APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1993, p. 899–904
0099-2240/93/030899-06$02.00/0

Vol. 59, No. 3

Differential Responses of Nitrogen-Fixing Cyanobacteria to Salinity and Osmotic Stresses

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Received 8 July 1992/Accepted 10 November 1992

Two nitrogen-fixing *Anabaena* strains were found to be differentially tolerant to salinity and osmotic stresses. *Anabaena torulosa*, a brackish-water, salt-tolerant strain, was relatively osmosensitive. *Anabaena* sp. strain L-31, a freshwater, salt-sensitive strain, on the other hand, displayed significant osmotolerance. Salinity and osmotic stresses affected nitrogenase activity differently. Nitrogen fixation in both of the strains was severely inhibited by the ionic, but not by the osmotic, component of salinity stress. Such differential sensitivity of diazotrophy to salinity-osmotic stresses was observed irrespective of the inherent tolerance of the two strains to salt-osmotic stress. Exogenously added ammonium conferred significant protection against salinity stress but was ineffective against osmotic stress. Salinity and osmotic stresses also affected stress-induced gene expression differently. Synthesis of several proteins was repressed by salinity stress but not by equivalent or higher osmotic stress. Salinity and osmotic stresses induced many common proteins. In addition, unique salt stress- or osmotic stress-specific proteins were also induced in both strains, indicating differential regulation of protein synthesis by the two stresses. These data show that cyanobacterial sensitivity and responses to salinity and osmotic stresses are distinct, independent phenomena.

Cellular adaptation to environmental stress is a major process that protects organisms from the deleterious effects of various stresses like heat, salinity, drought, and heavy metals, etc. Among the various environmental stresses, salinity and osmotic stresses are the major deterrents of agricultural productivity, globally. Elucidation of the mechanisms of salinity and osmotolerance is, understandably, the focus of intense research efforts. In general, salinity and osmotic stresses are considered to be similar (12, 13, 33) and the two terms have often been used more or less as synonyms. Thus, in higher plants osmoregulators like glycine betaine and proline betaine accumulate during osmotic stress, which is achieved by applying increasing concentrations of NaCl (13, 20, 29, 31, 33). Similarly, in the extensively studied enteric bacteria, such as *Escherichia coli*, and in plants, elevated osmotic stress is achieved by increasing the NaCl concentration in the medium, and this induces osmoresponsive genes (12, 16, 24, 29). However, unlike purely osmotic stress, salinity stress has two constituent components, namely, the osmotic and ionic components. The individual effects of these ionic and osmotic components have not been distinguished or characterized in detail.

Cyanobacteria are a unique group of photoautotrophic bacteria, some of which also fix atmospheric nitrogen. They are known to have survived a wide spectrum of environmental stresses (10, 15). Having originated three billion years ago (9), they are considered to be good model systems for studying plant responses to environmental stresses. Many cyanobacteria show considerable tolerance to salinity stress, and several physiological mechanisms underlying such tolerance have been identified (7, 15, 17, 19, 26). Curtailment of Na^+ influx is a major mechanism of salt tolerance in nitrogen-fixing cyanobacteria (3, 4). However, such a mechanism has no relevance during exposure to a purely osmotic stress. The accumulation of inorganic (K^+ ions) or organic (sugars, polyols, and quaternary amines, etc.) osmoregulators is a mechanism adequate to provide protection against both salinity and osmotic stresses. Such osmoregulators are present in almost all of the organisms studied (13, 31, 33), including cyanobacteria (6, 17, 19, 21, 26, 27).

In cyanobacteria, the responses to salinity and osmotic stresses have not been distinguished and are generally treated as similar. In this communication, we report on the differential effects of ionic and osmotic stresses on growth, nitrogen fixation, and protein synthesis in two cyanobacterial strains. The results demonstrate that *Anabaena* sp. strain L-31 (a freshwater isolate) and *Anabaena torulosa* (a brackish-water saline form) respond to salinity and osmotic stresses differently and independently.

MATERIALS AND METHODS

Organisms and growth conditions. Two filamentous heterocystous nitrogen-fixing cyanobacteria, *Anabaena* sp. strain L-31 (a freshwater isolate) (32) and *A. torulosa* (a sporulating brackish-water form) (14), isolated in this laboratory were used under axenic conditions. Cyanobacteria were usually grown in combined nitrogen-free BG-11 liquid medium at pH 7.0 (11). When required, KNO_3 was added at 10 mM and NH_4Cl was added at 3 mM. Salinity stress was applied as NaCl, and osmotic stress was applied as sucrose, both at required concentrations. For growth on plates, medium was supplemented with Difco Bacto Agar (1.2%) and 10-μl aliquots of cyanobacterial cultures were spotted on the agar surface and grown for 5 days. All cultures were grown photoautotrophically in an orbital incubator shaker at 25°C under continuous illumination (2.5 mW/cm^2) with shaking (100 rpm; for liquid cultures only). Growth was measured as the content of chlorophyll *a* as described earlier (18).

Each treatment consisted of three replicates; the results presented are mean values. Variation among replicates was less than 10%. Each experiment was repeated five or six

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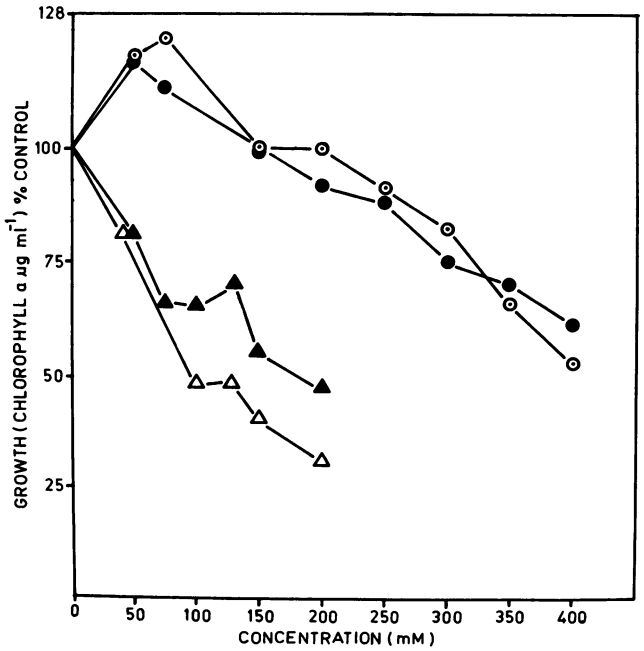

FIG. 1. Effects of salinity (△, ▲) and osmotic (⊙, ●) stresses on growth of *Anabaena* sp. strain L-31. Growth of liquid cultures was measured as content of chlorophyll *a* 3 (▲, ●) and 5 (△, ⊙) days after inoculation.

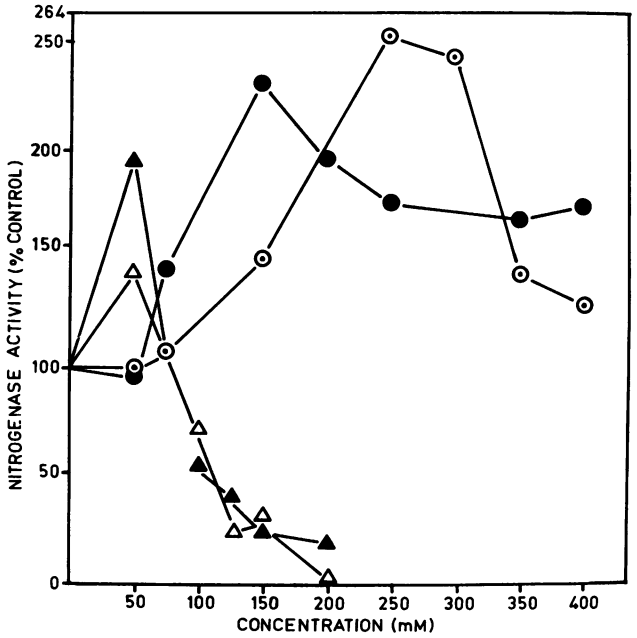

FIG. 2. Effects of salinity (▲, △) and osmotic (⊙, ●) stresses on nitrogen fixation by *Anabaena* sp. strain L-31. Nitrogenase activity was measured as acetylene reduction in 2-ml aliquots 3 (▲, ●) and 5 (△, ⊙) days after inoculation as described earlier (3).

times; results from a representative experiment are presented.

Measurement of nitrogenase activity. Nitrogenase activity was measured by the acetylene reduction technique at appropriate time intervals. Cyanobacteria grown either in liquid cultures (2-ml aliquots) or on petri plates (agar blocks spotted with cyanobacteria) were transferred to 5-ml Vacutainers and incubated with 0.1 atm (1 atm = 101.29 kPa) of acetylene for 30 min under growth conditions. Assays were conducted as described earlier (3).

In vivo radiolabeling, electrophoresis, and autoradiography of proteins. Stress-induced or -repressed proteins were analyzed as described earlier (1). Logarithmic-phase (3-day-old) liquid cultures were treated for 30 min with 130 mM NaCl–350 mM sucrose for *Anabaena* sp. strain L-31 and 200 mM NaCl–250 mM sucrose for *A. torulosa*. Filaments were pulse-radiolabeled in vivo with [^{35}S]methionine during the last 5 min of stress. Proteins were extracted and electrophoresed on sodium dodecyl sulfate–5 to 14% polyacrylamide gradient gels. Gels were dried and autoradiographed.

RESULTS

Exposure of *Anabaena* sp. strain L-31 to increasing salinity (NaCl) or osmotic (sucrose) stress affected its growth differently (Fig. 1). Measurements done both early (3 days) and late (5 days) in the logarithmic phase of growth showed that growth of this freshwater strain was very sensitive to salinity stress, being inhibited by more than 50% between 100 and 125 mM NaCl. In contrast, equiosmolal sucrose (200 to 300 mM) inhibited growth by only 10 to 20%. The concentration of sucrose required to inhibit growth by 50% (GI_{50}) was higher than 350 mM; the GI_{50} for NaCl was 130 mM (Fig. 1). Simultaneous exposure to combined salt and sucrose (each applied at the respective GI_{50}) was most inhibitory and reduced growth to less than 5% of the control level (data not shown).

Figure 2 shows the response of the nitrogenase activity of *Anabaena* sp. strain L-31 cultures exposed to salinity and osmotic stresses. Although a low salt concentration (50 mM) somewhat stimulated nitrogenase activity, at higher concentrations nitrogen fixation was severely inhibited. In sharp contrast to this, exposure to osmotic stress (sucrose) alone had no inhibitory effect on nitrogenase activity but instead enhanced it significantly (2.2- to 2.5-fold). While 130 mM NaCl inhibited nitrogenase activity by 50% or more, no inhibition of activity by sucrose was observed, even at 400 mM sucrose. In fact, nitrogenase activity, even at 400 mM sucrose, remained higher than in unstressed controls. Simultaneous addition of both salt and sucrose (each at the respective GI_{50}) caused inhibition of nitrogenase activity (data not shown) similar to that observed during exposure to salt stress alone.

Figure 3 shows the kinetics of the response of *Anabaena* sp. strain L-31 to the GI_{50}s of salt (130 mM) and sucrose (350 mM). The inhibitory effects of both salinity and osmotic stresses on growth were observed right from day 1, and cultures in the late logarithmic phase were more sensitive. The stimulation of nitrogenase activity by sucrose similarly ensued right from day 1 and was maximum on day 3. Salinity stress was much more inhibitory to nitrogenase activity and diazotrophic growth than was osmotic stress throughout the growth phase (Fig. 3).

Addition of exogenous combined nitrogen sources has earlier been shown to protect cyanobacteria from salinity stress (3, 25). However, exogenously added nitrate or ammonium differently affected *Anabaena* sp. strain L-31 exposed to salinity or osmotic stress. Nitrate protected against both salinity and osmotic stresses (Table 1). Ammonium, while protecting *Anabaena* sp. strain L-31 against salinity stress, was ineffective against osmotic stress (Table 1).

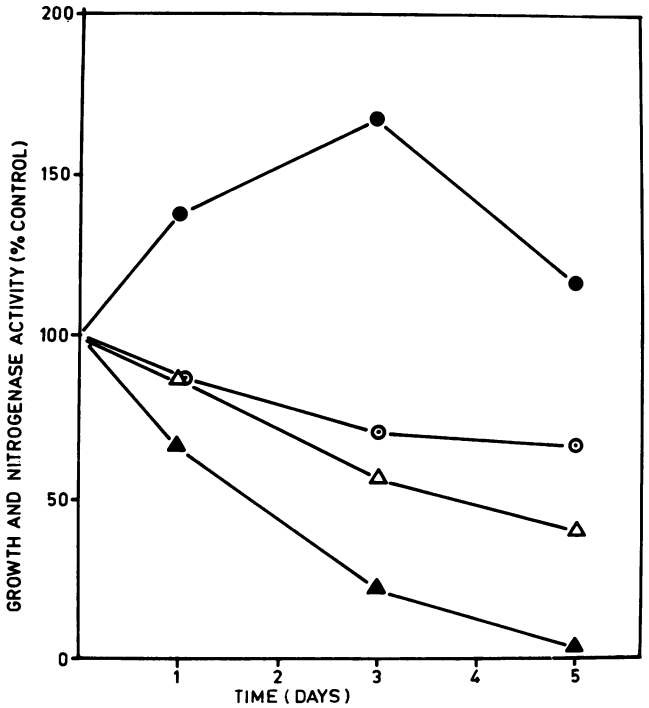

FIG. 3. Kinetics of response of growth (△, ⊙) and nitrogen fixation (▲, ●) by *Anabaena* sp. strain L-31 to GI_{50}s of NaCl (▲, △) and sucrose (⊙, ●).

To ascertain that the different responses to salinity and osmotic stresses were not a unique feature or peculiarity of the particular strain (i.e., *Anabaena* sp. strain L-31), a similar experiment was carried out with a known salt-tolerant strain, *A. torulosa* (3, 4). For proper comparison, this experiment was conducted on solid medium (medium BG-11 plus 1.2% agar) supplemented with appropriate concentrations of NaCl or sucrose and both strains were inoculated on the same plates as 10-μl spots. Comparison of the growth and nitrogen fixation of the two strains 5 days after inoculation (Fig. 4) revealed the following. (i) *Anabaena* sp. strain L-31 displayed salt sensitivity and osmosensitivity similar to those observed in liquid cultures (Fig. 1 and 2), with an NaCl GI_{50} of 130 mM and a sucrose GI_{50} of >350 mM. (ii) *A. torulosa* showed better salt tolerance than

TABLE 1. Effects of combined nitrogen sources on growth of *Anabaena* sp. strain L-31 grown under salinity or osmotic stress

Treatment[a]	Growth (% of control)[b]
Control	100
NaCl	38
Sucrose	60
Nitrate	95
NaCl + nitrate	60
Sucrose + nitrate	93
Ammonium	87
NaCl + ammonium	76
Sucrose + ammonium	60

[a] Combined nitrogen-free BG-11 medium was supplemented, as required, with NaCl (130 mM), sucrose (350 mM), KNO_3 (10 mM), and NH_4Cl (3 mM).
[b] Growth was measured at 5 days after inoculation as content of chlorophyll *a* (in micrograms per milliliter) as previously described (18).

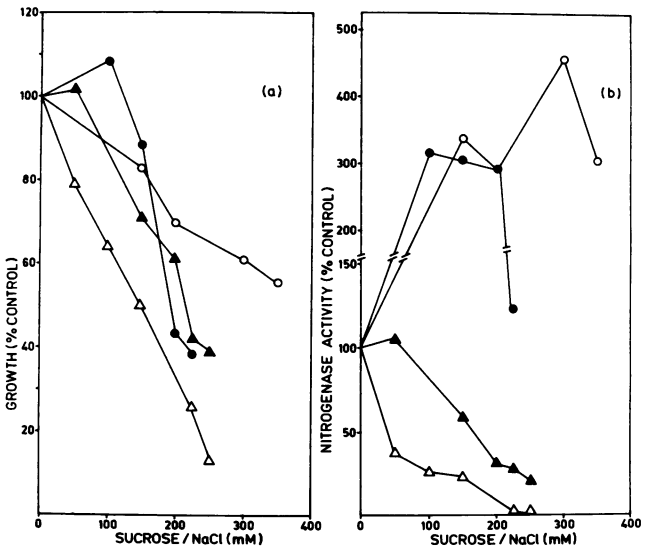

FIG. 4. Differential effects of salinity (▲, △) and osmotic (●, ○) stresses on *Anabaena* sp. strain L-31 (△, ○) and *A. torulosa* (▲, ●). Growth (a) and nitrogen fixation (b) were measured 5 days after inoculation on solid medium as described in Materials and Methods.

Anabaena sp. strain L-31 but was quite osmosensitive. The NaCl GI_{50} for this strain was 250 mM, and the sucrose GI_{50} was 225 mM (Fig. 4). Similar results were obtained with *A. torulosa* in liquid cultures (data not shown). (iii) The nitrogenase activities of both of the strains were quite sensitive to salinity stress but were significantly enhanced by osmotic stress (Fig. 4). The magnitude of enhancement of the nitrogenase activity of *Anabaena* sp. strain L-31 by sucrose on solid medium was even higher (4.5-fold) than that observed in liquid medium (2.5-fold) (Fig. 2 and 3).

Figure 5 shows that cyanobacterial exposure to salinity and osmotic stresses elicited distinct protein synthesis responses in the two *Anabaena* strains. Synthesis of several proteins, in both strains, was repressed by salinity stress but not by osmotic stress (Table 2). In *Anabaena* sp. strain L-31, most of the peptides induced by salinity and osmotic stresses were identical (5). However, few new peptides were observed only under salinity stress and not under osmotic stress (Fig. 5 and Table 2). In *A. torulosa* also, while certain proteins were induced by both salinity and osmotic stresses, proteins were also induced under salinity or osmotic stress only (Fig. 5 and Table 2).

DISCUSSION

The two *Anabaena* strains examined in the present study showed distinct differences in their responses to salinity and osmotic stresses (Fig. 4). The salt-sensitive freshwater form *Anabaena* sp. strain L-31 was considerably osmotolerant (Fig. 1 to 3). This suggests that the salt sensitivity of this osmotolerant strain arises from its sensitivity to the ionic component of salt stress. In contrast, the salt-tolerant species *A. torulosa* was quite osmosensitive (Fig. 4). This is unusual, since osmotic stress is an essential component of salt stress and tolerance to a certain level of NaCl stress is expected to be matched by tolerance to at least equivalent osmotic stress. The osmotolerance of *A. torulosa*, however, did not match even its own salt tolerance. Thus, the growth responses of these strains to salinity and osmotic stresses

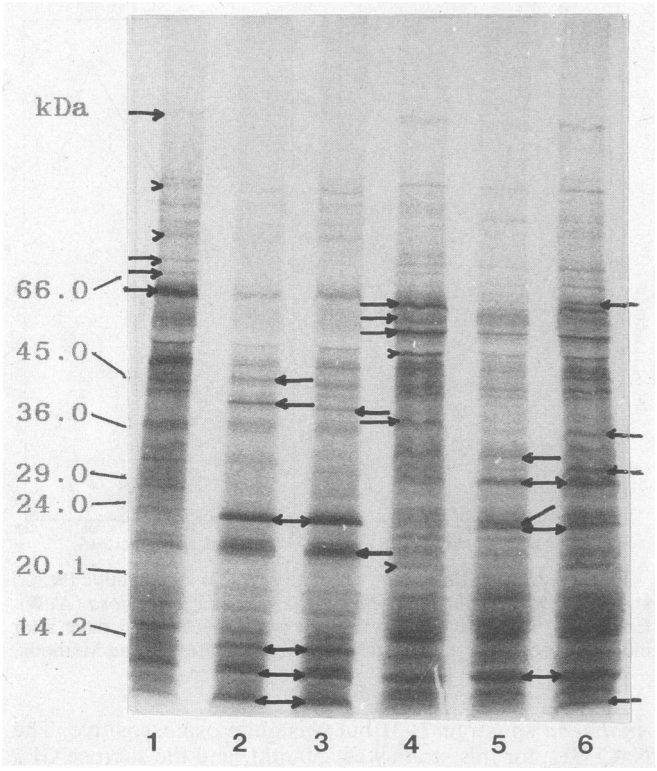

FIG. 5. Salinity stress- and osmotic stress-induced proteins in *Anabaena* sp. strain L-31 (lanes 1 to 3) and *A. torulosa* (lanes 4 to 6). Exponentially grown cultures were exposed to salinity stress (NaCl; lanes 2 and 5) or osmotic stress (sucrose; lanes 3 and 6) at the respective GI_{50}s for 30 min. Proteins were pulse-labeled with [^{35}S]methionine (60 μCi/ml) in vivo for 5 min and then extracted and electrophoresed on sodium dodecyl sulfate–5 to 14% polyacrylamide gradient gels. Proteins were visualized by autoradiography. Proteins induced by both stresses are shown by two-headed arrows, and those induced by NaCl or sucrose are shown by single-headed arrows. Unstressed cultures (lanes 1 and 4) served as controls. The numbers on the left show the molecular masses of standard proteins. In control lanes 1 and 4, proteins repressed by both stresses (single-headed arrows) or by NaCl (arrowheads) only are indicated.

TABLE 2. Modification of protein synthesis in *Anabaena* strains exposed to salinity or osmotic stress

Strain	Modification	Stress	Protein molecular masses (kDa)
Anabaena sp. strain L-31	Repression	Salinity	84, 110
		Osmotic	
		Salinity + osmotic	64, 70, 78, 150
	Induction	Salinity	42, 52
		Osmotic	22, 41.5
		Salinity + osmotic	13, 15, 19, 23
A. torulosa	Repression	Salinity	21, 52
		Osmotic	
		Salinity + osmotic	40, 56, 60, 63
	Induction	Salinity	23, 34
		Osmotic	11.5, 26, 38, 66
		Salinity + osmotic	13, 25, 30

show neither a qualitative nor a quantitative relationship to each other. These data establish that tolerance to salinity stress and tolerance to osmotic stress are apparently unlinked, distinct phenomena in cyanobacteria. Selection of cyanobacterial strains with possible biofertilizer applications in saline environments or drought situations has, therefore, to be pursued independently.

The most striking differential response of *Anabaena* strains to salinity and osmotic stresses is exemplified by nitrogenase activity. Nitrogenase activity in both of the strains was sensitive to salinity stress but completely insensitive to osmotic stress (Fig. 2 to 4). The deleterious effects of salinity stress on nitrogen fixation are, therefore, a consequence of the ionic component rather than the osmotic component. Interestingly, simultaneous exposure of cyanobacteria to the GI_{50}s of both NaCl and sucrose elicited the same response of nitrogenase activity as did salinity stress alone (data not shown). The stimulatory effect of osmotic stress, thus, does not overcome the adverse effect of ionic stress. In previous studies, we have established that in cyanobacteria exposed to salinity stress the diversion of cellular energy towards osmoregulation, especially Na^+ efflux, may be the main cause of loss of nitrogenase activity. This is supported by the fact that inhibition of Na^+ influx, and consequently efflux, enhances nitrogenase activity during salt stress (3).

Inhibition or stimulation of nitrogenase activity by salinity and osmotic stresses was observed irrespectively of the inherent differences in the tolerance of the two strains. The reasons for the significant enhancement of nitrogenase activity by osmotic stress are not clear, but the available evidence suggests that unlike soil salinity, drought situations are not very deleterious to cyanobacterial nitrogenase. Indeed, NifH protein has been detected in desiccated cyanobacteria, which also show almost instantaneous reactivation of nitrogenase proteins upon rehydration (22).

Several forms of exogenously added combined nitrogen sources (nitrate, ammonium, glutamine, and glutamate, etc.) are known to protect cyanobacteria during exposure to salinity stress primarily by curtailing the influx of Na^+ (3, 25). Other compounds, such as glycine and proline, which do not inhibit Na^+ influx are ineffective against salinity stress (25). It was of interest, therefore, to determine whether combined nitrogen sources can alleviate the inhibitory effects of a purely osmotic stress like sucrose. This search revealed a very interesting difference; i.e., while nitrate was osmoprotective, ammonium was not (Table 1), although both protected *Anabaena* sp. strain L-31 against salinity stress. These results show that protection from ionic and osmotic stresses depends on independent, unrelated mechanisms. While nitrate can contribute to or aid both of these mechanisms, ammonium does not. These findings have interesting implications for agricultural practices wherein cyanobacterial biofertilizer is applied along with basal levels of chemical N fertilizer. Obviously, such a combination (especially of nitrate and cyanobacteria) may greatly help the salinity or drought tolerance of cyanobacteria and enhance their nitrogen biofertilizer potential (after chemical N has been degraded) during exposure to such stresses.

Almost all organisms respond to environmental stresses by synthesis of certain stress-induced proteins (23, 28, 30). This is also true of cyanobacteria, which synthesize stress-induced proteins in response to salinity and osmotic stresses and heat shock, etc., by transcriptional activation of certain genes (5). While many of the proteins induced by salinity and osmotic stresses are common, unique stress-specific pro-

teins were also observed in both *Anabaena* strains; i.e., the proteins were induced by salinity or osmotic stress but not by both (Fig. 5). This is especially true of salinity stress, which represses synthesis of several proteins while osmotic stress does not. This demonstrates that ionic and osmotic stresses regulate cyanobacterial gene expression differently.

As mentioned earlier, historically, salinity and osmotic stresses have generally been considered to be similar, if not identical, by many researchers (8, 13, 16, 21, 24, 26, 29, 31). Indeed, in many organisms effects produced by these two stresses are usually indistinguishable (12, 13, 20, 33). Since osmotic stress is an obligatory constituent of salinity stress, there is no a priori reason to believe that the two stresses would elicit entirely different responses. However, as demonstrated in this study, at least cyanobacteria seem to sense and respond to these two stresses independently. This conclusion is borne out by the following results. (i) Osmotolerance and salt tolerance are not linked—a salt-tolerant strain can be osmosensitive and vice versa. (ii) Osmotolerance and salt tolerance of strains are related neither in quality nor in quantity. (iii) Nitrogen fixation is differently influenced by salinity (inhibition) and osmotic (stimulation) stresses; while the ionic stress severely inhibits nitrogenase activity, the osmotic component has no inhibitory effect. (iv) Exogenously added combined nitrogen sources (nitrate and ammonium) show differences in the ability to protect against salinity and osmotic stresses. (v) Salinity and osmotic stresses regulate gene expression differently. Unique repression and induction of specific proteins are elicited by salinity and osmotic stresses.

That a freshwater strain should be osmotolerant but not necessarily salt tolerant is perhaps expected; i.e., during dry seasons, such strains would be subjected to drought but exposure to salinity is relatively uncommon. The brackish-water strain, similarly, must be salt tolerant but may not normally experience purely osmotic stress. The observed behaviors of these strains therefore seem to be in accordance with their respective ecological niches. The molecular basis of such differences seems to lie in the genetic material. Recently, we probed both *Anabaena* strains with salinity stress-induced RNA isolated from each strain by a subtractive RNA hybridization technique (2). These studies showed that *Anabaena* sp. strain L-31 either does not possess genes similar to the salt stress-induced genes of *A. torulosa* or at least does not express them. This may account for its salt sensitivity. The molecular basis for the osmosensitivity of *A. torulosa* is not clear, but salt-induced RNA from *Anabaena* sp. strain L-31 hybridized to *A. torulosa* DNA only poorly. This may indicate that the genes responsible for osmotolerance are absent from *A. torulosa*. Thus, the different observed stress tolerances of strains are most likely a consequence of differences in genetic makeup and/or differential gene expression during stress.

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