Genetically modified cyanobacterium *Nostoc muscorum* overproducing proline in response to salinity and osmotic stresses

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In the parent *Nostoc muscorum* an active proline oxidase enzyme is required to assimilate exogenous proline as a fixed nitrogen source. Cyanobacterial mutants, resistant to growth inhibitory action of proline analogue L-azetidine-2-carboxylate (Ac-R), were deficient in proline oxidase activity, and were over-accumulators of proline. Proline over-accumulation, resulting either from mutational acquisition of the Ac-R phenotype, or from salinity-induced uptake of exogenous proline, confirmed enhanced salinity/osmotic tolerance in the mutant strain. The nitrogenase activity and photosynthetic O_2 evolution of the parent were sensitive to both salinity as well as osmotic stresses than of Ac-R mutant strain. In addition, the mutation to Ac-resistant phenotype showed no alteration in salinity inducible potassium transport system in the cyanobacterium

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1. Introduction

Osmoadaptable organisms, like bacteria and plants, respond to varying levels of inorganic/organic osmotica by synthesizing and accumulating a few selective compatible organic solutes like trehalose, sorbitol, mannitol, proline, and glycine-betaine which help these organisms to tolerate and grow under such special environmental stress situations (Csonka 1989; Bartels and Nelson 1994; Lucht and Bremer 1994). The problem of salt/osmo adaptation has been analysed genetically in the members of enterobacteria were *kdpABCDE* genes required for inducible K^+ uptake, shown involvement of signal transduction pathway for adaptation to salt/osmotic stress (Walderhaugh *et al* 1987). This primary osmotic intracellular signal activates genes functioning specifically either in the transport, or in the synthesis and accumulation of compatible organic solutes. The product of salinity-inducible *proU* and *proP* genes offer protection by inducing transport of betaine and proline (Cairney *et al* 1985a,b). Proline biosynthetic genes, *proABC*, control production of proline from its parent amino acid glutamate and it is known that mutation in *proA* gene overproduce of proline. Such mutants no longer showed feedback inhibition control of its activity by proline, and that is why proline overproducing, *proA*, mutant, selected for resistant to proline analog L-azetidine-2-carboxylate, show salinity/osmotic resistant phenotype (Mahan and Csonka 1983, Jakowec *et al* 1988). Similarly, *otsA* and *otsB* genes are also osmo-inducible and, thus, synthesized trehalose as an osmo-protectant under osmotic stress condition (Giaever *et al* 1988). The *omp* genes of *Escherichia coli* produce characteristic porin proteins as a function of osmotic-stress and salinity-stress conditions (Csonka and Hanson 1991).

Cyanobacteria are oxygenic photosynthetic prokaryotes and little knowledge is available on their molecular characteristic of salt/osmo adaptation. Some recent studies have

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Abbreviations used: AAS, Atomic absorption spectroscopy; Ac-R, L-azetidine-2-corboxylate resistant; CFUs, colony forming units; Chl *a*, chlorophyll *a*.

shown trehalose, sucrose, glucosyl-glycerol functioning as osmobalancer in either fresh water or marine cyanobacteria and glycine-betaine as osmobalancer in hypersaline cyanobacteria (Mackey *et al* 1984; Warr *et al* 1988). Genetic engineering of salt/osmo tolerance for both nitrogenase activity and diazotrophy has been achieved in *Klebsiella pneumoniae* (Le Rudulier *et al* 1982; Le Rudulier and Bouillard 1983). Blumwald and Tel-Or (1982) reported that sucrose function as osmo-regulator in the diazotrophic heterocystous cyanobacterium *Nostoc muscorum* PCC 7119 apparently without any increase in free-proline content. Similar results were obtained in the case of fresh-water *Gloeocapsa* and *Anabaena* c*ylindrica* (Borowitzka 1981). In few marine cyanobacteria glucosyl-glycerol is the major osmolyte (Erdmann *et al* 1992).

Accumulation of proline in response to osmotic stress has been documented in higher plants (Dix and Pearce 1981; Voteburg and Stewart 1984; Bhaskaran *et al* 1985; Kapuya *et al* 1985; Handa *et al* 1986; Roosen *et al* 1999), algae (Brown and Hellebust 1980) and bacteria (Csonka 1989), but not in cyanobacteria. Many studies indicate a correlation between the degree of osmotic stress and the levels of accumulated proline. It is suggested that during osmotic stress accumulation of proline results in balancing the osmotic strength of the cytoplasm with that of the environment (Yancey *et al* 1982). Furthermore, proline can also prevent damage from cellular dehydration by increasing the water-binding capacity of protein (Schobert and Tschesche 1978).

In this study, the proline analog L-azetidine-2-carboxylate (Ac) has been used to examine the possibility of spontaneous occurrence of analog-resistant mutant which is capable of overproducing proline and simultaneously showing tolerance to salinity as well as to osmotic stress.

2. Materials and methods

2.1 *Organism and maintenance of the culture*

In the present study, the experimental organism was *N. muscorum* (a freshwater cyanobacterium), grown axenically in Chu No. 10 medium (Gerloff *et al* 1950) without any combined nitrogen source. Cultures were incubated at 28°C ± 2°C and illuminated with daylight fluorescent tubes having a photon fluence rate of 50 µmol m^{-2} s^{-1}. The culture medium was buffered to pH 7.5 with 10 mol m^{-3} HEPES-NaOH.

2.2 *Estimation of percent survival*

Nutrient agar plates at different graded concentration of the inhibitor were prepared. Exponentially growing diazotrophic cultures of the cyanobacterium in quantities of 5×10^7 colony forming units (CFUs) were inoculated on to each plate. A control plate containing no inhibitor was also inoculated. After 6 days of growth, the number of CFUs appearing on each plate was compared with respect to the control and expressed as percent of the control. The total number of CFUs survival on the control plate was presumed to show 100% survival.

2.3 *Isolation of L-azetidine-2-carboxylate resistant mutant of the parent strain*

L-azetidine-2-carboxylate (Ac) is a growth toxic analog 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 *N. muscorum* in quantities of 5×10^7 CFUs were inoculated onto the diazotrophic medium containing 1 mol m^{-3} Ac, a dose 10-fold more lethal 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 analog.

2.4 *Growth, chlorophyll* a *and protein measurement*

In liquid culture medium, *N. muscorum* cells were homogeneous; therefore, a direct absorbance change at 663 nm was proportional to its multiplication and was used for measurement of growth. Whereas the chlorophyll *a* (Chl *a*) contents was estimated by the method of Mackinney (1941), the protein contents was estimated by the method of Lowry *et al* (1951) by using lysozyme (Sigma, St. Louis, MO, USA) as a standard.

2.5 *Estimation of ^{14}C-proline transport activity*

The method of Gour *et al* (1997) was used for measurement of ^{14}C-proline uptake. The cyanobacterial cells were centrifuged, washed and resuspended in 10 mol m^{-3} HEPES-NaOH buffer (pH 7.5) and equilibrated for 30 min at 28°C at a photon fluence rate of 50 μmol m^{-2} s^{-1}. ^{14}C-proline was then added to the incubation mixture to a final concentration of 0.05 mol m^{-3} (specific activity 7.5 KBq mol^{-1}). Samples were withdrawn at regular time intervals and reaction was terminated by the addition of 0.1 ml of 2 N HCl. A 2.5 ml of scintillation cocktail containing four parts of 0.8% PPO (2,5-diphenyloxazole) plus 0.01% POPOP [1,4-bis

(4-methyl-5-phenyl-2-oxazole)-benzene] in toluene and three parts of ethanol was added to each sample vial. ^{14}C-proline transport activity was counted in liquid scintillation counter (Wallac 1409, Finland). The activity was expressed in terms of mmol proline g^{-1} Chl *a*.

2.6 *Determination of proline oxidase activity*

Proline oxidase activity was measured by the method of Dendinger and Brill (1970) with slight modification as described by Spence and Stewart (1986). Exponentially growing cultures were harvested and permeabilized in 1 cm^3 of 100 mol m^{-3} sodium cacodylate buffer (pH 6.6) for 5 min using 0.3 cm^3 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 trichloro acetic acid (TCA). The reaction mixture was incubated in dark for 10 min and then centrifuged at low speed and the absorbency of the supernatant was read at 443 nm.

2.7 *Estimation of K^+ transport activity*

K^+ uptake was measured under stressed and unstressed conditions by atomic absorption spectroscopy (AAS). Ten mol m^{-3} HEPES-NaOH buffer (pH 7.5) wss used as a medium to estimate K^+ uptake by exponentially growing cyanobacterial cultures under the given growth conditions. The experimental samples were equilibrated with unstressed- or stressed-buffer medium for 30 min before the addition of 5 ppm (0.128 mol m^{-3}) KCl. The stressed salinity condition was created by the addition of 50 mol m^{-3} NaCl to the buffer medium. K^+ uptake was estimated by determining the K^+ contents of various supernatants at given time intervals by AAS, and then subtracting these values from the control value.

Heterocyst frequency, nitrogenase activity, photosynthetic O_2 evolution and intracellular proline contents were measured as described previously by Bhargava *et al* (2003).

3. Results

The spontaneously occurring Ac-R mutant arose with a mutation frequency of $0.6 - 0.8 \times 10^{-7}$, suggesting that the resulting mutant phenotype is a product of single mutation in the chromosomal gene. The parent *N. muscorum* showed no heterocysts/nitrogenase activity in growth medium containing 1 mol m^{-3} proline or 1 mol m^{-3} NH_4Cl, suggesting that *N. muscorum* utilizes proline as a readily assimilable nitrogen source for growth and multiplication. The Ac-R mutant clones was compared with its parent for photoautotrophic growth, heterocyst frequency, nitrogenase activity and proline oxidase activity (table 1). The result suggests that mutant strains showed almost similar level of photoautotrophic growth either in the diazotrophic medium or in the medium containing 1 mol m^{-3} proline or 1 mol m^{-3} NH_4Cl. However, the mutant showed normal heterocyst frequency and nitrogenase activity in the medium containing 1 mol m^{-3} proline, in which its parent never did. Furthermore, heterocyst frequency and nitrogenase activity of mutant strains are similar to that of parent when grow in 1 mol m^{-3} NH_4Cl medium. Thus, while Ac-R mutant of cyanobacterium has lost genetically proline-repression control of heterocyst and nitrogenase activity, has not lost its NH_4^+- repression control of heterocyst and nitrogenase activity during the course of its mutation to Ac-R phenotype. 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

Table 1. Growth (OD change at 663nm), heterocyst frequency (HF%, number of heterocysts per 100 vegetative cells), nitrogenase activity (nmol C_2H_4 formed mg^{-1} Chl *a* h^{-1}) and proline oxidase activity (mmol proline oxidized g^{-1} Chl *a* h^{-1}) of the parent *N. muscorum* and its Ac-R mutant strains in different nitrogen growth medium.

	Parent				Ac-R mutant			
Treatment	Growth	HF %	Nitrogenase activity	Proline oxidase activity	Growth	HF (%)	Nitrogenase activity	Proline oxidase activity
N_2-medium	0.76 ± 0.05	7–8	12.28 ± 0.27	2.46 ± 0.12	0.77 ± 0.04	7–8	12.14 ± 0.52	0–0
+ 1 mol m^{-3} NH_4Cl	0.80 ± 0.06	0–0	0–0	2.23 ± 0.10	0.80 ± 0.06	0–0	0–0	0–0
+ 1 mol m^{-3} proline	0.82 ± 0.09	0–0	0–0	3.98 ± 0.09	0.82 ± 0.08	7–8	11.82 ± 0.27	0–0

One mol m^{-3} proline grown cultures were source of inocula for the experiments. Such inocula were grown for six days in respective growth media and then used for estimation of their characteristics. Each reading is an average (±SEM) of three independent experimental determinations.

Table 2. Effect of salinity stress and osmotic stress on nitrogenase activity (nmol C_2H_4 formed mg^{-1} Chl *a* h^{-1}) and photosynthetic O_2 evolution (μmol O_2 evolved mg^{-1} Chl *a* h^{-1}) of the parent *N. muscorum* and its Ac-R mutant strain.

	Nitrogenase activity		Photosynthetic O_2 evolution	
Treatment	Parent	Ac-R mutant	Parent	Ac-R mutant
Control	12.28 ± 0.28	12.14 ± 0.26	524 ± 18.6	520 ± 20.4
+ 25 mol m^{-3} NaCl	8.26 ± 0.16	12.03 ± 0.21	488 ± 20.3	518 ± 16.2
+ 50 mol m^{-3} NaCl	6.80 ± 0.21	11.78 ± 0.16	320 ± 19.8	522 ± 15.3
+ 75 mol m^{-3} NaCl	2.20 ± 0.09	11.14 ± 0.22	128 ± 10.3	478 ± 18.5
+ 100 mol m^{-3} sucrose	8.56 ± 0.27	11.76 ± 0.24	512 ± 22.5	506 ± 19.2
+ 150 mol m^{-3} sucrose	5.60 ± 0.22	12.02 ± 0.12	318 ± 16.6	477 ± 12.3
+ 200 mol m^{-3} sucrose	1.50 ± 0.08	11.78 ± 0.19	100 ± 5.6	372 ± 15.6

Six-day-old diazotrophically grown cultures of the two strains were used as source of inocula for the experiments. Each reading is an average (±SEM) of three independent experimental determinations.

suggest that *N. muscorum* is capable of assimilating proline as a nitrogen source and that its mutation to Ac-R phenotype has resulted in loss of this ability. Furthermore, analyses of the two cyanobacterial strains with respect to their proline-catabolizing enzyme, proline oxidase, reveal the presence of this enzyme in the parent, but its absence in the Ac-R mutant. In addition, 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 parent and its Ac-R mutant were further compared with respect to their nitrogenase activity and photosynthetic O_2 evolution under normal and salinity/osmotic stress conditions (table 2) which shows that nitrogenase activity and photosynthetic O_2 evolution were all less affected by given NaCl and sucrose stresses in the mutant strain.

The two strains were also compared with respect to their intracellular level of proline under non-stress, and stress conditions (table 3). The intracellular proline level always remained about 3–4-fold higher in the Ac-R mutant strain than in the parent strain. However, both strains showed lack of sali-nity/osmotic-inducible increase in intracellular proline level.

Studies on salinity resistance or sucrose resistance level of Ac-R mutant in comparison to its parent showed that the degree of survival under unstressed condition in diazotrophic growth medium was considered to be 100%. A dose of 100 mol m^{-3} NaCl or 250 mol m^{-3} sucrose for 12 h was lethal to the parent strain. In comparison, the Ac-R strain showed nearly 88% salinity survival or 92% 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 (figure 1). Exogenous proline was counteracted salinity/osmotic-stress lethality in the parent strain, while the Ac-R mutant strain remained uninfluenced.

Table 3. Intracellular levels of proline (μmol g^{-1} protein) in N_2 medium and in medium containing 75 mol m^{-3} NaCl and 200 mol m^{-3} sucrose in the parent and its Ac-R mutant strain.

Treatment	Parent	Ac-R mutant
N_2 medium	9.60 ± 0.28	34.80 ± 2.1
+75 mol m^{-3} NaCl	12.20 ± 0.32	39.20 ± 2.6
+200 mol m^{-3} sucrose	10.80 ± 0.26	40.60 ± 1.8

Six-day-old diazotrophically grown cultures were used as source of inocula in the present series of experiments. NaCl and sucrose were added to the experimental cultures before 3 h of the start of the experiment. Each reading is an average (±SEM) of three independent experimental determinations.

For the role of salinity and osmotic stress in regulation of proline uptake and accumulation in the two strains (figures 2 and 3); proline uptake and accumulation were extremely sensitive to the uncoupler (CCCP) of photophosphorylation, suggesting that the cyanobacterial proline uptake and accumulation process is an active energy-requiring process. Salinity stress of 75 mol m^{-3} NaCl or osmotic stress of 200 mol m^{-3} 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 salt/osmo stimulable and its mutation to Ac-R phenotype has left its salt/osmo stimulable proline uptake process almost unaffected. Thus, the Ac-R mutant does not seem to have suffered genetic damage in its salt/osmo-regulated proline-transport activity.

Figure 4 represents comparison of the parent and its Ac-R mutant for the activity of their salinity-inducible K^+ transport system. Mutant and the parent under normal diazotrophic condition exhibited a K^+ transport activity, which increase many folds under the NaCl stress condition.

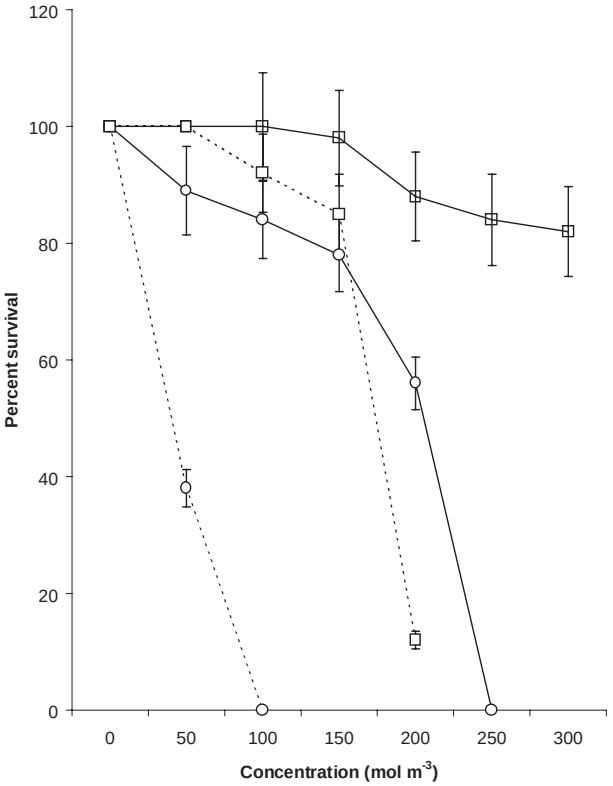

Figure 1. Percent survival of the parent *N. muscorum* (○) and its Ac-R mutant strain (□) to increasing concentration of NaCl (-------) and sucrose (———). Mean values from three independent determinations are shown ± SEM, where these exceed the dimensions of the symbols.

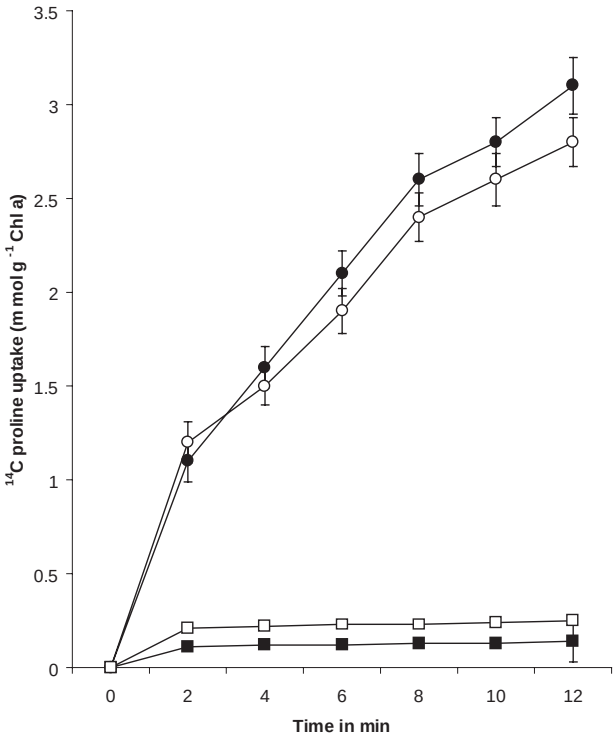

Figure 2. Effect of 75 mol m^{-3} NaCl (○), 200 mol m^{-3} sucrose (●), without stress **(□)** and 0.01 mol m^{-3} CCCP (■) on uptake of ^{14}C- proline in the parent *N. muscorum*. Mean values from three independent determinations are shown ± SEM, where these exceed the dimensions of the symbols.

Evidently both the parent and its Ac-R mutant strains are almost similar in respect of the activity of their salinity-inducible K^+ transport system. Thus mutation to Ac-R phenotype in cyanobacterium does not seem to alter the activity of salinity-inducible K^+ transport system.

4. Discussion

Osmoadaptable organisms belonging to bacteria (Csonka and Hanson 1991), cyanobacteria (Warr *et al* 1988), algae (Brown and Hellebust 1980), fungi (Jennings and Burke 1990) and higher plants (Bartels and Nelson 1994) accumulate one or more low-molecular weight organic compounds known as compatible solutes to maintain osmotic balance of their cytoplasm against high osmolarity of the environment. Christian (1950) showed a role of proline in protection of bacteria against salinity or osmotic stress. This led to the study of genes involved in proline production from its parent amino acid glutamate in enterobacteria. Such studies discovered three genes viz. *proABC* controlling proline production from glutamate. Further studies showed feed back inhibition of the first enzyme of proline biosynthesis, i.e. of γ-glutamyl kinase by proline in which *proA* gene coded for the first enzyme of the pathway. Mutation in the *proA* gene led to production of γ-glutamyl kinase which was no longer capable of feed back inhibition by proline. As a result, such mutants were over-producers of proline. Similar proline over-producing mutants were generated in enterobacteria by selecting them for resistance to growth-inhibitory action of proline analog L-azetidine-2-carboxylate. Thus, mutation in the *proA* gene resulted in over-production of proline and acquisition of salinity/osmotic resistance in enterobacteria (Csonka 1981; Csonka and Hanson 1991). A similar mechanism of proline over-production and osmotolerance is demonstrated in higher plants (Bartels and Nelson 1994). The mechanism involved in over production of proline by bacterial mutant resistance to L-azetidine 2-carboxylate has been the result of loss of feed back inhibition of γ-glutamyl kinase by proline. Since Ac is an analog of proline and analog toxicity is a function of cellular proline level. The proline over producer strain naturally exhibits resistance to proline analog in which relatively much higher level of intracellular level of proline prevents the inhibitory effect of its analog in the enterobacteria (Csonka 1989). There is a previous report

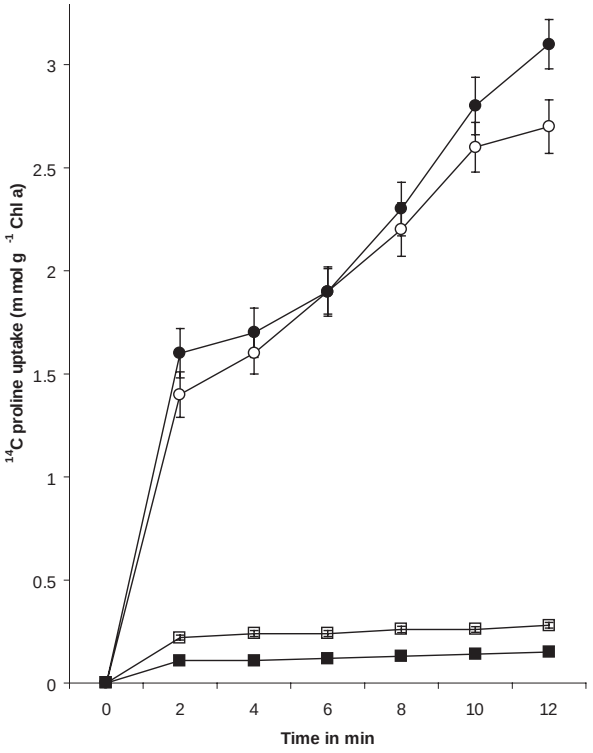

Figure 3. Effect of 75 mol m^{-3} NaCl (○), 200 mol m^{-3} sucrose (●), without stress (□) and 0.01 mol m^{-3} CCCP (■) on uptake of ^{14}C-proline in the Ac-R mutant strain of *N. muscorum.* Mean values from three independent determinations are shown ± SEM, where these exceed the dimensions of the symbols.

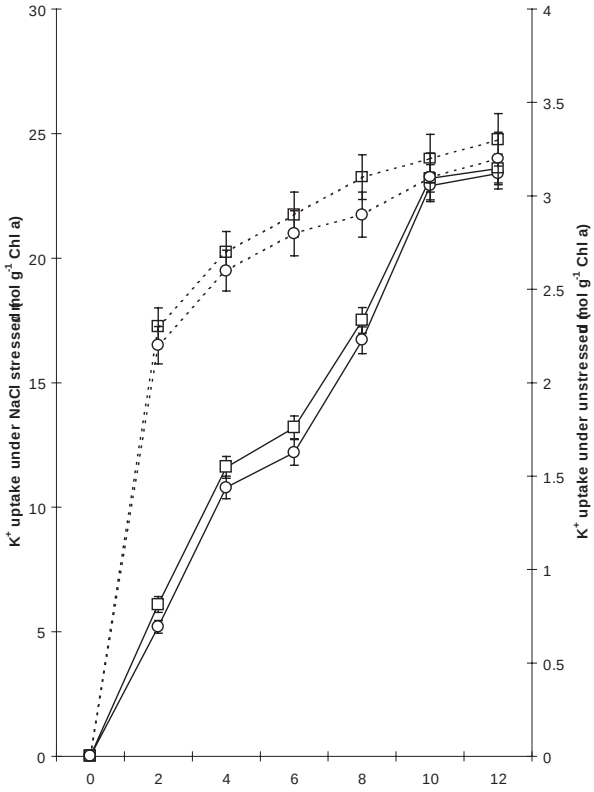

Figure 4. K^+ uptake pattern in the parent *N. muscorum* (○) and its Ac -R mutant strain (□) under 75 mol m^{-3} NaCl stressed (———) condition and unstressed (-----) condition. Mean values from three independent determinations are shown ± SEM, where these exceed the dimensions of the symbols.

on proline over producing Ac-R strain of *N. muscorum* where proline over-accumulation due to mutational loss of proline oxidase (Singh *et al* 1996), the enzyme used to assimilate proline as a nitrogen source. An organism resistant to amino acid analog can arise by mutational alteration of the parent system in the following ways:

(i) A mutant may become resistant by mutational loss of permeability to amino acid analog.
(ii) The mutant may become resistant to amino acid analog by producing an enzyme system which degrades the analog.
(iii) The mutant can alter the first enzyme of the biochemical pathway of the amino acid that no longer exhibits feed back inhibition by the amino acid under this situation the analog resistant phenotype is the result of the production of excess proline which comparatively dilutes the inhibitory effect of analog on the given organism.
(iv) Mutant can lead to loss of proline catabolizing enzyme system, thus leading to over accumulating of proline and, thereby, exhibiting apparent resistance against the amino acid analog.

The present Ac-R cyanobacterial strain is also the over-producer of proline, and the mechanism of resistance to proline analog appears to result from relatively higher levels of intracellular proline. The fact that analog-resistant mutant being a product of permeability mutation would be ruled out on the basis that if it is a permeability mutant strain, it can not be a proline over-producer merely because the latter condition would require an additional mutation to occur in the pathway of proline metabolism. Similar condition and reasoning would rule out enzymatic detoxification of proline analog as a cause of the cyanobacterium resistant to it.

In the present study it has been shown that mutational loss of proline oxidase results in loss of the ability to assimilate proline as a nitrogen source and such mutants were found proline over accumulating, heterocyst forming and N_2 fixing. In addition they were also found salinity as well as osmotic tolerant in terms of nitrogenase activity (Singh *et al* 1996), photosynthetic O_2 evolution, without influencing in any way the salinity inducible potassium transport system. Therefore it would be interesting to generate proline over-producing cyanobacterial mutant strains lacking osmo/salinity inducible K^+ transport system because study of such mutant will decide whether proline

over production still depends on this salinity/osmotic signal. In *Anabaena torulosa* and *E. coli* while potassium deficiency enhanced the synthesis of certain osmotic-induced proteins, addition of K^+ repressed the synthesis of these proteins (Alahari and Apte 2004). In enterobacterial system *proU* gene is restricted to media of high osmolarity, while the *kdp* (K^+ uptake) system activates in media of low osmolarity. In addition, the presence of proline in the growth media at high osmolarity reduces the expression of *proU*, but has no effect on expression of *kdp* system. High K^+ concentrations repress the Kdp system, whereas they are essential for the expression of the ProU system. This comparison of *kdp* and *proU* expression signals indicates that the expression of *proU* genes depend on the accumulation of potassium in the cytoplasm (Sutherland *et al* 1986). Briefly stated, cyanobacterium *N. muscorum* like enterobacteria uses proline as compatible solutes for salinity/osmotic protection without influencing adversely the cyanobacterial heterocyst frequency and nitrogenase charactersitic. This finding can be applied for generating biofertilizer strain of cyanobacteria for saline and user soils.

5. Conclusions

(i) The Ac-R mutant of the cyanobacterium is a proline over-accumulating strain and shows tolerance to salinity stress as well as osmotic stress.
(ii) Mutation to the Ac-R phenotype results from the loss in proline oxidase activity, leading to over-accumulation of proline. A definite role of proline oxidase in regulating nitrogen nutrition or osmo-protective function of proline is evidenced in the cyanobacterium.
(iii) The parent and its Ac-R mutant strains are almost similar in respect of the activity of their salt/osmo-inducible K^+ transport system. Thus mutation to proline-analog-resistant phenotype in cyanobacterium does not seem to alter the activity of salt/osmo-inducible K^+ transport system.

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