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EXPERIMENTAL ARTICLES

Regulation of Calcium Ion and Its Effect on Growth and Developmental Behavior in Wild Type and *ntcA* Mutant of *Anabaena* sp. PCC 7120 under Varied Levels of $CaCl_2$[1]

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Abstract—A study of calcium ion regulation in *Anabaena* 7120 and its derivative mutant (CSE2) strain impaired in *ntcA* gene were investigated in terms of altered morphological and physiological responses against various levels of calcium stress (0–100 mM). Calcium concentration of 10 mM was found to be inhibitory while 100 mM proved lethal for both wild type and mutant strain. The involvement of Ca^{2+} in the regulation of cellular processes has been described in terms of an influx or efflux of Ca^{2+} from the cytosol. A biphasic calcium uptake with difference in calcium influx and efflux rate was responsible for differential amount of remaining calcium which followed a decreasing trend both for wild type and mutant. Low $K_{s\,0.5}$ and high V_{max} in mutant suggest heavy and less restricted influx of calcium ion. Further, the interactive effect of calcium influx/efflux rate, remaining Ca^{2+} and intracellular levels of Na^+ and K^+ may be attributed for the degree of membrane damage and growth sustenance during exogenous supply of calcium salt. Widening in heterocyst spacing pattern, decreased heterocyst frequency and formation of abnormal cell structures at higher concentration (100 mM $CaCl_2$) suggest that calcium mediated regulatory process modulate heterocyst frequency and maintenance of cell structure. Further, poor regulation of calcium ion homeostasis in *ntcA* suggests that the calcium level and *ntcA* gene expression are inter-related.

Keywords: calcium ion regulation, influx, efflux, intracellular Na^+ and K^+, developmental pattern

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Cyanobacteria are one of the most diverse groups of oxygenic photosynthetic prokaryotes exhibiting versatile physiology and wide ecological tolerance that contributes to their competitive success over a wide range of environments. Many of them are also capable of fixing N_2, a process that requires dinitrogenase enzyme complex to be protected from oxygen. In order to overcome the barrier of oxygen-sensitive nitrogenase, certain filamentous forms evolved mechanisms for adapting themselves both structurally and functionally, by differentiating 5–10% of their vegetative cells into heterocysts which provide anaerobic site for the synthesis of nitrogenase enzyme complex [1]. These heterocysts differentiated at nearly regular intervals along the filaments, generating a spacing pattern [2]. The process of heterocyst differentiation is repressed by presence of fixed nitrogen in the medium such as nitrate [3] or glutamine [2, 4] by switch on of the number of genes involved in heterocyst differentiation, nitrogen fixation and switch off the genes involved in carbon fixation. In recent past, number of genes have been identified which participate in heterocyst differentiation and nitrogen fixation, the earliest documented gene that is activated by nitrogen deprivation happens to be *ntcA*, which belongs to the CRP (cyclic AMP receptor protein)/FNR (fumarate and nitrate reduction regulator) family of transcriptional regulators [5]. NtcA (the global transcriptional regulator) plays critical role in nitrogen and carbon homeostasis as well as heterocyst development in cyanobacteria [6]. So, NtcA is not only essential for *glnA* (encoding glutamine synthetase) but also for *hetR* and *hetC* genes. HetR and HetC are induced during the early course of nitrogen starvation [7]. Initiation of heterocyst development is controlled mainly by NtcA and HetR, which are positively autoregulated and mutually dependent on each other for upregulation; HetR also has autoproteolytic activity. HetR has been considered as the master regulator of heterocyst development. NtcA and HetR collaborate to reduce the levels of CcbP in the differentiating cell, the former by directly inhibiting transcription and the latter by proteolysis, resulting in release of free calcium. Two genes *hetF* and *patA* appear to positively control heterocyst differentiation and pattern formation by modulating the expression of HetR protein. Other heterocyst-specific genes whose products are required for heterocyst differentiation and nitrogen fixation are *hetP*, *hglb, hglCD*, *hglE, hglK, devABC, hepA,B,C* and *hepK, Xis* and *nif* genes [2]. In recent past, *patS* gene has been discovered

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whose products control intercellular inhibition of heterocyst development [8].

Calcium (Ca^{2+}) plays a pivotal role in the physiology and biochemistry of organisms ranging from prokaryots to eukaryots. Just like other elemental cycles a separate geochemical calcium cycle is operated by microorganisms that maintain neutral conditions on earth [9]. Further, a number of cellular processes in cyanobacteria mediated by calcium are reported such as heterocyst differentiation and nitrogen fixation [10, 11], cyanobacterial calmodulin [12], PS II activity [13], phosphate activity, trichome motility [14]. Of the most recent studies include acclimation to nitrogen starvation [15] and calcium transients evoked by salinity and osmotic stress [16] underline the role of calcium as intracellular second messenger in response to environmental stimuli. However, there is a scarcity of reports pertaining calcium ion regulation especially its kinetics and interaction with other ions like Na^+ and K^+. Influence of calcium ion on morphological attributes, heterocyst development and spacing pattern of wild type *Anabaena* 7120 and its mutant strain CSE2 impaired in ntcA gene [17] has been presented. The purpose of taking a mutant strain impaired in ntcA is an indirect way of answering the question whether there is a relation between *ntcA* gene and calcium ion. Thus, the present communication deals with the mechanism(s) involved in regulation of calcium ion and the requirement of specific concentration (critical concentration) of calcium ion in controlling heterocyst development and spacing pattern.

MATERIALS AND METHODS

Cyanobacterial strains and culture conditions. Heterocyst-forming cyanobacterium, *Anabaena* sp. PCC 7120 and Het^- derivative strain CSE2 (an insertional mutant of the *ntcA* gene) received from Prof. E. Flores, Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, E-41092 Seville, Spain, have been used in the present investigation. Wild type and mutant strains both were grown in a culture room under a moderate light intensity (50 μmol m^{-2} s^{-1}) provided with cool-white fluorescent lamps at 28°C in BG11 medium, pH 7.4. Mutant (CSE2) strain was maintained in the medium supplemented with streptomycin (2 μg mL^{-1}) in order to maintain transposons (Tn5). NH_4Cl (0.5 mM) was used as nitrogen source.

Experimental design. 8–10 days old cultures of wild type and mutant strains were harvested by centrifugation and were further inoculated in a medium containing different levels of calcium chloride (0–100 mM).

General methods protein determination. Protein content in wild type and mutant strains grown under varied levels of calcium chloride was estimated as per the protocol of Lowry et al., 1951 [18] with Bovine Serum albumin (BSA) as a standard.

Growth behavior. Cyanobacterial cells growing at a concentration of 35 μg protein mL^{-1} were inoculated in sterilized Erlenmeyer flasks containing 120 mL sterilized BG 11 medium supplemented with different concentrations of $CaCl_2$ (0, 1, 10 and 100 mM). The cultures were maintained at standard growth conditions. 3 mL cultures were withdrawn from the flask at every alternate day and growth was measured at 663 nm up to 30th day of incubation using Systronics Double Beam Spectrophotometer 2203.

Heterocyst differentiation and spacing pattern. Heterocyst frequency was calculated in terms of heterocysts present per 100 vegetative cells under the microscopic field [19]. A minimum of 1000 vegetative cells ware counted for each determination. Vegetative cells between two heterocysts were also counted to study the interheterocyst spacing pattern [4].

Ca^{2+} Ion Regulation

Calcium uptake and intracellular calcium ion concentration. The sterilized growth medium with or without supplementation of different concentration of $CaCl_2$ (0, 1, 10, and 100 mM) was prepared in the sterilized 250 mL flasks. The exponentially grown cultures (1 mL) were transferred in sterilized flasks and the experiment was set at standard growth conditions. Cultures (1 mL) of both, wild type and mutant strain was taken from the different flasks supplemented with different levels of calcium at different time intervals (0, 1, 2, 3, 5, 10, 15, 30 min) and centrifuged at 10000 rpm for 10 minutes. The supernatant was transferred into another tube and its absorbance was determined at 422.7 nm using atomic absorption spectrophotometer (Perkin Elmer model 2380) [20]. Decrease of calcium in the medium represents the uptake of calcium by the cyanobacteria cells. The uptake of Ca^{2+} increased linearly and proportionately with time but deviated after 3 min both in wild type and mutant strains. Therefore, the uptake rate for Ca^{2+} was calculated at 3 minutes and expressed in terms of μmol Ca^{2+} μg $protein^{-1}$ min^{-1}.

For determination of intracellular calcium ion concentration, inocula from 8 days old cyanobacterial cultures (1 mL) were transferred in 50 mL of sterilized BG11 medium supplemented with or without different concentrations of $CaCl_2$ (0, 1, 10 and 100 mM) and incubated for 5 min at the standard growth conditions. Then, cultures were centrifuged at 10000 rpm for 10 min and the obtained pellet was rinsed with aerated iso-osmotic solution of sorbitol to remove the adhering ions. Further, the pellet was digested with HNO_3 : $HClO_4$ (10 : 1 v/v) in a boiling water bath for 30 min. Samples were cooled at room temperature and were centrifuged at 10000 rpm for 10 min. The supernatant was taken for the determination of intracellular Ca^{2+} content in terms of μmol Ca^{2+} μg $protein^{-1}$ and

its absorbance was determined at 422.7 nm using atomic absorption spectrophotometer (Perkin Elmer model 2380).

Calcium efflux and remaining calcium. Wild type and mutant strains were incubated in $CaCl_2$ deplete and replete (0, 1, 10, and 100 mM) BG11 medium at standard growth conditions for 5 min. Then, the centrifugation was done at 10000 rpm for 10 min and the pellets were rinsed with an aerated isoosmotic solution of sorbitol for removal of adhering ions. The obtained pellets were again re-suspended in 5 mL sterilized Ca^{2+} free isotonic medium for 5 min, then recentrifugation was done at 10000 rpm for 10 min and absorbance of the supernatant was taken for the determination of rate of Ca^{2+} efflux in terms of μmol Ca^{2+} μg protein^{-1} using atomic absorption spectrophotometer (Perkin Elmer model 2380). Remaining calcium ion content (after efflux) of the cyanobacterial cells was calculated by deducting calcium ion effluxed from the intracellular calcium ion content of the cells of cyanobacteria.

Intracellular cation concentration of sodium and potassium. Cyanobacteria cultures were grown for 8 d at standard growth conditions in two different sets of BG11 medium i.e. medium without Na^+ and medium without K^+ and then after the above mentioned media were used for determination of the intracellular Na^+ and K^+ respectively. The intracellular cation content (Na^+ and K^+) at different concentrations of $CaCl_2$ was measured after 5 min of incubation at standard growth conditions with the addition of the respective cation and different concentrations of $CaCl_2$ (0, 1, 10, and 100 mM) in the above medium deprived of such cation. The concentration of Na^+ and K^+ in the experimental medium was same as used with standard growth conditions. Then after cultures were centrifuged at 10,000 rpm for 10 min and the obtained pellets were treated similarly as for the estimation of intracellular Ca^{2+} content. Concentration of Na^+ and K^+ was measured in terms of μmol μg protein^{-1} by using atomic absorption spectrophotometer (Perkin Elmer model 2380).

Estimation of electrolyte leakage. Electrolyte leakage was measured as per the protocol of Dionisio-Sese and Tobita [21]. Inocula (1 mL) were transferred from 8 d old cyanobacterial cultures in 50 mL of sterilized BG11 medium supplemented with or without different concentrations of $CaCl_2$ (0, 1, 10, 100 mM) and incubated at standard growth conditions. Cultures were centrifuged at 10000 rpm for 10 min after 8 d of incubation. Fresh pellet (0.5 mL) was taken and washed with deionized water to remove surface-adhered electrolytes. The washed pellet was placed in test tubes containing 10 mL distilled deionized water and incubated for 2 h in water bath at 32°C. The initial electrical conductivity of the above medium (EC1) was measured. The same sample was autoclaved at 121°C for 20 min and cooled. All the electrolytes released in the medium were measured as the final electrical conductivity (EC2). The electrolyte leakage (EL) was calculated using the formula EL = (EC1/EC2) × 100.

Statistical analysis. In all the graphs, bars indicate the standard error of the three replicates (n = 3). Results were subjected to two-way ANOVA in order to assess the significance of quantitative changes in the experimental parameters because of different $CaCl_2$ treatments and strains. SPSS software (SPSS Inc., version 16.0) was used to perform all the statistical tests (Table 1).

RESULTS

Effect of Calcium Chloride on Growth Behavior

Growth characteristics (in terms of cell density) of wild type and mutant strain grown in different levels of calcium chloride (i.e. 0, 1, 10, 100 mM) was observed with maximum being in 1 mM $CaCl_2$ followed by control (0 mM). Calcium concentration of 10 mM was found to be inhibitory while 100 mM proved to be lethal for both wild type and mutant strain. A histogram constructed for the growth of wild and mutant at 8th day of incubation reveals inhibition of growth by 62.96% at 100 mM in wild type strain and 71.7% in mutant strain compared to the control. At 1 mM concentration of $CaCl_2$, growth increased by 17.28% for wild type and 13.17% for the mutant strain. At 10 mM of $CaCl_2$, growth was inhibited by 13.86% in wild type while 15.11% reduction in growth was observed for the mutant strain compared to the control (0 mM). These observations suggest that both, wild type and mutant strain have a variable degree of response towards $CaCl_2$ (Fig. 1a).

Developmental Behavior

Microscopic analysis of the wild and mutant strains reveals a clear cut difference in response of the treated cultures to different levels of exogenously supplied calcium chloride salt. An increased supply of exogenous calcium salt resulted in gradual swelling, enlargement and granularization of vegetative cells as well as vacuolation and formation of abnormal cell structures at 10 and 100 mM concentration while cultures grown in 1 mM of calcium chloride showed normal and healthy cells. Fragmentation of filaments increased with the increased exogenous supply of calcium (data not shown). Wild type filaments grown in 10 mM $CaCl_2$ supplemented medium reduced the filament size considerably from ~50–60 cells to ~35–40 cells while filaments treated with 100 mM $CaCl_2$ showed marked reduction in filament length i.e. ~15–20 cells per filament. As was the case with wild type, the mutant strain also showed extensive fragmentation in filaments which ultimately reduced the filament length. Reduc-

Table 1. Results of ANOVA (analysis of variance) for repeated measures of $CaCl_2$ concentrations (Treatments), Strains and their interactions for Ca^{2+} uptake, intracellular Ca^{2+}, efflux Ca^{2+}, remaining Ca^{2+}, intracellular K^+, intracellular Na^+ and Electrolyte Leakage

Parameters	Treatment	Strain	Treatment × Strain
Uptake Ca^{2+}	0.001695***	745.787***	116.602***
Intracellular Ca^{2+}	0.0008131***	0.00001113***	0.0001931***
Efflux Ca^{2+}	0.004528***	0.005851***	929.262***
Remaining Ca^{2+}	0.0002602***	0.0002421***	0.009613***
Intracellular K^+	436.455***	0.774^{ns}	284.232 ***
Intracellular Na^+	0.001622***	0.0003063***	0.003860***
Electrolyte Leakage	0.0009161***	733.738***	107.421***

Level of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant.

tion in the filament size was more pronounced at higher calcium concentration. Further, it was also noticed that cell degradation was more exhaustive at 100 mM concentrations of $CaCl_2$ in both wild and mutant strain compared to the other levels of $CaCl_2$.

In order to evaluate the influence of different levels of $CaCl_2$ on heterocyst differentiation, the heterocyst differentiation was observed in 8th day old wild type filaments grown in nitrogen free medium supplemented with different concentrations of calcium chloride (Fig. 1b). The heterocyst frequency at 1 mM calcium chloride salt was found to be approximately two fold higher compared to control (0 mM). At the same time the heterocyst frequency decreased gradually with increase in calcium chloride concentrations (≥10 mM). The decrease in heterocyst frequency at 10 mM of calcium chloride was ~ 15% while 100 mM calcium chloride caused ~32% reduction in heterocyst frequency compared to control (0 mM). Results suggest that 1 mM of calcium chloride was stimulatory for heterocyst development. Further, changes in the inter-heterocyst spacing pattern were also examined in the filaments of wild type under nitrogen starved condition supplemented with various levels of calcium chloride (Fig. 1c). Out of 300 filaments taken for inter-heterocyst spacing (number of vegetative cells between two heterocysts), the wild type filaments in case of 1 mM calcium chloride concentration showed maximum frequency of 20–25 vegetative cells between two heterocysts i.e. 25% in 0 mM $CaCl_2$, 23% in 1 mM $CaCl_2$, 21% in 10 mM $CaCl_2$ and 15% in 100 mM $CaCl_2$. As mutant strain impaired in *ntcA* gene that control heterocyst differentiation and nitrogen fixing genes, *glnA* gene, *nir* and *nor* operon and other genes and therefore mutant strain unable to differentiate heterocyst and perform nitrogen fixation.

Calcium Uptake and Kinetics

The uptake of calcium ion in both, wild and mutant strain was calculated at different time intervals (0, 1, 2, 3, 5, 10, 15, 30 min) (data shown up to 15 min of incubation). The rate of uptake increased linearly upto 3 minutes. Thereafter the linearity deviated and saturation was observed after 15 minute of incubation and therefore the uptake rate was calculated at 3 minute of incubation. A biphasic pattern of calcium uptake (in terms of μmoles μg protein^{-1} min^{-1}) was observed both for wild and mutant strain (Figs. 2a, 2b). The calcium ion uptake occurs rapidly in the first 3 minute followed by a slow uptake process that takes about 15 minutes. In the first 3 minutes binding of calcium to the cyanobacterial cells is much faster at 10^{-1} M followed by 10^{-2} M and least at 10^{-3} M. The pattern of calcium uptake reflected its dependence on its concentration as well as the exposure time (0–15 minutes). The uptake rate (μmoles μg protein^{-1} minute^{-1}) was higher in mutant strain compared to wild type *Anabaena* sp. PCC 7120. With the increasing supply of exogenous calcium, the uptake rate was also increased; the uptake being highest at 100 mM calcium ions in the medium.

The calcium uptake rate followed Michaelis-Menton Kinetics and exhibits substrate dependent calcium uptake; $K_{s\ 0.5}$ for wild type (1.3663 mM) was higher than mutant strain (1.1896 mM). V_{max} followed a reciprocal relationship with respect to $K_{s\ 0.5}$ and was higher for mutant (0.1621 μmoles μg protein^{-1} min^{-1}) compared to wild type (0.1010 μmoles μg protein^{-1} min^{-1}) (Supplementary Table) Low $K_{s\ 0.5}$ and high V_{max} in mutant strain suggests heavy and less restricted inflow of calcium inside cellular system (Fig. 3a).

Maintenance of Cellular Calcium Status: Intracellular, Efflux and Remaining Calcium Ions

With the increase in uptake at different concentrations of calcium, internal calcium concentration (in terms of μmoles μg protein^{-1}) also increased in the cells of the cyanobacterium *Anabaena* sp. PCC 7120 (Fig. 3b). The intracellular calcium ion concentration was higher in mutant strain (24.6346 μmoles μg pro-

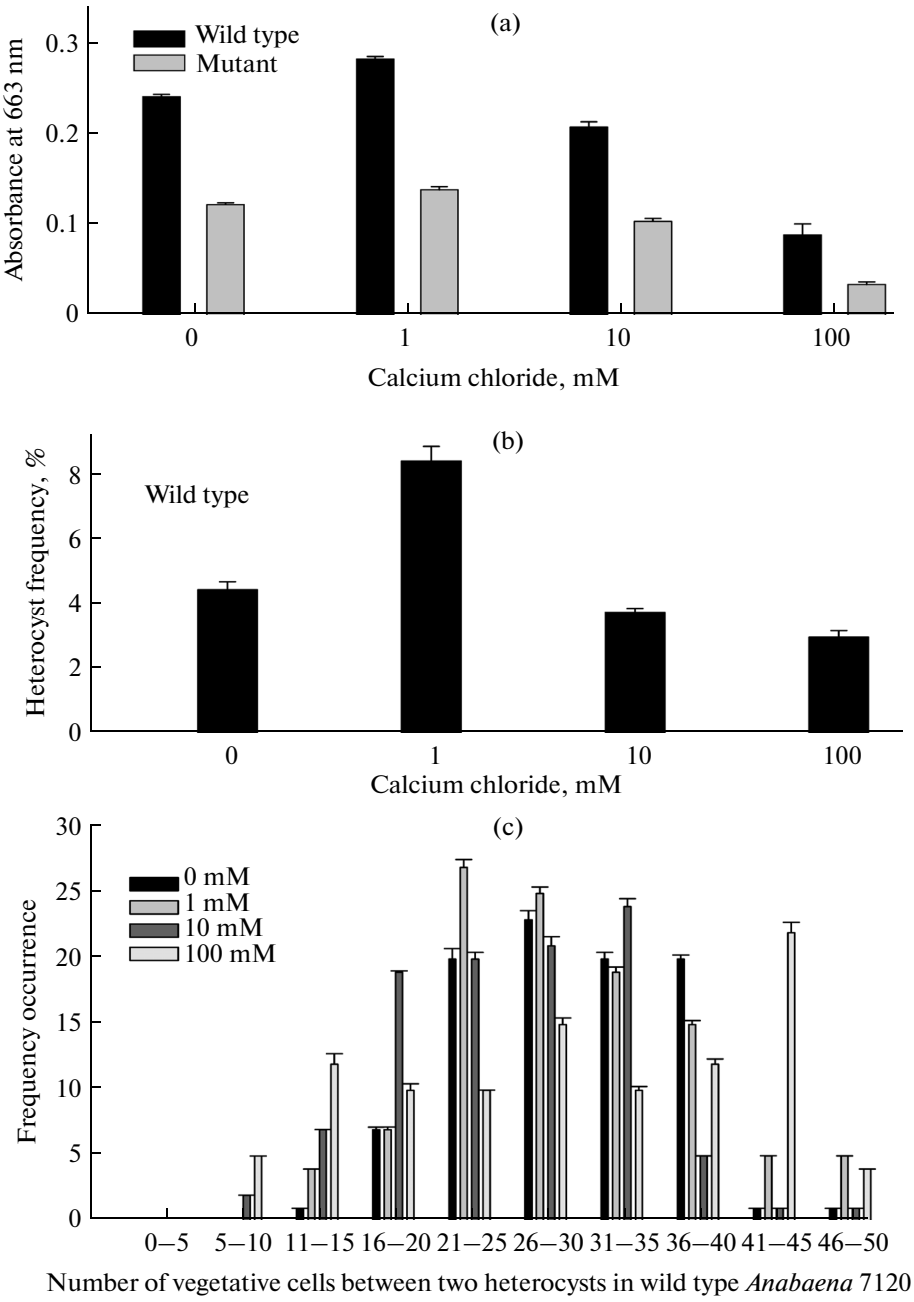

Fig. 1. Effect of different concentrations of calcium chloride on (a) growth behaviour of wild type and mutant strain of *Anabaena* sp. PCC 7120, (b) heterocyst frequency in wild type *Anabaena* sp. PCC 7120 and (c) heterocyst spacing pattern in wild type *Anabaena* sp. PCC 7120.

tein^{-1}) compared to the wild type (9.1228 μmoles μg protein^{-1}) at 100 mM $CaCl_2$.

An increase in calcium efflux rate was also observed as was the case of calcium ion uptake for the wild and mutant strain. Between the two strains studied, efflux was much higher for mutant (24.0700 μmoles μg protein^{-1}) compared to the wild type (8.9193 μmoles μg protein^{-1}) at 100 mM $CaCl_2$ (Fig. 3c).

Similarly, remaining calcium ion content in the cells of wild type and mutant strain was also calculated. Results clearly showed a decrease in remaining calcium content with increase in calcium chloride and attained minimum value at 100 mM of the calcium salt (Fig. 3d).

Intracellular Nutrient Status of Na^+ and K^+ during Exogenous Calcium Supplementation

To present a much clearer picture of calcium uptake process, intracellular level of two most impor-

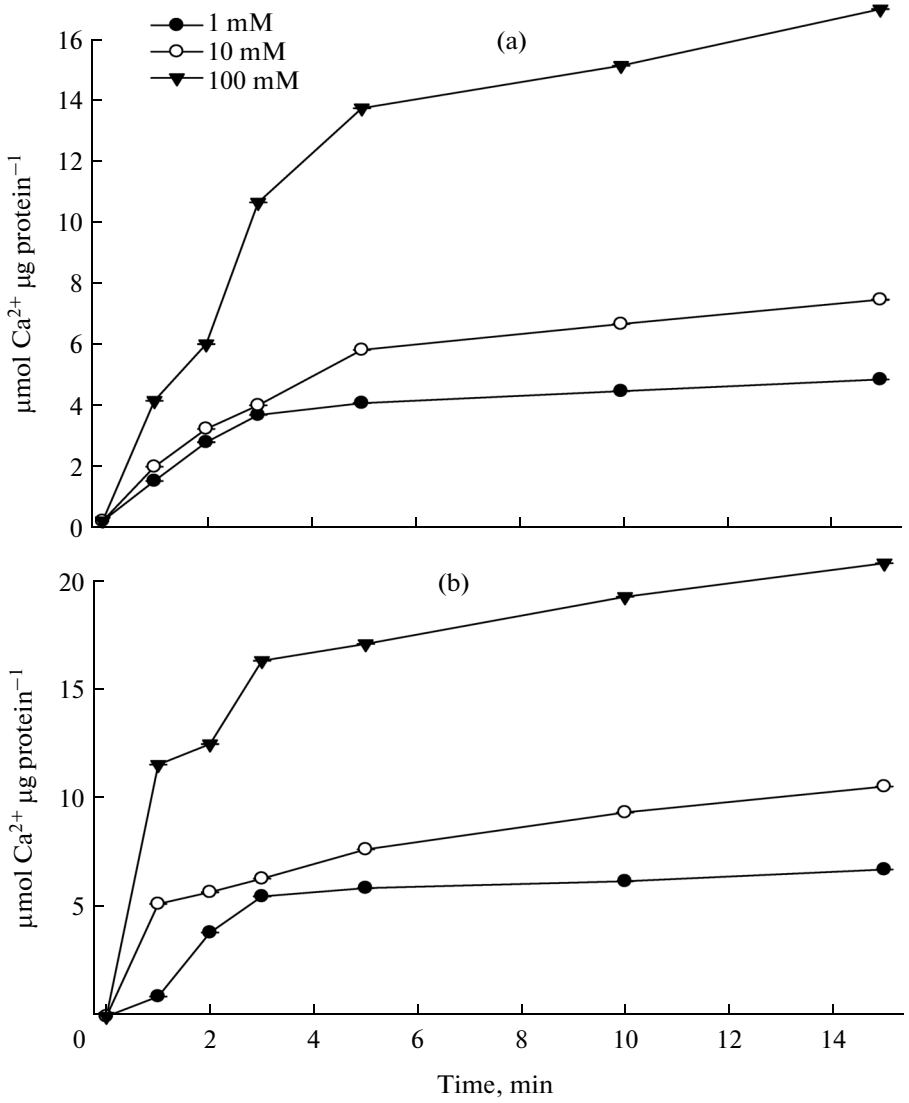

Fig. 2. Calcium ion (Ca^{2+}) uptake in (a) wild type and (b) mutant strain of *Anabaena* sp. PCC 7120 in relation to varying calcium chloride concentrations as a function of time.

tant ions Na^+ and K^+ have also been assessed in the wild type as well as in mutant strain (Figs. 4a, 4b). Observations on intracellular level of sodium ion (Na^+) at different concentrations of exogenously supplied calcium salt showed a distinct correlation among sodium ion, calcium ions and *ntcA* mutant incapable of utilizing nitrate as nitrogen source. Almost a contrasting correlation with respect to intracellular level of sodium in response to exogenously supplied calcium was obtained between the wild type and mutant strain of the cyanobacterium *Anabaena* sp. PCC 7120. In the mutant, the sodium ion concentration increased continuously on the other hand in the wild type the sodium ion concentration varying steeply with respect to increasing concentration of calcium ion.

There was an increase in intracellular K^+ concentration in both, the wild type as well as in mutant strain when exposed to the different levels of $CaCl_2$ (0–100 mM). The intracellular level of K^+ was highest at 100 mM in the mutant strain (0.030 µmoles µg protein^{-1} min^{-1}) while lowest was recorded at 0 mM (0.0130 µmoles µg protein^{-1} min^{-1}). A reduced intracellular level about 43.5% was observed in the wild type compared to mutant at higher concentration of calcium (100 mM).

Electrolyte Leakage

Measurement of electrolyte leakage (%) in wild type and mutant strain indicates maximum leakage in 100 mM calcium treated mutant cells (83.4%) fol-

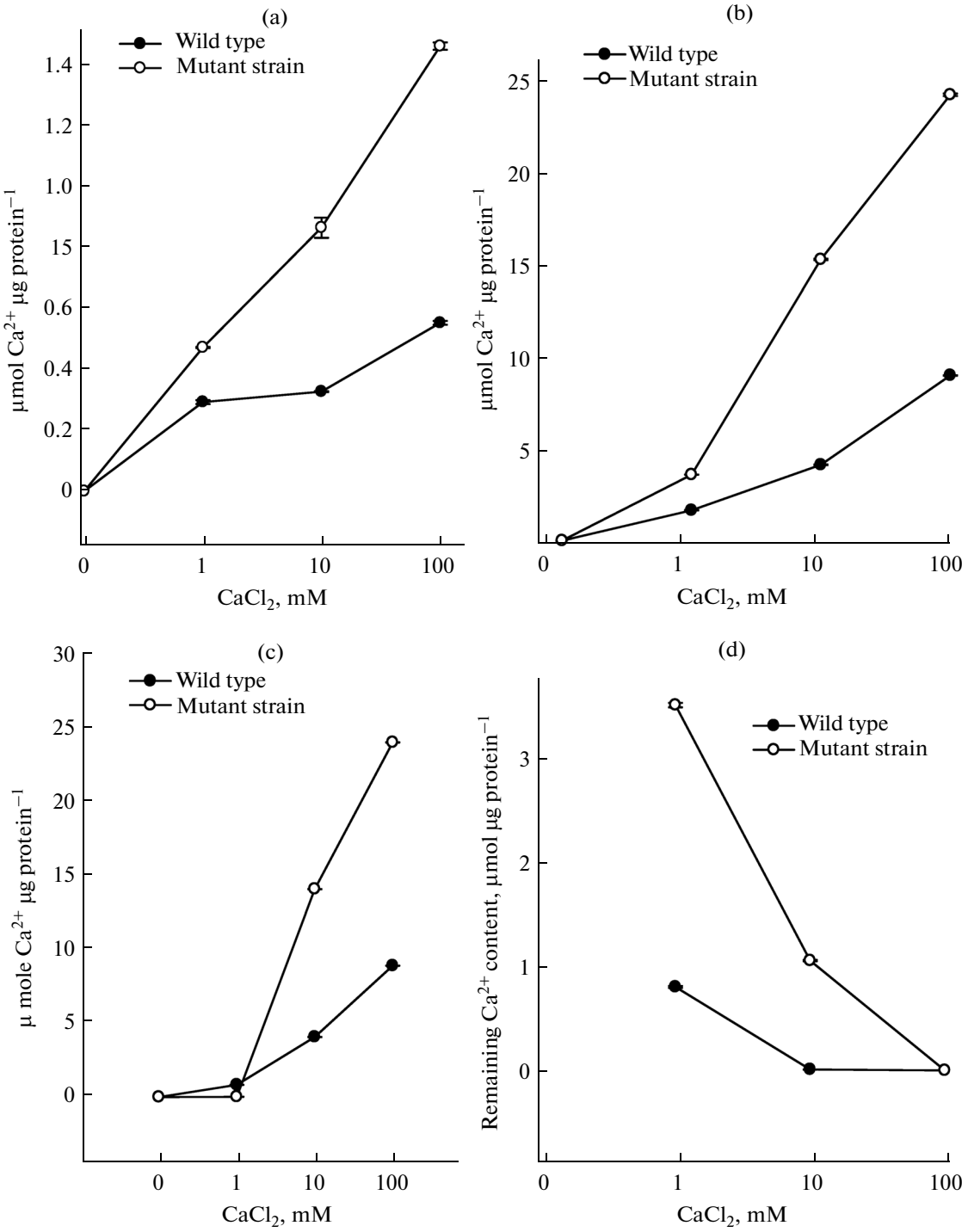

Fig. 3. Calcium ion regulation in relation to varied calcium chloride concentration in wild type and mutant strain (a) Uptake of Calcium ion, (b) Intracellular Ca^{2+} content, (c) efflux of calcium ion (d) remaining calcium ion content.

lowed by the wild type (78%) at the same calcium concentration. At 10 mM calcium concentration electrolyte leakage for wild type was found to be 66 and 72% in mutant strain (Fig. 4c). Electrolyte leakage of 5% and 7% was observed respectively for both wild type and mutant strain at 0 mM of calcium salt. Least electrolyte leakage was obtained at 1 mM of calcium salt in wild type (i.e. 2.01%) and mutant strain (i.e. 3%).

Results of Two-way ANOVA (analysis of variance) for repeated measures of $CaCl_2$ concentrations (Treatments), Strains and their interactions for Ca^{2+} uptake, intracellular Ca^{2+}, efflux Ca^{2+}, remaining Ca^{2+}, K^+

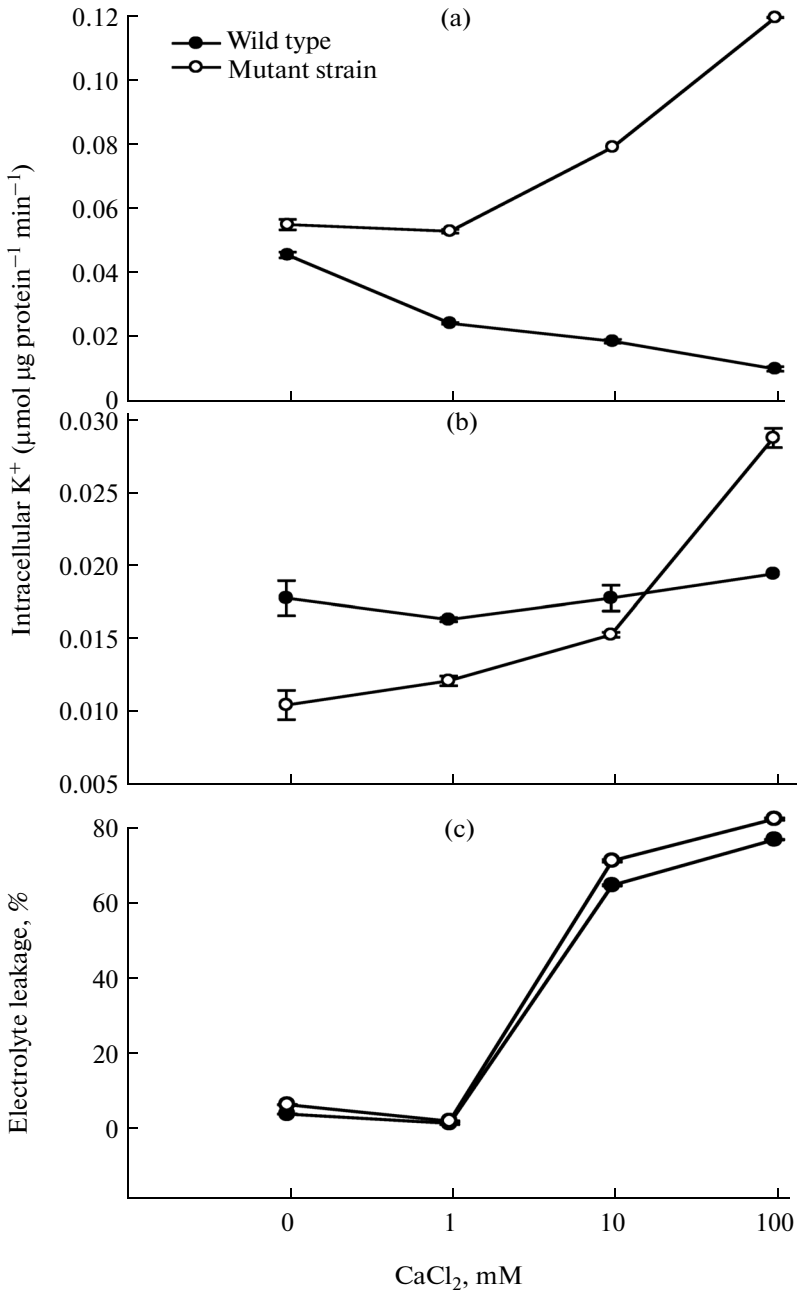

Fig. 4. Effect of Calcium chloride on (a) intracellular Na^+ content, (b) intracellular K^+ content, (c) electrolyte leakage in wild type and mutant strain of *Anabaena* sp. PCC 7120.

intracellular, Na^+ intracellular, Electrolyte Leakage revealed that all the tested parameters taken into consideration were significant and were mainly due to the effect of treatment and strains (Table 1).

DISCUSSION

Cyanobacteria are equipped with several mechanisms that allow them to survive under different environmental conditions which impose varied forms of stresses and the same time these cyanobacteria respond in a specific manner to combat stresses and to protract their existence. Calcium, a highly versatile intracellular signal operates over a wide temporal range to regulate many different cellular processes. Recently, it has been reported that salinity tolerance and other stress tolerance phenomenon in cyanobacteria inevitably depend on calcium ions [16]. In cyanobacteria, a number of processes such as photosynthesis, heterocyst differentiation and nitrogen fixation are mediated through calcium. At the same time global transcriptional regulator NtcA [22] has been

Table 2. The apparent steady state kinetics parameters for calcium uptake in wild type and mutant strain of *Anabaena* 7120

Strains	1/[S], mM^{-1}	$1/v$, (μmole μg $protein^{-1}$ $min^{-1})^{-1}$	$K_{s\,0.5}$, mM	V_{max}, (μmole μg $protein^{-1}$ $min^{-1})^{-1}$
Wild type	0.0000	0.0000	1.3663	0.1010
	1.0000	0.1330		
	0.1000	0.1220		
	0.0100	0.0400		
Mutant	0.0000	0.0000	1.1896	0.1621
	1.0000	0.0840		
	0.1000	0.0732		
	0.0100	0.0283		

implicated for heterocyst differentiation and nitrogen fixation involving calcium ion regulation. This paper deals with mechanism(s) involved in calcium ion regulation as well as alterations (if any) in heterocyst developmental behavior in wild type as well as *ntcA* mutant strain while subjected to different levels of calcium chloride.

Microscopic examination has revealed fragmentation of filaments, swelling of vegetative cells and formation of abnormal cell structures. Increased fragmentation of filaments (frequency) with increased level of calcium in response to fixed nitrogen deprivation suggests that increased level of calcium chloride possibly dismantle the intercellular communication between adjacent cells which ultimately disturbed the movement of carbon fixed product as well as nitrogen fixed product from one cell to another. Nayar et al. (2007) [23] has reported fragmentation of filaments is the result of increased expression of *fra* gene (required for the maintenance of cell integrity) under nitrogen starved condition. Similar observations have also been reported by number of workers [4, 24]. Vacuolation evident under very high calcium concentration represents a glimpse of the last survival strategies adopted by the cyanobacterial cells under calcium stress. All this probably indicates a defence mechanism against high calcium supplementation and nutrient limitation both in wild type and the mutant strain of the cyanobacterium.

The influence of various levels of $CaCl_2$ on heterocyst frequency was also examined in the filaments of wild type. The heterocyst frequency was found to be maximum at 1 mM calcium concentration while further increase in calcium concentrations (≥10 mM) caused decrease in heterocyst frequency suggests that the elevated levels of calcium chloride might be responsible for the over-expression of CcbP (Calcium calmodulin binding protein) which binds with free Ca^{++} available in excess amount in the medium and thus suppressing early gene (*hetR*) responsible for the heterocyst differentiation [25]. At the same time, calcium deficiency (0 mM) also brought about a delay in heterocyst differentiation as reflected by lower heterocyst frequency; this result being similar to sodium deficiency reported by Roussard-Jacquemin 1989 [26]. Thus, the results are interpreted as favouring the hypothesis that a calcium mediated regulatory process modulates heterocyst frequency. A change in heterocyst spacing pattern in response to varied levels of calcium concentrations can be correlated with the change in the heterocyst frequency. At 1 mM $CaCl_2$, the heterocyst frequency was more meaning thereby heterocyst differentiation in the filament are closely spaced while widening of the heterocyst pattern with lesser heterocyst frequency was evident at higher concentration suggesting lower concentration of calcium chloride was stimulatory while its higher concentration becomes inhibitory or toxic. It has been reported that widening in the heterocyst pattern at higher concentration probably be caused by a slower rate of heterocyst differentiation relative to growth rate [27].

To understand calcium ion regulation in wild type as well as in NtcA mutant; calcium uptake (influx), its accumulation and efflux was established at four concentrations (0, 1, 10, 100 mM) of calcium chloride. The uptake of calcium was rapid and increased linearly upto 3 minutes which represents rapid saturation of the uptake machinery. This was in accordance to the results reported for other N_2 fixing organisms in which a plateau is achieved rapidly [28]. Possibly, elevated levels of $CaCl_2$ establish an electrochemical gradient which might have been responsible for increased calcium uptake. Simultaneously, a quick rise in intracellular calcium ion supported the fact that calcium uptake was a passive process. Since cyanobacteria respond to almost any kind of external stimuli altering change in cytoplasmic free calcium concentration assessing the intracellular and remaining calcium ion was important [29].

Biphasic calcium uptake where the cyanobacterial cells removed calcium from the ambient medium in two possible ways, first involving rapid binding/uptake of calcium (first 3 minute) followed by a slower second phase at least upto 15 minutes. The pattern of calcium uptake reflected its dependence on calcium concentration as well as exposure time. In cyanobacteria as well as in other organisms, the intracellular calcium concentration $[Ca^{2+}(i)]$ is regulated through the processes of calcium influx and efflux [30]. Generally, cyanobacteria maintain 0.1–0.2 μM levels of intracel-

lular Ca^{2+} which is very less (in order to avoid toxicity to the cell metabolism), but the transient levels may accumulate upto 5 μM [16]. If the external Ca^{2+} concentrations are higher than that, as in our case, calcium uptake may involve low passive permeability and/or Ca^{2+} sensitive trans-membrane channels. So in the present case, with increased level of calcium in the external medium i.e. 0, 1, 10, 100 mM Ca^{2+} uptake must have occurred passively mediated by calcium specific trans membrane channels. A comparison of influx of calcium, its accumulation, efflux and residual calcium ion (after efflux) both in wild type and mutant strains provides a clear picture of the mechanisms participating in the transportation and regulation of calcium ion i.e. influx and efflux.

After the influx of calcium ions efflux occurred indicating that there must be some active Ca^{2+} efflux mechanism. Extrusion mechanisms for calcium have been reported in several bacterial systems [31] and variety of secondary exchangers and energy-dependent antiport systems have also been demonstrated [29, 32]. Differences in the influx or efflux rate of wild and mutant strain might be responsible for the varied level of remaining calcium which would be really the required Ca^{2+} concentration for the sustenance and survival of the strain. Since, above a critical level accumulated level of calcium was deleterious to the cyanobacterial physiology the efflux rate increased with increase in calcium concentration beyond 1 mM. This efflux rate increased much steeply in the mutant compared to the wild type. As compared to wild type poor growth and survival in the mutant at different calcium levels might be the result of higher uptake and extrusion of calcium which demanded more cellular energy. Possibly, extreme energy crisis during calcium stress could be implicated along with minimization of nutrient and metabolite transport along with protein synthesis, energy and lipid metabolism. Similar observations were obtained during NaCl stress condition in *Azolla-Anabaena* system [28] and *Frankia* strains [20].

Low $K_{s\ 0.5}$ and high V_{max} in mutant suggest heavy and less restricted inflow of calcium inside cellular system. The calcium uptake exhibits a hyperbolic response and the difference in $K_{s\ 0.5}$ and V_{max} of the wild type and the mutant is indicative of difference in affinity systems of the two strains. Exogenous supply of calcium salt had profound influence on electrolyte leakage in the wild type and mutant cells; thus indicating that time and concentration dependent increase of calcium was likely to be correlated with increase in electrolyte leakage as has also been reported by Galhano et al., 2011 [33].

In order to determine whether sodium level is influenced by calcium ions or not, the intracellular sodium status was evaluated which clearly demonstrated an almost opposing trend in the wild type and the mutant. In the wild type the sodium level [34] continuously declining with minimum sodium ion at 100 mM of calcium concentration, on the other hand in the *ntcA* mutant (unable to grow on nitrate source) with the increase in calcium the intracellular sodium level went on an increase attaining its maximum level at 100 mM of calcium. Results suggest that the *ntcA* mutant shows a specific response to the calcium ion (a signalling molecule) that triggers more uptake of sodium ion in contrast to the wild type where sodium exclusion is preferred. Such a behavior of mutant may be attributed to its inability to use nitrate because nitrate uptake has a strong dependence on Na^+ [35]. This result is in consistent with earlier observations of Ren et al., 2001 [34].

A continuous increase in intracellular potassium ion in response to increasing calcium ion up to 100 mM suggest that the enzymatic and metabolic processes of cells being well adapted to this high internal concentration [36]. In the *ntcA* mutant a high internal potassium concentration was observed which may be attributed to high sodium ion accumulation leading to high osmolarity [37, 38] due to the involvement of K^+-dependent Na^+/Ca^{2+} antiporters [39]. Further, calcium is known to modify cell surfaces and these modified surfaces act as efficient barriers for H then for Na and least for K [40]. All these experimentations also prove that calcium seems especially important in maintaining membrane selectivity toward K^+ and Na^+ [41].

Any nutrient when in excess disturbs the normal ionic balance and homeostasis of cell and lead to altered morphology and physiology and in most adverse cases to death of the organism [42]. In the present study also similar response to differential level of calcium ion was noticed. As we know that the basic skeleton and meshwork is contributed by calcium ion and further that the first and foremost impact occurs on the cell membrane. The increment in the electrolyte leakage on increasing calcium chloride ($CaCl_2$) was a measure of extent of stress on the membrane stability of *Anabaena* sp. PCC 7120 strains as reported in other organisms [43]. Compared to wild type, the mutant strain was much more prone to membrane damage as reflected by higher percentage of electrolyte leakage at all the tested concentrations. Greater electrolyte leakage at higher calcium chloride concentration may be attributed for poor growth. Loss of membrane integrity leads to solute leakage which was evident in the form of increase in electrolyte leakage from lower to higher calcium chloride concentration.

In view of the above experimental findings we conclude that (i) $CaCl_2$ has a concentration dependent dual role; its lower concentration is stimulatory while its higher concentrations is either inhibitory or toxic, (ii) wild type and the mutant strain differed in their ability to regulate Ca^{2+} ion when supplemented with varied levels of $CaCl_2$, the possible explanation could be attributed to their basic difference i.e. presence of functional heterocyst in the wild type *Anabaena* sp. PCC 7120 whereas complete absence in the mutant

strain and the role of *ntcA* gene in regulating calcium ion could be interpreted.

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