intracellular proline and since it exhibited cross-resistance relationship with *NaCl-R* mutant strain which too over-accumulated intracellular proline, the latter seems to be the common physiological mechanism of cross-resistance relationship between these two mutant strains. These studies further endorse the osmotic nature of stress produced in the cyanobacterium by both NaCl and sucrose.

Proline is metabolized like a nitrogen source in cyanobacteria (Spence & Stewart 1986; Singh et *al.* 1991). The genetics and molecular biology on utilization of proline as a nitrogen source or as a osmo(salinity)-protectant has been studied in detail in members of enterobacteriaceae (Csonka 1989; Lucht & Bremer 1994). The product of *putA* gene and proline together control *putP* and *putk* genes - the two key genes required for assimilation of proline as nitrogen source. Inactivation of *putP* gene results in loss of proline uptake by salinity/osmotic sensitive proline porter. Evidently, *putP*-encoded porter is an osmosensitive porter for proline in members of enterobacteriaceae. Loss of *put* A gene results in loss of the enterobacterial ability to oxidatively assimilate proline as

nitrogen source. There are two other proline porters, one encoded by the proP gene which is osmoinducible and the other encoded by the *proV* operon complex which is predominantly involved in glycinebetaine uptake and accumulation under osmotic stress condition (Lucht & Bremer 1994). In comparison, nothing is known about the multiplicity of proline porters in any cyanobacterium as yet. The present results about exogenous proline uptake under normal and NaCl/sucrose stressed condition suggests that *N. muscorum* like the enterobacterial system might have two proline porters, one osmo(salinity)-sensitive and involved in proline utilization as nitrogen source and the other osmo(salinity)-stimulable and involved in protection of the cyanobacterium against salinity and osmotic stresses. It is probably because of this reason the parent strain metabolized proline like a fixed nitrogen source under control conditions causing repression of heterocyst-nitrogenase system and used proline as an effective osmoprotectant under salinity or osmotic stress conditions permitting it to differentiate heterocysts and fix atmospheric N_2. Apparently, utilization of exogenous proline in the cyanobacterium as osmoprotectant seems to be osmoregulated through osmotic inhibition control of its catabolism. It is interesting to note that in enterobacteria exogenous proline uptake has been shown to be osmoinducible (Measures 1975), while in *Rhizobium meliloti* depending on the osmolarity of the growth medium prolinebetaine (a quaternary ammonium compound) is used as a nitrogen source or as an osmoprotectant (Gloux & Le Rudulier 1989). Since the salinity resistant mutant strains are defective in normal proline uptake and are proline over-accumulating strains, and since they are products of a single mutational event, it could be only suggested at the moment that the salinity resistant mutants are pleiotropic in nature with a genetic linkage between the gene regulating proline uptake and utilization as nitrogen source and the genes involved in

over-production of intracellular proline. Since, the mutant strains continued producing N -fixing heterocysts despite having higher intracellular proline level, one can infer that proline *per se* is not the repressor of heterocyst formation and nitrogenase activity in the cyanobacterium.

Mutational acquisition of osmotolerance in the cyanobacterium thus opens out the possibility of identifying specific cyanobacterial genes and their products in conferring adaptation to osmotic and salinity stresses. Apte & Haselkorn (1990) in an interesting study showed NaCl inducible transcription of about a hundred genes in the salt tolerant cyanobacterium *Anabaena torulosa* thus suggesting a polygenic nature of salinity stress. *A. torulosa* is a brackish-water form which grows under salt stress condition and it is therefore likely that the genes activated under salt stress may not only be functioning in generating compatible osmolytes but also producing enzymes and proteins required for normal growth of the cyanobacterium under such a situation and their specificity in conferring salt adaptation depends upon demonstrating directly the essentiality of their products in such a process. In this context, it is worth noting that all osmoinducible transcripts in enterobacteriaceae are not essential for acquisition of osmotolerance by them (Csonka & Hanson 1991; Csonka 1989). The present findings thus assume significance, mainly because earlier reports found no evidence for the involvement any single gene product in regulation of various osmogenes in bacteria (Higgins et *al.* 1987) or the salt resistant genes in the cyanobacterium *A. torulosa* (Apte & Haselkorn 1990).

In the present study, responses of the cyanobacterium to salinity and osmotic study seems to be similar and our results do not subscribe to the view that cyanobacterial responses to salinity stress and osmotic stress are physiologically distinct phenomena (Fernandes et *al.* 1993). Physiologically similar responses have been reported to occur in *A. variabilis* following induction of stress using NaCl, sucrose or sorbitol (Reed & Stewart 1985). NH -nitrogen has been shown to protect cyanobacteria against salinity stress by curtailing Na influx (Apte et *al.* 1987; Fernandes et *al.* 1993). Since, N -fixing cultures as well as NH -assimilating cultures of *N. muscorum* exhibited almost similar level of sensitivity to NaCl stress, it is concluded that NH -nitrogen does not seems to offer any protection to this cyanobacterium against NaCl or sucrose stress lethality. Since Na uptake and accumulation is more pronounced in NH -grown cultures of the parent strain than in its diazotrophic cultures, hence it is further emphasized that NH -nitrogen plays no role in curtailment of Na influx and appears to be in direct conflict with earlier reports (Fernandes et *al.*, (1993).

Since NaCl comprises two components (the ionic and osmotic (non-ionic)), the present *NaCl-R* mutant strains are primarily the result of sequential single mutational events showing cross-resistance with the *Sucrose-R* and most essentially showing the compatible osmolyte based mechanism of osmoadaptation by over-accumulating proline and differs from a yet another class of *NaCl-R* mutant strain (described in Chapter-6) which is the result of a single mutational event resulting from the effect of ionic component of NaCl.

Since over-accumulation of proline has been shown to confer salinity and osmotic stress tolerance in *N. muscorum* it was thought worthwhile to carry out further studies on proline

metabolism and analyze its role as a nitrogen source and as an osmoprotectant in the cyanobacterium. The next chapter deals with isolation and characterization of a yet another mutant strain of *N. muscorum* resistant ot L-azetidine-2-carboxylate (a toxic analogue of L-proline) and the results have been discussed from this perspective.

Chapter-5

STUDIES ON PROLINE UPTAKE AND PROLINE METABOLISM IN A PROLINE OVER-ACCUMULATING STRAIN OF THE CYANOBACTERIUM *NOSTOC MUSCORUM*

5.1 INTRODUCTION

Osmoadaptable organisms like bacteria and plants respond to increased environmental osmolarity or salinity by synthesizing and accumulating a few compatible solutes like trehalose, sorbitol, mannitol, proline and betaine (glycinebetaine) which function as osmoprotectants and thus constitute the biochemical basis of their osmoadaptation (Csonka 1989; McCue & Hanson 1990; Lucht & Bremer 1994; Bartels & Nelson 1994). The genetic basis of osmotic adaptation in enteric bacteria has been studied in detail which includes *kdp*A-E genes required for inducible K uptake (Walderhaug et *al.* 1987), proU and proP genes required for transport of betaine and proline (Cairney et *al.* 1985), proA, B & C required for synthesis of proline (Jakowec et *al.* 1985; Mahan & Csonka 1983), otsA & otsB genes required for synthesis of trehalose (Giaver et *al.* 1988) and *bet* A, B & C genes required for transport of choline and synthesis of betaine from choline (Styrvold et *al.* 1986). *omp* genetic system has also been shown to contribute to osmotic adaptation through control of synthesis of porin proteins of bacterial plasma membrane (Csonka 1991).

Cyanobacteria are oxygenic photosynthetic prokaryotes and recent studies have identified trehalose, sucrose or glucosyl-glycerol as osmoregulators characteristic of fresh water or marine forms and betaine (glycinebetaine) as osmoregulator characteristic of hypersaline forms (Mackay et *al.* 1984; Warr et *al.* 1988). Genetic engineering of osmotolerance for nitrogenase activity and diazotrophy has been achieved in *Klebsiella pneumoniae* (Le Rudulier et *al.* 1982). It has been previously reported that sucrose functions as osmoregulator in

the diazotrophic heterocystous cyanobacterium *N. muscorum* strain 7119 apparently without any increase in free-proline content (Blumwald & Tel-or 1982). Similar results were obtained in the case of fresh water *Gloeocapsa* spp. and *A. cylindrica* (Borowitzka 1981). It is also known that in few marine cyanobacteria glucosyl-glycerol is the major osmolyte (Erdmann et *al.* 1992), whereas in *Aphanothece halophytica* (Tindall et *al.* 1977) and *Plectonema tomasiniarum* isolated from salt lakes an increase in the cellular content of different amino acids (not including proline) appears to be responsible for osmoadaptation (Borowitzka 1980).

Accumulation of proline in response to osmotic stress has been well-documented in higher plants (Dix & Pearce 1981; Voetberg & Stewart 1984; Bhaskaran et *al.* 1985; Kapuya et *al.* 1985; Handa et *al.* 1986), algae (Brown & Hellebust 1980) and bacteria (Csonka 1989) but not in cyanobacteria. Many studies indicate a clear correlation between the degree of osmotic stress and the levels of proline accumulated. It is suggested that proline accumulation is a cellular adaptation to osmotic stress preventing damage from cellular dehydration by balancing the osmotic strength of the cytoplasm with that of the environment (Yancey et *al.* 1982). Proline may also prevent damage from cellular dehydration by increasing the water binding capacity of protein (Schobert & Tsechesche 1978).

Since the role of proline as osmo(salinity)-protectant has not so far been analyzed genetically or physiologically in any cyanobacterium, we approached this problem with the method already successful in bacteria (Csonka 1981) algae (Vanlerberghe & Brown 1987) and cyanobacteria (Riccardi et *al.* 1983) by isolating mutant clones of the cyanobacterium *N. muscorum*

resistant to growth inhibitory action of the L-proline analogue, L-azetidine-2-carboxylate (AC) and examining it for intracellular proline level, salinity and osmotic stress tolerance characteristics and nature of proline uptake process. The results presented in Chapter-4 assume significance in view of its being the first report for a role of proline in osmoprotection reported in any cyanobacterium. Further, evidences are presented in this chapter to show that cyanobacterial L-azetidine-2-carboxylate resistant (*Ac-R*) mutant is a proline accumulating strain and shows tolerance to salinity as well as osmotic stress (resulting from mutational inactivation of proline oxidase being the apparent cause of proline accumulation), and that osmo(salinity)-stimulable increase in the uptake of exogenous proline by the parent strain is the cause of its osmotic(salinity) tolerance in proline medium. Evidence is also presented to show that betaine (glycinebetaine) offers no protection to the cyanobacterium against salinity or osmotic stresses and is assimilated like a nitrogen source. In other words, proline has been found to be the sole salinity and osmotic protectant in *N. muscorum*. The results also clearly show a single gene-controlled nature of salinity and osmotic stress tolerance in the cyanobacterium. We also believe that this procedure may confirm use of classical mutational methods for genetic engineering of this trait in other cyanobacteria.

5.2 MATERIALS AND METHODS

Axenic clonal cultures of *N. muscorum* strain was maintained in combined nitrogen-free medium called diazotrophic medium as described in Chapter-2 (Section 2.1).

5.2.1 Isolation of L-Azetidine-2-carboxylate resistant *(Ac-R)* mutant c the parent Strain

L-Azetidine-2-carboxylate (AC) is a growth toxic analogue of L-proline and has been used in bacteria to isolate

L-Azetidine-2-carboxylate resistant (*Ac-R*) mutants defective in proline metabolism (Csonka 1981). Exponentially growing diazotrophic cultures of the cyanobacterium in quantities of 5 x 10 colony forming units (CFUs) were inoculated onto the diazotrophic medium containing 1 mol m AC, a dose ten-fold more toxic to the cyanobacterial diazotrophic growth. Inoculated plates along with the control plates were incubated in the growth chamber and colonies appearing on the nutrient plates after three weeks of such growth incubation were checked for the *Ac-R* phenotype following the method of Singh et *al.*, (1989). One such mutant colony was isolated and maintained on nutrient slant containing the proline analogue.

5.2.2 Physiological characterization of the parent and mutant strains

The cultures of the parent and *Ac-R* mutant were grown diazotrophically or with 1 mol m proline. Such cultures were assesed for growth, chlorophyll a, protein, heterocyst formation and heterocyst frequency, survival characteristics and nitrogenase activity as described in Chapter-2 (Sections 2.4, 2.5, 2.6, 2.7, 2.8 & 2.9 respectively). The method of Bates et *al.* (1971) was used to estimate intracellular levels of proline under normal, osmotic stress and salinity stress conditions, while ^{14}C-proline was used to measure its uptake as already described in Chapter-4 (Sections 4.2.3 & 4.2.4 respectively).

5.2.3 Determination of proline oxidase activity

Proline oxidase activity was measured by the method of Dendinger & Brill (1970) with slight modification as described by Spence & Stewart (1986). 6-day old exponentially growing cultures were harvested and permeabilized in 1 cm of 100 mol m sodium cacodylate buffer (pH 6.6) for 5 min using 0.3 cm of

toluene at 30 C and then centrifuged at low speed. The pellet was resuspended in 1 cm^3 of sodium cacodylate buffer to which 1 cm^3 of 1000 $mol\ m^{-3}$ L-proline and 0.2 cm^3 of 50 $mol\ m^{-3}$ aminobenzaldehyde were added. The reaction was terminated with TCA. The reaction mixture was incubated in dark for 10 min and then centrifuged at low speed and the absorbance of the supernatant read at 443 nm.

5.2.4 Chemicals used

^{14}C-proline was obtained from Board of Radiation and Isotope Technology (BRIT), India. All other chemicals used in the present study were obtained either from M/s Sigma Chemical Co., USA or British Drug House (BDH), India.

5.4 RESULTS

Availability of bacterial mutants resistant to various proline analogues has provided incontrovertible evidence for the role of proline as sole source of carbon, nitrogen or salinity or osmotic protectant in members of enterobacteria (Csonka & Hanson 1991). The spontaneous mutant of *N. muscorum* resistant to the growth inhibitory action of L-azetidine-2-carboxylate *(Ac-R)* arose with a frequency of 0.8 × 10 . One of the *Ac-R* clones thus obtained was grown in bulk for comparison with its parent in respect of growth, heterocyst frequency, nitrogenase activity and proline oxidase activity (Tables 5.1a & b) . As shown in Table-5.1b, it grew slightly better diazotrophically and less well with proline than its parent (Table 5.1a) under parallel growth conditions. A microscopic examination revealed the *Ac-R* mutant strain producing heterocysts both during growth with N_2 or proline as nitrogen source with almost similar magnitude, while its parent showed no heterocysts in growth medium containing proline as nitrogen source (Plates I & II) . The *Ac-R* strain also showed characteristic nitrogenase activity associated with heterocyst formation in growth medium containing either N_2 or proline, while the parent strain showed such an activity in

Table 5.1a

Growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells), nitrogenase (N_2ase) activity (m mol C_2H_4 formed g^{-} Chl *a* h~) and proline oxidase (Prox.) activity (m mol proline oxidized g^{-1} Chl *a* h^{-1}) of the parent *N. muscorum* in different nitrogen growth media.

Medium	Growth	HF%	N_2ase activity	activity
N_2-medium	0.68 ± 0.06	5 - 6	9.8 ± 0.7	2.61 ± 0.24
+ 1 mol m^{-3} NH_4Cl	0.71 ± 0.03	0.0	0.0	2.33 ± 0.2
+ 1 mol m~^{3} Betaine	0.64 ± 0.03	0.0	0.0	2.4 t 0.24
+ 1 rool m^{-3} Proline	0.84 ± 0.06	0.0	0.0	4.3 ± 0.27

1 mol m proline grown cultures were source of inocula for the experiments. Such inocula were grown for six days in respective media and then used for estimation of their characteristics. Each reading is an average (± SEM) of three independent experimental determinations.

Table

Growth (optical density at 663 nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells), nitrogenase (N_2ase) activity (m mol C_2H_4 formed g^{-} Chl a h^{-}) and proline oxidase (Prox.) activity (m mol proline oxidized g^{-1} Chl a h^{-1}) of the *Ac-R* mutant strain of *N. muscorum* in different nitrogen growth media.

Medium	Growth	HF%	N_2ase activity	Prox. activity
N_2-medium	0.71 ± 0.05	5 - 6	10.4 ± 1.0	0.0
+ 1 mol m^{-3} NH_4Cl	0.72 ± 0.03	0.0	0.0	0.0
+ 1 mol m^{-3} Betaine	0.65 ± 0.04	0.0	0.0	0.0
+ 1 mol m^{-3} Proline	0.78 + 0.07	5 - 6	9.6 + 1.3	0.0

1 mol m^{-} proline grown cultures were source of inocula for the experiments. Such inocula were grown for six days in respective media and then used for estimation of their characteristics. Each reading is an average (± SEM) of three independent experimental determinations.

Plate I
Filaments of parent *N. muscorum* in diazotrophic (N_2) medium (A) and in growth medium containing 1 mol m^{-3} proline (B).

1 mol m NH -grown cultures were the source of inocula. Such inocula were grown for six days in diazotrophic medium (A) and in growth medium containing 1 mol m proline (B), before microscopically examining them.

Plate-1

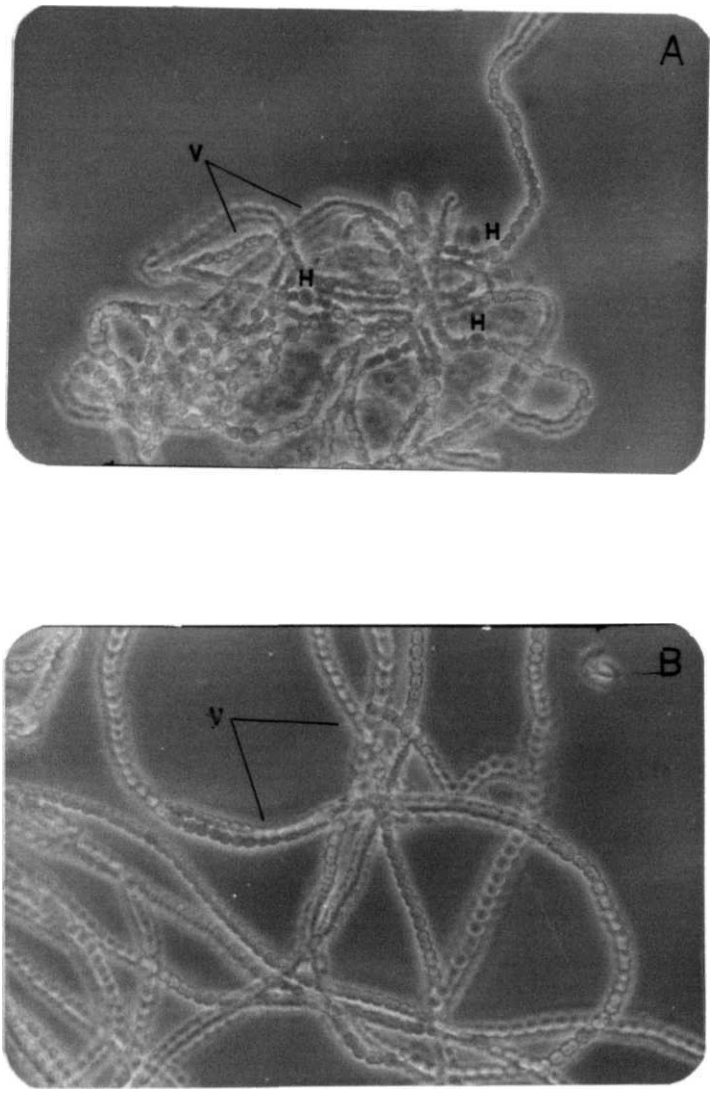
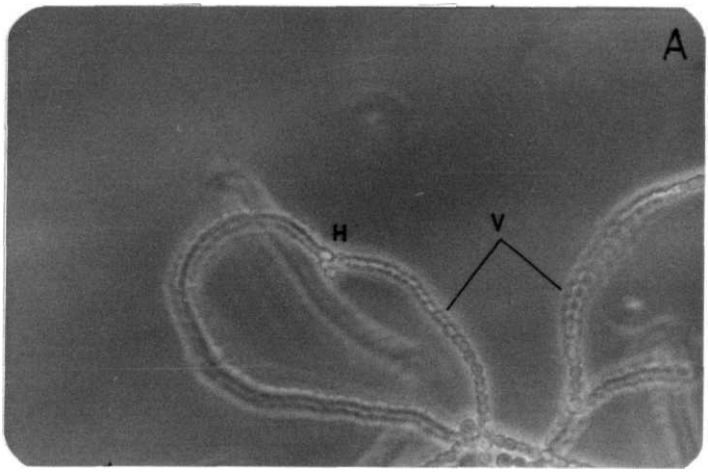


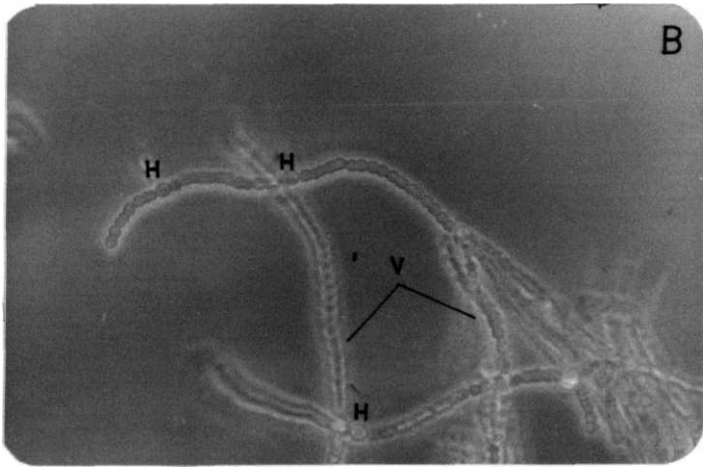
Plate II

Filaments of the *Ac-R* mutant strain of *N. muscorum* in diazotrophic (N_2) medium (A) and in growth **medium** containing 1 **mol m** proline (B).

1 **mol** m~ NH_4^+-**grown** cultures were the source of inocula. Such inocula were grown for six days in diazotrophic medium (A) and in growth medium containing 1 mol m proline (B) , before microscopically examining them.

Plate-II





diazotrophic medium alone (Tables 5.1a & b) . Evidently, lack of heterocyst and nitrogenase activity in proline grown culture of **the parent strain** coupled with its ability to **grow** better than its *Ac-R* strain in proline medium suggests that *N. muscorum* is capable of assimilating proline as nitrogen source and that its mutation to *Ac-R* phenotype has resulted in loss of this ability. Further, analysis of the two cyanobacterial strains in respect of proline catabolizing enzyme proline oxidase revealed the presence of this enzyme in the parent and its absence from the *Ac-R* mutant. Also, the mutational lack of proline oxidase activity associated with the lack of proline assimilation in the *Ac-R* strain strongly implicates a definite role of the oxidase enzyme in cyanobacterial assimilation of proline as nitrogen source.

The next series of experiments were conducted to estimate the intracellular level of proline in diazotrophically grown cultures of the parent and *Ac-R* strains under normal growth conditions as well as under salinity stress condition. As shown in Fig. 5.1, the intracellular proline level always remained about 3-4 fold higher in the *Ac-R* mutant strain than in the parent strain. However, salinity or osmotic stress did not seem to cause significant variation in the intracellular level of proline in either strain. Hence, the first conclusion is that salinity or osmotic stress does not seem to substantially influence the intracellular level of proline in the cyanobacterium, and second, that mutational loss of proline oxidase activity apparently seems to be the reason for about 3-4 fold **higher** intracellular level of proline in the *Ac-R* strain. This also indicates a role of proline oxidase activity in regulation of intracellular proline level in the cyanobacterium and third since both the parent and the mutant strain exhibit similar level of heterocyst formation and nitrogenase activity despite the latter having a 3-4 fold higher level of

Fig. 5.1
Intracellular levels of proline in **diazotrophic medium (——)** and in diazotrophic medium containing 75 **mol m NaCl (——)** in the parent ***N. muscorum*** (0) and in its ***Ac-R*** mutant **strain** (D).
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

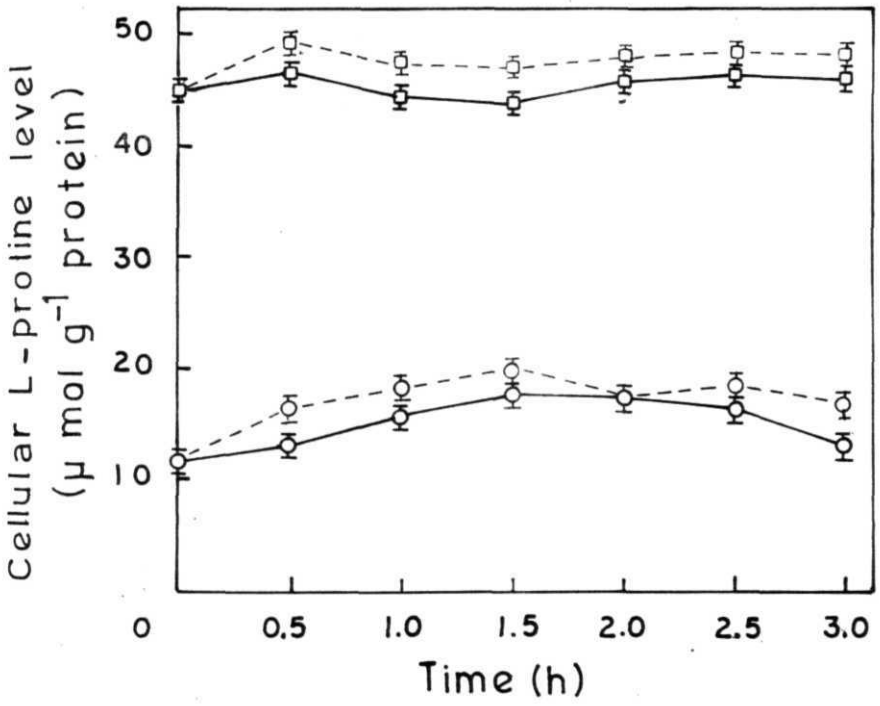

Cellular L-proline level
(μ mol g^{-1} protein)
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
Time (h)

Fig.5.1

intracellular proline internal proline *per se* is not the heterocyst-nitrogenase inhibitor in the cyanobacterium.

Table 5.2 and Fig 5.4 shows the degree of survival of the parent and the *Ac-R* strains to salinity and osmotic stresses under different nitrogen treatments. The degree of survival under unstressed conditions on diazotrophic growth medium was considered to be 100%. A dose of 100 mol m~ NaCl or 250 mol m~ sucrose was lethal to the parent strain. In comparison, the *Ac-R* strain showed nearly 90% salinity survival or 94% osmotic survival under such stress conditions. The mutation to *Ac-R* phenotype thus appears to have caused development of both salinity tolerance and osmotic tolerance in it. This also means that the mechanism causing salinity tolerance and osmotic tolerance in the cyanobacterial mutant strain is apparently same. Exogenous proline was found very effective in counteracting salinity/osmotic stress lethality in the parent strain. In this regard, the *Ac-R* mutant strain remained uninfluenced by addition of proline. The apparent lack of osmoprotective effect of proline in the *Ac-R* mutant might be the result of endogenous accumulation of an osmoprotectant like proline.

Subsequently, experiments were conducted to examine the role of salinity and osmotic stress in regulation of proline uptake and accumulation in the two strains. As shown in Fig. 5.2, proline uptake and accumulation were extremely sensitive to the uncoupler (CCCP) of photophosphorylation thus suggesting that the cyanobacterial proline uptake and accumulation process is an active energy-requiring process. Salinity stress of 75 mol m^{-} NaCl or osmotic stress of 200 mol m sucrose resulted in rise of proline uptake activity by about 7-fold in the *Ac-R* strain and by about 9-fold in the parent strain over a period of 10 min. Evidently, the proline uptake system in *N. muscorum* is osmo(salinity)-stimulable and its mutation to *Ac-R* phenotype has left its osmo(salinity)-

Table 5.2

Per cent survival of the parent and ***Ac-R*** **mutant** strains of ***N. muscorum*** in different nitrogen **media** under salinity and osmotic stress conditions.

Medium	Parent	***Ac-R***
N_2-medium	100	100
+ 100 mol m~^{3} **NaCl**	0	90
+ 100 **mol m^{-3}** NaCl + 1 **mol m** Betaine	1	90
+ 100 mol **m^{-3}** NaCl + 1 **mol m** Proline	87	95
+ 250 **mol** m~ Sucrose	2	94
+ 250 **mol m** Sucrose + 1 **mol** m Betaine	5	95
+ 250 **mol m** Sucrose + 1 mol m~ Proline	93	95

6-day old diazotrophically grown cultures of the two strains were used as source of inocula for the experiments.
Each reading is an average of three independent experimental **determinations.**

Fig 5.2

Effect of 75 mol m^{-3} NaCl, 200 mol m^{-3} sucrose and 0.01 mol m^{-3} CCCP on uptake of ^{14}C-proline in the parent *N. muscorum* and in its *Ac-R* mutant grown diazotrophically.

(O——O), Untreated cultures of parent strain

(●——●), Parent strain stressed with NaCl

(●——•) , Parent strain stressed with sucrose

(O——O), Parent strain treated with CCCP

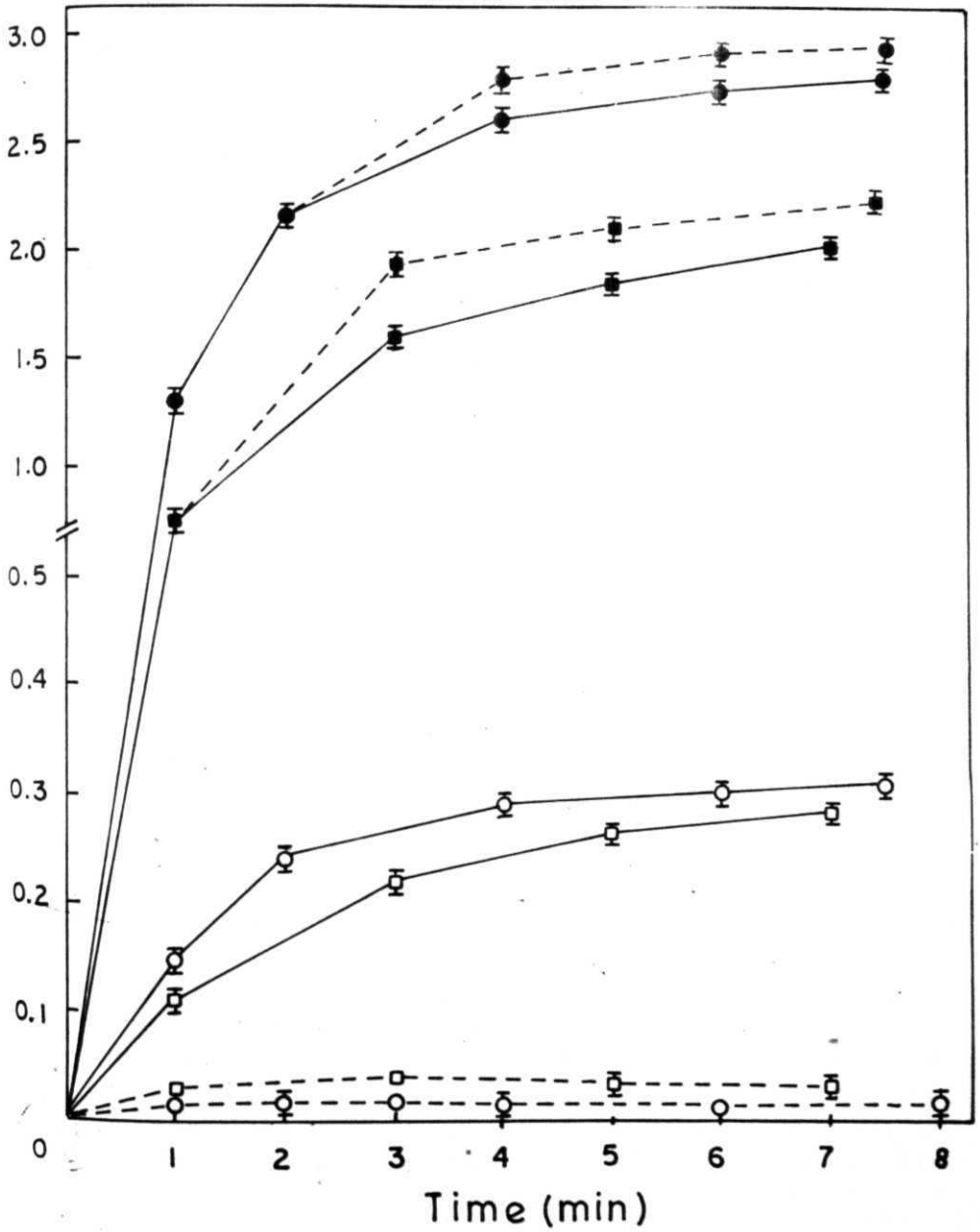
(□——o), Untreated cultures of the *Ac-R* mutant strain

(■——•), *Ac-R* mutant strain stressed with NaCl

(■——•) , *Ac-R* mutant strain stressed with sucrose

(□——□), *Ac-R* mutant strain treated with CCCP

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


3.0
2.5
2.0
1.5
1.0
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
Time (min)

stimulable proline uptake process almost unaffected. Thus, the *Ac-R* mutant does not seem to have suffered genetic damage in its normal or osmo(salinity)-regulated proline transport activity.

The two cyanobacterial strains were also investigated for the response of their nitrogenase activity to salinity stress of 75 mol m NaCl and osmotic stress of 200 mol m sucrose (Fig. 5.3). While the enzyme activity in the parent declined with duration of salinity/osmotic treatment and reached almost zero value by 6 h of such treatment, that in the *Ac-R* strain remained almost uninfluenced by such treatment. Clearly, the mutation to *Ac-R* phenotype in the cyanobacterium has been accompanied by loss of the inhibitory effect of salinity/osmotic stress on its nitrogenase activity. Since the *Ac-R* strain unlike its parent, accumulates proline, the resistance of its nitrogenase activity to salinity and osmotic stresses is quite expected in view of the known role of intracellularly accumulated proline in salinity and osmotic protection.

The most interesting results came with studies on betaine (glycinebetaine). The parent strain while differentiating heterocyst and showing nitrogenase activity in N -medium did not show the same in medium containing either NH , betaine or proline, thus suggesting that betaine like NH and proline is being metabolized like a nitrogen source. This is further confirmed by the absence of nitrogenase activity in the parent strain growing in medium containing NH , betaine or proline. *Ac-R* strain however while showing heterocysts and nitrogenase activity in proline medium associated with loss in proline oxidase activity was nonetheless similar to the parent strain in assimilation of betaine or NH as a nitrogen source (Table 5.1a). Addition of betaine to the growth medium did not counter the lethal action imposed by salinity or osmotic stresses

Fig. 5.3
Effect of 75 mol m^{-3} NaCl and 200 mol m^{-3} sucrose on nitrogenase activity of the parent *N. muscorum* and its *Ac-R* mutant.
(●——•) Parent strain stressed with NaCl
(●——•) Parent strain stressed with sucrose
(■——•) *Ac-R* mutant strain stressed with NaCl
(■——•) *Ac-R* mutant strain stressed with sucrose
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

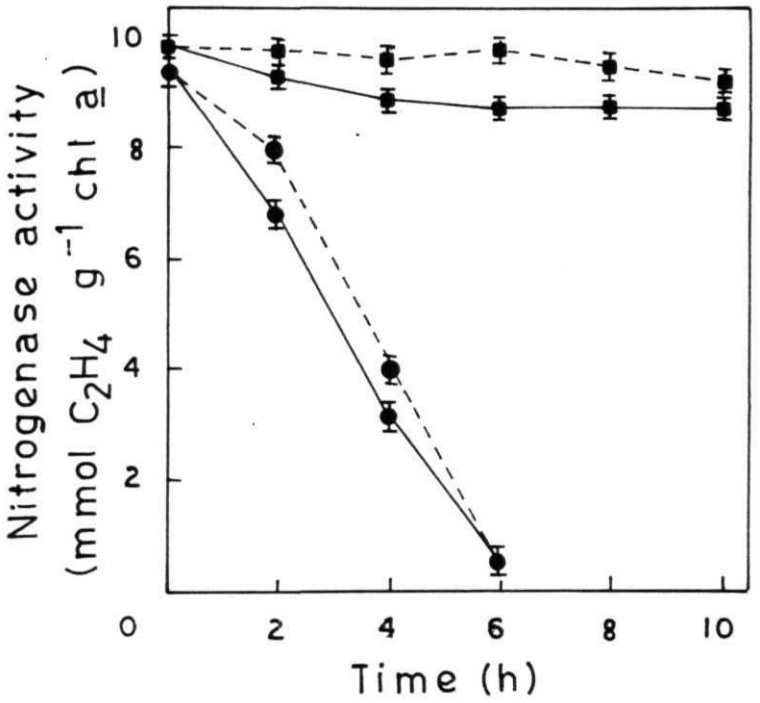

Nitrogenase activity
(m mol C_2H_4 g^{-1} chl a)
10
8
6
4
2
0
2
4
6
8
10
Time (h)

Fig.5.3

Fig. 5.4
Per cent survival of the parent *N. muscorum* (0), its *NaCl-R* mutant (a), *Sucrose-R* mutant (A) and *Ac-R* mutant (▼) strains to increasing concentrations of NaCl (——) and sucrose (——). Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

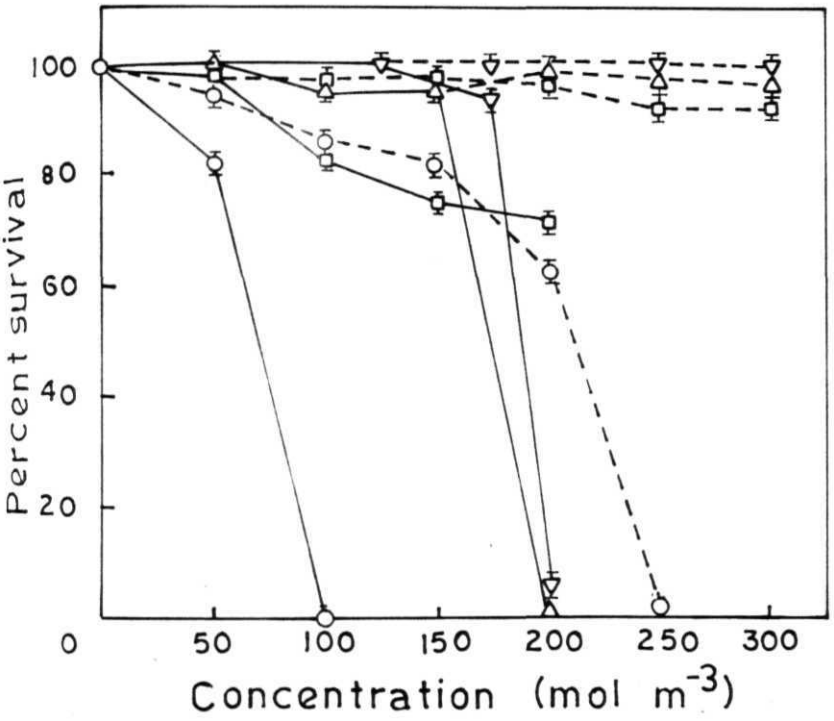

Percent survival
100
80
60
40
20
0
50
100
150
200
250
300
Concentration (mol m^{-3})

Fig. 5.4

in a way proline did to the parent strain while the *Ac-R* strain remained uninfluenced (Table 5.2). Thus, exogenous betaine fails to function as an osmo(salinity)-protectant in the cyanobacterium.

Since both betaine and proline are transported by the ProP uptake system in bacteria (Csonka 1989) and since exogenous proline in the parent strain and internal proline content in the *Ac-R* strain was found to offer protection to them under salinity and osmotic stress conditions, it was thought proper to study the effect of exogenous betaine on proline uptake characteristics. The uptake of ^{14}C-proline was followed upto 10 min. However, for clarity and easy comprehension, values of uptake at the 10th min alone were presented. For experiments involving preincubation with betaine, they were incubated for 30 min after which ^{14}C-proline was added and uptake followed for 10 min. As can be seen from Tables 5.3a & b, betaine pretreatment or simultaneous treatment of the samples did not affect the proline uptake process under unstressed and salinity/osmotic stressed conditions, thus suggesting lack of a common transport for betaine and proline in the cyanobacterium.

Mutant strains of the cyanobacterium selected for resistance to salinity and osmotic stresses as described in Chapter 4 (section 4.2.1) were compared with the parent and *Ac-R* mutant strains in respect of percent survival and percent nitrogenase activity (Table 5.4). The survival and nitrogenase activity of the four strains under normal diazotrophic growth conditions was taken as 100%. *NaCl-R* strain like *Ac-R* strain was also resistant to osmotic inhibition. Similarly, the *Sucrose-R* strain like the *Ac-R* strain was found resistant to salinity stress while the parent strain was extremely sensitive to both salinity as well as osmotic stresses. Evidently, mutational production of salinity/osmotic resistant strains of the cyanobacterium is possible and that salinity tolerant and osmotic

Uptake of ^{14}C-proline (m mol proline g^{-1} Chl a) by parent *N. muscorum* under unstressed, salinity stressed (75 mol m^{-3} NaCl) and osmotic stressed (200 mol m sucrose) conditions.

Treatment	Unstressed	NaCl stress	Sucrose stress
N$_2$-medium	0.25 ± 0.002	2.3 ± 0.016	2.5 t 0.12
+ 0.05 mol m Betaine	0.27 i 0.004	2.3 ± 0.22	2.1 ± 0.24
+ 1 mol m Betaine	0.24 ± 0.045	2.6 ± 0.17	2.4 ± 0.21
+ 0.05 mol m~ Betaine (preincubated)	0.22 ± 0.02	2.3 ± 0.17	2.4 ± 0.22
+ 1 mol m Betaine (preincubated)	0.27 ± 0.022	2.4 ± 0.27	2.9 ± 0.26
+ 0.01 mol m CCCP	0.012 ± 8 x 10~^{5}	-	-

6-day old diazotrophic cultures were the source of inocula. Salinity or osmotic stress conditions were induced at zero time. Treatment with betaine or CCCP and addition of C-proline to such cultures were also done at zero time. For experiments involving preincubation of cultures with betaine, the duration of preincubation was for 30 min.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 5.[illegible]

Uptake of ^{14}C-proline (m mol proline g Chl a) by the *Ac-R* mutant strain of *N. muscorum* under unstressed, salinity stressed (-3 mol m NaCl) and osmotic stressed (200 mol m^{-3} sucrose) conditions.

Treatment	Unstressed	NaCl stress	Sucrose stress
N$_2$-medium	0.22 ± 0.003	1.6 ± 0.029	2.2 ± 0.12
+ 0.05 mol m^{-3} Betaine	0.25 ± 0.001	1.7 ± 0.12	1.6 ± 0.18
+ 1 mol m^{-3} Betaine	0.20 ± 0.015	1.6 ± 0.17	1.6 ± 0.05
+ 0.05 mol m^{-3} Betaine (preincubated)	0.23 ± 0.006	1.8 ± 0.15	1.7 ± 0.11
+ 1 mol m^{-3} Betaine (preincubated)	0.25 ± 0.023	1.8 ± 0.13	1.6 ± 0.08
+ 0.01 mol m^{-3} CCCP	0.018 ± 6 x 10^{-5}		

6-day old diazotrophic cultures were the source of inocula. Salinity or osmotic stress conditions were induced at zero time. Treatment with betaine or CCCP and addition of ^{14}C-proline to such cultures were also done at zero time. For experiments involving preincubation of cultures with betaine, the duration of preincubation was for 30 min.
Each reading is an average (± SEM) of three independent experimental determinations.

Table 6.

Cross-resistance relationship between the various strains of *N. muscorum* (in terms of per cent survival and per cent nitrogenase activity relative to the parent strain) under unstressed, salinity (100 mol m NaCl) and osmotic stressed (250 mol m sucrose) diazotrophic growth conditions.

Strains and characteristics	unstressed	NaCl stress	Sucrose stress
Parent			
% survival	100	0	2
% nitrogenase activity	100	0	0
Ac-R			
% survival	100	85	92
% nitrogenase activity	100	88	90
NaCl-R			
% survival	100	100	95
% nitrogenase activity	100	96	97
Sucrose-R			
% survival	100	90	100
% nitrogenase activity	100	92	94

About 10 CFU's per nutrient plate were inoculated for estimation of per cent survival under various growth conditions. Nitrogenase activity was measured in 6-day old diazotrophically grown cultures treated with and without NaCl or sucrose for 6 h and then expressed as per cent of control.
Each reading is an average of three independent experimental determinations.

tolerant strains are cross-tolerant in terms of their survival and diazotrophic characteristics.

5.4 DISCUSSION

Many organisms belonging to bacteria (Csonka & Hanson 1991), cyanobacteria (Warr et *al.* 1988), algae (Brown & Hellebust 1980), fungi (Jennings & Burke 1990) and higher plants (Bartels & Nelson 1994) intracellularly accumulate one or more low-molecular weight organic compounds called compatible solutes, so as to maintain osmotic balance of their cytoplasm against high osmolarity of the environment. The role of proline as an effective protectant of bacteria against osmotic or salinity stress was first shown by Christian (1955) and since then genetic evidence has been obtained in bacteria (Csonka 1981) and in plants (Sumaryati et *al.* 1992) for the participation of single genes in intracellular accumulation or overproduction of proline leading to generation of their osmotolerant strains. The spontaneous mutational frequency of the *Ac-R* phenotype suggests that a single mutational event is the cause of its origin in the cyanobacterium. The other characteristics associated with the *Ac-R* phenotype in the cyanobacterium include loss of L-proline oxidase activity, lack of proline inhibitory effect on heterocyst differentiation and nitrogenase activity and intracellular accumulation of proline and genetic acquisition of salinity tolerance.

The parent strain grew better in proline medium than in N_2-medium and showed more activity of proline oxidase enzyme in growth medium containing proline than in the diazotrophic medium. The ability of the parent strain to show more proline oxidase activity without producing N_2-fixing heterocysts in N_2 + proline medium than in N_2-medium where it produces N_2-fixing heterocysts

suggests that the cyanobacterium utilizes proline as sole source of nitrogen through a pathway involving proline oxidase enzyme and that such a pathway of proline utilization functions in proline inhibition of heterocyst formation and nitrogenase activity. The lack of proline oxidase activity in the *Ac-R* mutant combined with its inability to utilize proline as a nitrogen source as well as its inability to show proline inhibition of heterocyst formation and nitrogenase activity clearly show the essentiality of proline oxidase in assimilation of proline as nitrogen nutrient in the cyanobacterium. That proline oxidase enzyme is essential for proline assimilation as nitrogen source leading to repression of heterocyst formation and nitrogenase activity in the cyanobacterium is fully supported by the observed behaviour of grwoth of the proline oxidase deficient *Ac-R* mutant in growth medium containing proline producing heterocysts and nitrogenase activity. Such an essential role of proline oxidase in proline catabolism has already been reported in *Anabaena* PCC 7120 (Spence & Stewart 1986). Proline has also been shown to be assimilated as a fixed nitrogen source causing inhibition of both heterocyst formation and nitrogenase synthesis in *N.* muscorum (Singh et *al*. 1991). Since the *Ac-R* mutant forms N -fixing heterocysts even in growth medium containing proline, N remains the sole source of nitrogen for its growth under such a condition.

An organism resistant to an amino acid analogue may overcome the toxic effect of the antimetabolite by developing a discrimanatory mechanism that either prevents the entry into the cell of the analogue or that selects at the level of protein synthesis an amino acid against the analogue. Alternatively the resistant cell may degrade the analogue or it may alter the regulation of the pathway leading to biosynthesis of the parental amino acid. The latter case results in overproduction of amino acid in the cell. Overproduction of amino acids is a common feature of microbial resistance to analogues of amino acids

(Fowden et *al.* 1967; Umbarger 1971; Yamada et *al.* 1972). The possible cause of *Ac-R* phenotype in various biological system include:

a) impairment in uptake system of AC (Fowden et *al.* 1967);

b) proline overproduction by regulatory alterations in its biosynthetic pathway (Csonka 1989) or in its degradative pathway (Stewart 1977) leading to dilution of the growth inhibitory effect of AC;

c) enzymatic degradation of AC (Fowden et *al.* 1967);

d) impairment in AC metabolizing pathway that generates a toxic product from AC (there is no previous example published).

The cyanobacterial *Ac-R* mutant is a product of a single mutational event and its ability to grow in hyperosmotic (hypersaline) medium demonstrates that the osmotic (salinity) stress tolerance of the mutant is not necessarily dependent on the interactions of a large array of gene products, but simply results from a single mutational event that causes loss of proline oxidase enzyme activity leading to a rise in intracellular proline level. Mutational inactivation of proline oxidase enzyme of the proline catabolic pathway is thus one of the mechanisms of proline accumulation and thereby of osmotic (salinity) tolerance in *N. muscorum.* A similar mechanism of proline accumulation and osmotolerance and salinity tolerance has been reported in barley leaves (Stewart et *al.* 1977). Single gene *Ac-R* mutants of *Salmonella typhimurium* have been found to be proline overproducing as well as osmotolerant and salinity tolerant and the mechanism involved in such overproduction has been shown to result from mutational loss of the inhibitory effect of proline on γ-glutamyl kinase, the first enzyme of its biosynthetic pathway from glutamate (Csonka 1981; Csonka, 1989). A similar mechanism of proline overproduction and osmotolerance has been demonstrated in higher plants as well (Bartels &

Nelson 1994). L-Azetidine-2-carboxylate is a growth toxic analogue of proline and the absence of its toxicity in the *Ac-R* strain of *N. muscorum* might result from dilution of its inhibitory effect by higher level of intracellular proline accruing from absence of the proline catabolic enzyme proline oxidase, in it. Although high intracellular proline level in the present case is the cause of resistance to L-Azetidine-2-carboxylate toxicity in the mutant strain, it differs from earlier studies in that the present strain shows a high intracellular level of proline resulting from mutational block in proline catabolism leading to overaccumulation of proline, while in the earlier studies it is the result of overproduction of proline resulting from loss of feed-back inhibition on the biosynthetic pathway of proline by proline. The *Ac-R* phenotype in S. *typhimurium* has already been shown to result from overproduction of proline by the biosynthetic pathway (Csonka 1981).

Intracellular accumulation of proline can result from increased net synthesis, from genetic or physiological inhibition of catabolism or by enhanced uptake from the medium. Hypersalinity does not affect the intracellular level of proline in parent or *Ac-R* strain of *N. muscorum* but does stimulate considerably the uptake of exogenous proline by both the strains. Since exogenous proline is found reversing almost completely the NaCl lethality in the parent strain, osmo(salinity)-stimulated uptake **and** accumulation of exogenous proline is apparently the mechanism of salinity and osmotolerance in the cyanobacterium. Osmo- or salinity-stimulated uptake and accumulation of proline leading to acquisition of osmo and salinity tolerance in enterobacteria is a well known mechanism of osmoadaptation (Csonka 1989). The impact of osmo(salinity)-stimulated uptake of exogenous proline on osmotic/salinity survival of **the** *Ac-R* mutant is apparently not seen here, as it accumulates proline intracellularly to a level sufficient to overcome salinity stress

of 150 mol m^{-3} NaCl and osmotic stress of 250 mol m^{-3} sucrose. This is further confirmed by the fact that while nitrogenase activity of parent strain declines to almost zero value with 75 mol m^{-3} NaCl stress for 6 h or 200 mol m^{-3} sucrose for 6 h, that of the *Ac-R* strain continues functioning almost normally under similar stress conditions.

Betaine, more popularly known as glycinebetaine (N,N,N-trimethylglycine) is a major osmolyte in a number of photosynthetic organisms including both eukaryotes and prokaryotes (Storey & Wyn Jones 1977; Hanson & Hitz 1982; Gorham et *al*. 1985; Reed et *al*. 1986). Betaine is not used as a carbon or nitrogen source in enetric bacteria (Le Rudulier & Bouillard 1983), while it can be used as both only in media of low osmolarity (Smith et *al*. 1988). Few prokaryotes are able to carry out *de novo* synthesis of betaine (Reed & Stewart 1985; Imhoff 1986) and is shown to be regulated by osmotic stress (Brouquisse et *al*. 1989). Others are dependant on transport of this compound for its accumulation. Genetic engineering of betaine pathway of important crops has been suggested to be one of the possible solutions to osmotic stress problem afflicting them (McCue & Hanson 1990). The transport of betaine is mediated by the proP and proU systems which also mediate transport of proline (Csonka 1989). Le Rudulier & Bouillard (1983) have shown that nitrogenase activity of *K. pneumoniae* is enhanced by exogenous betaine at high osmolarity. Reed et *al*., (1984) have shown betaine to be accumulated as a major osmolyte in the highly halotolerant cyanobacterial forms when challenged with osmotic stress. However, the regulation of betaine accumulation as well as its metabolism in cyanobacteria are very poorly understood. Since in the present study exogenous proline was found to offer Protection to the cyanobacterium against both salinity and osmotic stresses, it was thought proper to assess the role of

betaine as well in this cyanobacterium. The results clearly show that exogenous betaine doesnot offer any protection to the cyanobacterium under hyperosmotic or hypersalinity conditions and is utilized as nitrogen source under normal conditions. Such osmoprotective function of betaine could be possible only when the organism in question is unable to metabolize it like a nitrogen source. There has been no previous report on the utilization of proline as a nitrogen source in any cyanobacterium and it is in this light the present study assumes significance. In this regard, it is interesting to note that prolinebetaine (a quaternary ammonium compound) is found to function as a nitrogen nutrient or as an osmoprotectant in *R. meliloti* depending on the osmolarity of the growth medium (Gloux & Le Rudulier 1989). There seems to be no differences between *£. coli* and S. *typhimurium* with respect to the genetics of betaine and proline uptake. Furthermore, there appears to be a general consensus among workers in the field that the main permease of the proline catabolism encoded by the *putP* gene is not involved in the osmotic control of proline uptake (Csonka 1989). In enterobacteria, betaine is transported by the transport systems encoded by proP and proU which also transport proline and are osmoinducible systems. Since nothing is known about the multiplicity of proline porters in any cyanobacterium, it is still premature to make any significant conclusion except that proline and betaine do not share a common transport system in this cyanobacterium.

Increased synthesis of proline (Csonka 1981) or betaine (Landfald & Strom 1986; Styrvold et *al.* 1986) or the uptake of these compounds supplied exogenously (Cairney et *al.* 1985a 1985b; Christian 1955; Csonka 1989; Le Rudulier & Bouillard 1983; Perroud & Le Rudulier 1985) can markedly enhance growth rates at high osmolarities. Proline overproduction has been reported to increase osmotolerance in mutants of *Salmonella* (Csonka 1981), *Serratia* (Sugiura & Kisumi 1985) and the cyanobacterium *Spirulina*

(Riccardi et *al.* 1983). In contrast, proline overaccumulation did not result in enhanced osmotolerance in the green alga *Nannochloris* (Vanlerbeghe & Brown 1987). In higher plants it is still debatable whether or not higher proline levels can confer increased osmotolerance (Bhaskar et *al.* 1985; Dix & Pearce 1981; Riccardi et *al.* 1983; Kueh & Bright 1982; Kapuya et *al.* 1985). Levels of **free-proline** have been proposed as a selection criterion to produce more salt and/or drought resistant cultivars (Wyn Jones et *al.* 1984). It is still unclear whether such an approach would be useful. To date most of the studies have been carried out on organisms which normally accumulate proline in response to osmotic or salinity stresses. The studies with prokaryotes (Csonka 1981; Sugiura & Kisumi 1985; Riccardi *et al.* 1983) suggest that a better approach may be to select proline accumulating cell lines from organisms which do not normally accumulate proline in response to osmotic stress and increased osmotolerance may be confered on such cell lines as a result.

Chapter-6

EVIDENCE FOR AN ENHANCED H^+-GRADIENT DEPENDENT ALKALI CATION EFFLUX SYSTEM IN CONFERING RESISTANCE AGAINST ALKALI METAL STRESS AS WELL AS ALKALINE pH STRESS IN THE CYANOBACTERIUM *NOSTOC MUSCORUM*

6.1 INTRODUCTION

Response of alkali metals in general is discriminatory exhibiting growth requirement for some and inhibitory to others. While Na is essential for cellular transport of solutes (Padan & Schuldiner 1994), high concentrations of NaCl is known to cause both ionic and osmotic stresses to biological systems (Csonka 1989). Na is an essential requirement for diazotrophy (Thomas & Apte 1984) and autotrophy (Allen & Arnon 1955; Apte & Thomas 1987; Miller et *al.* 1984) in cyanobacteria. The autotrophic requirement of Na for cyanobacterial photosynthesis has been shown to be due to its role in transport of bicarbonate ions (Espie et *al*. 1988; Kaplan et *al*. 1989). The primary targets sensitive to Na starvation are three independent anion transporters (symports) namely, HCO_3^- (Padan & Vitterbo 1988; Kaplan et *al*. 1989), NO_3^- (Lara et *al*. 1993) and PO_4^{3-} (Walker & Sanders 1991). A Na requirement for chloride transport has also been reported in cyanobacteria (Ritchie 1992). Studies have also demonstrated a role for Na in cyanobacterial pH homeostasis in addition to its role in cyanobacterial growth and photosynthesis (Miller et *al* 1984). However, recently an inducible Na -independent HCO_3^- transport system has been shown in the cyanobacterium *Synechococcus* UTEX 625 (Espie & Kandasamy 1992). Li ions are toxic to *E. coli* cells resulting mainly from its inhibitory effect on the activity of pyruvate kinase enzyme (Umeda et *al*. 1984). Li^+ has also been found to inhibit cyanobacterial growth by inhibiting competitively the Na^+-bicarbonate symport system (Espie et *al*. 1988).

K is an essential nutrient for plants and microbial growth, pH homeostasis and in osmoregulation (Schahtmann & Schroeder 1995). Studies on molecular aspects of Na^+ and K requirement for cyanobacterial growth have been wanting in detail. K is known to be essential for cyanobacterial growth and this essentiality has been attributed to its role as general activator of enzyme activity and regulation of cell osmolarity (Reed & Walsby 1985; Reed & Stewart 1985) . There is one report that suggests the replacement of K by Rb in the growth of cyanobacterium *Anacystis nidulans* (Kumar & Purohit 1972). Another study, after examining the similarity in the mechanism of K transport and Rb transport, has concluded that there are two K transport systems, one with low affinity showing almost negligible discrimination against Rb and the other with high affinity showing considerable discrimination against Rb (Reed et *al*. 1981). Cs is a known growth inhibitor of microbial and cyanobacterial systems (Avery et *al*. 1991; Singh et *al*. 1994).

Alkali cations are involved in maintenance of optimal membrane potential and cytoplasmic pH as also the cell turgor (Booth 1985). This is usually facilitated by coupling of their fluxes with H . Detailed studies on molecular biology of Na /H antiporters in *E. coli* and other bacterial systems have shown importance of increased activity of such antiporters in bacterial adaptation to Na^+ or alkaline pH stress and in signal transduction (Padan & Schuldiner 1994). In this connection, it is worth mentioning that a majority of cyanobacteria growing and multiplying in nature are known to prefer alkaline pH habitats. Na^+ requirement for general growth of cyanobacteria might arise from its role in cyanobacterial pH homeostasis under alkaline

conditions. Since Li resistant mutants of bacterial systems have thrown considerable light on the role of Na^+/H^+ antiporters in pH homeostasis under alkaline pH conditions (Padan & Schuldiner 1994), it was thought proper to isolate Li^+, Na^+, Rb^+ and extreme alkaline pH resistant mutants of *N. muscorum* and to use such mutants in understanding the problem of alkali metal/H^+ antiporter activity in relation to alkaline pH tolerance. This chapter describes the characteristics of such mutants in relation to their ability of tolerance to Li^+, Na^+, K^+ and Rb^+ and to alkaline pH 11.0 stress. CCCP, an inhibitor of H -gradient formation, has been used to ascertain the role of H -gradient in driving the alkali metal /H antiporter activity in the cyanobacterium.

6.2 **MATERIALS AND** METHODS

Axenic clonal cultures of the two strains of *N. muscorum* were grown and maintained as described in Chapter-2 (Section 2.1).

6.2.1 Isolation of alkali cation mutant strains

The parent strain did not survive beyond a concentration of 10^{-3} mol m LiCl, 10^{-3} mol m NaCl or 15^{-3} mol m RbCl. Accordingly, its diazotrophically grown cultures in the strength of 5.8 x 10^8 colony-forming units (CFUs) were seeded per diazotrophic nutrient plate containing 15 mol m LiCl or 100 mol m~ NaCl or 20 mol m^- RbCl to select out spontaneously occurring alkali cation resistant mutant clones. Colonies appearing on the respective nutrient plates containing the inhibitor were tested for their stability by streaking them on fresh inhibitor-containing nutrient plates. Stable lithium chloride resistant (Li^+-R), sodium chloride resistant (Na^+-R) and rubidium chloride resistant (*Rb -R)* mutant clones thus obtained were grown and maintained like the parent strain under parallel diazotrophic growth conditions for and used for further

experiments described below.

6.2.2 Isolation of *pH,, -R* mutant strain

Since it was found that *Li -R* mutant clones showed a hundred percent survival at pH 11.0, selection for pH 11.0 tolerance was made as follows. The parent strain showed a poor growth at pH 11.0 and a survival of about 44%. A preliminary examination showed that few pin-head sized colonies grew better on pH 11.0 than the others. Accordingly, one such colony was selected and streaked onto a fresh nutrient plate of pH 11.0 and scored for its survival. From this plate another better growing colony was selected and the procedure repeated. After five such consecutive transfers, a single colony was grown in bulk. Accordingly, its diazotrophically grown cultures in the strength of 5.8 × 10 CFUs were seeded per diazotrophic nutrient plate containing 15 mol m LiCl to selectively eliminate any possible parent clones in them.

6.2.3 Determination of per cent survival

Per cent survival characteristics of the parent and its variuous mutant strains on graded concentrations of LiCl, NaCl, KC1, RbCl or at pH 11.0 under diazotrophic growth conditions were determined as described in Chapter-2 (Section 2.8).

6.2.4 Determination of intracellular Na

The extrusion of Na was estimated as follows. Diazotrophic cultures of the parent and its mutant strains were separately pre-equilibrated with NaCl (carrier free specific activity 7.4 MBq ml^{-1}) at 0.5 mol m~ strength for 12 h in the Na^{+}-free diazotrophic growth medium. Such cultures were harvested, sampled and then transferred to different

concentrations of LiCl, NaCl, KC1 or RbCl (all buffered to pH 7.5 with Tris-HCl) and incubated under growth condition for 5 min. At the end of the incubation period, the samples were harvested by centrifugation and counted for intracellular level of the radiolabel as described in Chapter-3 (Section 3.2.2). CCCP at a final concentration of 0.01 mol m^{-3} was used to treat the $^{22}NaCl$ pre-equilibrated samples for 20 min which were then suspended in various concentrations of buffered-NaCl for 5 min, harvested and finally examined for intracellular level of the radiolabel.

6.2.5 Determination of intracellular Rb

The extrusion of Rb^+ was determined exactly as described for Na^+ except that $^{86}RbCl$ (specific activity 55.5 MBq mol^{-1}) at 0.4 mol m^{-3} strength replaced $^{22}Na^+$ (See Section for 6.2.4 for other details).

The pH of normal diazotrophic growth medium was maintained at pH 7.5 by addition of 1 mol m^{-} HEPES buffer. Growth medium pH 8.0 was also achieved by addition of 1 mol m HEPES buffer, while pH 9.0 was achieved by addition of AMPSO, pH 10.0 by addition of CAPSO and pH 11.0 by addition of CAPS buffer. Chlorophyll a was estimated by the method of Mackinney as described in Chapter-2 (Section 2.5).

6.2.6 Chemicals used

$^{22}NaCl$ was obtained from Amersham International plc, UK while $^{86}RbCl$ from BRIT, India. All other chemicals used in the present study were purchased from M/s Sigma Chemical Co. USA or from BDH chemicals Co., India and were of analytical grade.

6.3 RESULTS

N -fixing cultures of the parent *N. muscorum* showed a decrease in survival with increase in the concentration of alkali salts and reached almost a zero per cent survival with 10 mol m

LiCl 100 mol m^{-3} NaCl, 70 mol m^{-3} KC1 or 20 mol m^{3} RbCl (Fig.6.1). A previous study on Cs^{+} toxicity on the diazotrophic cultures of *N. muscorum* has already shown that the cyanobacterium does not survive beyond a CsCl concentration of 1.5 mol m^{-} (Singh et *al*. 1994). On the basis of the response of the cyanobacterium to various alkali cation stresses, it is quite clear **that K** and Na salts become toxic at considerably higher levels than the salts of Li , Rb or Cs^{+}. It is also evident that the various alkali cations differ in their degree of toxicity to the cyanobacterium in the following order $Cs^{+}>Li^{+}>Rb^{+}>K^{+}>Na^{+}$.

Attempts were made to understand the nature of cyanobacterial tolerance to various alkali cations by looking **for** sponatneous mutants resistant to growth inhibitory action of one or the other of the alkali cations and then examining their cross-tolerance relationship. Spontaneous mutants resistant to growth inhibitory action of LiCl (Li^{+}-*R*), NaCl (Na^{+}-*R*) and RbCl *(Rb -R)* were separately isolated and the frequency with which each one of them arose was in the range 1.2 - 2.1 x 10 Although 70 mol m~ ' KC1 was found lethal to the parent strain, spontaneous mutant clones resistant KC1 could not be obtained. Hence studies were confined to spontaneous mutant clones resistant to other alkali cations. A class of CsCl resistant (Cs^{+}-*R*) mutant clones differed from rest of the alkali cation resistant mutant clones in showing an obligate requirement for Cs^{+}/Rb^{+} for growth and diazotrophy (studies on its characterization have been described in Chapter 7). Since, pH 11.0 **of the** growth medium was also found growth inhibitory, colonies capable of good growth on such a pH were isolated **as described** in materials & methods. The frequency with which such

Fig. 6.1
Effect of increasing concentrations of LiCl (0), NaCl (•), KCl (□), RbCl (•) and CsCl (▲) on per cent survival of the parent *N. muscorum* on diazotrophic medium of pH 7.5.
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

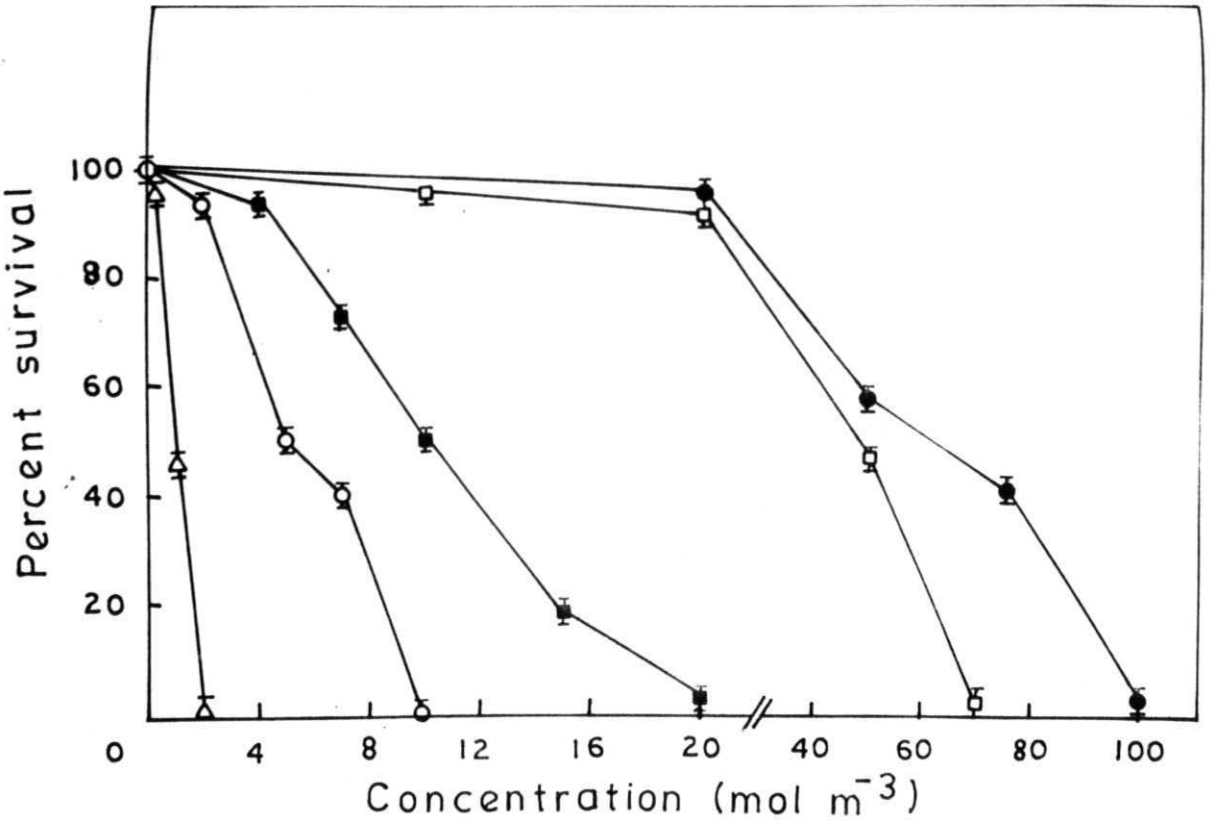

Percent survival
100
80
60
40
20
0
4
8
12
16
20
40
60
80
100
Concentration (mol m^{-3})

Fig. 6.1

mutant clones arose was 0.5 x 10^{7}. The pH 11.0 tolerant mutant was grown in bulk to examine for its tolerance to various alkali cations by the method used for examining the alkali metal tolerance characteristics of the parent strain. The results are shown in Fig.6.2 (a, b, c & d).

As shown in Fig.6.2a, *Li -R* mutant showed almost hundred percent survival at 10 mol m^{-} LiCl, a concentration at which the parent survival was found to be zero. The upper limit of tolerance to LiCl of the *Li*-R* mutant was 30 mol m Evidently, such increased tolerance of a stable nature to Li toxicity is a result of heritable change and the frequency characteristic of the mutant suggests it to be a product of a single mutational event. *Li -R* while showing increased tolerance to LiCl also showed similarly increased tolerance to NaCl, KCl, RbCl and CsCl than the parent strain. The simple conclusion from this result is that mutation to *Li -R* in the cyanobacterium is found simultaneously associated with acquisition of resistance to Na , K , Rb and Cs cations. In other words, *Li -R* mutant is a case of multiple alkali metal resistant phenotype.

The mutant strain of *N. muscorum* resistant to growth inhibitory action of NaCl (100 mol m) was also isolated, grown in bulk and examined for its tolerance characteristics to LiCl, NaCl, KCl, RbCl and CsCl. The results have been shown in Fig.6.2b. Like the *Li -R* mutant of the cyanobacterium its *Na -R* mutant also exhibited increased tolerance to other alkali cations. Evidently, the mechanism involved in confering multiple alkali tolerance in Li^{+}-*R* and Na -*R* mutants is most likely to be the same.

20 mol m Rb^{+}-*R* mutant like *Li -R* and *Na -R* mutant strains of the cyanobacterium exhibited incresaed tolerance to other alkali cations (Fig.6.2c). It is therefore concluded, that a common mechanism operates in the cyanobacterium for controlling

Fig. 6.2a

Effect of increasing concentrations **of LiCl (0), NaCl** (D), **KC1 (•)**, **RbCl** (A) and **CsCl** (A) on per cent survival of **the** *Li -R* mutant strain of *N. muscorum* on diazotrophic **medium** of pH 7.5. Mean values from three independent experimental determinations **are** shown ± SEM, where these exceed **the dimensions** of the symbols.

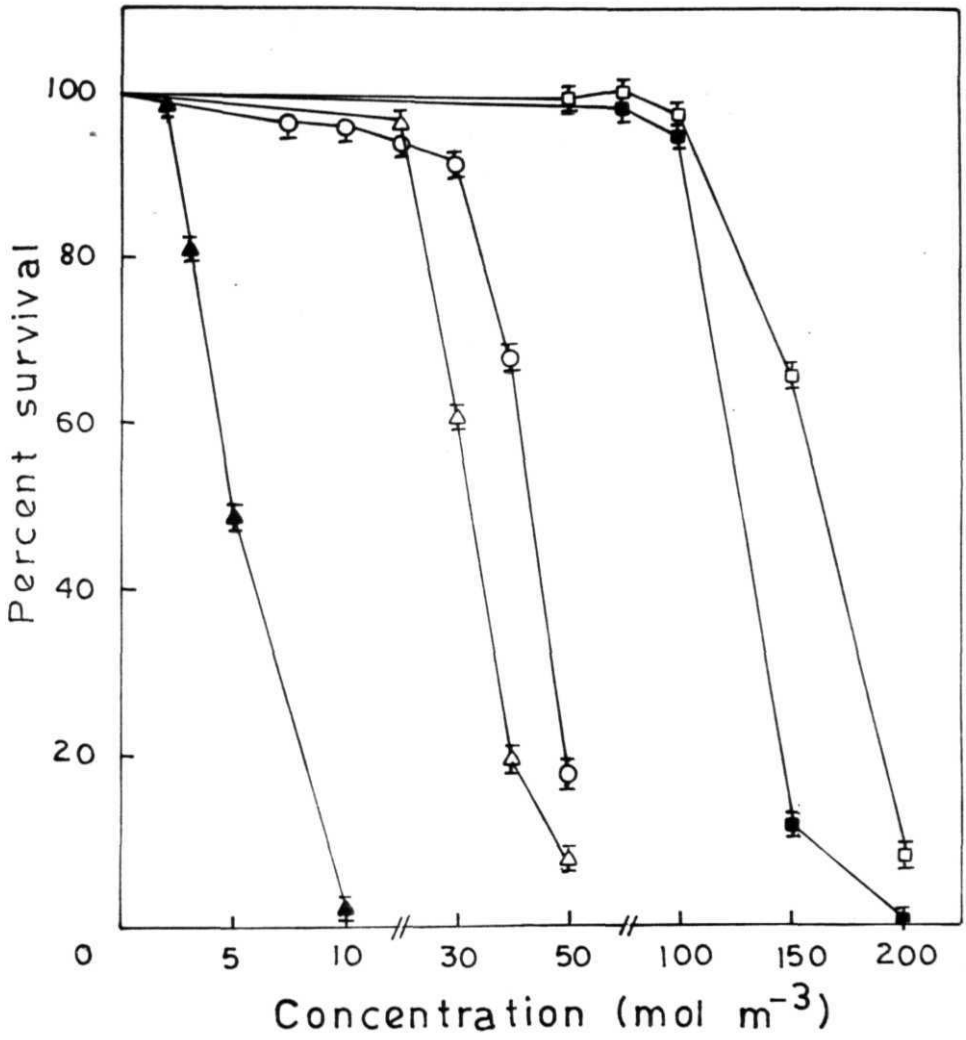

100
80
60
40
20
0
Percent survival
5
10
30
50
100
150
200
Concentration (mol m^{-3})

Fig.6.2a

Fig. 6.2b
Effect of increasing concentrations of **LiCl** (0), **NaCl** (a), KC1 (•), **RbCl** (A) and **CsCl** (A) on per cent survival of the ***Na*$^{+}$*-R*** mutant strain of *N. muscorum* on diazotrophic medium of pH 7.5. Mean values from three independent experimental determinations are shown 1 SEM, where these exceed the dimensions of the symbols.

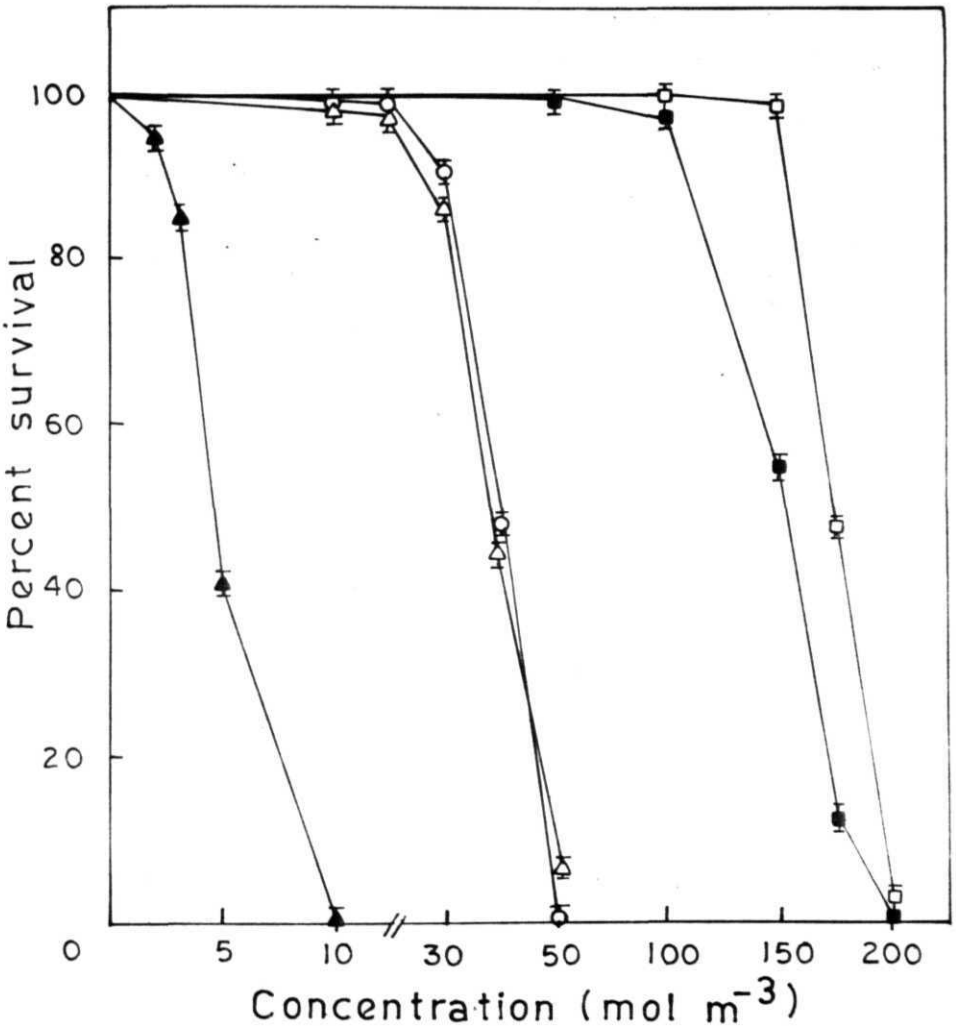

Percent survival
100
80
60
40
20
0
5
10
30
50
100
150
200
Concentration (mol m^{-3})

Fig.6.2b

Fig. 6.2c
Effect of increasing concentrations of LiCl (0), NaCl (D), KC1 (•), RbCl (▲) and CsCl (▲) on per cent survival of the Rb^{+}-*R* mutant strain of *N. muscorum* on diazotrophic medium of pH 7.5. Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

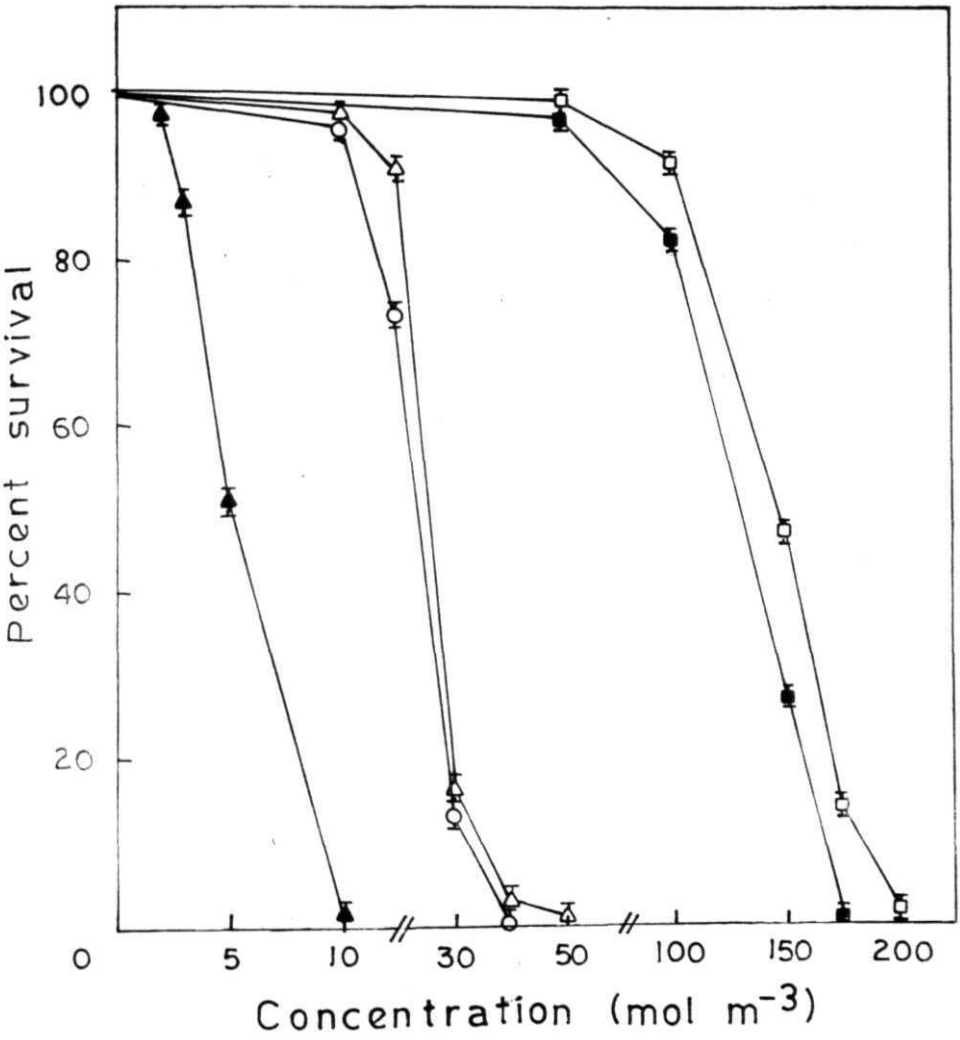

100
80
60
40
20
0
Percent survival
5
10
30
50
100
150
200
Concentration (mol m^{-3})

Fig.6.2c

Fig. 6.2d
Effect of increasing concentrations of **LiCl (O)**, **NaCl** (D), KC1 (•), **RbCl** (A) and **CsCl** (A) on per cent survival of the *pH*$_{1}$ $_{\alpha}$-*R* **mutant** strain of *N. muscorum* on diazotrophic **medium** of pH 7.5. Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

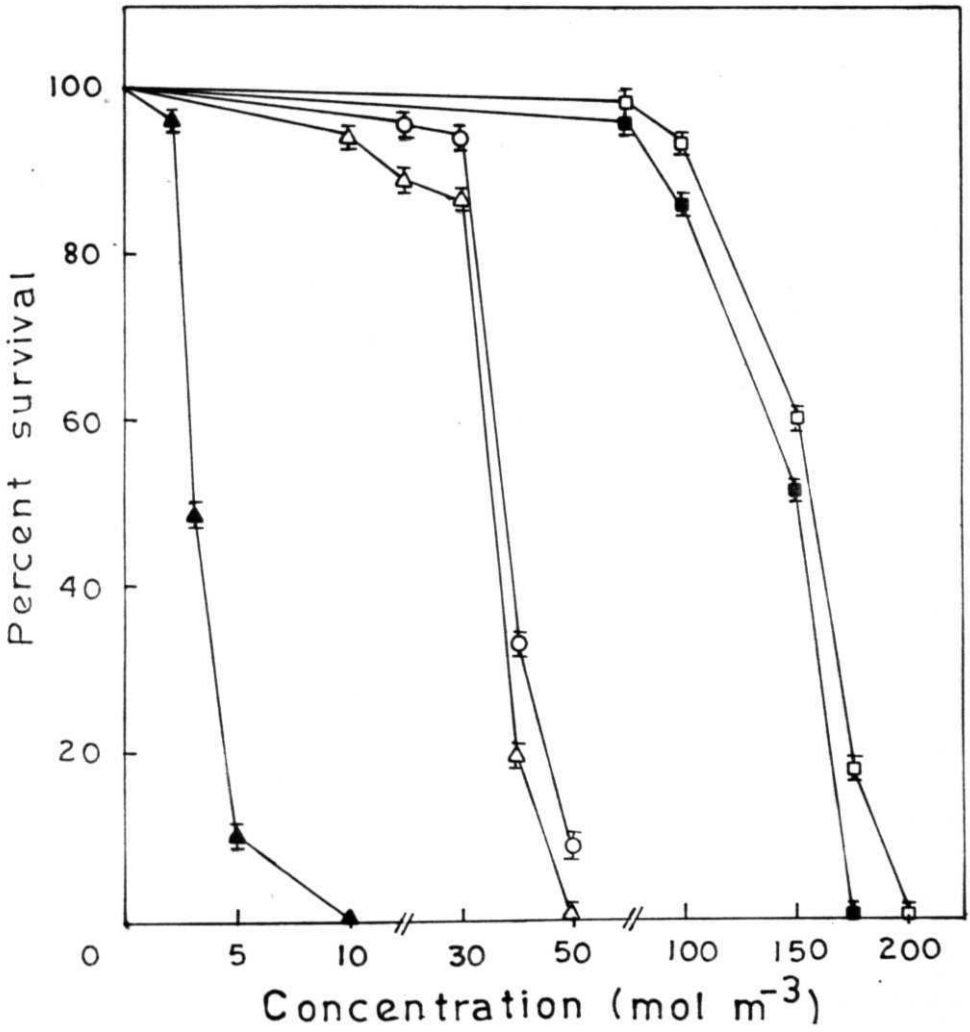

100
80
60
40
20
0
Percent survival
5
10
30
50
100
150
200
Concentration (mol m^{-3})

Fig. 6.2 d

its tolerance characteristic to various alkali cations.

Experiments were conducted to find out the alkali metal survival characteristics of the pH_{11} *-R* mutant strain as well. Like the Li^+*-R*, Na^+*-R* and Rb^+*-R*, the pH,, $_O$*-R*mutant also showed increased resistance to growth inhibitory action of Li^+, Na^+, K^+, Rb and Cs (Fig.6.2d). Apparently, the mechanism that seems to regulate cyanobacterial tolerance to various alkali metals is also the mechanism that confers tolerance against pH 11.0 stress. An obvious implication of the above findings is that cyanobacterial mechanism of alkali metal resistance and alkaline pH tolerance has a common physiological basis.

Table 6.1 compares the pH survival characteristics of the parent and its various alkali cation resistant mutant strains under different pH growth conditions. As the results indicate, pH tolerance characteristics of the parent and its various mutant strains remained indistinguishable upto pH 9.0 of the growth medium, above which the survival of the parent strain started declining with increase in pH and was about 44% at pH 11.0. However, the pH survival characteristics of the *Li -R*, *Na -R* and *Rb -R* mutant strains at growth medium pH 11.0 remained almost hundred per cent. These findings further indicate the operation of a common physiological mechanism regulating multiple alkali metal tolerance and alkaline pH tolerance in the cyanobacterium.

Table 6.2 compares the diazotrophic characterstics in terms of nitrogenase activity of the parent and its alkali metal resistant mutant strains to differential pH stress. As can be seen while the parent strain showed a progressive reduction in nitrogenase activity, the mutant strains were comparatively more

Table 5.4

Per cent survival of the different alkali cation resistant mutant strains of *N. muscorum* under varying pH stress under diazotrophic growth conditions.

pH	Strains			
	Parent	Li^+-R	Na^+-R	Rb -R
7.0	100	100	100	100
8.0	97	100	100	100
9.0	88	100	100	100
10.0	69	100	98	94
11.0	44	100	98	83

6-day old diazotrophic cultures growing at pH 7.0 were the source of inocula for the experiments.
Each reading is an average of three independent experimental determinations.

Table 6.2

Nitrogenase (N_2ase) activity of the different alkali cation resistant mutant strains of *N. muscorum* under varying pH stress under diazotrophic growth conditions.

	Strains			
	Parent	Li^+-R	Na^+-R	Rb^+-R
7.0	9.8 ± 1.3	9.4 ± 1.05	9.5 ± 0.5	8.6 ± 0.88
8.0	9.5 ± 0.45	9.7 ± 0.4	10.1 ± 1.45	9.4 1 1.32
9.0	7.6 ± 0.5	9.4 ± 1.2	9.2 ± 0.6	9.3 ± 0.03
10.0	5.5 ± 0.22	9.0 ± 0.5	8.5 ± 0.31	7.1 ± 0.53
11.0	4.2 ± 0.26	8.8 ± 0.4	7.4 ± 0.4	7.1 ± 0.46

6-day old diazotrophic cultures growing at pH 7.0 were the source of inocula for the experiments.
Each reading is an average (± SEM) of three independent experimental determinations.

stable in maintaining their activities under various pH stress conditions. These findings further endorse the opinion of the presence of a common physiological mechanism governing the diazotrophy in alkali cation resistant mutants and alkaline pH tolerance characteristics of diazotrophy in them.

NaCl inducible stress in plant and microbial cells consists of two components - the ionic stress component resulting from cellular accumulation of Na ions to toxic levels and the osmotic (water stress) component resulting from cellular loss of water (Csonka 1989). Since the NaCl-resistant clone (here termed *Na -R)* is also resistant to other alkali cation induced stresses, one would expect it to be also resistant to sucrose induced osmotic stress. Hoever, the mutant strain was found less tolerant to sucrose induced osmotic stress than the parent strain. Hence, it was concluded that the present NaCl-resistant clone belongs to a different class of mutant and differs from the *NaCl-R* mutant clone described in Chapter-4. Further, the *Li -R*, *Rb -R* and pH_{11} *-R* mutant strains were also found to be sensitive to sucrose induced osmotic stress (Table 6.3). What then is the cause for alkali metal resistant phenotype in the present cyanobacterial mutant strains? Clearly, the organic osmolyte based mechanism of osmoprotection is not the mechanism of multiple alkali metal resistant phenotypes.

Is the multiple alkali metal tolerance in the mutant strains then the result of enhanced efflux or diminished influx activity of its alkali metal transport system? This question was analyzed by comparing the rate of efflux of the radiolabel from pre-equilibrated cultures of the parent and its mutant strains with $^{22}Na^{+}$ or $^{86}Rb^{+}$ as a function of various alkali cations in the external medium. As shown in Fig.6.3, alkali metal induced decrease in intracellular $^{22}Na^{+}$ content occurred at a several-fold higher rate in the Li^{+}*-R* mutant than in the parent strain. Such a decline in the radiolabel is obviously due to its

Table 3

Effect of sucrose induced osmotic stress on the per cent survival of parent *N. muscorum* and its various mutant strains under diazotrophic growth conditions (pH 7.5)

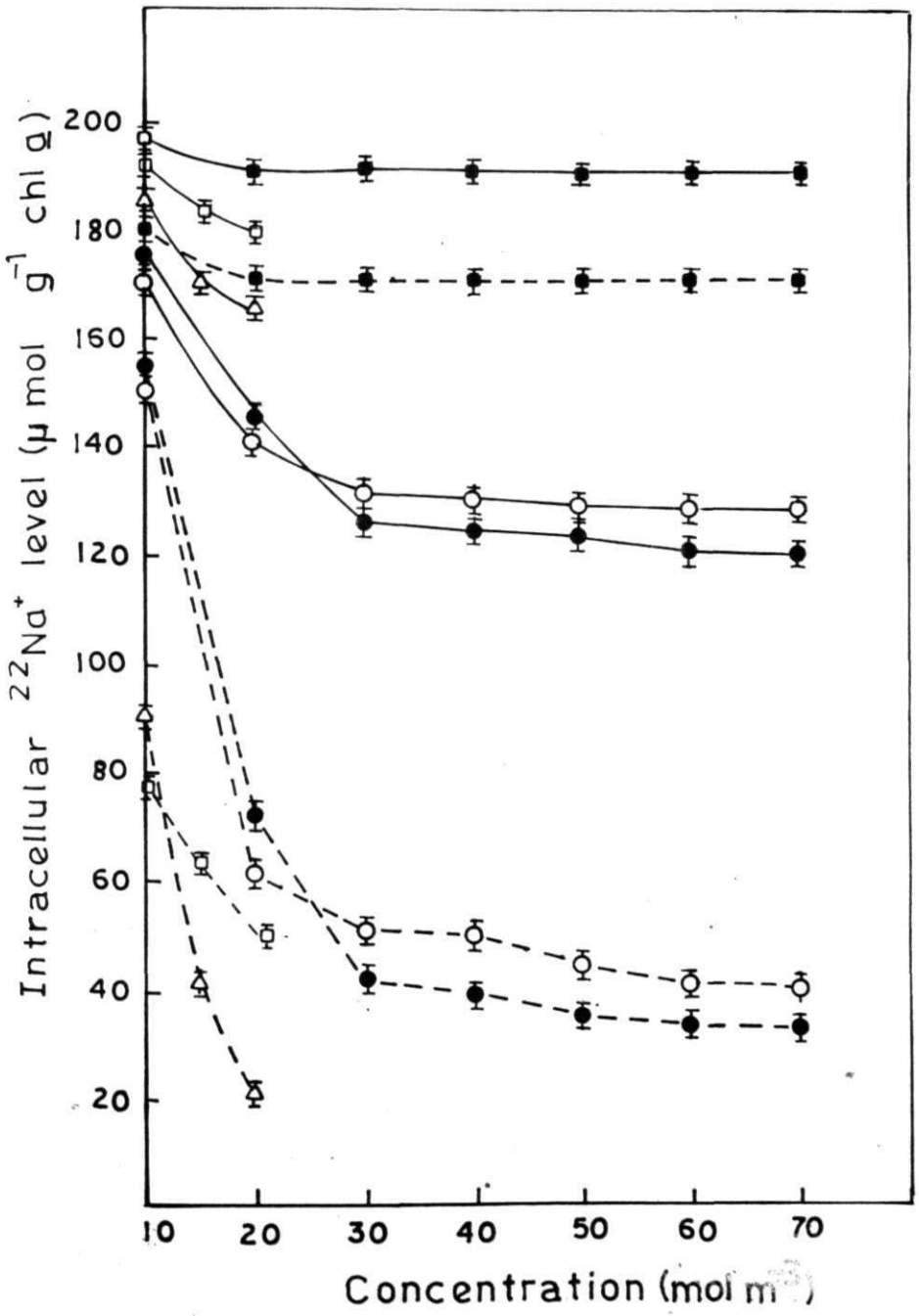
Strains	Concentration of sucrose (mol m^{-})				
	Unstressed	100	150	200	250
Parent	100	95	90	40	0
Li^{+}*-R*	100	100	96	45	0
Na^{+}*-R*	100	100	100	56	1
Rb^{+}*-R*	100	97	70	34	0
pH$_{11\ 0}$*-R*	100	90	75	21	0

6-day old diazotrophic cultures were the source of inocula for the experiments.
Each reading is an average of three independent experimental determinations.

Fig. 6.3

Effect of increasing concentrations of **LiCl** (A), **NaCl** (0), KC1 (•) and **RbCl (□),** on the intracellular level of $^{22}Na^{+}$ in parent *N. muscorum* (——) and its *Li -R* mutant strain (——). Effect of CCCP (•) on the intracellular level of Na was **examined** by pretreating the Na equilibrated **samples** with **0.01 mol m** of inhibitor for 20 **min.**

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


Intracellular $^{22}Na^{+}$ level (μ mol g^{-1} chl g)
200
180
160
140
120
100
80
60
40
20
10
20
30
40
50
60
70
Concentration (mol m^{-3})

efflux by an alkali cation activable Na extrusion system. The observation that *Li -R* mutant has a considerably more efficient alkali cation activable system of Na^+ extrusion explains why it is more tolerant to NaCl stress than the parent. A similar pattern of extrusion of Na was observed with the other mutant strains (Table 6.4a).

The influence of increasing concentrations of LiCl, NaCl, KC1 and RbCl on Rb was also similarly examined (Fig.6.4 & **Table** 6.4b). The mutant strains exhibited considerably more active alkali cation dependent Rb extrusion than the parent strain. The considerable increase in Rb extrusion in the mutant strains thus also appears to be the reason for its being more tolerant to RbCl stress than the parent. Since the mutant strains exhibited tolerance to LiCl, KC1 and CsCl stress equally well, it is also suggested that efficient extrusion of toxic Li or K or Cs ions from the cells is the mechanism of their tolerance to LiCl or KCl or CsCl induced lethality as well. Thus the ability of the mutant strains to behave like multiple alkali metal resistant strains is because of their having a more efficient and active system of multiple alkali cation extrusion. CCCP pretreatment resulted in complete abolition of the Na extrusion in the *Li -R* mutant strain (Fig.6.4).

6.4 DISCUSSION

Stress induced by alkali metal salts in plants and in microbial cells consists of two components, the ionic stress component resulting from cellular accumulation of alkali cations to toxic levels and the osmotic (water) stress component resulting from cellular loss of water (Csonka 1989). Plants and

Table 6.4a

Effect of **LiCl** (20 mol m^{-3}), NaCl (75 rool m^{-3}), **KCl** (70 mol m^{-3}) and **RbCl** (20 **mol** m^{-3}) on the level of intracellular $^{22}Na^{+}$ (**μmol Na** g **chl** a) in the parent and its various alkali cation resistant strains as well as on the $pH_{11.0}$-*R* mutant strain.

	Treatment				
	Control	LiCl	NaCl	KCl	**RbCl**
Parent	180.6 (11.2)	165.3 (9.4)	160.3 (9.3)	158.1 (11.1)	143.9 (6.4)
Li^{+}-*R*	160.7 (14.1)	20.1 (0.66)	48.0 (1.8)	42.1 (1.6)	31.6 (1.3)
Na^{+}-*R*	160.1 (10.4)	31.1 (2.3)	35.9 (2.1)	36.4 (2.4)	38.4 (2.6)
Rb^{+}-*R*	150.6 (9.4)	34.8 (2.1)	31.7 (1.8)	30.2 (0.9)	22.0 (1.2)
$pH_{11.0}$-*R*	180.4 (9.3)	36.1 (4.0)	36.1 (3.1)	25.2 (2.5)	28.8 (1.5)

6-day old exponentially growing diazotrophic cultures were the source of inocula for the experiments.
Values in parentheses are ± SEM of three independent experimental determinations.

Fig. 6.4
Effect of increasing concentrations of LiCl (•), NaCl (O), KCl (•) and RbCl (□), on the intracellular level of Rb in parent *N. muscorum* (——) and its *Li -R* mutant strain (——).
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

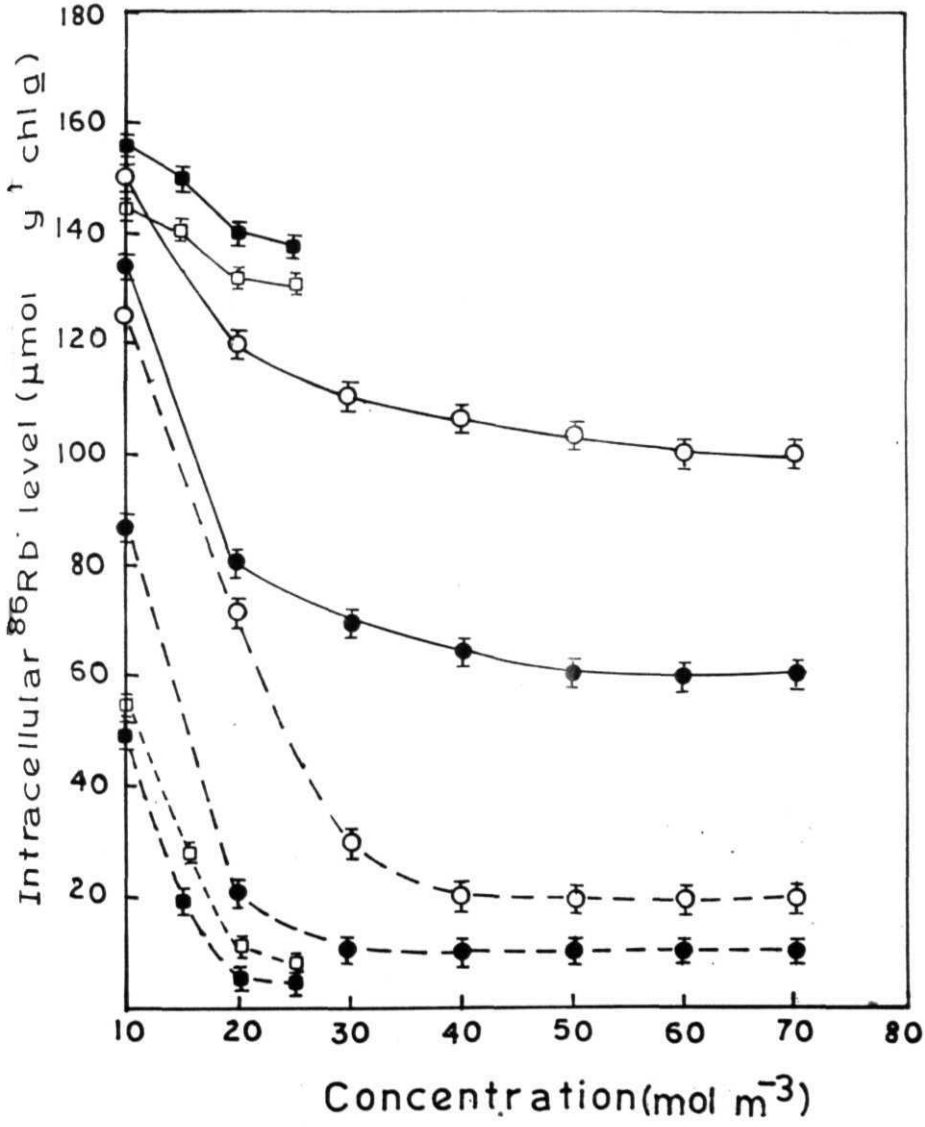

Intracellular $^{86}Rb^{+}$ level (μmol g^{-1} chl g)
180
160
140
120
100
80
60
40
20
10
20
30
40
50
60
70
80
Concentration(mol m^{-3})

Fig. 6.4

Table 6. 4b

Effect of **LiCl** (20 **mol** m^{-3}), **NaCl** (75 mol m^{-3}), KC1 (70 **mol** m^{-3}) and **RbCl** (20 **mol** m^{-3}) on the level of intracellular Rb^+ (**μmol** Rb **g chl** a) in parent *N. muscorum* and its various alkali cation resistant strains as well as on the $pH_{11.0}$-*R* strain.

	Treatment				
	Control	**LiCl**	**NaCl**	KC1	RbCl
Parent	165.4 (10.4)	140.4 (10.3)	124.1 (9.4)	79.6 (6.3)	84.3 (8.1)
Li^+-*R*	138.5 (9.3)	7.5 (0.55)	24.2 (1.5)	15.4 (0.9)	18.1 (0.64)
Na^+-*R*	140.3 (8.2)	9.6 (0.65)	18.2 (1.1)	12.2 (0.71)	13.1 (0.33)
Rb^+-*R*	155.0 (11.1)	15.1 (0.88)	15.1 (0.84)	9.4 (0.61)	5.3 (0.24)
$pH_{11.0}$-*R*	166.3 (12.1)	15.4 (1.05)	15.2 (1.1)	18.4 (1.1)	7.8 (0.44)

6-day old exponentially growing diazotrophic cultures were the source of inocula for the **experiments.**
Values in parentheses are ± SEM of three independent experimental determinations.

microbes can overcome the ionic stress by having an efflux system actively functioning in effluxing out alkali cations (Blumwald et *al*. 1984). Similarly, they can overcome water stress component by actively accumulating compatible organic osmolytes such as sugars, betaines, proline etc. (Warr et *al*. 1988; Bartels & Nelson 1994). The mechanism of adaptation to NaCl stress have been studied in bacterial systems and the results suggest a specific definite role of Na /H antiporter activity in bacterial adaptation to such alkali metal stress (Padan & Schuldiner 1994). The activity of Na /H antiporter has also been found to be one of the mechanisms of salt adaptation in the cyanobacterium *Synechococcus* PCC 6311 (Blumwald et *al*. 1984). However, no detailed study has been made about the general mechanism of adaptation to various alkali metal salts in any cyanobacterium. A knowledge of how monovalent (alkali) cations influence each other's transport, accumulation and toxicity in cyanobacteria would be extremely useful in understanding the mechanisms involved in regulation of their alkali cation nutrition and toxicity. In this connection, it is important to mention here that it has been shown recently that Li or Na stimulates K uptake and accumulation, and Li , Na or K inhibits Cs uptake and accumulation, by individually influencing the activity of K transport system in the cyanobacterium *Synechocystis* PCC 6803 (Avery et *al*. 1991). However, nothing is known about the mechanism of physiological adaptation to alkali salt stress in any cyanobacterium.

The multiple alkali metal resistance nature of the *Li -R*, *Na -R*, *Rb -R* and $pH_{,,\ O}$*-R* mutant strains clearly suggest, the occurrence of a common physiological mechanism for adaptation to alkali metal stress in *N. muscorum*. Since the ability of cyanobacterial or bacterial systems to adapt to NaCl stress is known to result from enhanced Na /H antiporter activity, one can infer a role of alkali cation/H antiporter activity in cyanobacterial adaptation to one or the other alkali metal

stresses. The observed increased efflux of Na or Rb from alkali metal resistant mutants as well as from *pH*$_{,,}$ $_{-}$*-R* mutant does suggest a role of increased efflux system in confering tolerance against alkali metal stress and alkaline pH stress to the cyanobacterial systems. The next question is whether this increased efflux system is a H -gradient driven antiporter like the already known Na /H antiporter or something different? Since CCCP is an inhibitor of the formation of H gradient, and since pretreatment of the cultures with the inhibitor results in complete inhibition of Na extrusion, a role of H gradient as a driving force for extrusion of Na through the activity of an Na /H antiporter system is thus evident. Similarly, CCCP pretreatment inhibited Rb extrusion, thus implying a role of H -gradient driven Rb efflux and since the *Li -R* mutant strain is also resistant to Cs and K , it is suggested that a mutationally amplifiable H -gradient driven alkali cation efflux system is the mechanism of multiple alkali metal resistant phenotype in the mutant. Such a multiple alkali cation specific antiporter has to be genetically and physiologically distinct from the Na -specific H -antiporter already reported from bacterial and cyanobacterial systems (Padan & Schuldiner 1994; Blumwald et *al*. 1984), as the former according to the present finding functions in cyanobacterial adaptation to NaCl, KCl, RbCl or CsCl stress. The latter is known to be specific for adaptation to only Na stress. Bacterial systems have been reported to contain Na /H antiporters as well as other antiporters that do not discriminate amongst alkali metals and is known to exchange one or the other alkali metal for H (Karpel et *al.* 1991). The multiple alkali metal resistant nature of *Na -R* or Rb^{+}*-R* cyanobacterial mutant strains can thus also be similarly explained.

Na /H antiporter in bacterial and cyanobacterial systems is not only the mechanism of adaptation to Na stress but also a mechanism of resistance to alkaline pH stress (Bluwald et *al*. 1984; Karpel et *al* . 1991). Since the activity of multiple alkali cation efflux system in the cyanobacterium is H -gradient dependent and since H -gradient dependent antiporter activity is a known mechanism of adaptation to both alkali metal stress and alkaline pH stress, one would expect that multiple alkali metal resistant cyanobacterial mutants should also exhibit resistance to alkaline pH stress and vice-versa. The finding that the *pH -R* mutant and the Li *-R*, *Na -R* and *Rb -R* mutants exhibit cross-tolerance is indicative of the existence of a role of H -gradient dependent multiple alkali cation specific efflux system functioning in adaptation of the cyanobacterium to both alkali metal-induced stress and alkaline pH stress. The present findings have a considerable ecophysiological implication for cyanobacterial population growing in habitats of alkali metal or alkaline pH stress and they by spontaneous mutation produce new population ecophysiologically fully adapted to such a habitat by showing operation of an enhanced mutiple alkali cation efflux system which scavenges them out.

It is important to point out that the NaCl resistant mutants described here (termed as *Na -R)* differs from the class of mutants described in Chapter-4, in that it fails to show the compatible osmolyte mechanism of osmoadaptation while showing an enhanced antiporter activity. Since NaCl-induced stress resistance could be the result of two components; osmotic and ionic, the present *Na -R* mutant strain is the result of mutation to NaCl resistance involving the ionic component alone and is found sensitive to sucrose-induced osmotic stress. Similarly, the other multiple alkali metal resistant mutant strains or the alkaline pH resistant mutant on preliminary examination were found to be sensitive to sucrose induced osmotic stress. It is

thus concluded that H -gradient driven alkali metal/H antiporter activity is primarily the mechanism of adaptation to salt or alkaline pH stress but not to osmotic stress. If this be so, how the cyanobacterial multiple alkali metal resistant mutants are able to overcome the osmotic stress imposed by considerably high concentrations of NaCl or KC1? In this context, it is important point out that a naturally occuring salt resistant cyanobacterium *Anabaena torulosa* has been shown to be sucrose sensitive (Apte & Haselkorn 1990) and the present results can only mean further investigation on the nature of mechanisms that can help the cyanobacterial mutant strains overcoming the osmotic stress component imposed by NaCl or KCl.

Chapter-7

EVIDENCE FOR AN OBLIGATE REQUIREMENT OF Cs^+/Rb^+ IN A *Cs^+-R* MUTANT OF THE CYANOBACTERIUM *NOSTOC MUSCORUM*

7.1 INTRODUCTION

Growing pollution and consequent toxicity of natural environments are posing a serious threat to the existing ecosystems. There is a serious concern about the biological implications of pollution caused by Cs , arising from its continual discharge from nuclear industries into aquatic habitats in view of its high bioavailability and long half-life Cyanobacteria and algae are the main primary producers of the aquatic ecosystems and the biological consequences of Cs pollution on these primary producers needs to be investigated and understood at ecological, physiological and molecular level. Previous studies have demonstrated adverse effects of Cs on the physiology and growth of cyanobacteria and microalgae that function as primary producers in the aquatic food chain (Williams & Swanson 1958). Recent studies on the physiological reasons of Cs toxicity or lack of it to cyanobacteria and algae have shown that it results from cellular replacement of K by Cs associated with the inability of Cs to substitute functionally for K (Avery et *al*. 1991), that K , Na or NH mitigates/eliminates it by preventing the entry and accumulation of Cs (Avery et *al*. 1992 a&b; Avery et *al*. 1993; Singh et *al*. 1994) and that it arises specifically from Cs inhibition of N -fixation in the cyanobacterium *Nostoc muscorum* (Singh et *al*. 1994).

Earlier studies have shown that Cs toxicity in *N. muscorum* is diazotrophic specific and NH_4^+-repressible and that one class of caesium resistant *(Cs -R)* mutants of the cyanobacterium arise with about 50% impairment in their nitrogenase activity and diazotrophic growth (Singh et *al*. 1994). In the present study, experiments have been carried out to analyse the nature and consequences of caesium resistance *(Cs -R)*

associated loss in cyanobacterial diazotrophy in the presence and absence of alkali cations such as Na , K , Cs and Rb, more specifically, the latter two cations. An evidence is presented here to show that *Cs -R* mutant has suffered genetic damage of pleiotropic nature adversely influencing its growth, oxygenic photosynthesis, chlorophyll a content, nitrogenase activity and osmotolerance specific to diazotrophic mode of growth and that Cs or Rb alone is nutritionally capable of repairing fully such cyanobacterial mutational pleiotropy.

7.2 MATERIALS AND METHODS

Axenic clonal cultures of the parent *N. muscorum* used in the present study were routinely grown and maintained in combined nitrogen-free medium of Gerloff et *al*. (1950), as described in Chapter-2 (Section 2.1).

7.2.1 Composition of the nutrient medium

The original growth medium of Gerloff et *al*. (1950) was modified to make it free from Na and K for the experiments examining the role of Na , K along with Rb or Cs in the cyanobacterial diazotrophic nutrition. Accordingly, Na SiO , Na CO and K HPO were omitted from the original growth medium thus rendering the medium -Na^+ and -K^+. CaHPO and CaCO were added in place of CaCl and Na CO at equimolar concentration. Further, except for the experiments involving the study on the effect of individual cations on the dizotrophic growth medium of the parental strain, all other experiments have been conducted with the cultures of parent or mutant strains grown in modified Chu No. 10 medium containing 3 mol m NaCl, 5 mol m^{-3} KCl, 0.0574 mol m $CaHPO_4$ and 0.19 mol m $CaCO_3$. Addition of 1 mol m NH Cl to the growth medium has been termed as

NH_4^+-medium. The pH of all the media were adjusted to 7.5 using 1 mol m^{-3} HEPES buffer.

7.2.2 Osmotic survival studies

Osmotic survival studies were made on N - or NH -nutrient plates containing increasing concentrations of sucrose as described in Chapter 2 (Section 2.8). The inoculum size per nutrient plate was 500 CFUs. Role of Cs^+/Rb^+ in regulation of cyanobacterial osmotolerance was examined by scoring the osmotic survival characteristics in the presence or absence of either alkali cations.

7.2.3 Determination of intracellular $^{137}Cs^+$

Effect of Rb on the uptake and accumulation of Cs in the cyanobacterial strains was examined as follows. The diazotrophically grown cultures were harvested and sampled in 10 mol m Hepes buffer of pH 7.5. The various samples contained a fixed amount of radioactive Cs (0.2 mol m CsCl; specific activity 38.85 MBq mol) against increasing concentration of cold RbCl. The samples thus prepared were incubated for 10 min, then harvested and examined for intracellular radiolabel as described in Chapter-3 (Section 3.2.2). Similar method was employed to evaluate the influence of Na /K on the uptake and accumulation of Cs

7.2.4 Determination of intracellular $^{86}Rb^+$

Effect of Cs on the uptake and accumulation of Rb in the cyanobacterial strains was examined exactly as per the protocol given above for Cs^+ (Section 7.2.3) except that a fixed amount of radioactive Rb (0.4 mol m^{-3} RbCl; specific activity 55.5 MBq mol) was used against increasing concentrations of cold CsCl.

7.2.5 Assay of GS (biosynthetic) activity (EC 6.3.1.2)

GS was assayed under *in* vitro conditions. Cultures were

harvested by centrifugation at 2000 > g and washed twice with 50 mol m^{-} Tris-HCl buffer, pH 7.5 (Buffer-A) followed by washing and resuspension in Buffer-B (Buffer-A + 5 mol m $MgCl_2$, 10^{-3} mol m sodium glutamate, 5×10^{-3} mol m β-mercaptoethanol, 1 mol m EDTA), pH 7.5. The cells in the suspension were then broken in liquid N and the cell-free extract thus obtained was centrifuged at 36,000 x g for 30 min at 4 °C. The supernatant was used for Mg -dependent biosynthetic assay according to the procedure of Sampaio *et al*. (1979) as given below.

The method monitors the oxidation of NADH coupled to ADP production from ATP. The assay mixture comprised:

REAGENTS	CONCENTRATION ($\times 10^{-6}$ moles per 0.2 cm^3)
Tris-HCl (pH 7.5)	150
Sodium glutamate	10
ATP	3
NH_4Cl	200
KC1	150
$MgCl_2$	150
NADH	0.45
PEP	0.5
LDH	20 units
PK	8 units

To 1 cm^3 of the enzyme extract, 0.2 cm^3 each of the reagents is added. The optical density was measured at 340 nm in a Hitachi spectrophotometer. The activity of the enzyme was expressed in $\mu mol\ g^{-1}\ protein\ min^{-1}$

Growth, Chlorophyll a, protein and nitrogenase activity were estimated as described in Chapter-2 (Sections 2.4, *2.S, 2.6* & 2.9). Oxygenic photosynthesis as described in Chapter-4 (Section 4.2.2)

7.2.6 Chemicals used

CsCl and RbCl were obtained from Board of Radiation and Isotope Technology (BRIT), India. All other chemicals used in the present study were purchased either from M/s Sigma Chemical Co., USA or BDH Chemicals Co., India and were of analytical grade.

7.3 RESULTS

The spontaneously occurring cyanobacterial Cs resistant *(Cs -R)* mutant under diazotrophic growth condition arose with a frequency of 0.3 - 0.7 ↗ 10 . The diazotrophic growth medium devoid of both Na and K was used as basal growth medium to analyse the nutritive role of various alkali cations on growth of parent *N. muscorum* (Fig. 7.1). The optimal growth with individual cations occurred at 3 mol m^{-3} NaCl, 5 mol m^{-3} KC1 or 3 mol m^{-3} RbCl. However, growth was always significantly better with Na and K or Na and Rb together, than with Na , K or Rb alone. Cs (used as CsCl) on the other hand, was extremely growth inhibitory and lethal at 1.5 mol m^{-3}. Neither Na (3 mol m^{-3} NaCl) nor K (5 mol m^{-3} KC1) was found to mitigate the Cs toxicity to the cyanobacterium. These findings demonstrate clearly the nutritive role of Rb like that of Na or K **and** inhibitory role of Cs in the cyanobacterial diazotrophic growth.

The *Cs^{+}-R* mutant capable of growth in the presence or absence of 2 mol m^{-3} CsCl in the diazotrophic growth medium **was** examined along with its parent for diazotrophic growth characteristics (Fig. 7.2). The mutant grew very slowly in Cs -

Fig. 7.1
Effect of Na^+ (3 mol m^{-3} NaCl), K^+ (5 mol m^{-3} KC1), Rb^+ (3 mol m^{-3} RbCl), Cs^+ (1.5 mol m^{-3} CsCl), $Na^+ + K^+$ (3 mol m^{-3} NaCl + 5 mol m^{-3} KC1) and $Na^+ + Rb^+$ (3 mol m^{-3} NaCl + 3 mol m^{-3} RbCl) on the growth of parent *N. muscorum* in diazotrophic medium.
(O), Control (no addition of alkali cations)
(•), Na^+
(□), K^+
(•), Rb^+
(A), Cs^+
(▲), $Na^+ + K^+$
(x), $Na^+ + Rb^+$
Alkali cations were added the point marked by an arrow.
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

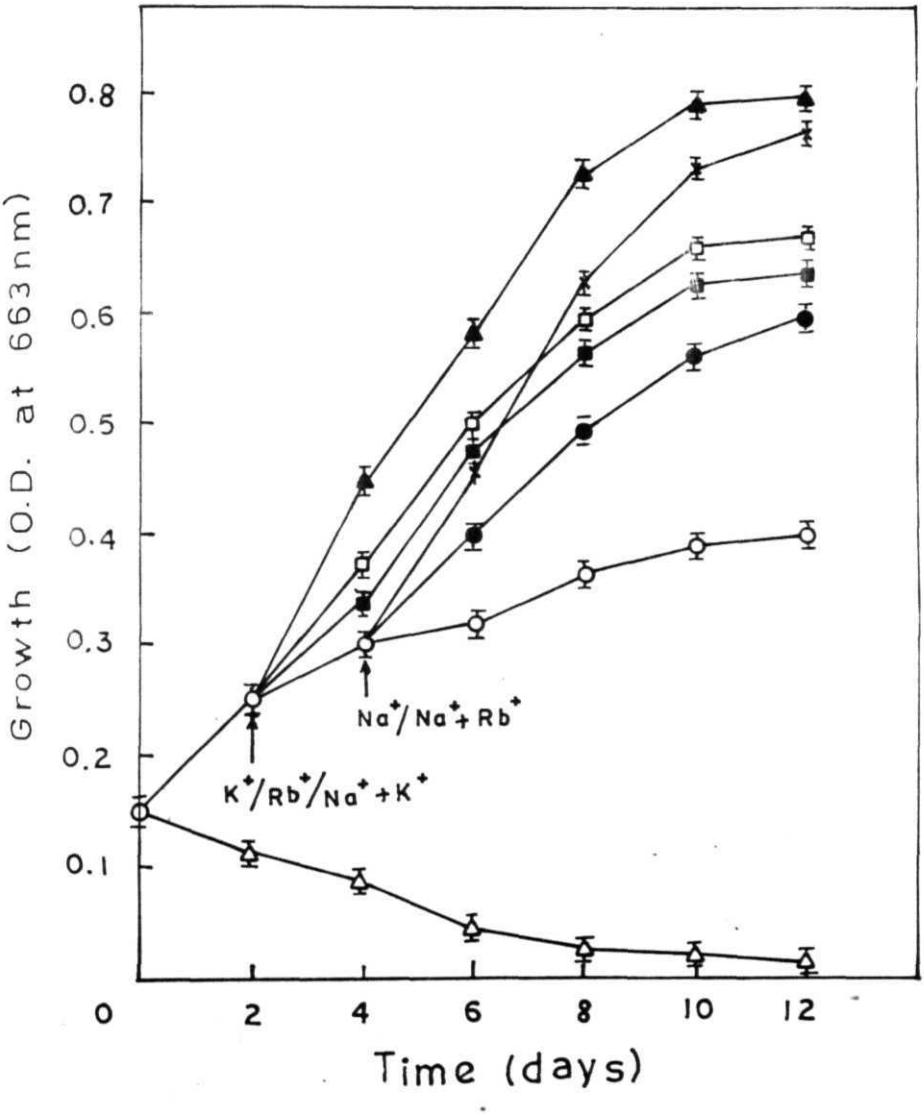

Growth (O.D. at 663 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
Time (days)
Na+/Na+ + Rb+
K+/Rb+/Na+ + K+

Fig.7.1

Fig. 7.2
Growth of parent *N. muscorum* in diazotrophic medium (○——○) and in diazotrophic medium containing 2 mol m CsCl (Cs , ●——•) as well as of its Cs -*R* mutant strain in diazotrophic medium (□——•) and in diazotrophic medium containing 2 mol m CsCl (Cs , ■——•) or 3 mol m~ RbCl (Rb , ■——•) . Cs /Rb were added at the point marked by an arrow.
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

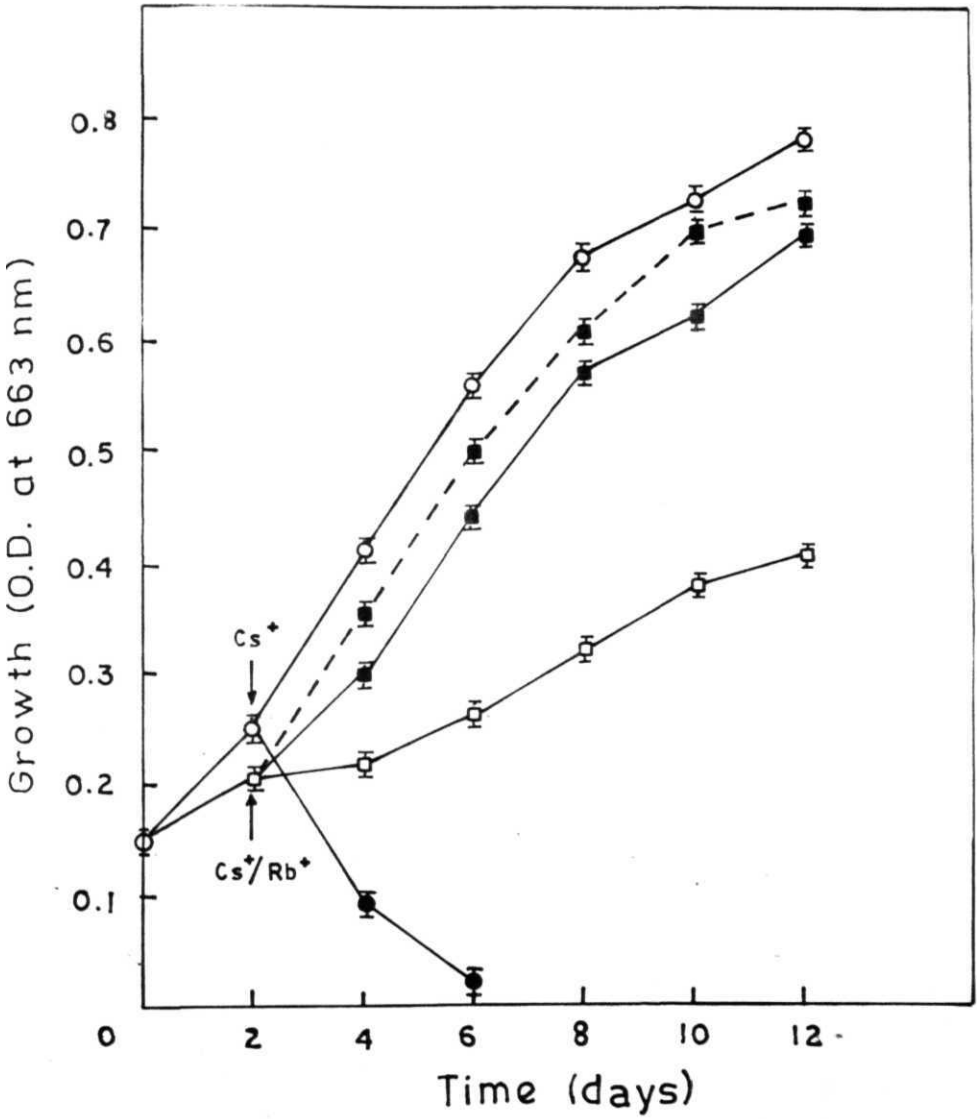

Growth (O.D. at 663 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
Time (days)
Cs+
Cs+/Rb+

Fig.7.2

or Rb -free diazotrophic growth medium and in quantitative terms, its estimated diazotrophic growth at the end of 12-days period was about 50% lower to that shown by the parental strain. Clearly, mutation to *Cs*-R* phenotype has resulted in about 50% impairment of the cyanobacterial diazotrophic growth. Interestingly, addition of 2 mol m~ CsCl or 2 mol m~ RbCl to the diazotrophic growth medium caused almost complete absence of mutationally impaired diazotrophy. In other words, Cs , the inhibitor of diazotrophic growth in the parent, became a nutritional requirement for normal diazotrophy in the mutant strain. In addition, Rb was found effectively substituting for such Cs nutritional requirement. In comparison, Na (3 mol m NaCl) or K (5 mol m KC1) in the growth medium did not repair the mutational damage. Thus, it can be concluded that Cs or Rb is a specific nutritional requirement for restoration of normal diazotrophy in the *Cs -R* mutant strain. It must be mentioned here that the parent and the mutant strains, both grew equally well in 1 mol m NH_4Cl medium with or without Cs or Rb . Evidently, cyanobacterial mutational damage is apparently diazotrophy specific and NH -repressible.

Under diazotrophic growth condition without Cs /Rb supplement, the mutant cyanobacterial samples looked greenish-yellow. The question whether such Cs or Rb nutritional requirement is specific for chlorophyll a content (Fig. 7.3) and nitrogenase activity (Fig. 7.4) or for both under diazotrophic growth condition was further investigated. In these experiments the source of inocula for the two strains was NH_4^+-grown cultures. As expected, nitrogenase activity of the two strains developed after a lag period of about 2-days, thereafter it increased differentially with a rate nearly 2-fold higher in the parent than in the mutant strain. Addition of Cs or Rb

Fig 7.3

Chlorophyll a content in diazotrophic medium of the parent *N. muscorum* lacking Cs /Rb (O——0) and of its *Cs -R* mutant strain lacking Cs /Rb (□——•) or containing Cs /Rb (■—•). **The** inocula for the experiments was 1 mol m^{-} NH_4Cl grown cultures of either strain transferred to diazotrophic medium. Cs /Rb were added at the point marked by an arrow.

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

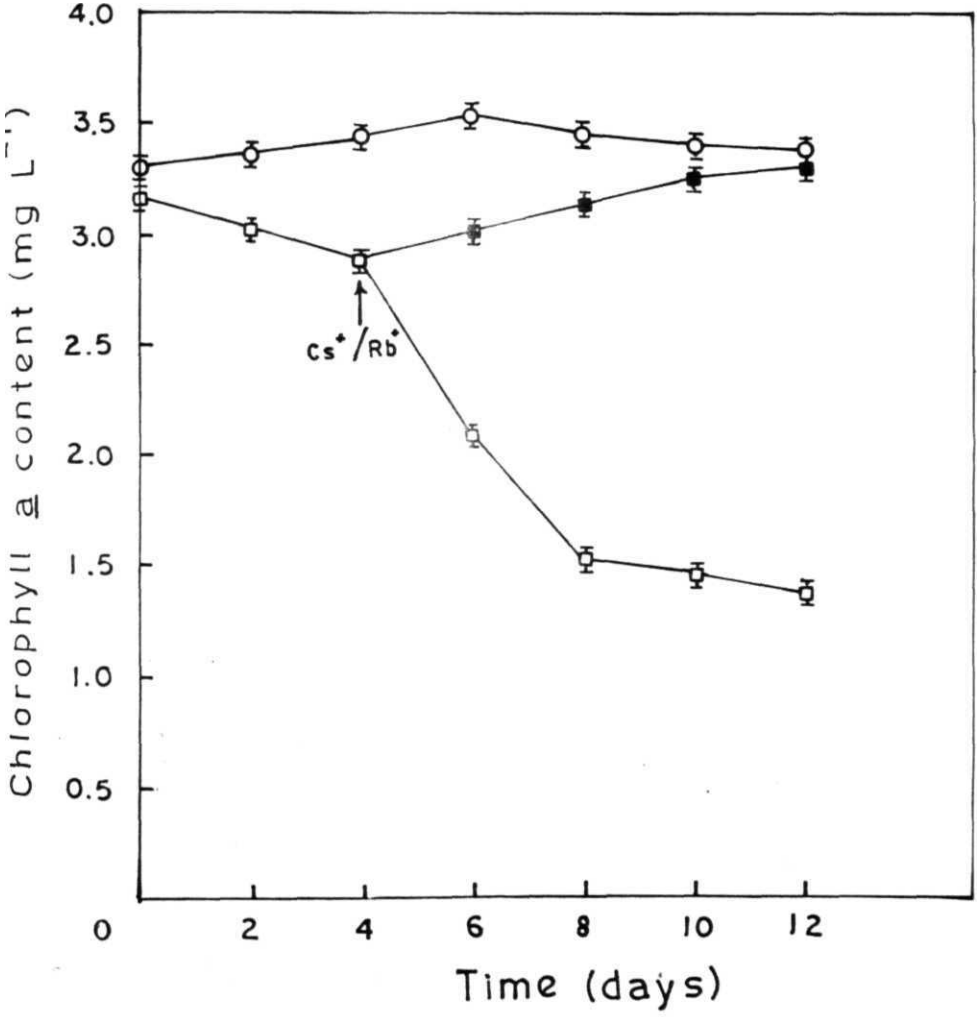

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
2
4
6
8
10
12
Chlorophyll a content (mg L⁻¹)
Time (days)
Cs⁺/Rb⁺

Fig. 7.3

Fig. 7.4

Nitrogenase activity in diazotrophic medium of the parent *N. muscorum* lacking Cs /Rb^+ (O——0) and of its *Cs -R* mutant strain lacking Cs^+/Rb^+ (□——□) or containing Cs^+/Rb (■—•). The inocula for the experiments was 1 mol m NH_4Cl grown cultures of either strain transferred to diazotrophic medium. Cs /Rb was added at the point marked by an arrow.

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

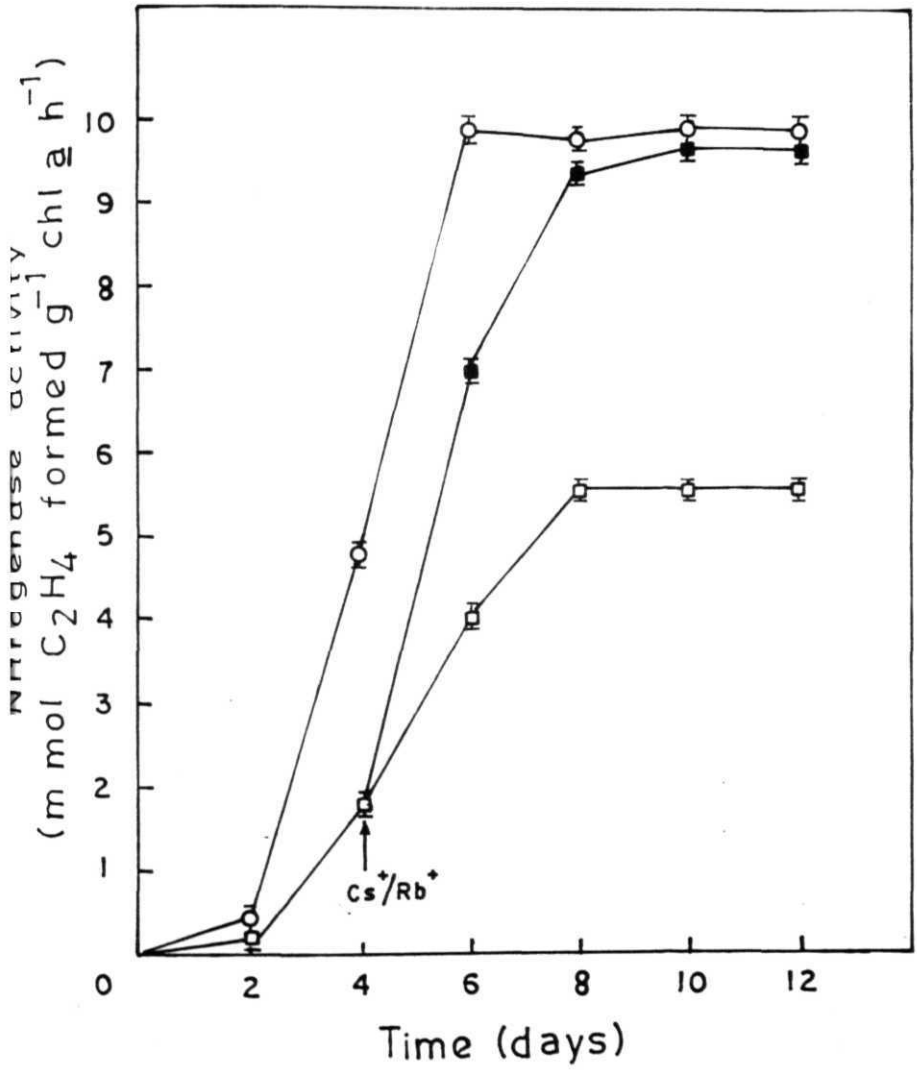

Nitrogenase activity
(m mol C_2H_4 formed g^{-1} chl a h^{-1})
0
1
2
3
4
5
6
7
8
9
10
2
4
6
8
10
12
Time (days)
Cs^+/Rb^+

Fig.7.4

restored nitrogenase activity of the mutant to almost parental level. Chlorophyll a content like nitrogenase activity, also required Cs or Rb to maintain its normal level. Thus mutation to *Cs -R* phenotype, appears to have adversely influenced the common cellular target essential for maintenance of normal nitrogenase activity and chlorophyll a content in the cyanobacterium.

The cyanobacterial strains grew diazotrophically at the expense of oxygenic photosynthesis and ammonium assimilatory activity of GS. It was therefore natural to investigate the effect of *Cs -R* mutation on these two aspects and the role of Cs or Rb in such mutational damage. The results show that photosynthetic O -evolution in the parent decreased with increasing concentration of Cs^+ and was zero at 2 mol m^{-3} CsCl (Table 7.1). In comparison, none of the Rb concentrations influenced this process significantly. This does suggest that in parent while Cs is inhibitory to oxygenic photosynthesis, Rb is not. In comparison, oxygenic photosynthesis of the *Cs -R* strain was nearly 50% of the parent in the absence of Cs or Rb . Gradual increase in concentration of Cs or Rb was found increasing the oxygenic photosynthetic activity of the mutant proportionately. In contrast, GS (biosynthetic) activity was not much adversely affected with increasing concentrations of Cs or Rb in the parent or mutant strain. The mutation to *Cs -R* phenotype thus seems to have adversely affected mainly oxygenic photosynthesis of the cyanobacterium.

The next series of experiments were conducted to examine the role of nitrogen source or Cs /Rb in the regulation of osmotolerance in the parent and *Cs -R* strains. As shown in Table 7.2, the osmotic survival rate of the parent in diazotrophic medium or NH_4^+-medium remained almost similar with increasing doses of sucrose and reached zero value with 250 mol m^{-3} sucrose. Clearly, the nature of the nitrogen source

Table 7.1

Effect of graded concentrations of Cs^+ (CsCl) and Rb^+ (RbCl) on photosynthetic O_2-evolution (m mol O_2 evolved g~ Chl a) and on GS (biosynthetlc) activity (μ mol NADH oxidized g^{-1} protein min~) in the parent and *Cs^+-R* mutant strains of *N. muscorum.*

Treatment	Parent strain		*Cs -R* strain	
	evolution	*GS* -activity	evolution	*GS* activity
Control	610 ± 12.5	86 ± 8.2	292 ± 15.2	82 ± 6.4
+ Cs^+ (mol m~^{3})				
0.25	436 ± 18.4	82 ± 6.7	316 ± 12.3	81 ± 6.5
1.0	313 ± 21.5	76 ± 5.8	412 ± 8.6	79 ± 3.6
1.5	36 + 2.3	72 ± 5.5	562 t 17.4	81 ± 2.5
2.0	0.0	75 ± 5.3	586 ± 6.3	83 ± 5.1
+ Rb^+ (mol m~^{3})				
1.0	590 ± 36.0	82 ± 5.2	422 ± 14.5	81 ± 2.7
1.5	602 ± 21.3	79 t 5.7	518 i 7.2	81 ± 1.5
2.0	586 ± 29.4	82 ± 5.5	594 ± 36.6	81 ± 6.6

Cultures of both the strains were grown diazotrophically with or without the various treatments for 72 h before using them for the various experimental determinations.
Each reading is an average (± SEM) of three independent experimental determinations.

Osmotic survival characteristics of the parent and *Cs -R* mutant strains of *N. muscorum* on diazotrophic (N_2) medium and on NH_4^+-medium (1 mol m^{-3} NH_4Cl) supplemented with increasing concentrations of sucrose. The N_2-medium with or without Cs^+/Rb^+ (2×10^{-3} mol m^{-3} CsCl or 3×10^{-3} mol m^{-3} RbCl) was used to score the survival of *Cs -R* strain in order to examine the role of Cs /Rb in its osmoprotection.

Treatment	Parent strain		*Cs -R* strain			
			N_2 medium			NH_4^+
	medium	+ medium	+Cs	+Rb	-Cs /-Rb	medium
Basal medium	100	100	100	100	100	100
+ Sucrose (mol m^{-3})						
100	65 (± 4.5)	68 (± 5.1)	70 (± 2.5)	66 (± 6.1)	36 (± 3.5)	68 (± 4.5)
200	42 (± 3.6)	45 (± 4.5)	40 (± 3.4)	44 (± 4.2)	2 (0.13)	43 (± 2.5)
250	0.0	0.5 (± 0.03)	1 (0.06)	1 (0.05)	0.0	1 (0.05)

The inoculum size per nutrient plate was about 500 colony forming units (CFU's). The efficiency of survival on control medium was taken as 100%.
Values in parentheses are ± SEM of three independent experimental determinations.

or nutrition does not seem to influence the osmotolerance characteristic of the parent cyanobacterium. The osmotic survival pattern of *Cs -R* mutant in the NH_4-medium was almost similar to that of the parental strain under parallel condition. However, it was not so in diazotrophic medium lacking Cs /Rb where its percent survival decreased much faster with rise in sucrose concentration and reached a zero value at 200 mol m sucrose. Addition of Cs /Rb in the diazotrophic medium resulted in restoration of the osmotolerance of the mutant strain to almost parental level. Rb like Cs also caused restoration of osmotolerance in the mutant. Thus, Rb is also required for repair of the osmotolerance in the mutant strain.

The pattern of Cs or Rb uptake was also studied in the two strains grown diazotrophically as well as in the NH medium. DCCD at a concentration of 20 mg L was used to pretreat the parental diazotrophic culture for 30 min in order to examine the energy dependence of Cs uptake. As shown in Fig. 7.5 & 7.6, the rate of Cs uptake was slightly higher than the rate of Rb uptake in the parent. The uptake pattern of both the cations was similar in the mutant but was slightly higher compared to the parent. DCCD pretreated diazotrophic cultures of the parent lacked Cs uptake and accumulation. NH -grown cultures of the parent similarly lacked Rb uptake and accumulation or Cs uptake and accumulation. NH_4-grown cultures of the mutant also lacked Rb or Cs uptake and accumulation. DCCD pretreated diazotrophic cultures of the mutant like that of its parent were similarly deficient in Cs or Rb uptake **and** accumulation. Evidently, both cyanobacterial strains contain NH -repressible DCCD sensitive Cs or Rb uptake process of more or less equal magnitude. In other words, mutation to *Cs -R* phenotype does not seem to have altered **the** Cs or Rb uptake

Fig. 7.5

Uptake of Cs in parent *N. muscorum* grown diazotrophically (O——0) and in 1 mol m NH_4Cl medium for 96 h (●——•) and of its Cs^+-*R* mutant strin grown diazotrophically (o) and in 1 mol m~ NH Cl medium for 96 h (•). DCCD at a strength of 20 mg L was used to pretreat the parent strain (O——0) to examine the role of energy metabolism on $^{137}Cs^+$ uptake. Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

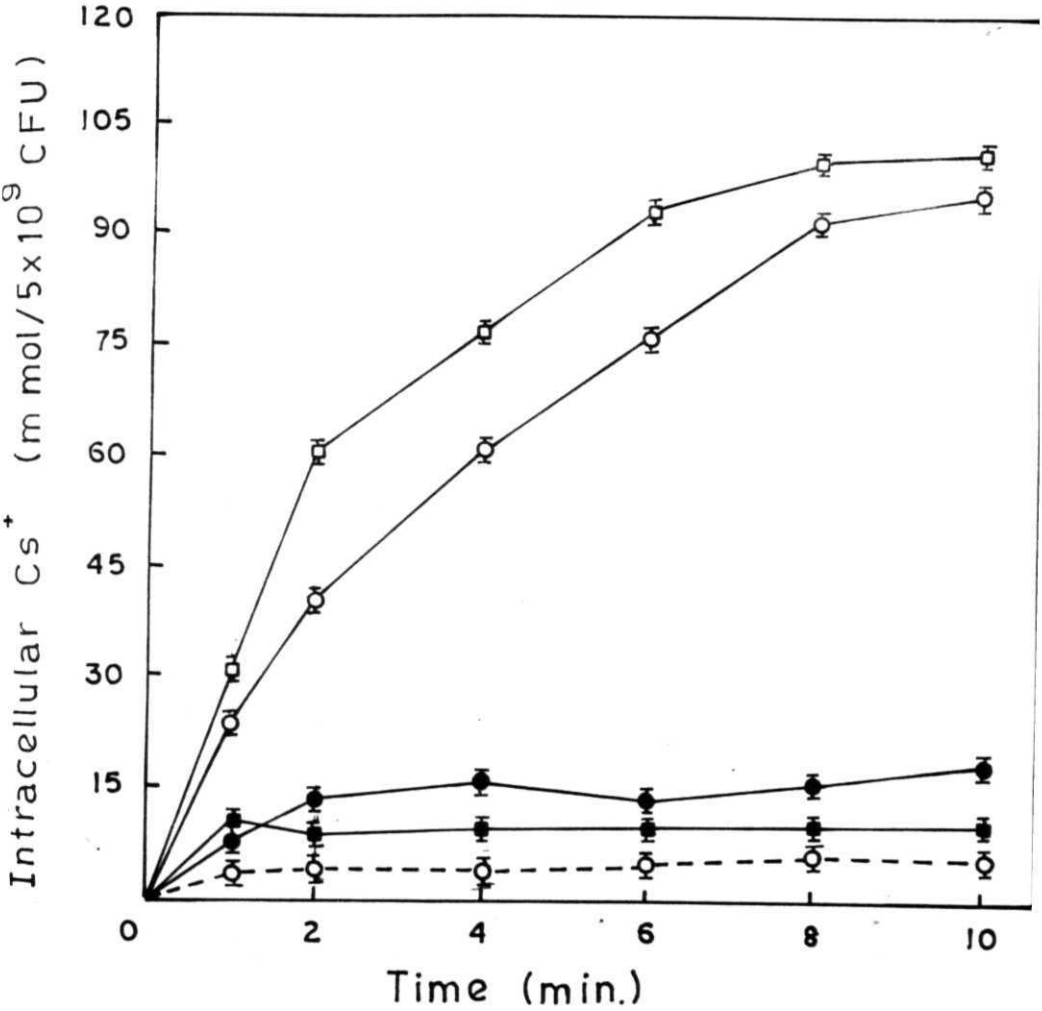

Intracellular Cs^+ (m mol/5×10^9 CFU)
120
105
90
75
60
45
30
15
0
2
4
6
8
10
Time (min.)

Fig. 7.5

Fig. 7.6

Uptake of $^{86}Rb^{+}$ in parent *N. muscorum* grown diazotrophically (O——O) and in 1 mol m~ NH_4Cl medium for 96 h (•——•) and of its *Cs -R* mutant strin grown diazotrophically (a) and in 1 mol m~ NH_4Cl medium for 96 h (•). DCCD at a strength of 20 mg L^{-1} was used to pretreat the parent strain (O——O) to examine the role of energy metabolism on $^{86}Rb^{+}$ uptake.

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

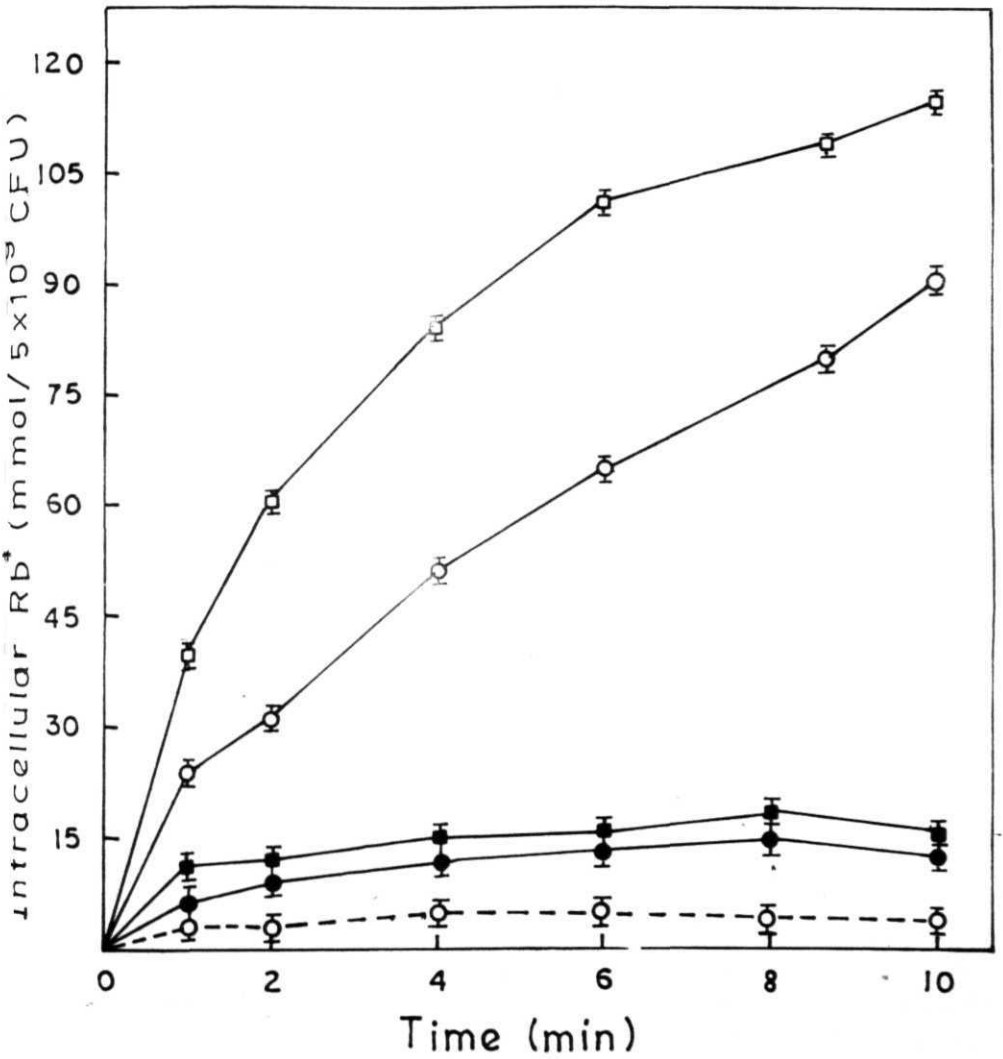

Intracellular Rb^+ (mmol/5×10^9 CFU)
120
105
90
75
60
45
30
15
0
2
4
6
8
10
Time (min)

Fig. 7.6

process in the diazotrophic cultures of the two cyanobacterial strains.

Experiments were also conducted to examine the influence of exogenous Cs on the uptake activity of Rb transport system and of exogenous Rb on the uptake activity of Cs transport system. As shown in Fig. 7.7 & 7.8, when both the cations are present simultaneously in the growth medium, uptake of one cation got inhibited progressively at increasing concentration of the other cation and vice-versa. These results do suggest that the two cations affect the uptake and accumulation of the other.

The effect of increasing concentrations of K (KCl) and Na (NaCl) on the intracellular content of Cs was also examined. As shown in Fig. 7.9 & 7.10, neither Na upto 5 mol m NaCl nor K upto 10 mol m KCl could influence the intracellular level of Cs in the parent or in the *Cs -R* strain. However, with further rise in Na or in K concentrations there was a corresponding decline in Cs accumulation which became almost negligible at an external concentration of 20 mol m NaCl or 50 mol m KCl.

7.4 DISCUSSION

Diazotrophic growth results in the parent strain suggest that Rb can replace for K functionally. Parallel studies with the mutant suggest K cannot substitute functionally for Rb while Cs can do so very effectively. These findings imply a role of the cyanobacterial genetic determinant in controlling the specificity of nutritional interchangeability of alkali cations from K /Rb to Rb /Cs in the cyanobacterium. K is required by microbes as an enzyme activator, as an osmotic regulator and as a regulator of internal pH (Booth 1985; Walderhaug *et al.* 1987). It is therefore essential to examine specifically the role of Rb in various known cellular functions

Fig. 7.7

Influence of increasing concentrations of RbCl on the uptake of Cs in parent *N. muscorum* (0) and its *Cs -R* mutant strain (D). Diazotrophic cultures of the two strains grown with Cs for 48 h were used in the present study.

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

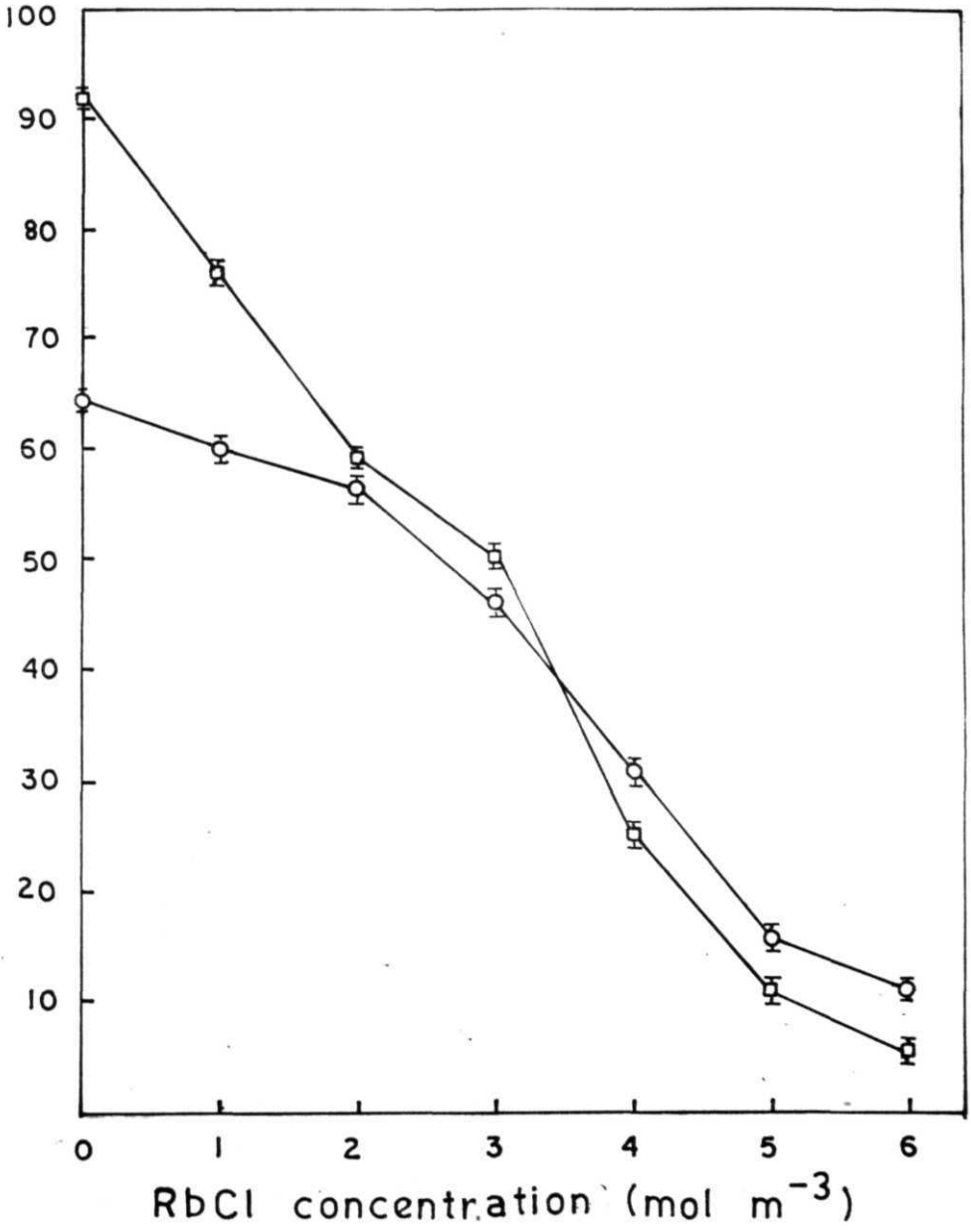

100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
RbCl concentration (mol m^{-3})

Fig. 7.7

Fig. 7.8
Influence of increasing concentrations of **CsCl on the uptake** of **Rb** in parent *N. muscorum* (**O**) and its *Cs* **-*R* mutant strain** (a). Diazotrophic cultures of the two strains grown **with** Cs **for 48** h were used in the present study.
Mean values **from** three independent experimental **determinations are** shown ± SEM, where these exceed the **dimensions** of **the** symbols.

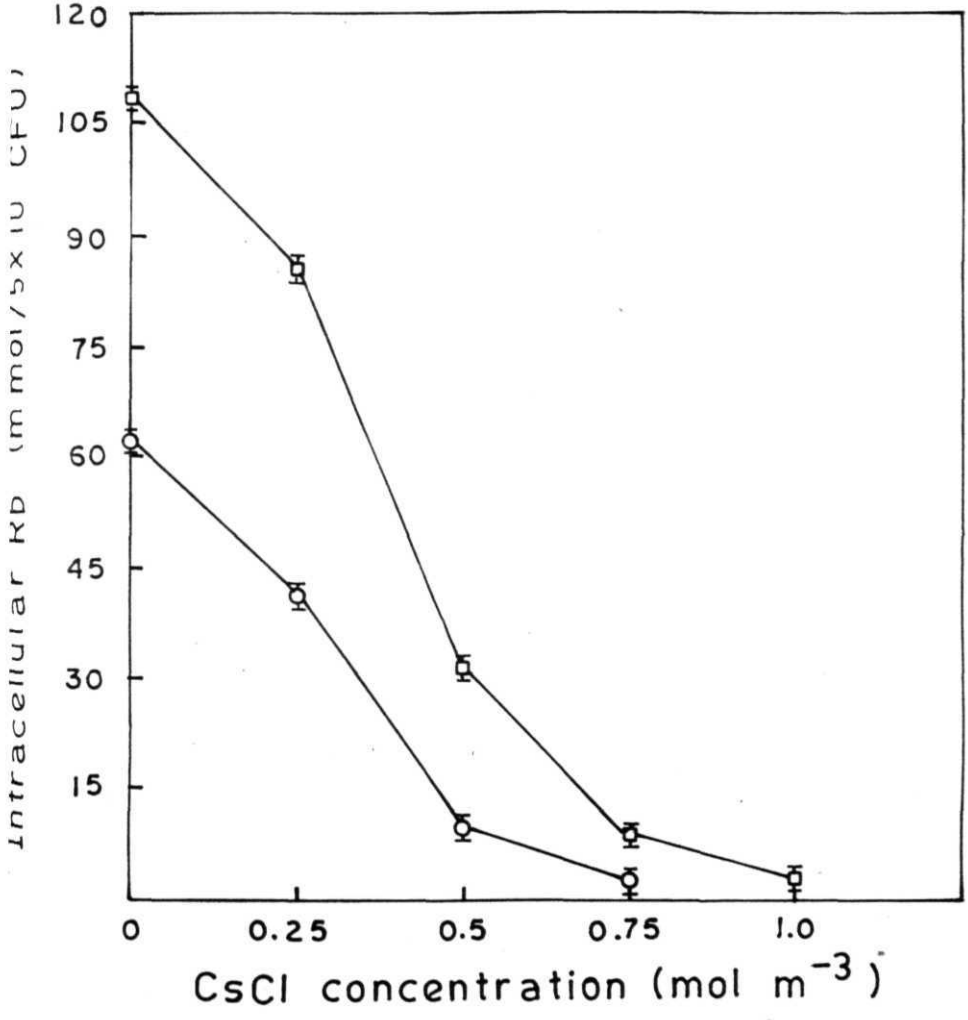

120
105
90
75
60
45
30
15
0
0.25
0.5
0.75
1.0
CsCl concentration (mol m^{-3})

Fig.7.8

Fig. 7.9
Influence of external Na (as **NaCl**) on the cellular level of Cs in parent *N. muscorum* **(0)** and its *Cs* **-*R* mutant strain (D)**. Mean values from three independent **experimental** determinations **are** shown ***t* SEM**, where these exceed the dimensions of the symbols.

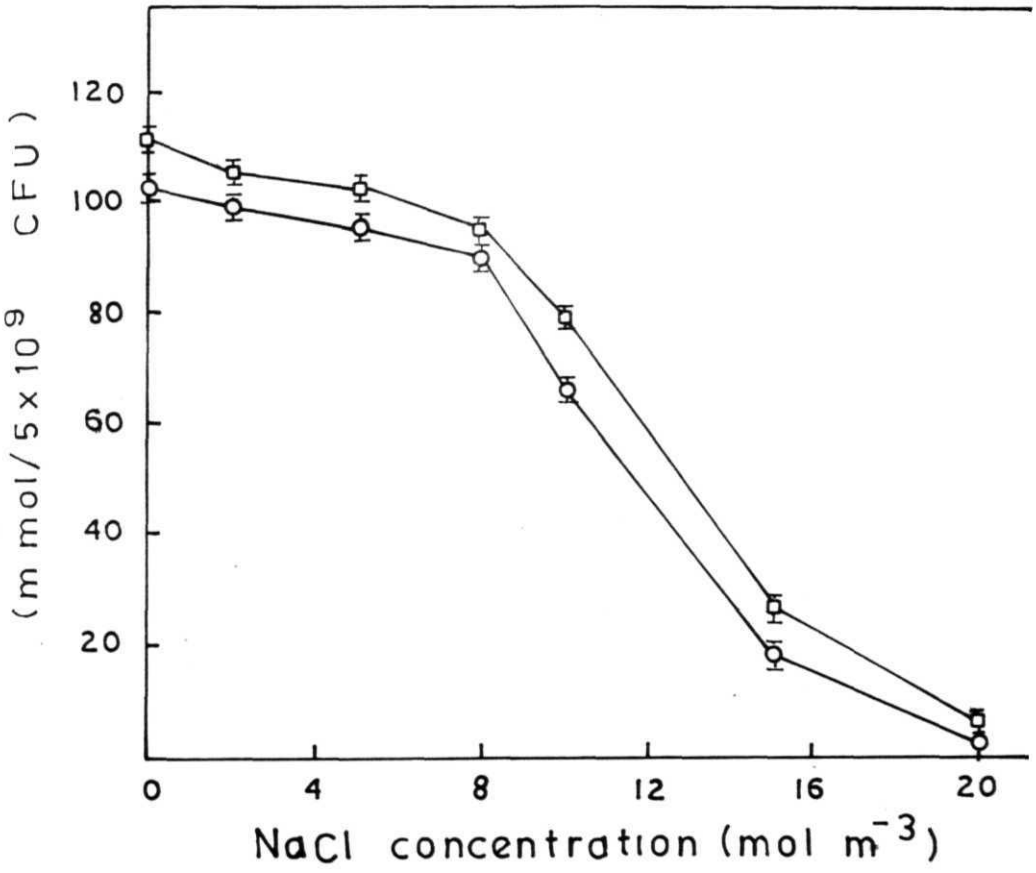

120
100
80
60
40
20
(m mol/5x10^9 CFU)
0
4
8
12
16
20
NaCl concentration (mol m^-3)

Fig. 7.9

Fig. 7.10
Influence of external K^+ (as KC1) on the cellular level of $^{137}Cs^+$ in parent *N. muscorum* (0) and its *Cs -R* mutant strain (a).
Mean values from three independent experimental determinations are shown ± SEM, where these exceed the dimensions of the symbols.

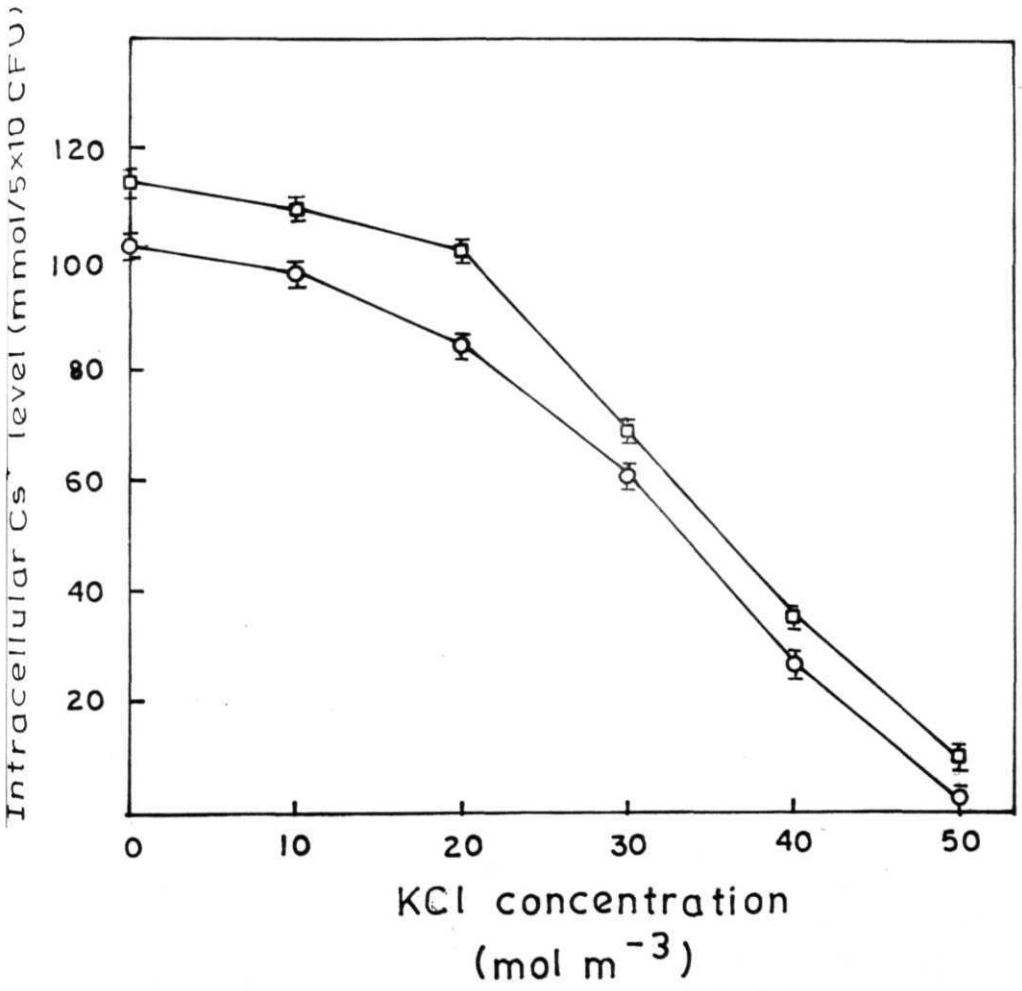

Intracellular Cs+ level (mmol/5×10 CFU)
120
100
80
60
40
20
0
10
20
30
40
50
KCl concentration
(mol m-3)

Fig.7.10

of K by modern techniques of molecular biology before accepting or rejecting its biological functional equivalence to K .

Cs toxicity to cyanobacterial diazotrophic cultures is because such cultures contain both active NH -repressible Cs transport system and Cs sensitive intracellular **target(s)**. In cyanobacteria, mutational alteration of the NH -repressible Cs transport system has been shown to be one genetic mechanism of *Cs -R* phenotype (Avery et *al*. 1992). The other genetic mechanism of *Cs -R* phenotype has been the mutational acquisition of resistance by the Cs sensitive intracellular target against Cs (Singh et *al*. 1994). The present finding that Cs^+/Rb^+ uptake and accumulation is NH -repressible in the parent as well as in the mutant strain while confirming earlier conclusion of Cs transport being NH -repressible, further shows that Rb transport like Cs transport is also NH -repressible in the cyanobacterium. Since the observed mutational frequency of the *Cs -R* phenotype falls within the range characteristic of a single mutational event in chromosomal genes, it is concluded that the present cyanobacterial *Cs -R* mutant is also a product of a single mutational event. Since the *Cs -R* mutant showed impaired diazotrophy, oxygenic photosynthesis, chlorophyll a content and **osmotolerance** which are fully repairable by exogenous Cs , it is concluded that the *Cs -R* mutant is a pleiotropic mutant and that its apparent resistance to Cs is because of the requirement of this cation for its normal diazotrophy. The mutational pleiotropy is expressed only under diazotrophic growth condition and never under nitrate or ammonium nutrition growth condition thus suggesting the mutant phenotype to be the result of mutation in a genetic determinant co-ordinating cyanobacterial oxygenic photosynthesis, N -fixation and osmotolerance. Rb like Cs is found equally effective in physiological restoration of the

cyanobacterial mutational pleiotropy. Since neither Na /K^+ is found capable of repairing the Cs /Rb repairable mutational phenotype, the obvious inference is that Cs /Rb requirement cannot be substituted by Na /K in the cyanobacteriuiti. The question as to why chlorophyll a content declines in the absence of provision for Cs /Rb under diazotrophic growth condition can simply be explained on the basis of the dependence of diazotrophic growth of the mutant on exogenous Cs /Rb . Since the cyanobacterial Cs -*R* mutant seems to be a product of single mutational event in a genetic determinant of regulatory nature that controls oxygenic photosynthesis, N -fixation and osmotolerance, it would be interesting to find out how such a regulatory genetic determinant co-ordinates the three major cyanobacterial processes. In recent years, *P* protein has been shown to modulate photosynthetic carbon and nitrogen metabolism in unicellular non-diazotrophic cyanobacteria (Allen 1992; Tsinoremas *et al*. 1991).

Osmoremedial bacterial mutations where mutant phenotypes are expressed in medium of low osmolarity but not expressed in medium of elevated osmolarity are already known (Csonka 1989). Some bacterial mutants also show specific requirement for NaCl to correct their mutational defects (Kohno & Roth 1979) . The present osmosensitive pleiotropic cyanobacterial mutant belongs to a different category in that, it requires specifically Cs /Rb at low non-osmotic concentrations for the repair of osmosensitive mutant phenotype. Since Cs /Rb also repairs the other phenotypes of the pleiotropic cyanobacterial mutant, it is logical to infer that such specific requirement of Cs /Rb results from their corrective role in the mutant phenotype.

A knowledge of how monovalent (alkali) cations influence each other's transport, accumulation and toxicity in cyanobacteria would be extremely useful in understanding the

cyanobacterial mechanism(s) involved in regulation of their alkali cation nutrition and toxicity. It has been shown recently, that Li^+/Na^+ stimulates K^+ uptake and accumulation and Li^+, Na^+ or K^+ inhibit Cs^+ uptake and accumulation by individually influencing the activity of K^+ transport system in *Synechocystis* PCC 6803 (Avery et *al*. 1991).

Cs^+ toxicity in cyanobacterium has been suggested to result from cellular replacement of K^+ by Cs^+ which cannot substitute functionally for K^+ in cyanobacterial physiology (Avery et *al*. 1991, 1992a & b). The present finding that K^+ is unable to repair the Cs^+/Rb^+ repairable mutant phenotype in the cyanobacterium implies that K^+ also cannot substitute functionally for Cs^+/Rb^+ in cyanobacterial diazotrophic physiology. Since Cs^+ is found to be more efficient in inhibiting Rb^+ uptake and accumulation than Rb^+ in inhibiting Cs^+ uptake and accumulation and since Na^+ or K^+ at many fold higher concentration, also inhibits Cs^+ uptake and accumulation, it is suggested that Na^+, K^+ and Rb^+ individually or in combination would determine the intracellular level of Cs^+ in cyanobacterial population growing under Cs^+ polluted habitats. The one most obvious ecological implications of the present finding is that, Cs^+ requiring mutants of the given type likely to arise spontaneously in Cs^+ polluted habitats would not survive in such habitats rich in Na^+/K^+ because of the inhibitory effect of the latter on the availability of exogenous Cs^+ to the cyanobacterial mutant. The other implication of the present work concerns the application of such Cs^+/Rb^+ requiring cyanobacterial mutants in identification and removal/recovery of Cs^+/Rb^+ from habitats rich in either.

Conclusions

CONCLUSIONS

The results of the studies done exclusively on *Nostoc muscorum* and described in this thesis led to the following conclusions:

1) A class of tungsten resistant (*W-R*) mutants of the cyanobacterium were found severely defective in Mo-transport activity and required vanadium (V) for growth on N_2 or NO_3^- as nitrogen source. The requirement of V for nitrate nutrition is the first novel finding reported for any microbial system.
2) *N. muscorum* can make or regulate V-dependent nitrogenase in the absence of. active V-dependent nitrate reductase (NR) and *vice versa* as it makes or regulates its Mo-nitrogenase or Mo-NR independently.
3) The physiology of salinity stress on Mo- or V-dependent diazotrophic growth appears to be similar.
4) Cyanobacterial sensitivity to salinity and osmotic stresses is primarily because of its greater sensitivity to its N -fixation process than to its photosynthetic activity.
5) Mutation to salinity or osmotic stress resistance in the cyanobacterium results in severe curtailment in Na influx.
6) NH_4^+-nitrogen does not offer any protection to the cyanobacterium against salinity or osmotic stress induced lethality and plays no role in regulating Na transport.
7) Exogenous proline is used as a nitrogen source under unstressed conditions while it serves as an osmoprotectant under salinity or osmotic stressed conditions in the cyanobacterium.
8) Physiological responses of the cyanobacterium to osmotic and salinity stresses appear to be similar.

9) The cyanobacterial L-azetidine-2-carboxylate resistant (*Ac-R*) mutant is a proline overaccumulating strain and shows tolerance to salinity and as well as osmotic stresses.
10) Mutation to the *Ac-R* phenotype is accompanied by a loss in proline oxidase activity associated with inability to assimilate proline as a nitrogen source. A definite role of proline oxidase in regulating nitrogen nutrition or osmoprotective function of proline is evidenced in the cyanobacterium.
11) Mutational loss in proline oxidase activity seems to be the reason for overaccumulation of proline (leading to high intracellular level of proline) in the *Ac-R* mutant strain.
12) Proline *per se* is not the repressor of heterocyst formation and nitrogenase activity in the cyanobacterium.
13) Exogenous betaine functions as a nitrogen source in the cyanobacterium and fails to function as an osmo(salinity)-protectant in it.
14) Betaine does not share a common transport with proline in the cyanobacterium.
15) Single gene mutations confer genetic acquisition of osmo(salinity)-stress resistance in the cyanobacterium (as evident from the *NaCl-R*, *Sucrose-R* and *Ac-R* phenotypes).
16) Spontaneous cyanobacterial mutants resistant to growth toxic effects of alkali metals (Li^+, Na^+ & Rb^+) and alkaline pH (pH 11.0) show an enhanced H^+-gradient dependent multiple alkali cation efflux system and are found sensitive to sucrose-induced osmotic stress.
17) The cyanobacterium *N. muscorum* shows a definite requirement for Na^+ and K^+/Rb^+ for optimal growth under diazotrophic growth conditions.

18) Cs uptake and toxicity is diazotrophy-specific and NH_4^+-repressible.
19) Mutation to caesium resistance phenotype *(Cs -R)* results in physiological pleiotropy manifest in the form of impaired diazotrophic growth, oxygenic photosynthesis, chlorophyll a content, nitrogenase activity and osmotolerance.
20) Cs /Rb alone is found capable of restoring fully the physiological pleiotropy of the *Cs -F* mutant strain to its normal level.

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Appendices

STUDIES ON DIAZOTROPHY AND ITS REGULATION BY SALINITY STRESS AND OSMOTIC STRESS IN THE CYANOBACTERIUM *NOSTOC MUSCORUM*

SYNOPSIS

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Studies on diazotrophy and its regulation by salinity stress and osmotic stress in the cyanobacterium *Nostoc muscorum*

A majority of cyanobacteria, are diazotrophs deriving their carbon and nitrogen requirements from CO_2 and N_2 for growth and multiplication at the expense of photosynthesis. Their significance in regulation of nitrogen budget in natural ecosystems as well as in agricultural ecosystems is enormous in view of their oxygenic photosynthetic diazotrophic mode of nutrition. In India, diazotrophic cyanobacteria, native to water-logged rice-field ecosystems are cultivated and distributed on a large scale for use as biofertilizers and reports indicate considerable positive impact of such practices on the quality and quantity of rice agriculture. It is therefore natural to understand the genetics and physiology of cyanobacterial diazotrophy in relation to various environmental stresses like herbicide treatment, dessication, salinity etc. Very few studies of this applied nature have been made in past. Ammonia excreting laboratory strains of diazotrophic cyanobacteria have been demonstrated to supply fixed nitrogen to the crop systems under laboratory conditions. This practice has not gone to field level as yet. There is little information at molecular level about salinity and osmotic regulation of cyanobacterial diazotrophy under laboratory and field conditions.

There are three genetically distinct nitrogenases in

Azotobacter vinelandii. Growing evidence for wide spread occurrence of similar nitrogenases in other bacterial systems are also available. This aspect has not been studied in detail in diazotrophic cyanobacteria. In view of the ability of cyanobacterial forms to grow and adapt to various ecological niches, it is important to know and find out the chemical nature of such habitats and of the cyanobacterial forms growing there. A knowledge of vanadium (V) in various soil or aquatic habitats in nature is required in order to infer operation of V-nitrogenase in diazotrophic cyanobacterial forms growing in such habitats, as V like molybdenum (Mo) is being considered to play a key role in nitrogen cycle in nature (Robson *et al* 1986). In the present study, *Nostoc muscorum* has been physiologically and mutationally analyzed for requirement of V in nitrogen fixation and in nitrate assimilation. The reason for the present study, was the finding that tungstate resistant (*W-R*) mutants of *Nostoc muscorum*, no longer required Mo for diazotrophy and nitrate assimilation (Singh *et al* 1978). The same technique was used to isolate a fresh *V-R* mutants of parent *Nostoc muscorum* and of its non-nitrogen fixing and non-nitrate assimilating mutant strains. Such mutants were then separately examined for their ability to grow with the respective nitrogen source in the presence or absence of Mo/V. The results of these critical studies clearly show that, a class of *W-R* mutants deficient in Mo-transport activity acquire the need for V in place of Mo for growth on N_2 or NO_3 as a nitrogen source. The V-requiring N_2- or NO_3^-- assimilating mutant did not exhibit any alteration in their

nitrogen regulatory characteristics of nitrogen regulation of diazotrophy and NO_3^--assimilation. While the demonstration of V-dependent nitrogenase activity in cyanobacteria is a characteristic already known in other diazotrophic bacterial forms, the present demonstration of V-dependent nitrate assimilation is the first novel report of this kind for any microbial system. In view of the observed similarity in the nitrogen regulation of diazotrophy in both the parent strain (a Mo-dependent diazotroph) and in the mutant strain (a V-dependent diazotroph) further studies on salt/osmotic regulation of nitrogen fixation in the cyanobacterium was thus restricted mainly to the parental strain.

Studies were conducted to isolate mutants of *Nostoc muscorum* resistant to growth inhibitory action of salinity (NaCl) and osmotic (sucrose) stress. The salinity resistant mutant was found lacking in normal proline transport activity while retaining the salinity-stimulable uptake process. The salinity stress resistant (*NaCl-R*) mutant and the osmotic stress resistant mutant (*Sucrose-R*) were checked for their cross-resistance relationship and were found to exhibit such a relationship. This finding suggested possible use of mutational methods for generating cyanobacterial strains that are capable of growth and multiplication in salinity/osmotically stressed habitats of agricultural ecosystems. All the mutants obtained were of spontaneous nature with a frequency characteristic of a single mutational event. It thus appears that single mutational

activity and ... N_2- fixing heterocysts in proline medium. The parent strain under normal growth conditions assimilated proline as a fixed nitrogen source. Thus, the *Ac-R* proline overproducing cyanobacterial mutants were also salinity/osmo -tolerant demonstrating for the first time a role of proline as a salinity/osmo -protectant in cyanobacteria.

Experiments were also conducted to examine whether proline transport system in cyanobacteria like that in other bacterial systems is salinity/osmo -regulated. Both the parent and the *Ac-R* mutant strains showed salinity/osmo -stimulable proline transport system. This led us to examine the role of exogenous proline on salinity/osmo -protection in the cyanobacterium. The results showed that under salinity stress in the presence of proline, the parent strain produced heterocysts, showed nitrogenase activity and exhibited increased tolerance to salinity/osmotic stress. Also, under similar conditions the intracellular proline level rose considerably. These results suggest that overaccumulation of proline resulting either from increased uptake, increased synthesis or from decreased degradation is the physiological mechanism of cyanobacterial adaptation to salinity/osmotic stress.

Glycinebetaine like proline is a known salinity/osmo -protectant in bacterial forms (Rhodes fc Hanson, 1993). Cyanobacterial forms isolated from hypersaline habitats accumulate predominantly glycinebetaine to overcome

hypersalinity/hyperosmotic stress. Experiments were conducted to find out whether exogenous glycinebetaine would offer protection to the cyanobacterium against salinity stress and whether salinity-stimulable proline transport system would in anyway get affected by glycinebetaine. Glycinebetaine like proline under normal physiological conditions was utilized like a fixed nitrogen source leading to repression of heterocyst formation and nitrogenase activity. Exogenous glycinebetaine failed to offer protection against salinity/osmotic stress. Salinity/osmo -stimulable proline transport system remained uninfluenced with exogenous glycinebetaine.

Li has been shown to be growth toxic to bacterial (Umeda *et al* 1984) and cyanobacterial (Avery *et al* 1991) systems and the bacterial lithium resistant mutants (*Li-R*) are known to exhibit increased adaptability to Li , Na and Rb (Padan & Schuldiner, 1994). This alkali cation has not been studied for its physiological effects except for inorganic carbon transport in any cyanobacterium. Spontaneous mutants of the cyanobacterium resistant to lethal action of lithium chloride (LiCl) were also isolated to find out the mechanism of resistance to Li as well as to other alkali cations. The lithium resistant (*Li -R*) mutant was found resistant to growth inhibitory action of higher concentrations of NaCl, KCl, RbCl and CsCl. Detailed studies of such multiple alkali metal resistant strain suggested that the mutation to the Li^{+}-*R* phenotype has resulted from the activation of an efflux system specific to the various alkali cations. This

mutant was also found relatively more resistant to pH 11.0 than its parental strain. Studies with the inhibitors of H^+-gradient formation suggested the multiple alkali metal efflux system to be H -gradient driven process. This finding has provided us a knowledge for constructing diazotrophic cyanobacterial strains that are resistant to salinity stress and thus be useful for use as biofertilizer strains in rice agriculture. Similarly a sodium chloride resistant strain (*Na -R),* rubidium chloride resistant mutant strain (*Rb -R)* and an alkalotolerant strain ($pH_{11.0}$-R) were also isolated, which showed cross resistance to Li , Na , K^+, Rb^+ and Cs^+ induced lethality as well as tolerance to alkaline pH stress.

137 +

Cs is a regular radioactive pollutant discharged from nuclear waste and has been found to be very toxic to the primary producers including cyanobacteria. Cs has been shown to be transported in cyanobacteria through the ammonium transport system and its toxicity seems to result by its replacing intracellular K (Avery et *al* 1991). Cs toxicity has been found to be ammonium repressible in the cyanobacterium *Nostoc muscorum* (Singh *et* al 1994). In the present study, a class of Cs resistant *(Cs -R)* mutants were isolated under diazotrophic conditions which showed definite requirement of Cs /Rb for normal growth. This is a very novel finding with obvious implications in the sense that Cs requiring mutants in diazotrophic cyanobacteria can arise during their growth under natural conditions in habitats polluted with Cs . The results of

the interaction of Na^+, K^+ on nutritive role of Cs^+/Rb^+ suggested that Na and K at higher concentrations would result in cellular replacement of Cs^+/Rb^+ thus inhibiting the growth of Cs^+/Rb^+ requiring mutant under growth conditions containing excess of Na* or K . Further analysis of Cs^+ requiring diazotrophic mutant suggested it to have acquired sensitivity to salinity/osmotic stress under diazotrophic growth conditions. Thus the Cs^+ requiring diazotrophic mutant of *Nostoc muscorum* is salinity/osmo -sensitivity both of which are found NH_4^+-repressible.

The important conclusions from the present work are hereby cited below :

1) A class of tungsten resistant mutants of the cyanobacterium *Nostoc muscorum* found defective in Mo-transport activity, were found to show a requirement on V for growth on N_2 or NO_3 as nitrogen source.
2) Salinity stress resistant mutants and osmotic stress resistant mutants showed a cross-resistance relationship.
3) Exogenous proline offered complete protection to the parent strain against salinity/osmotic stress induced lethality. The salinity-stimulable nature of proline uptake process appears to be the alternative exclusive mechanism of salinity/osmotic protection by proline.
4) Mutation to salinity resistance has inactivated the normal proline uptake process while leaving the salinity-stimulable proline transport system unaffected.

5) Spontaneous mutants resistant to L-Azetidine-2-carboxylate (AC) were found to show increased tolerance to salinity/osmotic stress. A loss in proline oxidase activity in the *Ac-R* mutant strain was found associated with a rise in its intracellular proline level.

5) Glycinebetaine is metabolized like a fixed nitrogen source and like NH_4^+-nitrogen, does not offer any protection to the cyanobacterium against salinity/osmotic stress.

6) The *Li -R*, Na^+-*R*, Rb^+-*R* and the $pH_{11\ 0}$-*R* mutant strain all were found resistant to lethal action of Li , Na , K , Rb and Cs and to alkaline pH 11.0 stress, thus suggesting a common physiological basis for such a resistance.

7) A role of H -gradient driven alkali metal efflux system in conferring multiple alkali metal resistance and to pH 11.0 has been evidenced.

8) Resistance to NaCl can result either from overaccumulation of proline (*NaCl-R*, salinity stress resistance) or from enhanced activity of H^+-gradient dependent alkali cation efflux system {*Na -R,* multiple alkali cation resistance).

9) A class of Cs^+-*R* mutants were found to show a definite requirement of Cs^+/Rb^+ for normal diazotrophic growth and salinity/osmo tolerance.

On the whole, results presented in this study offer a feasible approach to mutationally generate salinity/osmotic stress resistant mutant strains, for use as biofertilizer in saline rice agriculture.

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List of publications of the candidate

1) **Modi, D.R., Singh, A.K., Rao, K.S., Chakravarthy, D. & Singh, H.N. (1991) Construction of multiple herbicide resistant ammonia excreting strains of the cyanobacterium *Nostoc muscorum*. *Biotechnology Letters* 13(11), 793-798.**

2) **Singh, R.K., Singh, H.N., Chakravarthy, D., Rao, K.S. & Singh, H.N. (1992) Mutational analysis of glutamine synthetase response to the ammonium analogue ethylene diamine** in **the** cyanobacterium *Nostoc muscorum. FEMS Microbiology Letters* 95, 43-48.

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