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

SYNOPSIS
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A majority of cyanobacteria, are diazotrophs deriving their carbon and nitrogen requirements from \( \text{CO}_2 \) and \( \text{N}_2 \) 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
Amotobacfr 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 were 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\textsubscript{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-i-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 11.4 than its parental strain. Studies with the inhibitors of H\textsuperscript{+}-gradient formation suggested the multiple alkali metal efflux system to be H\textsuperscript{-}-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\textsuperscript{-}R), rubidium chloride resistant mutant strain (Rb\textsuperscript{-}R) and an alkalotolerant strain (\textit{pH}_{11.0\textsuperscript{-}R}) were also isolated, which showed cross resistance to Li\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}, Rb\textsuperscript{+} and Cs\textsuperscript{+} induced lethality as well as tolerance to alkaline pH stress.

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\textsuperscript{+} (Avery et al 1991). Cs toxicity has been found to be ammonium repressible in the cyanobacterium \textit{Nostoc muscorum} (Singh et al 1994). In the present study, a class of Cs resistant (Cs\textsuperscript{-}R) mutants were isolated under diazotrophic conditions which showed definite requirement of Cs /Rb\textsuperscript{+} 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 $\text{Na}^+$, $\text{K}^+$ on nutritive role of $\text{Cs}^+$/Rb$^+$ suggested that Na and K at higher concentrations would result in cellular replacement of $\text{Cs}^+$/Rb$^+$ thus inhibiting the growth of $\text{Cs}^+$/Rb$^+$ requiring mutant under growth conditions containing excess of Na$^+$ or K$^+$. Further analysis of $\text{Cs}^+$ requiring diazotrophic mutant suggested it to have acquired sensitivity to salinity/osmotic stress under diazotrophic growth conditions. Thus the $\text{Cs}^+$ requiring diazotrophic mutant of $\textit{Nostoc muscorum}$ is salinity/osmo-sensitivity both of which are found $\text{NH}_3$-repressible.

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

1) A class of tungsten resistant mutants of the cyanobacterium $\textit{Nostoc muscorum}$ found defective in Mo-transport activity, were found to show a requirement on V for growth on $\text{N}_2$ or $\text{NO}_3$ 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$_{11}$O$^{-}$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.
References

List of publications of the candidate


