APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1980, p. 689-693 Vol. 40, No. 4
0099-2240/80/10-0689/05$02.00/0

Response of N_2-Fixing Cyanobacteria to Salt

ELISHA TEL-OR†
Desert Research Institute, Ben-Gurion University of the Negev, Sede Boqer, Israel

The effect of salt on photosynthetic activity, acetylene reduction, and related activities was examined in two species of cyanobacteria, *Nostoc muscorum* and *Calothrix scopulorum*. Photosynthesis was more resistant to high salt concentration than was N_2 fixation. The salt resistance of both activities increased after a period of exposure of the cells to salinity. The transfer of electrons via ferredoxin and ferredoxin-nicotinamide adenine dinucleotide phosphate reductase was found to be extremely sensitive to salt. In comparison, the transfer of reducing power by glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, and photosystem 1 was less affected by NaCl, whereas glutamine synthetase exhibited higher tolerance to salt.

The number of N_2-fixing cyanobacteria in saline environments is small (4, 5). It was stated that "nitrogen fixation as a process is more sensitive to variations and extremes of environmental conditions than photosynthesis or overall growth" (20). Earlier studies on growth, nitrogen fixation, and release of N-fixed compounds in response to salinity in axenic cultures of *Calothrix scopulorum* (conducted by Stewart and coworkers [8, 15, 16]) and recent reports on rapidly growing marine strains of *Anabaena*, found to fix nitrogen very vigorously in marine environments (7, 14), provide a better understanding of the relationship between the physiology and ecology of marine N_2-fixing cyanobacteria.

The present study is aimed at the understanding of limitations caused by the saline environment to the survival and productivity of N_2-fixing cyanobacteria. *Nostoc muscorum* (strain 7119) was selected as a freshwater strain and *C. scopulorum* was selected as a brackish water strain. The process of N_2 fixation was indeed found to be more sensitive than photosynthesis and reactions involved in the transfer of reducing power to nitrogenase and the assimilation of the ammonia produced. Evidence for acquired tolerance to salt of N_2 fixation in vivo and of the ferredoxin:ferredoxin-nicotinamide adenine dinucleotide phosphate (NADP) reductase enzyme couple, which is known to be a salt-sensitive system (6), is presented.

MATERIALS AND METHODS

Culture conditions. *N. muscorum* strain 7119 (18) and *C. scopulorum* Cambridge culture collection no. 1410 (16) were grown in Allen and Arnon medium (1) in batch cultures on a rotary shaker at 30°C, continuously illuminated, *I*, at 35 μEinsteins m^{-2} s^{-1}. It was harvested in the late logarithmic phase of growth.

Cell-free preparations. Cultures were harvested, washed, and suspended in buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH, pH 7.5), disrupted by sonication, and then centrifuged (27,000 × *g*, 30 min). The supernatant was used for assays of the following soluble enzyme activities: cytochrome *c* reductase, isocitric dehydrogenase (ICD), glucose-6-phosphate dehydrogenase (G6PD) and glutamine synthetase (GS). The pellet, suspended in buffer, was used for the measurement of methylviologen photoreduction. Protein was determined according to Lowry et al. (9). Chlorophyll was extracted in 80% acetone, and chlorophyll *a* was determined according to MacKinney (10).

Photosynthetic activities. Oxygen evolution was measured in cells which had been washed and suspended in growth medium, using a Clark-type electrode (YSI 4004, Yellow Springs Instrument Co., Yellow Springs, Ohio), at 30°C ($I = 500$ μEinsteins m^{-2} s^{-1}). Photosystem 1 reaction in vitro was measured with methylviologen as electron acceptor by following O_2 consumption with an oxygen electrode.

Acetylene reduction. Acetylene reduction was measured under 16% acetylene in air in cells which had been washed and suspended in growth medium in vials sealed with rubber stoppers. Samples were illuminated on a shaker at 30°C ($I = 100$ μEinsteins m^{-2} s^{-1}), and ethylene formation was followed with a Packard-Becker model 417 gas chromatograph provided with a Porapack-N column, as described earlier (18).

Enzyme assays. Reduction of cytochrome *c* was measured at 550 nm, using a Gilford model 250 spectrophotometer. For assay of ICD and G6PD activities, NADPH formation was followed at 350 nm. For assay of GS activity, the biosynthetic pathway of GS was measured (17, 19) by following the release of P_i.

RESULTS

Response of photosynthesis and acetylene reduction to salt *in vivo*. *Nostoc* and *Calothrix* cultures were harvested during late

† Present address: Department of Agricultural Botany, Faculty of Agriculture, Hebrew University, Rehovot, Israel.

logarithmic phase, and the intact filaments were assayed for the effect of salt on oxygen evolution and acetylene reduction. In all of the experiments, the photosynthetic activity was found to be more resistant to NaCl than the N_2-fixing activity. *Calothrix* cells grown in medium containing 0.4 M NaCl (Fig. 1b) were more tolerant to salt than cells growing without salt (Fig. 1a), as shown for both activities, indicating that the cells grown in saline medium acquire tolerance to salt. The data on nitrogenase activity of *Nostoc* cells cultured in the presence and absence of 0.2 M NaCl are summarized in Table 1. The salt-grown cells tolerated 0.2 M NaCl much better than did the control cells. NaCl at a concentration of 0.4 M stopped growth and N_2 fixation in *Nostoc* cells but did not stop photosynthesis. Salt-adapted cultures of *Calothrix* tolerated higher salt concentrations than salt-adapted cultures of *Nostoc*. This is in accordance with the natural distribution of the two strains.

Comparative studies on the effect of NaCl *in vitro*. (i) Reduction of cytochrome *c*. The reduction of cytochrome *c*, mediated by ferredoxin and ferredoxin-NADP reductase, was found to be extremely sensitive to salt when *Nostoc* and *Calothrix* extracts were assayed (Fig. 2a and b). This enzyme couple participates in the terminal steps of the photoreduction of NADP and transfers electrons to nitrogenase (2). The observed sensitivity of ferredoxin:ferredoxin-NADP reductase to salt is similar to the sensitivity of these enzymes isolated from higher plants (11). When the effect of salt was tested on *Nostoc* cultures grown with or without NaCl (Fig. 2a), it was evident that the enzymes in the salt-grown cultures tolerated higher ionic strength. Such acquired tolerance could involve the formation of salt-resistant forms of the enzymes involved. Various isozymes of the ferredoxin-NADP reductase or ferredoxin (6) are known, and possibly salt tolerance involves selective synthesis of salt-tolerant forms.

(ii) ICD and G6PD. ICD and G6PD are known to provide reducing power to nitrogenase, and alteration of their activities may affect nitrogenase activity (2, 13). It was, therefore, important to determine the degree of sensitivity of these soluble enzymes to NaCl. ICD activity in crude soluble preparations of *Nostoc* was moderately sensitive to NaCl, whereas no resistance was acquired during extended growth in medium containing 0.2 M NaCl (Fig. 3). A similar effect of salt on ICD activity was observed in preparations of *Calothrix* sp. The activity of G6PD was also found to be moderately sensitive to

TABLE 1. *Effect of salt on N_2 fixation in vivo in Nostoc cultures growing with or without salt*[a]

Growth medium	NaCl concn in assay medium (M)	C_2H_2 reduction (μmol mg of chlorophyll^{-1} h^{-1})
Control	0	6.5 ± 0.3
Control	0.2	0.3 ± 0.05
0.2 M NaCl in medium	0	5.0 ± 0.3
0.2 M NaCl in medium	0.2	2.0 ± 0.1

[a] Duplicate samples with cells containing 10 μg of chlorophyll *a* were assayed.

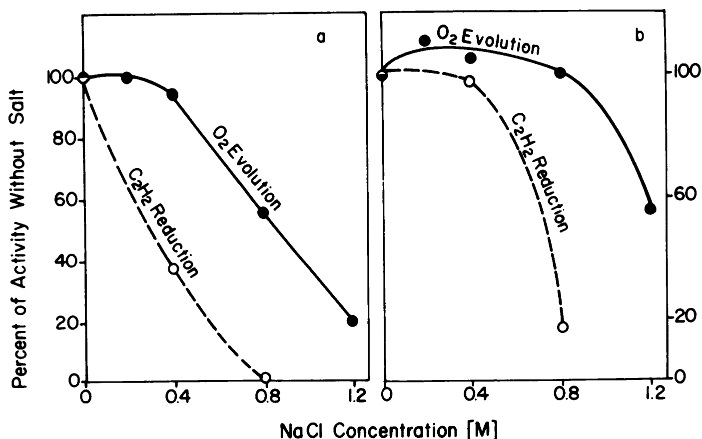

FIG. 1. *Effect of NaCl on photosynthesis and N_2 fixation in vivo with C. scopulorum. Photosynthesis was measured by O_2 evolution, and N_2 fixation was measured by C_2H_2 reduction with: (a) culture grown in Allen and Arnon medium; (b) culture grown in medium containing 0.4 M NaCl. Samples for O_2 evolution assay contained 10 μg of chlorophyll a, and those for C_2H_2 reduction contained 50 μg of chlorophyll a per 2 ml. 100% activity: 8.1 and 7.0 μmol of C_2H_4 formed mg of chlorophyll^{-1} h^{-1} for (a) and (b); 206 and 321 μmol of O_2 evolved mg of chlorophyll^{-1} h^{-1} for (a) and (b). Results are averages of duplicate samples.*

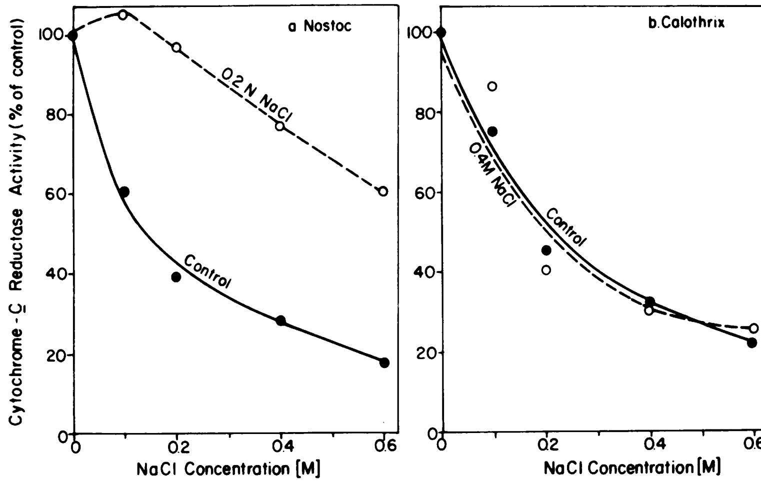

FIG. 2. *Cytochrome c reductase activity with Nostoc (a) and Calothrix (b) cultures. Soluble fractions of cells from control (Allen and Arnon medium [1]) and salt-containing medium cultures were tested for the reduction of cytochrome c in a 1-ml reaction mixture containing: HEPES-KOH (pH 7.5), 25 mM; $MgCl_2$, 5 mM; NADPH, 33 μM; cytochrome c horse heart (Sigma), 33 μM; and broken cell supernatant containing 1 to 2 mg of protein. 100% activity: 5 and 8.5 nmol of cytochrome c reduced mg of protein^{-1} min^{-1} for Nostoc, and 10.2 and 8.7 nmol of cytochrome c reduced mg of protein^{-1} min^{-1} for Calothrix in control and salt-grown cells, respectively.*

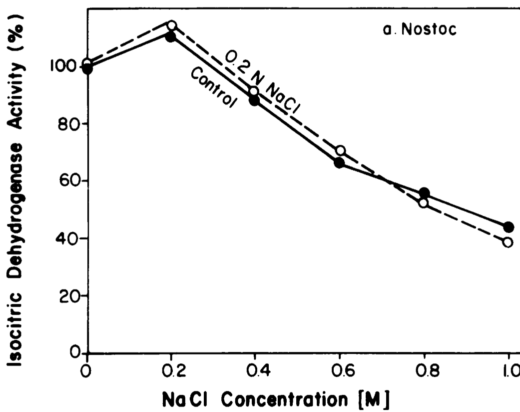

FIG. 3. *ICD with control and salt-grown Nostoc cultures. Samples in 1-ml reaction mixture contained: HEPES-KOH (pH 7.5), 50 mM; $MgCl_2$, 5 mM; sodium isocitrate, 5 mM; NADP, 0.33 mM; and soluble cell fraction containing 2.4 and 2.75 mg of protein for the control and salt-grown cultures, respectively. 100% activity: 11.4 and 7.4 nmol of NADPH formed mg of protein^{-1} min^{-1} for the control and salt-grown cultures, respectively.*

NaCl (Fig. 4), and the response to salt of *Calothrix* extracts prepared from cells grown in saline medium was similar to that of the control. There was no long-term adaptation of G6PD from *Nostoc* or *Calothrix* to salt.

(iii) Photosystem 1-mediated photoreduction of methylviologen. The sonicated cell particles, carrying the membrane-bound components of photosystem 1, were used in the study of the effect of salt on photoreduction of methylviologen supported by reduced dichlorophenolindophenol. The activity was insensitive to salt in both *Nostoc* and *Calothrix* preparations (Fig. 5). Electron transport, mediated by the membrane-bound carriers of photosystem 1, tolerated much higher concentrations of NaCl than the

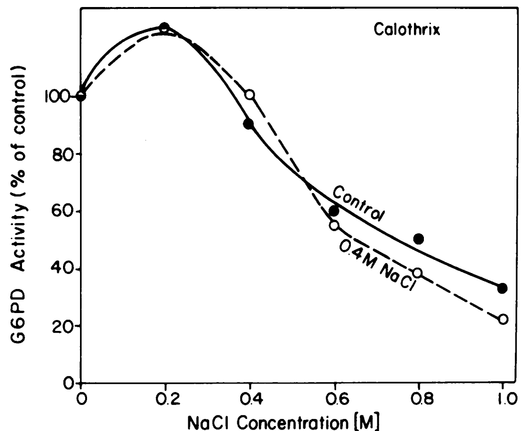

FIG. 4. *G6PD with control and salt-grown Calothrix cultures. Samples in 1-ml reaction mixture contained: HEPES-KOH (pH 7.5), 25 mM; $MgCl_2$, 5 mM; glucose-6-phosphate, 5 mM; NADP, 0.33 mM; and soluble cell fraction containing 0.2 mg of protein. 100% activity: 38 and 17 nmol of NADPH formed mg of protein^{-1} min^{-1} for the control and salt-grown cultures, respectively.*

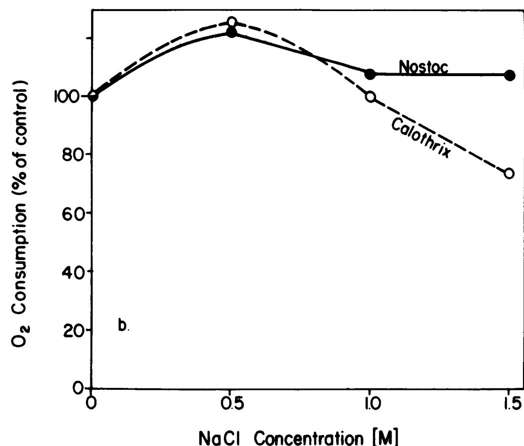

FIG. 5. *Photoreduction of methylviologen with Nostoc and Calothrix cultures. Samples in 4 ml contained: HEPES-KOH (pH 7.5), 50 mM; sodium ascorbate, 5 mM; dichlorophenolindophenol, 50 μM; methylviologen, 50 μM; sodium azide, 2.5 mM; and membrane preparations containing 30 μg of chlorophyll a. 100% activity: 520 and 380 μmol of O_2 consumed mg of chlorophyll^{-1} h^{-1} for Nostoc and Calothrix preparations, respectively.*

soluble carriers ferredoxin and ferredoxin-NADP reductase.

(iv) GS. The ammonia synthesized by nitrogenase is primarily assimilated in cyanobacteria by the GS and glutamate synthase (GOGAT) pathway (17, 19). The response of GS to NaCl as measured in the biosynthetic assay of GS was tested by using crude extracts of *Nostoc* and *Calothrix*. The activity was not inhibited by a high NaCl concentration (Fig. 6).

DISCUSSION

Few cyanobacteria from a saline environment have been shown to fix N_2. The reasons are uncertain, but this characteristic paucity in habitats poor in combined nitrogen is remarkable. The data presented here show that the activities of different enzymes involved directly or indirectly in N_2 fixation and photosynthesis are affected differentially by the saline environment.

The data obtained are summarized schematically in Fig. 7. It can be seen that: (i) photosynthetic activity is more tolerant to salt and growth limitation in a saline medium cannot be attributed to it and especially not to photosystem 1, which was salt tolerant; (ii) the ferredoxin:ferredoxin-NADP reductase, on the other hand, is extremely sensitive to salt in unadapted cells, but it adapts with time, suggesting that the formation of multiple forms of ferredoxin-NADP reductase (6) and forms I and II of ferredoxin (3) may be involved in the acquired salt tolerance of the pathways of reductant flow to nitrogenase; (iii) sensitivity to salt does not necessarily increase with complexity of structure of the enzyme since GS, which is a large polymer (12), is not affected by salt. Sensitivity to salt is not directly related to the soluble state of the enzyme as for ICD and G6PD, which are moderately sensitive to salt.

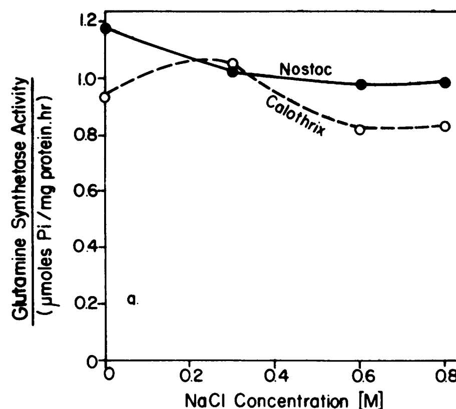

FIG. 6. *GS with Nostoc and Calothrix cultures. Samples in a volume of 1 ml contained: adenosine triphosphate, 5 mM; $MgCl_2$, 15 mM; NH_4Cl, 5 mM; sodium glutamate, 2.5 mM; HEPES-KOH (pH 7.5), 25 mM; and soluble cell fraction containing 2.4 and 3.0 mg of protein for Nostoc and Calothrix, respectively. Samples were incubated at 37°C for 30 min; reaction was stopped by the addition of the reagents for P_i determination.*

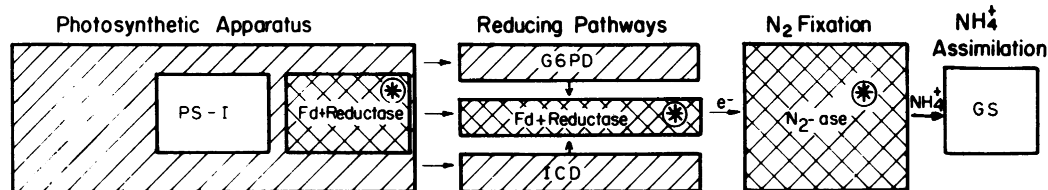

FIG. 7. *Schematic presentation of the response to salt in N_2-fixing cyanobacteria. Symbols: ▩, extremely sensitive; ▨, moderately sensitive; □, insensitive; ⊛, adaptable to NaCl.*

ACKNOWLEDGMENTS

I am grateful to A. E. Richmond for encouragement and to S. Tel-Or for assistance.

This research was supported by the Centre for Absorption in Science, the Ministry of Immigrant Absorption, State of Israel, and by the Bat-Sheva de Rothschild Fund.

LITERATURE CITED

1. **Allen, M. B., and D. I. Arnon.** 1955. Studies on nitrogen fixing blue green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. **30**:366-372.
2. **Apte, S. K., P. Rowell, and W. D. P. Stewart.** 1978. Electron donation to ferredoxin in heterocysts of the N_2-fixing alga *Anabaena cylindrica*. Proc. R. Soc. London Ser. B **200**:1-25.
3. **Dutton, J. E., and L. J. Rogers.** 1978. Isoelectric focussing of ferredoxin, flavodoxin and rubredoxin. Biochim. Biophys. Acta **537**:501-506.
4. **Fogg, G. E.** 1973. Physiology and ecology of marine blue-green algae, p. 368-378. *In* N. G. Carr and B. A. Whitton (ed.), The biology of blue green algae. University of California Press, Berkeley.
5. **Fogg, G. E., W. D. P. Stewart, P. Fay, and A. E. Walsby.** 1973. The blue green algae. Academic Press, London.
6. **Forti, G.** 1977. Flavoproteins, p. 222-226. *In* A. Trebst and M. Avron (ed.), Encyclopedia of plant physiology, vol. 5. Springer-Verlag, Berlin.
7. **Gotto, J. W., F. R. Tabita, and C. Van Baalen.** 1979. Isolation and characterization of rapidly growing, marine nitrogen fixing strains of blue green algae. Arch. Microbiol. **121**:155-159.
8. **Jones, K., and W. D. P. Stewart.** 1969. Nitrogen turnover in marine and brackish habitats. III. The production of extracellular nitrogen by *Calothrix scopulorum*. J. Mar. Biol. Assoc. U.K. **49**:475-488.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
10. **MacKinney, G.** 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. **140**:315-322.
11. **Nelson, N., and J. Newmann.** 1969. Interaction between ferredoxin and ferredoxin-nicotinamide adenine dinucleotide phosphate reductase in pyridine nucleotide photoreduction and some partial reactions. J. Biol. Chem. **244**:1926-1931.
12. **Sampio, M. J. A. M., P. Rowell, and W. D. P. Stewart.** 1979. Purification and some properties of glutamine synthetase from the nitrogen fixing cyanobacteria *Anabaena cylindrica* and *Nostoc* sp. J. Gen. Microbiol. **111**:181-191.
13. **Smith, R. G., R. J. Noy, and M. C. W. Evans.** 1971. Physiological electron donor systems to the nitrogenase of the blue green alga *Anabaena cylindrica*. Biochim. Biophys. Acta **253**:104-109.
14. **Stacey, G., C. Van Baalen, and F. R. Tabita.** 1977. Isolation and characterization of a marine *Anabaena* sp. capable of rapid growth on molecular nitrogen. Arch. Microbiol. **114**:197-201.
15. **Stewart, W. D. P.** 1962. Fixation of elemental nitrogen by marine blue green algae. Ann. Bot. (London) **26**:439-445.
16. **Stewart, W. D. P.** 1964. Nitrogen fixation by myxophyceae from marine environment. J. Gen. Microbiol. **36**:415-422.
17. **Stewart, W. D. P., and P. Rowell.** 1975. Effect of L-methionine-DL-sulphoximine on the assimilation of newly fixed NH_3, acetylene reduction and heterocyst production in *Anabaena cylindrica*. Biochem. Biophys. Res. Commun. **65**:846-856.
18. **Tel-Or, E., L. W. Luijk, and L. Packer.** 1977. An inducible hydrogenase in cyanobacteria enhances N_2-fixation. FEBS Lett. **78**:49-52.
19. **Thomas, J., C. P. Wolk, P. W. Shaffner, S. M. Austin, and A. Galonsky.** 1975. The initial organic products of fixation of ^{13}N-labelled nitrogen gas by the blue green alga *Anabaena cylindrica*. Biochem. Biophys. Res. Commun. **67**:501-507.
20. **Whitton, B. A., and C. Sinclair.** 1975. Ecology of blue green algae. Sci. Prog. (London) **62**:429-446.