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EXPERIMENTAL ARTICLES

Evidence for a Sodium-Dependent Proline and Glycine-Betaine Uptake in the Cyanobacterium *Nostoc muscorum*[1]

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Abstract—The cyanobacterium *Nostoc muscorum* is able to utilized proline and glycine-betaine as a nitrogen source under unstressed growth conditions. This cyanobacterium when grow in modified Chu No. 10 medium (without Na^+) unable to utilized proline and glycine-betaine as a nitrogen source. Spontaneously occurring mutant clones defective in Na^+ transport (Na^+-R) was isolated and analyzed for proline and glycine-betaine utilization. The mutant phenotype showed normal heterocyst frequency and nitrogenase activity even in the medium containing 1 mM proline or 1 mM glycine-betaine, indicates the role of Na^+ for proline/glycine-betaine uptake. The Na^+-R mutant showed 100% survival at pH 11 and was simultaneously able to uptake and utilize proline/glycine-betaine at higher alkaline pH. This indicates that proline and glycine-betaine uptake systems are more efficient at higher alkaline pH. Since, the hypersaline environments are rich in Na^+ contents and have alkaline pH, therefore it is suggested that the origin and evolution of specific compatible solutes may not depend only on the osmoregulatory role they play, but also on the other ecological factors operating simultaneously in the organism's niche.

Keywords: antiporters, cyanobacteria, glycine-betaine, *Nostoc muscorum*, proline, symporter.

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Organisms growing in hypersaline ecosystems have specific transport systems for the accumulation of compatible solutes. This adaptive strategy has significant ecological advantage in terms of reducing metabolic energy [1]. The bacterium *Desulfovibrio halophilus* normally synthesized trehalose as compatible solutes but can also accumulate glycine-betaine under hypersaline conditions [2]. The recycling of compatible solutes in hypersaline ecosystems provides a source of carbon and nitrogen for diazotrophs in microbial mat ecosystems [3]. Many heterotrophic and prototrophic microorganisms can utilize amino acids as a source of carbon, nitrogen and energy [4]. The catabolism of proline and glycine-betaine has also been reported in cyanobacteria and in anaerobic phototrophic bacteria [5, 6].

Number of sodium (Na^+) driven antiporters and symporters has been characterized to understand the movement of cation and organic solutes across the biological membranes [7]. These transport systems are mainly driven by a proton (H^+)-motive force generated by redox-reaction-coupled primary H^+ (Na^+) pumps and H^+ (Na^+)-translocating ATPases. The Na^+/substrate symport driven by the electrochemical gradient accumulates nutrients without disturbing the cell physiology [8, 9].

In cyanobacteria the ionic component of the stress factor is usually overcome by the export system drive by H^+ [10, 11]. Likewise, the equilibrium of osmotic potential of the cytoplasm is maintained either by accumulation or by synthesis of varieties of low molecular weight organic compounds known as compatible solutes [12–15]. The mechanism of synthesis of these organic osmolytes involved activation of genes promoting their synthesis while, simultaneously inactivating the genes promoting their catabolism [16].

In this study we proposed that under unstressed growth conditions, both proline and glycine-betaine accumulated as a nitrogen source, and their uptake regulated by the Na^+/solute symport.

MATERIALS AND METHODS

Organism and culture conditions. The cyanobacterium *Nostoc muscorum* used in the present study was fresh water strain. This cyanobacterial strain was considered as parental strain in the present study for generating spontaneously occurring mutant clones resistant to growth inhibitory action of NaCl (Na^+-R). The cultures was grown in bulk in the Chu No. 10 medium [17] for routine as well as for experimental studies in the growth chamber, with a light intensity at a photon fluence rate of 50 mol $m^{-2} s^{-1}$ and temperature of 28 ± 2°C.

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Table 1. Heterocyst frequency (HF%, number of heterocyst per 100 vegetative cells) and nitrogenase activity (μmol C_2H_4 formed g^{-1} Chl *a* h^{-1}) of the *N. muscorum* and its Na^+-R mutant strain in different nitrogen growth medium

Medium	Wild type		Na^+-R	
	HF%	Nitrogenase activity	HF%	Nitrogenase activity
N_2-medium	7–8	11.24 ± 1.1	7–8	10.84 ± 0.9
+1 mM NH_4Cl	0-0	0-0	0-0	0-0
+1 mM proline	0-0	0-0	7–8	10.84 ± 1.2
+1 mM glycine-betaine	0-0	0-0	7–8	10.84 ± 1.1

Note: 1 mM 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.

Cultures of the cyanobacterium *N. muscorum* were grown routinely in diazotrophic growth medium. Chu No. 10 medium was modified to make it free from Na^+ for the experiment examining the role of Na^+ in proline and glycine-betaine uptake. Therefore, Na_2SiO_3 and Na_2CO_3 from the Chu No. 10 medium were replaced by equimolar concentration of $CaCO_3$.

Isolation of Na^+-R mutant strain. NaCl at a concentration of 100 mM was found lethal to the diazotrophically grown cultures of the cyanobacterium *N. muscorum*. The NaCl-resistant (Na^+-R) mutant clones of the cyanobacterium resistant to growth inhibitory action of NaCl was isolated and checked for their stability by the method described previously [18].

Measurement of Na^+-influx. Wild type *N. mucorum* and its Na^+-R mutant strains were grown in Na^+-free Chu No. 10 medium for 72 h then examined for Na^+-influx by using Flame Photometer. NaCl stress at the concentration of 50 mM was given to both the cultures for 12 h in 10 mM HEPES-NaOH buffer (pH 7.5). Such NaCl treated cultures were harvested and centrifuged. The amount of Na^+ present in the buffer was measured with the help of Flame Photometer at given time intervals and then subtracting these values from the control value.

Heterocyst frequency, chlorophyll *a* contents [19], protein contents [20], nitrogenase activity and intracellular proline contents were also measured as described previously [18].

The respective pH (8, 9, 10, and 11) of the diazotrophic growth medium was maintained by addition of HEPES, AMPSO, CAPSO, and CAPS buffers.

RESULTS

Wild type *N. muscorum* did not produce heterocyst and showed no nitrogenase activity in the growth medium containing 1 mM NH_4Cl, 1 mM proline or 1 mM glycine-betaine. In fact, proline and glycine-betaine like that of NH_4Cl have been utilized as a nitrogen source in the wild type strain under normal growth conditions. The Na^+-R mutant clones of the cyanobacterium produced normal heterocyst frequency and nitrogenase activity in the medium containing 1 mM proline. Evidently mutation to Na^+-R phenotype seems to have rendered nitrogen fixing apparatus and nitrogenase activity in the medium containing proline. Wild type and its Na^+-R mutant clones were further characterized with regards to glycine-betaine accumulation. The glycine-betaine a well known compatible solute was utilized as a nitrogen source by the wild type strains under normal growth condition. The Na^+-R mutant clones showed normal heterocyst frequency and nitrogenase activity even in the growth medium containing 1 mM glycine-betaine. These finding suggested that in the investigated cyanobacterium proline and glycine-betaine follows the same route for their uptake (Table 1).

The intercellular proline contents of the wild type under NaCl stress conditions found to show no significant rise in proline. In comparison, the co-existence of proline in the growth medium enhanced intracellular proline contents about 3-fold. This is because of the increased uptake of exogenous proline under NaCl stress conditions. In comparison, Na^+-R mutant clones did not show any significant increase in proline contents in the diazotrophic growth medium or in the medium containing NaCl + 1 mM proline. It means the wild type strains have both normal proline uptake system (proline utilized as a nitrogen source) and salinity inducible proline uptake system. On the contrary, mutation leading to NaCl resistant phenotype lost normal as well as salinity inducible proline uptake system (Table 2).

Wild type and its Na^+-R mutant clones were further examined for their Na^+ influx pattern. The results as shown in the figure suggested that Na^+-R mutant clones as compare to the wild type showed reduction in the Na^+ influx pattern. This finding suggested that Na^+-R mutant clones are deficient in Na^+ uptake

mechanism; and are also deficient in proline/glycine-betaine uptake. Hence, it can be concluded that proline/glycine-betaine uptake in the Na^+-R mutant clones was carried out through Na^+/solute symport system. The diazotrophic growth medium, in addition to Na^+ also contains K^+, Ca^{2+} and Mg^{++}, therefore, it is suggested that these cations are not involved in proline/glycine-betaine uptake and accumulation.

The filaments of *N. muscorum* are unable to grow in the growth medium containing 100 mM NaCl, while Na^+-R mutant clones thrives well in the medium containing 100 mM NaCl. Since, Na^+-R mutants showed 100% survival at pH 11 (Table 3) we examined the uptake of proline and glycine-betaine at pH 11 in the Na^+-R mutants. Under this extreme alkaline pH conditions proline and glycine-betaine are utilized as a nitrogen source by the mutant clones. These results suggested that proline and glycine-betaine transporters are more active at alkaline pH.

DISCUSSION

The catabolism of proline and glycine-betaine has been reported from variety of microorganisms [2, 21]. In *E. coli* ProP and ProU encoded transport systems involved in transport of glycine-betaine, proline and ecotoine [22–25].

In addition to their osmolyte role compatible solutes are also known to play other ecological roles under unstressed condition, these compatible solutes are utilized by variety of microorganisms as carbon, nitrogen and energy source. Thus the selective pressure like mode of life, relative availability of nutrients determines the accumulation and synthesis of particular compatible solutes.

Any change in the medium osmolarity, activated or inactivated membrane transports system by altering physiological mechanisms [16]. In enterobacteria it has been reported that during the initial up shock accumulation of K^+ takes place through potassium transport systems *viz.* Kdp and Trk [26, 27] followed by accumulation/synthesis of compatible solutes.

Microorganisms have different transport systems functioning at high osmolarity and at high ionic concentration. The uptake systems of these microorganism's involved primary ABC-type transporters like ProU or secondary transporters have single transmembrane proteins. The ABC systems of primary transporters relay on the energy generated through ATP hydrolysis. The secondary transporter and the sodium solutes symporters facilitate passive transport [28].

The mechanism of accumulation of compatible solutes has been analyzed in *N. muscorum* and its spontaneously occurring Na^+-R mutant clones and the results suggested that the accumulation of compatible solute takes place through the Na^+-solute symporter family. These results supported by the results obtained from *Bacillus subtilis* [28]. A similar system for the betaine transport has been reported in *Aphanothece halophytica* [7].

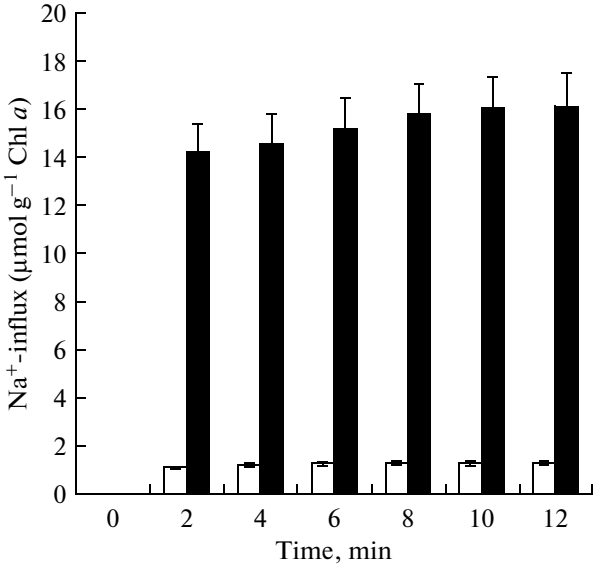
Table 2. Intracellular contents of proline (μmol proline g^{-1} protein) in the wild type and its Na^+-R mutant strain under unstressed and 3 h NaCl stressed conditions, following their incubation in diazotrophic growth medium or 1 mM proline containing growth medium

Medium	Wild type	Na^+-R
N_2-medium	9.56 ± 1.3	10.12 ±1.4
+ NaCl	10.28 ± 1.2	10.53 ± 1.9
+ Proline	27.34 ± 2.5	10.55 ± 1.7
+ NaCl + proline	97.56 ± 11.1	11.58 ± 1.8

Note: exponentially grown diazotrophically cultures of the two strains were the source of inocula. Each reading is an average (± SEM) of three independent experimental determinations.

The Na^+/H^+ antiporter activity in regulating Na^+ stress and alkaline pH stress (by maintaining H^+ gradient) has already been documented for cyanobacterial systems and for bacterial system [29], The Na^+-solute symporter and the Na^+/H^+ antiporter activities lead to accumulation of proline/glycine-betaine at alkaline pH and in the adjustment of H^+ gradient. Our interpretation of the cyanobacterium Na^+-R mutant is in quite agreement with the previous report on the *A. halophytica* [7].

The betaine transport in a cyanobacterium *A. halophytica* has takes place by betaine transporter (BetT) in



Comparison of Na^+-influx pattern in *N. muscorum* (dark bars) and its Na^+-R mutant strain (white bars). Mean values from three independent experimental determinations are shown ±SEM, where these exceed the dimension of the symbols.

Table 3. pH survival characteristics of the wild type *N. muscorum* and its Na^+-R mutant strain under varying pH stresses in the diazotrophic growth medium

pH	Wild type	Na^+-R
8.0	100	100
9.0	88 ± 10.2	100
10.0	66 ± 6.7	100
11.0	40 ± 3.8	100

Note: Exponentially grown 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.

the presence of Na^+ [7]. Similar studies on *E. coli* suggested the role of *proU* gene in transporting proline/betaine [30]. Our interpretation also proves that Na^+ plays an important role in the accumulation of exogenous betaine.

The hypersaline environments have higher concentration of NaCl and also have higher alkaline pH. These abiotic factors exert selective pressure for the origin and evolution of transport systems operated at high Na^+ concentration and alkaline pH. Thus, the origin and evolution of compatible solutes accumulation/synthesis in microbial world may have been influenced by the habitat in which they grow and multiply.

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