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Relationship between Sodium Influx and Salt Tolerance of Nitrogen-Fixing Cyanobacteria

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The relationship between sodium uptake and cyanobacterial salt (NaCl) tolerance has been examined in two filamentous, heterocystous, nitrogen-fixing species of *Anabaena*. During diazotrophic growth at neutral pH of the growth medium, *Anabaena* sp. strain L-31, a freshwater strain, showed threefold higher uptake of Na^+ than *Anabaena torulosa*, a brackish-water strain, and was considerably less salt tolerant (50% lethal dose of NaCl, 55 mM) than the latter (50% lethal dose of NaCl, 170 mM). Alkaline pH or excess K^+ (>25 mM) in the medium causes membrane depolarization and inhibits Na^+ influx in both cyanobacteria (S. K. Apte and J. Thomas, Eur. J. Biochem. 154:395–401, 1986). The presence of nitrate or ammonium in the medium caused inhibition of Na^+ influx accompanied by membrane depolarization. These experimental manipulations affecting Na^+ uptake demonstrated a good negative correlation between Na^+ influx and salt tolerance. All treatments which inhibited Na^+ influx (such as alkaline pH, K^+ above 25 mM, NO_3^-, and NH_4^+), enhanced salt tolerance of not only the brackish-water but also the freshwater cyanobacterium. The results indicate that curtailment of Na^+ influx, whether inherent or effected by certain environmental factors (e.g., combined nitrogen, alkaline pH), is a major mechanism of salt tolerance in cyanobacteria.

The salt tolerance exhibited by many cyanobacteria (26) has been exploited with some success in reclamation of brackish soils (24). The physiological-biochemical basis of osmotic adaptation is not adequately understood in cyanobacteria, although a few mechanisms have been identified. One such mechanism is the formation of internal osmoticum by the accumulation of inorganic ions (16) or organic solutes like carbohydrates, polyols, and quaternary ammonium compounds (6, 13, 18, 20, 27). The role of ion transport phenomena (2, 4, 19, 22, 26, 27) and metabolic adaptations (7, 26) in cyanobacterial halotolerance has, in comparison, received scant attention.

Although sodium is an important requirement for cyanobacterial growth and nitrogen fixation (1, 3), the element is the predominant agricultural deterrent in saline habitats. The features of Na^+ transport across the membrane have considerable bearing on cyanobacterial halotolerance. In general, cyanobacteria do not accumulate Na^+ (2, 4, 17), although a transient net Na^+ uptake may occur in response to hypersaline upshock (19). Recently, we reported the features of Na^+ transport in two nitrogen-fixing, heterocystous cyanobacterial strains, one salt sensitive and the other salt tolerant (4). *Anabaena torulosa*, the brackish-water strain, excludes Na^+ by lower influx and by efficient efflux of the cation. We report here experiments designed to inhibit Na^+ influx and to show that such manipulations enhance the salt tolerance of two isolates of cyanobacteria, from either fresh- or brackish-water origins.

MATERIALS AND METHODS

Organisms and growth conditions. Two filamentous, heterocystous, nitrogen-fixing cyanobacteria, *Anabaena torulosa* (a sporulating, brackish-water form [1, 11]) and *Anabaena* sp. strain L-31 (a nonsporulating, freshwater form [25]), isolated in this laboratory, were used under axenic conditions. Fivefold-diluted cyanophycean medium (CM/5) (10), free of combined nitrogen and containing 1 mM Na^+ at pH 7.0, was used for growth and maintenance of all cultures. When needed, nitrate was added (as KNO_3) at 10 mM and NH_4^+ was added (as NH_4Cl) at 3 mM final concentration. K^+ and Na^+ were added as chlorides. Liquid cultures were grown photoautotrophically at 25°C under constant illumination (2.5 mW cm^{-2}) and aeration (2 liters min^{-1}) and were harvested after 5 days (in the late-logarithmic phase) of growth.

Salt tolerance was examined on solid medium (1.5% agar in CM/5) adjusted to the desired pH (with 0.1 N LiOH HCl) and supplemented with the required concentrations of K^+, Na^+, NO_3^-, or NH_4^+. Cyanobacteria were grown for growth measurements in agar petri plates and for nitrogenase activity measurements on agar slants in 5-ml Vacutainer vials (plugged with sterile cotton) at 25°C and 2.5 mW cm^{-2} for up to 5 days. Under normal experimental conditions (controls), the mean generation time of *A. torulosa* and *Anabaena* sp. strain L-31 was 18 and 17 h, respectively.

Measurement of sodium influx, membrane potential, and intracellular volume. Na^+ influx and transmembrane electric potential ($\Delta\psi_m$) were determined by using radiotracers $^{22}Na^+$ and [phenyl-^{14}C]tetraphenylphosphonium, respectively, in assays as described earlier (4). Intracellular radioactivity was corrected for internal cell volume, which was estimated (23) by using [U-^{3}H]H_2O and $Mg^{35}SO_4$ (instead of a sugar) as described previously (4). The average intracellular volume and standard deviation of eight replicates was 0.426 ± 0.02 and 0.621 ± 0.01 μl μl^{-1} of packed cells for *A. torulosa* and *Anabaena* sp. strain L-31, respectively. Under the normal experimental conditions in the absence of salt stress, *A. torulosa* and *Anabaena* sp. strain L-31 exhibited Na^+ influx rates (along with standard deviation of five replicates) of 464 ± 10.8 and 842 ± 14.3 cpm μl^{-1} of internal cell volume min^{-1}, respectively.

Measurement of nitrogenase activity, chlorophyll, and protein. Nitrogenase assays were carried out with cyanobacteria grown on agar slants in 5-ml glass tubes. For assays, cotton plugs were replaced by rubber stoppers. The gas phase was

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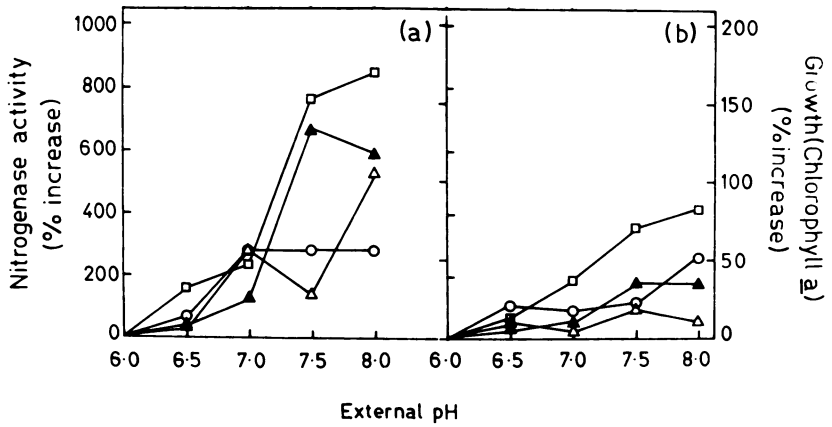

FIG. 1. Effect of variation of external pH on growth and nitrogenase activity of (a) *A. torulosa* and (b) *Anabaena* sp. strain L-31 exposed to salt stress. The desired initial pH (i.e., 6.0, 6.5, 7.0, 7.5, or 8.0) was adjusted with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–0.1 N LiOH. All the cultures contained (a) 170 mM NaCl or (b) 55 mM NaCl. Activities have been compared, with cultures where the initial pH was adjusted to 6.0 serving as controls. Growth (▲), measured as micrograms of chlorophyll *a*, is shown for day 5 after inoculation. Nitrogenase activity, estimated as micromoles of C_2H_2 reduced per milligram of chlorophyll *a* per hour, is shown for days 1 (○), 3 (□), and 5 (△) after inoculation. The starting level of chlorophyll *a* in the inoculum was 1.5 μg in all the cultures; it increased to (a) 4.6 μg and (b) 7.2 μg on day 5 in controls kept at pH 6.0. Nitrogenase activity in controls at pH 6.0 on days 1, 3, and 5, respectively, was (a) 1.2, 1.4, and 0.5 and (b) 5.1, 3.6, and 2.6 μmol of C_2H_2 reduced mg^{-1} of chlorophyll *a* h^{-1}.

adjusted to contain 0.1 atm (ca. 10.13 kPa) of acetylene in air, and the vials were incubated at 25°C and 2.5 mW cm^{-2} for 30 min. Assays were terminated by addition of ammoniacal $AgNO_3$, and the gas phase was analyzed for ethylene as described previously (9).

Growth was assessed by the content of chlorophyll *a* determined by the method of Mackinney (14). Total soluble protein was measured by the method of Lowry et al. (12).

Chemicals, radioisotopes, and gases. All inorganic chemicals were purchased from Sarabhai M. Chemicals, Baroda, or British Drug Houses, Bombay, India, and agar was obtained from Difco Laboratories, Detroit, Mich. $^{22}NaCl$, [U-^{3}H]H_2O, and $Mg^{35}SO_4$ were acquired from Amersham International, Amersham, England, and [phenyl-^{14}C]tetraphenylphosphonium bromide was obtained from New England Nuclear Corp., Boston, Mass. All other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Gases were supplied by Indian Oxygen Ltd., Bombay, except pure ethylene, which was obtained from Matheson Gas Products, East Rutherford, New Jersey.

RESULTS

Na^+ influx in *A. torulosa* and *Anabaena* sp. strain L-31 has earlier been shown to be a secondary active uptake, closely regulated by membrane potential ($\Delta\psi_m$) (4). Variation of external pH and external K^+ concentration, which modify $\Delta\psi_m$ and thereby affect Na^+ influx, was used here to assess the effect on salt tolerance. Tolerance to salt was estimated in terms of growth and nitrogenase activity since in the N-free medium used in these experiments N_2 fixation is a major determinant for growth. The response of nitrogenase to salt was therefore examined in detail on days 1, 3, and 5, corresponding to the early log, log, and near-stationary phases of growth, when specific activity of nitrogenase varies markedly.

Figure 1 shows response of *Anabaena* spp. to salt stress during variation of external pH. The salt concentrations used (170 mM NaCl for *A. torulosa* and 55 mM NaCl for *Anabaena* sp. strain L-31) corresponded to 50% lethal dose at pH 7.0. Tolerance to salt was compared with controls containing salt and maintained at pH 6.0. When the external pH was shifted towards 8.0 in the presence of salt, growth and nitrogenase activity of both cyanobacteria increased substantially. Such enhancement may arise from a beneficial effect of alkaline pH per se on growth and nitrogenase activity, rather than on salt tolerance. To ascertain this, the values of growth and nitrogenase activity in the presence of salt at each specific pH were compared with the respective controls maintained at the same specific pH but containing no salt (Fig. 2). At pH 6.0 the values of nitrogenase activity under salt stress were less than 30% of those for cultures without excess salt (Fig. 2). In both cyanobacteria this inhibition by salt was greatly relieved at alkaline pH, and in *Anabaena* sp. strain L-31 growth and nitrogenase activities approached or even exceeded the control values obtained during growth without excess salt.

In general, the effect of salt stress on growth was insignificant on day 1 and noticeable on day 3 (data not shown), whereas on day 5 growth was reduced substantially by salt. In contrast, nitrogenase activity was affected from day 1 itself. Nitrogenase specific activity on days 1, 3, and 5 was markedly different (see legends to Fig. 1 and 2), but the relative activities remained unchanged on all 3 days of measurement, showing that salt and pH did not delay induction of the enzyme. pH values below 7.0 caused greater inhibition, while those above 7.0 conferred protection of growth and nitrogenase activity against salt (Fig. 1 and 2). The enhanced salt tolerance at alkaline pH seems to be related to Na^+ transport. Na^+ influx has been shown to be reduced under such conditions, with the rates at pH 8.0 being only 24 and 28% of that at pH 6.0 for *A. torulosa* and *Anabaena* sp. strain L-31, respectively (4).

External K^+, above 25 mM, has been shown to inhibit Na^+ influx; at 100 mM external K^+, influx rates of Na^+ were only 49 and 33% of that at 1 mM K^+ for *A. torulosa* and *Anabaena* sp. strain L-31, respectively (4). Similar concentrations (25 to 100 mM) of K^+ also enhanced growth and nitrogenase activity during salt stress (Fig. 3). K^+, per se, even at 100 mM did not influence growth or nitrogenase

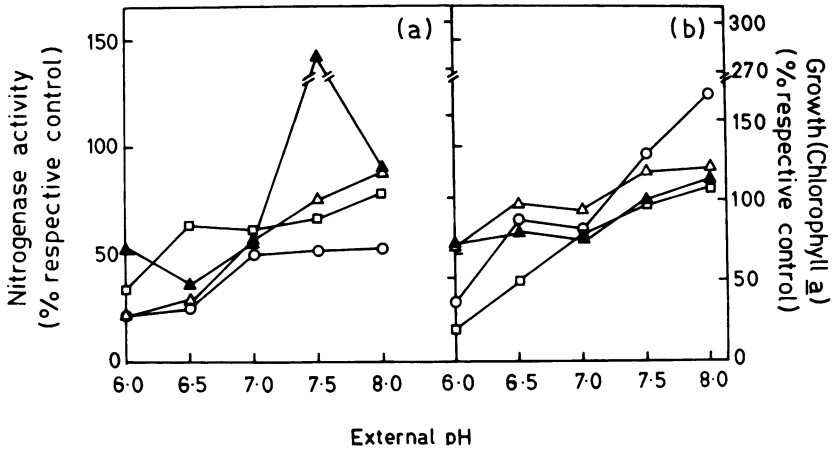

FIG. 2. Effect of variation of external pH on growth and nitrogenase activity of (a) *A. torulosa* and (b) *Anabaena* sp. strain L-31 grown in the absence or presence of salt. Two sets of cultures were grown at each specific pH (i.e., 6.0, 6.5, 7.0, 7.5, and 8.0). To one set NaCl was added at either (a) 170 mM or (b) 55 mM. The other set, maintained at each specific pH but containing no salt, served as the control for that pH. Activities of cultures exposed to salt have been plotted as percent of their respective pH controls without salt. The starting level of chlorophyll *a* in the inoculum was 1.5 μg in all the cultures. The mean chlorophyll *a* level on day 5 in controls kept at pH 6.0, 6.5, 7.0, 7.5, and 8.0 and containing no salt was (a) 6.6 ± 0.16 μg and (b) 10.0 ± 0.43 μg. Nitrogenase activities of these controls on days 1, 3, and 5, respectively, were (a) 9.64 ± 1.52, 7.18 ± 2.67, and 3.22 ± 0.61 and (b) 15.5 ± 2.96, 15.1 ± 3.16, and 5.1 ± 0.38 μmol of C_2H_2 reduced mg^{-1} of chlorophyll *a* h^{-1}. Other details were as described in the legend to Fig. 1.

activity in cultures without excess salt (Fig. 4; see legend to Fig. 4 for comparison of respective controls). Provision of K^+, however, resulted in considerable amelioration of the effect of salt stress. Again, the relative nitrogenase activities at different K^+ concentrations showed an identical pattern on all 3 days, indicating that salt and K^+ did not modify the induction of nitrogenase. In the presence of 100 mM K^+, salt did not affect the growth and N_2 fixation of *Anabaena* sp. strain L-31, while in *A. torulosa* the effect was only marginal (Fig. 3 and 4).

The presence of combined nitrogen during growth severely reduced Na^+ influx in both *Anabaena* spp. (Fig. 5). Such cells also showed a significant membrane depolarization (Table 1). Comparable depolarization of membrane has earlier been shown to cause significant inhibition of Na^+ influx in both *Anabaena* spp. (4). While NH_4^+ is known to depolarize the membrane, the mechanism by which NO_3^- (added as KNO_3) causes depolarization is not clear, but it is certainly not due to accompanying K^+, which does not affect Na^+ influx or $\Delta\psi_m$ at such low (10 mM) concentrations (4). Figure 6 shows that both NO_3^- and NH_4^+ also caused significant enhancement in the salt tolerance of not only the brackish-water strain (Fig. 6a) but also the freshwater strain (Fig. 6b). As expected, such cultures did not possess nitrogenase activity on account of its repression by combined nitrogen.

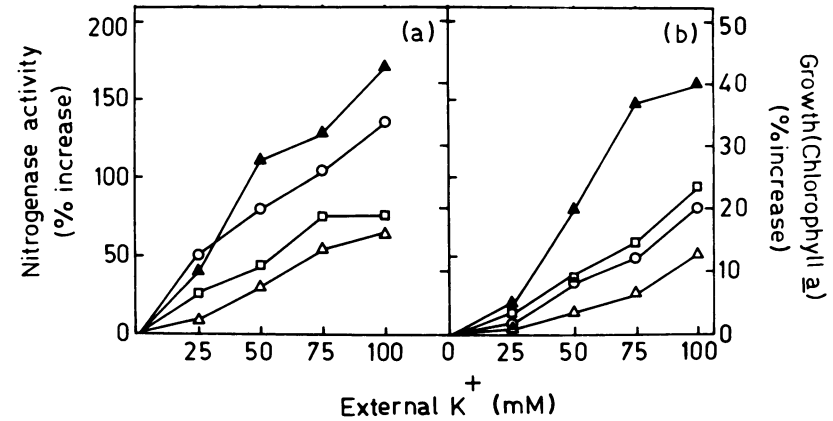

FIG. 3. Effect of variation of external K^+ concentration on growth and nitrogenase activity of (a) *A. torulosa* and (b) *Anabaena* sp. strain L-31 exposed to salt stress. Salt (NaCl) was added to all the cultures at either (a) 170 mM or (b) 55 mM. Activities at various K^+ concentrations have been compared with controls containing 1 mM K^+. Growth (▲), measured as micrograms of chlorophyll *a*, is shown for day 5 after inoculation. Nitrogenase activity, estimated as micromoles of C_2H_2 reduced per milligram of chlorophyll *a* per hour, is shown for days 1 (○), 3 (□), and 5 (△) after inoculation. The starting level of chlorophyll *a* in the inoculum was 1.5 μg in all the cultures and increased to (a) 5.6 μg and (b) 5.3 μg on day 5 in the controls containing 1 mM K^+. Nitrogenase activities in these controls on days 1, 3, and 5, respectively, were (a) 2.6, 7.9, and 6.0 and (b) 4.3, 13.0, and 12.4 μmol of C_2H_2 reduced mg^{-1} of chlorophyll *a* h^{-1}.

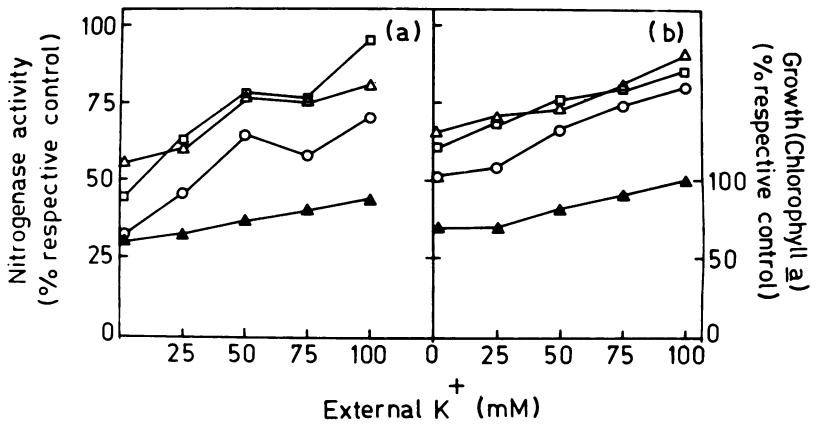

FIG. 4. Effect of variation of external K^+ concentration on growth and nitrogenase activity of (a) *A. torulosa* and (b) *Anabaena* sp. strain L-31 grown in the absence or presence of salt. Two sets of cultures were grown at each specific K^+ concentration. To one set NaCl was added at (a) 170 mM or (b) 55 mM. The other set, maintained at each specific K^+ concentration but containing no salt, served as the control for that K^+ concentration. Activities of cultures exposed to salt have been compared with their respective controls. The starting chlorophyll *a* level in the inoculum was 1.5 μg in all the cultures. Mean chlorophyll *a* levels on day 5 in controls containing 1, 25, 50, 75, and 100 mM K^+ were (a) 9.5 ± 0.23 μg and (b) 7.8 ± 0.27 μg. Nitrogenase activities of these controls on days 1, 3, and 5, respectively, were (a) 8.45 ± 0.75, 16.42 ± 1.74, and 11.36 ± 0.99 and (b) 8.80 ± 0.57, 23.75 ± 2.14, and 19.34 ± 0.70 μmol of C_2H_2 reduced mg^{-1} of chlorophyll *a* h^{-1}. Other details were as described in the legend to Fig. 3.

DISCUSSION

Osmotic adaptation in most organisms involves intracellular accumulation of low-molecular-weight organic solutes or inorganic ions. Among cyanobacteria, carbohydrates such as glucosylglycerol (8), sucrose (6), and trehalose (20), along with the quaternary ammonium compound glycine betaine (18), constitute the major internal osmotica during salt stress. The ionic relations of cyanobacteria are rather poorly understood, but there is evidence that a turgor-sensitive K^+ influx operates in cyanobacteria and that K^+ accumulation precedes accumulation of organic solutes during salt stress (22, 27). In contrast, cyanobacteria do not accumulate Na^+ (2, 4, 17); they pump it out by an active efflux which is not linked to K^+ accumulation (i.e., K^+/Na^+ exchange is inoperative) (4, 21). Although the basic mechanism of Na^+ influx is identical, there are inherent differences in flux rates and affinity of Na^+ carrier for the cation in freshwater and a brackish-water *Anabaena* spp. (4). Thus, halotolerant *A. torulosa* exhibits a 2.5- to 3-fold lower influx, a more efficient efflux, and a 9-fold lower Michaelis constant (K_m) for Na^+ than the freshwater *Anabaena* sp. strain L-31 and consequently accumulates less intracellular Na^+, especially during salt stress.

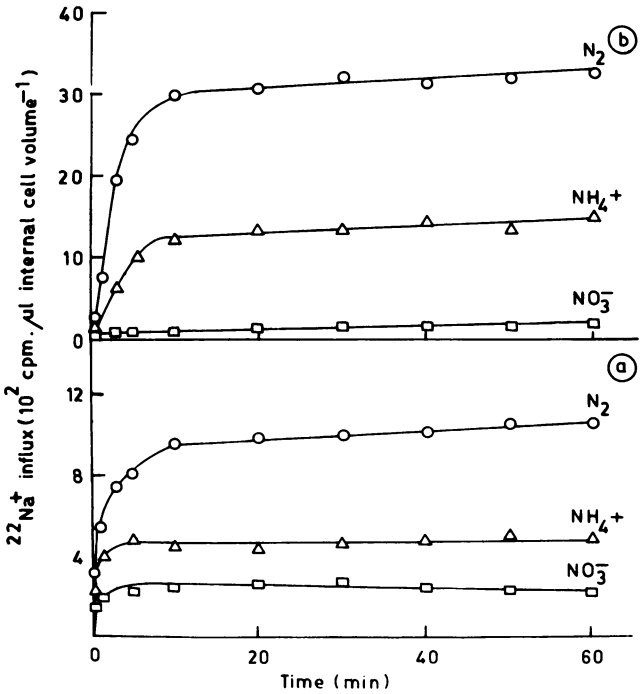

FIG. 5. Influx of sodium in (a) *A. torulosa* and (b) *Anabaena* sp. strain L-31 grown on either dinitrogen (○), 10 mM KNO_3 (□), or 3 mM NH_4Cl (△). The influx was initiated by the addition of 0.23 μCi of $^{22}NaCl$ (carrier-free) per ml of cyanobacterial suspension (20 to 25 μl of packed cells). Other details have been described previously (4).

Na^+ exclusion, achieved primarily by curtailment of net Na^+ uptake, appears to be an important mechanism of combating salt stress in cyanobacteria (4, 26). In conformity with this, all the treatments which inhibit Na^+ influx (i.e.,

TABLE 1. Membrane potential of cyanobacteria grown with different sources of nitrogen[a]

Nirogen source	Membrane potential (mV)[b]	
	A. torulosa	*Anabaena* sp. strain L-31
N_2	−66.2 ± 2.02	−64.3 ± 1.13
NO_3^-	−55.2 ± 2.09[c]	−53.3 ± 2.76[d]
NH_4^+	−58.0 ± 2.08[d]	−53.6 ± 1.90[c]

[a] Cyanobacteria were grown with either dinitrogen or 10 mM KNO_3 or 3 mM NH_4Cl in liquid cultures. Cells were suspended in CM/5 containing 20 mM HEPES–0.1 N LiOH at pH 7.0 and allowed to equilibrate with 5 μM [phenyl-^{14}C]tetraphenylphosphonium (specific activity, 19.2 Ci mol^{-1}) and 20 μM tetraphenylboron for 15 min. Intracellular radioactivity was corrected for internal cell volume, and $\Delta\psi_m$ was calculated by using the Nernst equation as described earlier (4).

[b] Values represent mean ± standard deviation of four replicates.

[c] $P < 0.001$.

[d] $P < 0.005$.

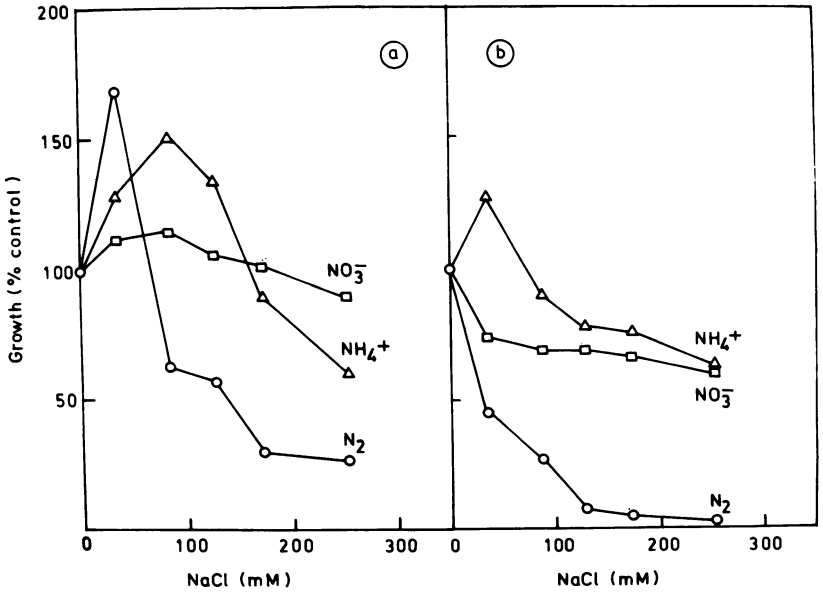

FIG. 6. Response of (a) *A. torulosa* and (b) *Anabaena* sp. strain L-31, grown on different sources of nitrogen, to salt stress. Cyanobacteria were grown (1.5% agar in CM/5) with the desired concentration of NaCl and with N_2 as nitrogen source (○) or supplemented with 10 mM KNO_3 (□) or 3 mM NH_4Cl (△) at pH 7.0. Growth was assessed by the content of chlorophyll *a* after 5 days. Control (no salt) values in the presence of N_2, NO_3^-, and NH_4^+ were 7.8, 5.9, and 9.4 μg for *A. torulosa* and 7.9, 6.1, and 10.3 μg for *Anabaena* sp. strain L-31, respectively.

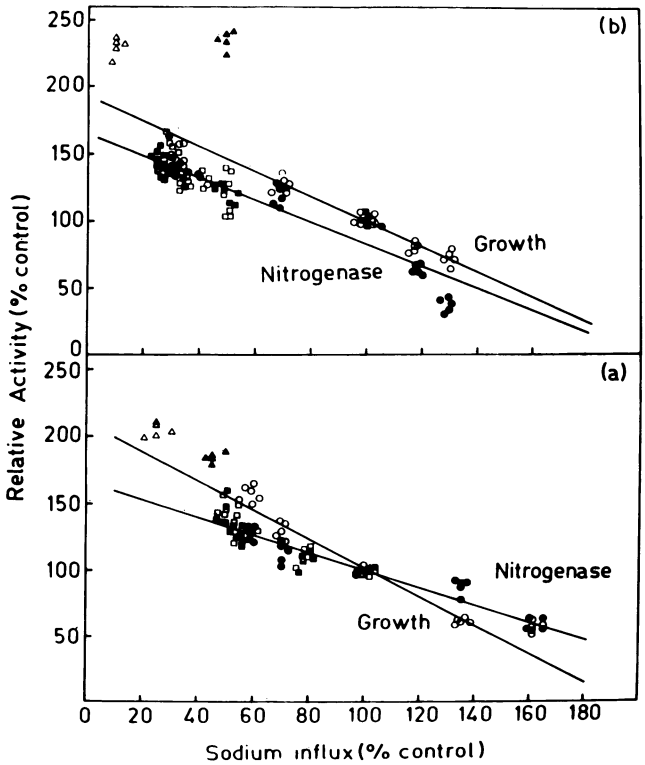

FIG. 7. Regulation of salt tolerance by sodium influx in nitrogen-fixing cyanobacteria: (a) *A. torulosa*; (b) *Anabaena* sp. strain L-31. Data on growth (open symbols) and nitrogenase activity (closed symbols) were pooled from experiments described in Fig. 1 through 6 on the effect of pH (○, ●), K^+ (□, ■) NO_3^- (△), and NH_4^+ (▲). Data on Na^+ influx during variation of external pH and external K^+ concentration have been described previously (4). The 100% values correspond to cultures grown in N-free CM/5 containing 1 mM K^+ at pH 7.0 and supplemented with either 1 mM Na^+ (for Na^+ influx; *x* axis) or 170 mM (a) or 55 mM (b) NaCl (for salt tolerance; *y* axis). The regression lines and correlation coefficients (*r*) were calculated using the programs available in a Texas Instruments TI-55 calculator. The calculated values of *r* for growth and nitrogenase activity were −0.93 and −0.94 for *A. torulosa* (a) and −0.86 and −0.92 for *Anabaena* sp. strain L-31 (b), respectively.

alkaline pH, excess K^+, NO_3^-, and NH_4^+) enhance the salt tolerance of both *Anabaena* strains (Fig. 1 through 6), whereas a shift towards acidic pH (i.e., <7.0), which increases Na^+ influx, decreases their salt tolerance (Fig. 1 and 2). The observed relationship between Na^+ influx and salt tolerance has been statistically assessed. Figure 7 shows that in both *Anabaena* strains a negative correspondence exists between Na^+ influx on the one hand and growth and nitrogenase activity on the other. The calculated correlation coefficients are close to unity, indicating a very good correlation. The close similarity of correlation lines of growth and nitrogenase activity clearly demonstrates that the salt tolerance (growth) under N-free conditions is promoted by the tolerance of N_2 fixation to salt stress.

The inhibition of Na^+ uptake may not entirely account for the observed halotolerance, and other possibilities exist. For example, (i) K^+ may accumulate intracellularly and provide a balancing osmoticum, as has been observed in certain cyanobacteria (22, 27); (ii) NO_3^- may accumulate and act as an internal osmoticum as in some higher plants (5); (iii) NO_3^- and NH_4^+ can aid the synthesis of nitrogenous osmoregulators like amino acids (15) and glycine betaine (18). However, the present results, especially the close correspondence indicated by the data presented in Fig. 7, clearly establish that curtailment of Na^+ influx plays a useful role in cyanobacterial halotolerance. It may contribute to salt tolerance significantly by reducing the need (i) to pump out excess Na^+, accumulated during salt stress, at a consider-

able energy cost and (ii) to tune the metabolism to function at higher intracellular salt concentrations.

The present data may help explain the relative abundance of cyanobacteria on saline alkali soils (24, 26) or the preponderance of unicellular and adiazotrophic forms among halotolerant cyanobacteria (6–8, 13, 16, 18–20, 27). It is tempting to speculate that in the former case alkaline pH, and in the latter case, provision of combined nitrogen, contributes greatly to their success. The ability of cyanobacteria to curtail Na^+ influx is probably genetically determined, but our data suggest that their ecological distribution is also influenced by modulation of Na^+ transport by the environmental factors. Thus *A. torulosa* is much more salt tolerant than *Anabaena* sp. strain L-31 under nitrogen-fixing conditions, but the latter can be raised to the status of a moderately halotolerant strain by inhibition of Na^+ uptake across its membrane at alkaline pH or in the presence of combined nitrogen.

ACKNOWLEDGMENT

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