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A Role for Osmotic Stress-Induced Proteins in the Osmotolerance of a Nitrogen-Fixing Cyanobacterium, *Anabaena* sp. Strain L-31

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The molecular basis of tolerance to osmotic stress was investigated with a cyanobacterium, *Anabaena* sp. strain L-31. The inherent osmotolerance of this strain (50% growth inhibition at 350 mM sucrose) was enhanced by adaptation with 100 mM sucrose for 30 min. Addition of 10 mM KNO_3 during growth also conferred significant osmoprotection, but addition of 3 mM NH_4Cl did not. Exposure of cells to 350 mM sucrose induced the expression of at least 12 osmotic-stress-induced proteins (OSPs) within 30 min, in the molecular mass range of 11.5 to 84 kDa. Exposure of cells to 100 mM sucrose or to 10 mM nitrate also induced all the OSPs, but addition of ammonium did not. The observed correspondence between the presence of OSPs and osmotolerance strongly suggests a role for OSPs in osmotolerance of *Anabaena* sp. strain L-31.

Among the abiotic stresses that limit crop productivity globally, salinity and drought rank as the most detrimental. Organisms which can resist such stresses are being intensely investigated. The ability of certain photoautotrophic, nitrogen-fixing cyanobacteria to tolerate drought or osmotic stress is especially important in tropical rice cultivation, in which cyanobacteria are naturally abundant, droughts are encountered frequently, especially in rain-fed agriculture, and cyanobacteria are used as biofertilizers to partially substitute for chemical nitrogen fertilizers. Selection and use of stress-tolerant cyanobacteria and understanding the mechanisms involved in such stress resistances are priority areas of contemporary cyanobacterial research. In contrast to most organisms, for which effects produced by salt or osmotic stress are usually indistinguishable, cyanobacteria sense and respond to the ionic and osmotic components of salinity stress differently (11). Particularly striking differences are seen in (i) the sensitivity of nitrogen fixation to the ionic component compared with purely osmotic stress, (ii) the ability of exogenous combined nitrogen to protect cyanobacteria against salinity or osmotic stress, and (iii) the expression of stress-induced proteins evoked by ionic and osmotic stresses (11).

Mechanisms underlying salinity and osmotic-stress tolerance in cyanobacteria, though not fully understood, appear, in general, to be similar to those in other organisms (10). These include (i) exclusion of Na^+ (3, 15) and (ii) accumulation of inorganic osmolytes such as K^+ (17) or organic osmolytes such as sugars, polyols, and quaternary amines (6, 7, 13, 16). In most organisms osmotic stress evokes an adaptive response, initiated by stress-regulated gene expression and culminating in certain structural and functional alterations suited for survival under osmotic stress (1, 10, 12, 19, 20). Salinity stress-induced genes have been cloned recently from a salt-tolerant cyanobacterium, *Anabaena torulosa* (2). The multitude of processes involved in osmoregulation necessitates reprogramming of protein syntheses. Indeed, many organisms, including cyanobacteria, respond to osmotic stress by elaborate modifications of the protein synthesis program regulated at the level of transcription (1, 4, 5, 12, 19). However, evidence linking such proteins with stress tolerance is lacking.

The possibility of modifying the inherent salt or osmotic tolerance of organisms has been revealed through phenomena such as adaptation and cross-protection (3, 11, 12, 15). This has opened up the possibility of investigating the correlation between the expression of osmotic-stress-induced proteins (OSPs) and cyanobacterial osmotolerance. In this study we have examined this correlation with reference to certain osmoprotective and osmosensitizing growth conditions. Our results show that expression of osmotic stress-induced proteins is correlated with osmotic adaptation in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain L-31.

The cyanobacterium *Anabaena* sp. strain L-31 (a freshwater strain), an isolate from this laboratory (20), was used under axenic conditions. The cyanobacterium was grown in BG-11 liquid medium free of combined nitrogen at pH 7.0 (8). When required, KNO_3 was added at 10 mM and NH_4Cl was added at 3 mM. Osmotic stress was applied by addition of sucrose. Cultures were grown photoautotrophically in an orbital incubator shaker at 25°C under continuous illumination (2.5 mW/cm^2) and with shaking (100 rpm). For evaluation of tolerance, cells in the logarithmic phase of growth (3 days old) were harvested, washed, and inoculated (at a chlorophyll *a* concentration of 1 μg/ml) in the experimental media with or without stress and compared with respect to growth and nitrogen fixation. Growth was assessed by measuring the chlorophyll *a* content (14), and nitrogenase activity was estimated by the acetylene reduction technique as described earlier (3). For identification of stress-induced proteins, 1-ml samples were pulse-labelled in vivo with [^{35}S]methionine (60 μCi/ml) for 5 min, under the usual growth conditions, as described earlier (1). Proteins were extracted, and equal amounts of trichloroacetic acid-precipitable radioactivity were electrophoresed on 5 to 14% polyacrylamide gradient gels containing sodium dodecyl sulfate (SDS). Gels were dried and autoradiographed as described earlier (1, 4).

Anabaena sp. strain L-31 is relatively osmotolerant (11). Low levels of osmotic stress (100 mM sucrose) did not affect growth (Table 1). Higher levels of osmotic stress (350 mM sucrose) inhibited growth but enhanced nitrogen fixation in this strain. Certain growth conditions modified the osmotolerance of *Anabaena* sp. strain L-31. A short (30-min) preexposure of the cyanobacterium to a permissive level of osmotic stress (100

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TABLE 1. Effects of adaptation and exogenous combined nitrogen on the inherent osmotolerance of *Anabaena* sp. strain L-31

Treatment (mM)	Growth[a] (%)		Nitrogenase activity[b] (%)	
	Day 3	Day 5	Day 3	Day 5
None (control)	100	100	100	100
Nitrate (10)	133	122	0	0
Ammonium (3)	114	106	0	0
Sucrose				
100	103	98	180	297
350	66	61	236	206
350 + nitrate (10)	92	96	0	0
350 + ammonium (3)	66	54	0	0
100→350[c]	70	95	141	301

[a] Measured as the amount of chlorophyll *a* per milliliter. The values for the unstressed controls on days 3 and 5 were 2.27 and 4.14 μg/ml, respectively.

[b] Measured as micromoles of C_2H_2 reduced per mg of chlorophyll *a* per h. The values for the unstressed controls on days 3 and 5 were 15.4 and 5.7 μmol/mg/h, respectively.

[c] Cells were exposed to 100 mM sucrose for 30 min and then to 350 mM sucrose.

mM sucrose) was found to enhance the osmotolerance of the strain during a subsequent exposure to 350 mM sucrose. Five days after transfer to 350 mM sucrose, the sucrose-preadapted culture exhibited no adverse effects of osmotic stress (Table 1). Exogenously added nitrate (10 mM) also significantly enhanced the osmotolerance of *Anabaena* sp. strain L-31, but the addition of ammonium did not (Table 1). Both nitrate and ammonium have earlier been shown to inhibit influx of Na^+ and protect cyanobacteria against salinity stress (3, 15). In the absence of an ionic (Na^+) component, osmoprotection conferred by nitrate alone indicated that osmoprotection involved an entirely different mechanism and that it was not triggered by ammonium.

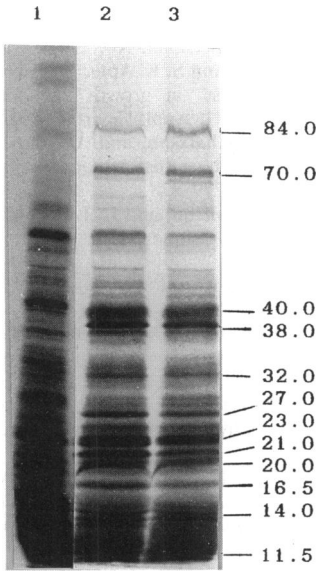

FIG. 1. Expression of OSPs in *Anabaena* sp. strain L-31. Exponentially grown filaments were exposed to either 100 mM sucrose (lane 2) or 350 mM sucrose (lane 3) for 30 min. Unstressed control cells were included for comparison (lane 1). During the last 5 min of the stress period, cells were pulse-labelled in vivo for 5 min with [^{35}S]methionine (60 μCi/ml). An equal amount of trichloroacetic acid-precipitable radioactivity was electrophoresed on 5 to 14% polyacrylamide gradient SDS gels and visualized by autoradiography of dried gels. The molecular masses (in kilodaltons) of OSPs are indicated on the right.

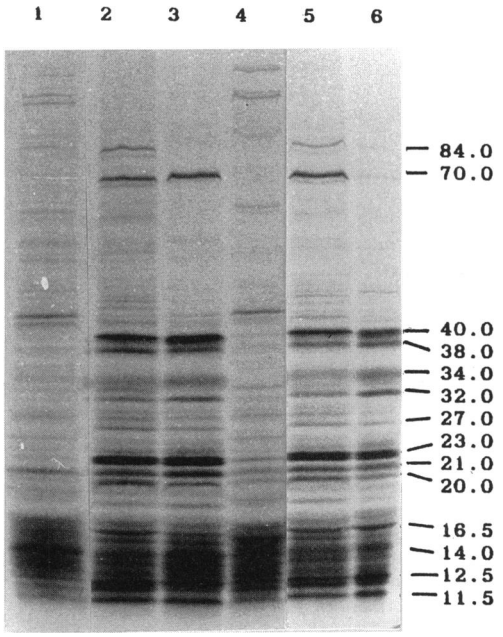

FIG. 2. Effect of exogenous combined nitrogen on the expression of OSPs in *Anabaena* sp. strain L-31. Exponentially grown cultures were exposed for 30 min to one of the following treatments: no stress (control) (lane 1), 350 mM sucrose (lane 2), 10 mM nitrate (lane 3), 3 mM ammonium (lane 4), nitrate plus sucrose (lane 5), and ammonium plus sucrose (lane 6). Other details were as described for Fig. 1.

Figure 1 shows the OSPs in nitrogen-fixing cultures of *Anabaena* sp. strain L-31. At least 12 peptides, ranging from 11.5 to 84 kDa, were found to be induced in this strain by a 30-min exposure to 350 mM sucrose. Each of these polypeptides was also equally induced during a 30-min exposure to the permissive level of osmotic stress (100 mM sucrose). The induction of OSPs during sucrose adaptation, thus, correlated well with the osmoprotection conferred by such treatment (Table 1), suggesting a possible role for OSPs in osmotolerance.

Figure 2 shows the effect of a short exposure to exogenously added combined nitrogen on the induction of osmotic stress proteins. Very interestingly, the addition of nitrate alone rapidly induced all the OSPs, except for an 84-kDa peptide, even in the absence of osmotic stress. Addition of ammonium failed to evoke this response (Fig. 2). However, when nitrate or ammonium was added along with 350 mM sucrose, all the OSPs were expressed. Thus, although ammonium per se did not induce OSPs, it did not rapidly prevent (within 30 min) induction of OSPs by sucrose. Prolonged growth in ammonium-sucrose-supplemented media, however, repressed OSP synthesis (Fig. 3).

Figure 3 shows the synthesis of OSPs by combined nitrogen-supplemented cultures of *Anabaena* sp. strain L-31 grown under prolonged osmotic stress for 5 days. Synthesis of many of the high-molecular-weight OSPs, induced in the first 30 min of osmotic stress (Fig. 1), declined after 5 days, although many of the low-molecular-weight OSPs (less than 40 kDa) continued to be synthesized actively, suggesting that these may be more important for long-term osmotic adaptation. Various cultures synthesized OSPs at rates which followed the order nitrate-sucrose > sucrose ≫ ammonium-sucrose. The last treatment did not support synthesis of many of the OSPs, or the cultures

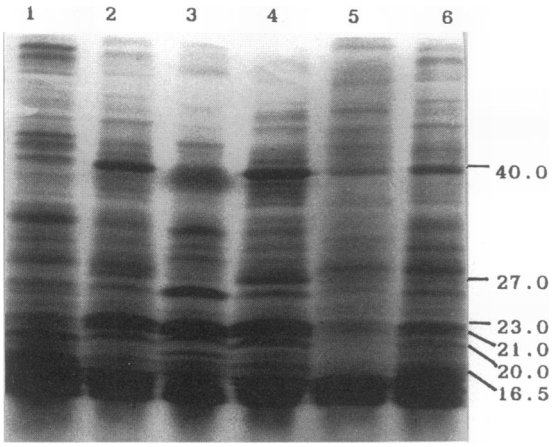

FIG. 3. Osmotic stress-induced protein expression in *Anabaena* sp. strain L-31 after prolonged exposure to 350 mM sucrose. Cells were grown for 5 days either without stress (lane 1) or in sucrose (lane 2), 10 mM nitrate (lane 3), sucrose plus nitrate (lane 4), 3 mM ammonium (lane 5), or sucrose plus ammonium (lane 6). Cells were pulse-labelled with 60 μCi of [^{35}S]methionine per ml for 5 min in vivo on day 5. Other details were as described for Fig. 1.

synthesized them at reduced rates. Particularly noticeable were OSP40, OSP27, OSP20, and OSP16.5, which were observed as strong bands in sucrose-nitrate (lane 4) and were either lacking or observed only as weak bands in ammonium-sucrose (lane 6) (Fig. 3).

Stress-induced proteins have been described for a variety of life-forms and occur in response to several stresses (1, 4, 5, 9, 18, 19). Whether such stress proteins play a role in osmotic adaptation has been a matter of much speculation. Studies of enteric bacteria have indicated a role for OSPs in osmotolerance. Thus, starvation has been shown to induce OSPs and provide cross-protection against osmotic challenge in *Escherichia coli* (12). At present, the available information on cyanobacterial OSP expression is scanty. With less sensitive techniques (such as Coomassie staining), thus far, only one or two OSPs could be shown for cyanobacteria (9, 18). The present study, using in vivo protein radiolabelling, has revealed the synthesis of at least 12 OSPs in *Anabaena* sp. strain L-31 (Fig. 1). A role for these OSPs in osmotolerance of *Anabaena* sp. strain L-31 is strongly suggested by the clear correlation between the presence of OSPs and osmotolerance on one hand and their absence and osmosensitivity on the other (Table 1; Fig. 1 to 3). Thus, OSPs are rapidly induced during osmotic stress (Fig. 1), and many of them continue to be actively synthesized throughout the stress period (Fig. 3). Adaptation with permissive levels of sucrose which induce expression of all the OSPs (Fig. 1 and 2) also causes significant osmoprotection. Nitrate appears to elevate the inherent osmotolerance by enhancing the expression of at least four OSPs (Fig. 2). Although ammonium does not immediately (within 30 min) repress expression of OSPs (Fig. 2), continued presence of ammonium (for 5 days) during osmotic stress reduces OSP expression to a minimal level (Fig. 3), resulting in impairment of osmotolerance (Table 1). Significantly reduced levels of at least four OSPs in ammonium-grown osmotically stressed *Anabaena* sp. strain L-31 (Fig. 3) may account for the increased osmosensitivity of such cultures.

The identities and functions of the individual OSPs are unknown at present. Recently, a 40-kDa OSP from *Anabaena* sp. strain PCC 7120 has been shown to cross-react immunologically with an antibody raised against barley dehydrins (9). Another desiccation-induced protein of a similar molecular mass (39 kDa) and products of its proteolytic degradation (37 and 33 kDa) have been identified for *Nostoc commune* (18). A 40-kDa protein, similar to cyanodehydrin in molecular mass (11), is synthesized throughout the osmotic stress and is one of the most actively synthesized OSPs in *Anabaena* sp. strain L-31. Its synthesis is enhanced by all osmoprotective treatments and decreased by ammonium (Fig. 1 to 3).

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