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CO_2 Fixation Rate and RuBisCO Content Increase in the Halotolerant Cyanobacterium, *Aphanothece halophytica*, Grown in High Salinities[1]

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ABSTRACT

The growth of the halotolerant cyanobacterium *Aphanothece halophytica*, previously adapted to 0.5 molar NaCl, was optimal when NaCl concentration in culture medium was in the range 0.5 to 1.0 molar. The growth was delayed at either too low or too high salinities with lag time of *ca.* 0.5 day in 0.25 molar NaCl and *ca.* 2 days in 2 molar NaCl under the experimental conditions. However, the growth rates at the logarithmic phase were similar in the culture media containing NaCl in the range 0.25 to 2.0 molar. The capacity of photosynthetic CO_2 fixation increased 3.7-fold in the cells at the logarithmic phase as NaCl concentration in the culture medium increased from 0.25 to 2.0 molar. The protein level of ribulose 1,5-bisphosphate carboxylase/oxygenase was also found to increase with increasing salinity using both an immunoblotting method and protein A-gold immunoelectron microscopy. These results indicate that high photosynthetic capacity and high ribulose 1,5-bisphosphate carboxylase/oxygenase content may entail an important role in betaine synthesis and adaptation of the *A. halophytica* cells to high NaCl level.

Salinity is one of the important elements to limit crop productivity. Since the synthesis and accumulation of low mol wt organic "compatible" solutes such as sugars and quaternary ammonium compounds are known to be essential for adaptability of plant cells to high salinity (10, 11, 22, 28, 29), it can readily be surmised that changes in carbon, nitrogen, and/or energy metabolisms are tightly connected to the overall process. The molecular basis of adaptation and tolerance to salinity in plants is not well understood.

Betaine (glycinebetaine) has previously been demonstrated to be a major osmoticum in a number of photosynthetic organisms, including both procaryotes and eucaryotes (10, 19, 22). In higher plants, some plant species in families such as Chenopodiaceae, Amaranthaceae, and Gramineae accumulate betaine in response to water or salt stress (10). Recently, Hanson and co-workers (12, 27) have reported that betaine synthesis occurs in chloroplasts from spinach leaves. Subsequently, Robinson and Jones (21) have reported the accumulation of betaine up to 0.3 M in spinach chloroplasts to provide osmotic adjustment during salt stress. It is also known that highly halotolerant cyanobacteria accumulate betaine as a major osmoticum, whereas less tolerant cyanobacteria accumulate either sucrose or glucosylglycerol (19). The unicellular cyanobacterium *Aphanothece halophytica* is a highly halotolerant organism that can grow at high external NaCl concentrations up to 3 M. It was recently demonstrated that betaine is accumulated as the major osmoticum inside *A. halophytica* cells in response to changes in external salinity (20).

We have previously reported (13, 15) that betaine masks inhibitory effect of Cl^- on the enzyme activity of RuBisCO[3] from *A. halophytica* and prevents the enzyme dissociation into constituent subunits. Therefore, betaine is involved in not only osmoregulation but also stabilization of enzymes in the cells grown in high salinities.

As a step to understanding the molecular basis of salt tolerance and, in particular, its relation to cellular metabolisms in photosynthetic cells, we studied salinity effects on growth, photosynthetic CO_2 fixation, and RuBisCO content of the *A. halophytica* cells and found that both the rate of photosynthetic CO_2 fixation and RuBisCO content in the cells increase with increasing external salinity, whereas growth rates are not changed.

MATERIALS AND METHODS

Growth of *Aphanothece halophytica*: *Aphanothece halophytica* cells obtained from Dr. G. A. Codd (University of Dundee, Scotland) were grown photoautotrophically at 30°C in BG 11 medium plus 18 mM $NaNO_3$ and Turk Island salt solution as previously described (7), except that NaCl concentration of the culture medium was adjusted to a range from 0.25 to 2 M as desired. Cotton-plugged 500 mL conical flasks containing 150 mL of medium each were used and shaken on a reciprocal shaker without supplementation of condensed CO_2 gas. The culture flasks were incubated at 30°C with illumination (70 $\mu E\ m^{-2}\ s^{-1}$).

Purification of RuBisCO and Its LSU. RuBisCO of *A. halophytica* was purified according to the method described previously (24). Purified RuBisCO protein was subjected to SDS-PAGE (16). The gel fragments containing the LSU protein (52 kD, *ca.* 300 μg) were directly used for preparation of its antibody as described below.

Quantitation of RuBisCO Protein. RuBisCO protein was quantitated by immunoblot analysis (3, 25). Crude extracts of *A. halophytica* (10 μg soluble protein) different salt concentrations were subjected to SDS-PAGE (16). The proteins on the gel were

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[3] Abbreviations: RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); LSU, large subunit of RuBisCO.

electrophoretically transferred to a membrane filter (Schleicher & Schuell, PH9, 0.1 μm). The membrane filter was then immunodecorated with the antiserum produced by injecting the purified LSU of *A. halophytica* RuBisCO into a rabbit. The filter was first washed for 1 h with PBS containing 0.1% Tween 20 and 2% defatted skim milk. This buffer is referred to as the washing buffer. After the washing the filter was incubated with RuBisCO LSU antiserum in the washing buffer for 1 h. The filter was washed twice for 30 min each with the washing buffer before incubating with alkaline phosphatase-conjugated second antibody for 1 h. The filter was washed with PBS and then immersed in alkaline phosphatase color development solution. The intensity of the color band was measured by a Shimadzu scanner CS-940.

RuBP Carboxylase and CO_2 Fixation. RuBP carboxylase activity was assayed radiometrically by measuring the incorporation of $^{14}CO_2$ into acid stable product as described previously (24).

Photosynthetic CO_2 fixation was measured as follows. The *A. halophytica* cells were first washed by centrifugation at 2000*g* for 5 min in a medium containing 50 mM Hepes-NaOH buffer (pH 8.0), 10 mM $MgCl_2$ and NaCl of various levels, and resuspended in the same buffer. A small aliquot (0.2 ml, 50 μg protein) was illuminated at 200 $\mu E\ m^{-2}\ s^{-1}$ for 5 min at 25°C. $NaH^{14}CO_3$ (10 mC/mmol) of various concentrations was added and the incubation was continued for another 10 min before the addition of 0.1 ml of acetic acid to stop the reaction. The whole mixture was dried under an electric lamp. The dried residues were suspended in 0.3 mL water followed by the addition of 3 mL of scintillation fluid and the radioactivity was counted by an Aloka liquid scintillation counter.

Immunocytochemical Protein A-Gold Labeling. The *A. halophytica* cells were treated for 2 h with fixative containing 4% paraformaldehyde and 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4). Postosmication was omitted. The cells were dehydrated in graded ethanol at −20°C and embedded in Lowicryl-K4M at −10°C under UV irradiation. Immunocytochemical protein A-gold labeling procedures were exactly the same as described by Yokota *et al.* (30). The sections made were examined under a Hitachi H 600 electron microscope at 100 kV.

Other Analytical Procedures. Protein was determined by the modified method of Lowry using BSA as a standard (2). Chl was determined in 80% acetone by the method of Mackinney (18). Cell number was determined in a Coulter counter.

RESULTS

Growth of *A. halophytica* under Various Salinities. The growth of *A. halophytica*, previously adapted to 0.5 M NaCl, in response to various NaCl concentrations, was studied by measuring the change in cell number in culture medium (Fig. 1). The growth was optimal when NaCl concentration was in the range 0.5 to 1.0 M under the experimental conditions. At either too low or too high NaCl concentrations the growth was delayed with lag time such as *ca.* 0.5 d at 0.25 M NaCl and *ca.* 2 d at 2.0 M NaCl. However, the growth rates at the logarithmic phase (3–8 d) were quite similar in the culture media containing NaCl in the range 0.25 to 2.0 M. It has been reported that in *Escherichia coli*, which also accumulates betaine as an osmoprotectant (23), inhibition of growth under high salinities can be reduced when the growth medium contains small amount of betaine (17). However, the supplementation of 1 mM betaine to the culture medium containing 2 M NaCl did not enhance the growth of *A. halophytica*, suggesting that *A. halophytica* does not possess any betaine uptake system.

Photosynthetic CO_2 Fixation of the *A. halophytica* Cells Grown under Various Salinities. As a step to compare capacities of photosynthetic CO_2 fixation in the cells grown under various salinity conditions, the response of photosynthetic CO_2 fixation to external $NaHCO_3$ concentrations was examined with the *A. halophytica* cells grown in 0.25, 0.5, or 2.0 M NaCl (Fig. 2). This is important since properties of inorganic carbon uptake and subsequent CO_2-concentrating systems in any halotolerant cyanobacteria have not so far been studied and they might be affected by external salinity. The apparent K_m ($NaHCO_3$) for photosynthetic CO_2 fixation was found to be nearly the same (0.5 mM) in 7 d-old-cells grown in 0.25 M, 0.5 M, or 2.0 M NaCl. The rate of photosynthetic CO_2 fixation was saturated at *ca.* 5 mM $NaHCO_3$ and consequently this concentration was used hereafter for the measurements of the capacity of photosynthetic CO_2 fixation. The V_{max} of photosynthetic CO_2 fixation was the highest in the cells grown in 2.0 M NaCl and it decreased with

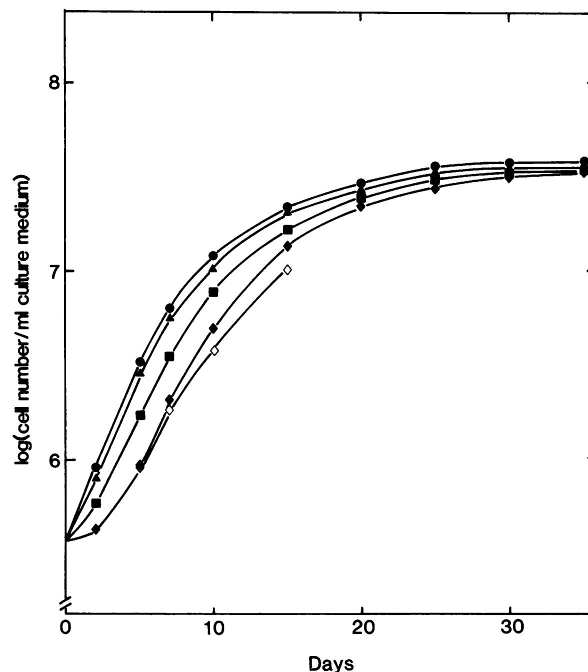

FIG. 1. Growth of *A. halophytica* under various salinity conditions. (■), 0.25 M NaCl; (●), 0.5 M NaCl; (▲), 1.0 M NaCl; (♦), 2.0 M NaCl, and (◇), 2.0 M NaCl + 1 mM glycinebetaine.

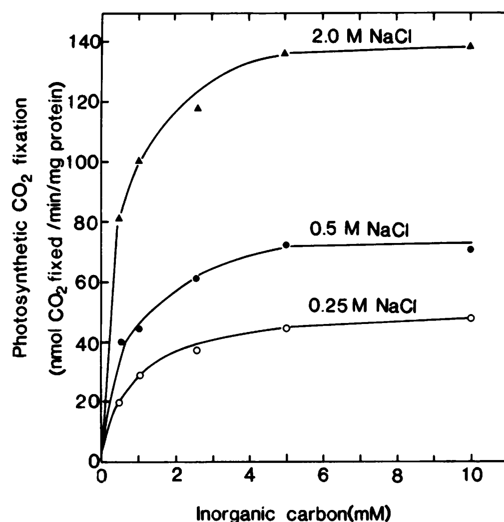

FIG. 2. Dependence of photosynthetic CO_2 fixation on $NaHCO_3$ concentrations in the *A. halophytica* cells grown in culture media containing NaCl of various levels. Experimental details are described in "Materials and Methods."

the decrease in external NaCl concentrations. Since photosynthetic capacity of the cells can vary depending on growth stage, it is necessary to compare the effect of external salinity on the photosynthetic capacity at various growth stages. As shown in Figure 3, all three salinities gave similar patterns of photosynthetic capacity during the growth period of 28 d. At the logarithmic phase the photosynthetic capacity remained at high levels and then decreased gradually. Furthermore it was found that the rate of CO_2 fixation is always increased with increasing salinity throughout the growth period tested.

RuBisCO Content of the *A. halophytica* Cells Grown under Various Salinities. As shown in Figure 4, extractable ribulose bisphosphate carboxylase activity was found to increase in the 7-d-old cells with increasing external salinity. However, since RuBisCO from *A. halophytica* was shown to be easily dissociated into constituent subunits resulting in enzyme inactivation under certain experimental conditions (14, 24), this made quantification of protein levels of RuBisCO by enzyme assay inadequate. Therefore, to estimate RuBisCO content in the cells grown under various salinity conditions, the immunoblotting methods using anti-LSU was employed. By immunoblotting and visualization with the alkaline phosphatase-conjugated second antibody, it was found that RuBisCO protein increases in the cells with increasing external salinity. Figure 5 shows the changes in RuBisCO content of the cells during growth in culture media containing NaCl of various levels. The RuBisCO content was high up to 13 days of growth in all three salinities and after 13 d the protein started to decline gradually. At each growth stage the protein level increased two- to threefold with the increase in salinity of growth medium from 0.25 M NaCl to 2.0 M NaCl. It is also noted that after the logarithmic phase, *i.e.* after 10 d of growth, the photosynthetic capacity decreased more drastically than RuBisCO content (*cf.* Figs. 3 and 5).

Distribution of RuBisCO in the *A. halophytica* Cells. The distribution of RuBisCO in the *A. halophytica* cells at logarithmic phase was studied by the protein A-gold immunoelectron microscopy. Figure 6 shows the labeling pattern observed with anti-LSU and nonimmunized IgG. Only a few particles were present in the cells treated with nonimmunized immunoglobulin G (Fig. 6, A and C). Anti-LSU by contrast densely labeled the cytoplasm region of the cells (Fig. 6, B and D). Table I compares the labeling of the cells grown in low (0.25 M NaCl) and high (1.0 M NaCl) salinities. The labeling density of gold particles was significantly higher by about 60% in the cells grown in 1.0 M NaCl than that in 0.25 M NaCl, suggesting again that RuBisCO content is increased in the cells grown in high external salinity, although the presence of exact linearity between protein content and

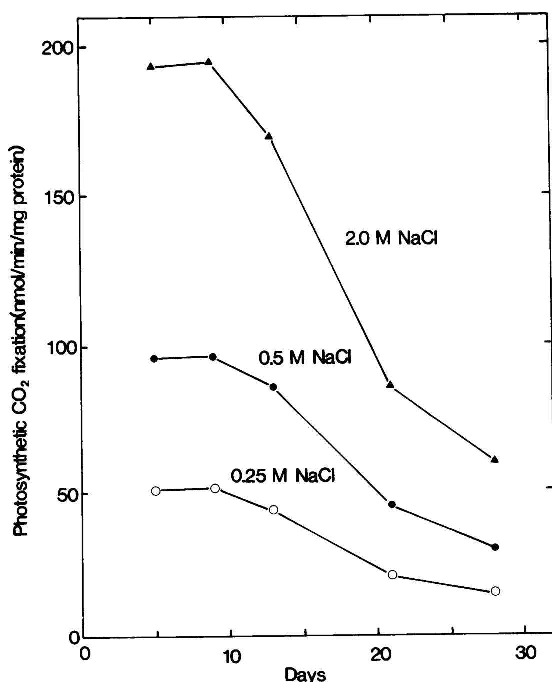

FIG. 3. Changes in photosynthetic CO_2 fixation in the *A. halophytica* cells during growth in culture media containing NaCl of various levels. Experimental conditions are exactly the same as in Figure 2, except that $NaHCO_3$ concentration is 5 mM.

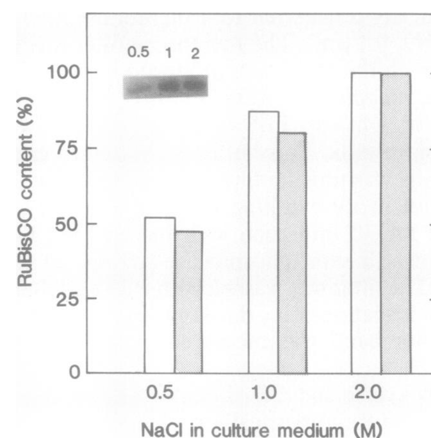

FIG. 4. RuBisCO activity and content of the 7-d-old *A. halophytica* cells grown in culture media containing NaCl of various levels. Experimental details are described in "Materials and Methods." (□) extractable ribulose bisphosphate carboxylase activity (100% control activity from the cells grown in 2.0 M NaCl was 131 nmol CO_2 fixed/mg protein·min). (▨) immunoblotting quantification of RuBisCO LSU (100% control is the band intensity with the crude extract of cells grown in 2.0 M NaCl). Inset shows the actual electrophoretogram after colour development.

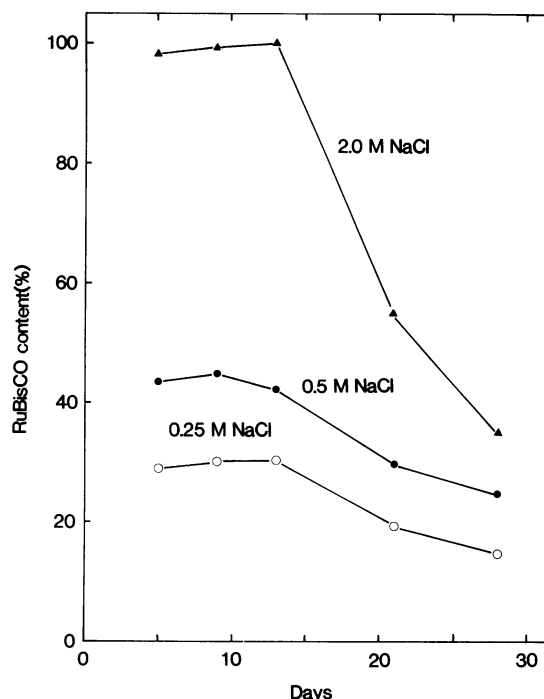

FIG. 5. Changes in RuBisCO content of the *A. halophytica* cells during growth in culture media containing NaCl of various levels. RuBisCO content was quantified by the immunoblotting method as described in Figure 4 (100% control is the band intensity of LSU with the crude extract of 13-d-old cells grown in 2.0 M NaCl).

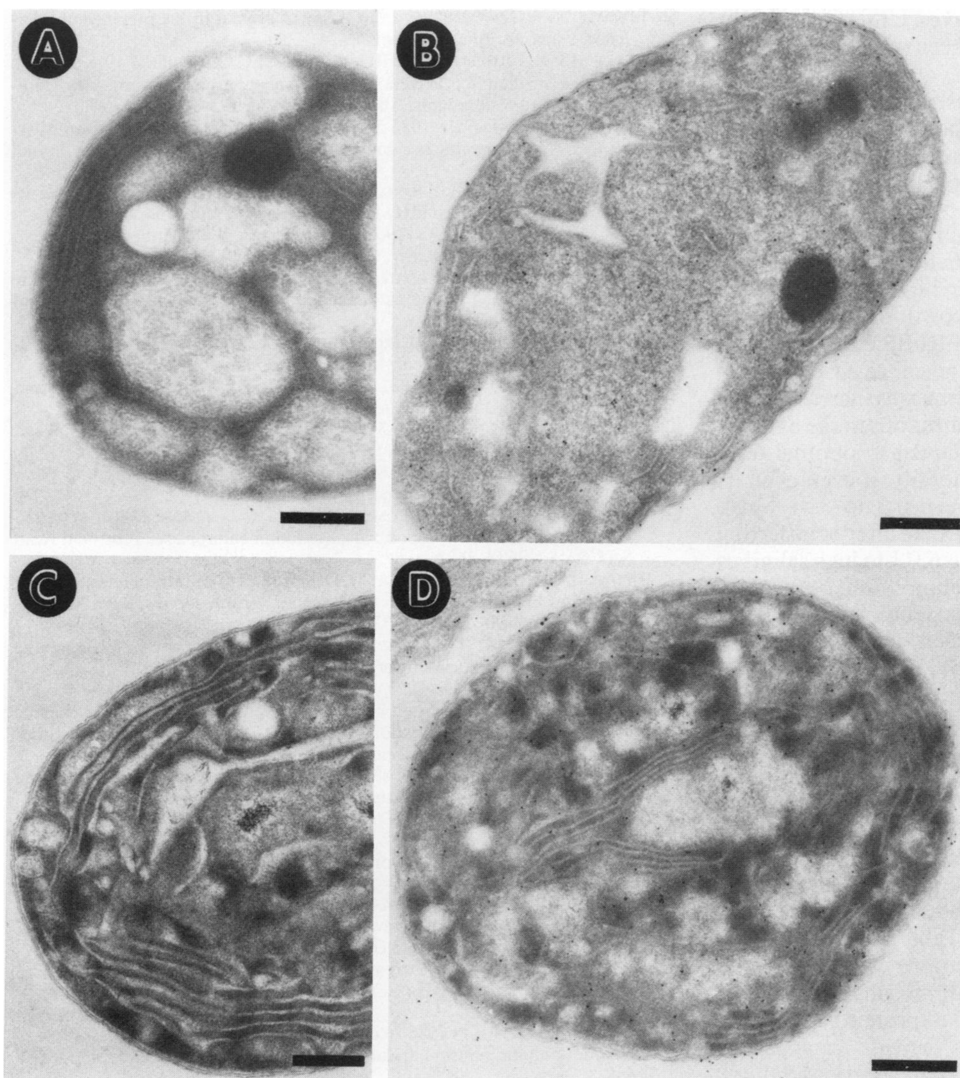

FIG. 6. Sections of Lowicryl K4M-embedded *A. halophytica* cells incubated with anti-RuBisCO LSU, followed by protein A-gold complex conjugation. Seven-d-old cells grown in 0.25 M NaCl (A and B) and 1.0 M NaCl (C and D). B and D, incubated with anti-RuBisCO LSU; A and C, incubated with immunoglobulin G fraction from nonimmunized rabbit. Original magnifications: A, ×28,000; B, ×26,000; C, ×24,000; D, ×26,000. Bars are 0.5 μm. Experimental details are described in the text.

Table I. *Density of Labeling in A. halophytica Grown in Low and High Salinities*[a]

NaCl Concentration in Culture Medium	Gold Particles per μm^2
M	
0.25	12.6 ± 1.4[b]
1.0	20.0 ± 3.0

[a] Five photographs evaluated. [b] ± SE.

Table II. *Protein and Chl Contents in A. halophytica Cells Grown in Media Containing NaCl of Various Levels*

NaCl Concentration in Culture Medium	Protein at Day of Culture:			Chl at Day of Culture:		
	5	14	21	5	14	21
M	*mg/10⁸ cells*			*μg/10⁸ cells*		
0.25	0.84	1.57	1.56	8.2	23.2	15.1
0.50	0.88	1.52	1.63	10.1	24.5	15.8
1.0	0.90	1.44	1.89	9.2	22.8	17.6
2.0	1.08	2.19	2.22	11.4	39.9	24.9

labeling density in the protein A-gold labeling method has not been generally accepted.

Protein and Chl Contents in the *A. halophytica* Cells under Various Salinities. Total protein and Chl contents in the *A. halophytica* cells were also determined on a per cell basis (Table II). Both protein and Chl contents increased by 30 to 40% and by 40 to 70%, respectively, when the external NaCl concentration increased from 0.25 M to 2.0 M.

DISCUSSION

In photosynthetic cells, carbon atoms of organic compatible solute molecules must be supplied from the photosynthates including both fixed carbon metabolites and reserve carbon such as starch and glycogen. It is known that in higher plants such as spinach and barley, betaine is synthesized in leaves from a two step oxidation of choline,

Choline→betainealdehyde→glycinebetaine.

The second enzyme has been purified and characterized (1, 27), whereas the enzymic nature of the first step is not well understood. Photosynthetic CO_2 fixation and betaine synthesis take place in the same organelle, the chloroplast (12, 21). In both unstressed and salt-stressed spinach leaves, the rate of betaine synthesis is estimated to be 20 and 70 nmol·mg $Chl^{-1}\cdot h^{-1}$, respectively (5, 12), being much lower than that of photosynthetic CO_2 fixation. Therefore, betaine synthesis is not a substantial

drain from fixed carbon in those plant leaves. Unfortunately, the capacity of betaine synthesis has not been well evaluated in halophytes in spite of its importance.

On the other hand, it has been previously reported that significant amount of carbon (about 20%) is photosynthetically incorporated into a compatible solute glycerol in the salt-stressed halotolerant green alga, *Dunaliella* (6, 26). In the dark, carbon transfer from starch to glycerol has also been reported to occur in *Dunaliella* cells (9). Furthermore certain photosynthetic algae have been reported to increase photosynthetic activity or extractable enzyme activity of RuBisCO under high salinities (4, 8). Gimmler *et al.* (8) have reported that growth at high salinities increased maximal extractable activity of RuBP carboxylase *ca.* twofold in the extremely halotolerant green alga, *Dunaliella parva*, when the external NaCl concentration was increased from 1 M to 3 M. The alga tolerates NaCl concentrations in the external medium virtually up to saturation. If adapted to optimal temperature, CO_2 concentration and light intensity, it even exhibits equal growth rates at all salinities between 0.3 to 3 M NaCl. Blumwald and Tel-Or (4) have reported that after transferring fresh water cyanobacterium *Synechococcus* 6311 to high salinities in the range 0.2 to 0.4 M NaCl the photosynthetic activity decreased initially but soon after it is transiently enhanced (*ca.* twofold). These authors have also argued that such enhancement in photosynthetic activity, even transiently, is beneficial for the accumulation of soluble sugars as compatible solutes and adaptation to high NaCl concentrations.

In the present study, we showed that the RuBisCO content and rate of photosynthetic CO_2 fixation on a total cellular protein basis increase under high salinities in *A. halophytica* whose cells accumulate betaine more than 1.5 M in response to external salinity (20). This was not due to a decrease of total protein per cell with increasing external salinity. In *A. halophytica* cells, the amount of total protein per cell was gradually increased with increasing external salinity as shown in Table II. On the other hand, the content of reserve carbon (glycogen) was found to be quite similar among the cells at logarithmic phase grown in various salinities (*ca.* 0.4 mg/mg cellular protein) (data not shown). In the *A. halophytica* cells, the increase in apparent photosynthetic rate at high external NaCl concentrations did not lead to an increase in growth rate (Fig. 1). Therefore, it can be surmised that the stimulation of photosynthetic CO_2 fixation by high external salinity entails an important role in osmoregulation (betaine synthesis and accumulation) and swift adaptation to a wide range of external NaCl concentrations in the *A. halophytica* cells.

Carbon partitioning in the *A. halophytica* cells under high salinities has to be studied in future experiments and it must be clarified further whether or not photosynthesis and osmoregulation (betaine synthesis and accumulation) are tightly regulated by external salinity in these nonvacuolated halotolerant photosynthetic cells.

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