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Iron-Stimulated Toxin Production in *Microcystis aeruginosa*

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Nitrate- and phosphate-limited conditions had no effect on toxin production by *Microcystis aeruginosa*. In contrast, iron-limited conditions influenced toxin production by *M. aeruginosa*, and iron uptake was light dependent. A model for production of toxin by *M. aeruginosa* is proposed.

It is well known that light intensity, temperature, and culture age influence toxin production in several cyanobacteria (1, 13–16, 18–20), while the effects of nutrients seem to be much more complicated than and not as obvious as the effects of light and temperature. It has been found that during peptide toxin production by *Microcystis aeruginosa* phosphorus-limited growth has little effect on toxicity (50% lethal dose [LD_{50}]), while nitrogen-limited conditions result in a decrease in toxicity (19). Peptide toxin production by *Oscillatoria* strains has been found to depend on both phosphorus and nitrogen levels (13). It is important to learn more about these matters if we want to understand the observed variations in toxin production by cyanobacteria.

All previous investigations on the effects of nutrients have been performed with batch cultures. Since growth conditions vary with the development of the culture during batch growth, we decided to examine the effects of different nutrients on toxin production in continuous cultures. In continuous cultures the growth conditions can be carefully controlled, and the effect of one factor at a time can be studied.

Organisms and growth conditions. The toxic strain *M. aeruginosa* CYA 228/1 and the nontoxic strain *M. aeruginosa* CYA 43 were obtained from the Norwegian Institute for Water Research.

The effects of nutrient-limited growth on toxin production by *M. aeruginosa* CYA 228/1 were studied by growing the organism axenically in the continuous culture system developed by Van Liere and Mur (17). The culture was fed with 02 medium (17). Phosphate-limited conditions were obtained by decreasing the concentration of K_2HPO_4 from 143.5 to 14.4 μM and providing the necessary potassium in the form of KCl. Nitrogen-limited conditions were obtained by decreasing the $NaNO_3$ concentration from 5.80 to 0.60 or 0.35 mM, and iron-limited conditions were obtained by decreasing the $FeCl_3$ concentration from 10 to 0.8 or 0.3 μM. The light, temperature, and aeration conditions used have been described previously (14).

The experiments in which we examined ^{59}Fe uptake as a function of light intensity were performed with *M. aeruginosa* CYA 228/1 and CYA 43 in 25-ml Erlenmyer flasks illuminated with Philips type TL AK 40 W/33 fluorescent tubes from one side.

The growth experiments in which we used different cellular iron concentrations were performed with 2-liter batch cultures of *M. aeruginosa* CYA 228/1. In these experiments iron uptake was inhibited by increasing the EDTA concentration in the growth medium to up to 10 times the "normal" concentration.

Analytical methods. Dry weights were determined by filtering 1- to 5-ml portions of cultures through preweighed membrane filters (type HA; pore size, 0.45 μm; Millipore). The filters were dried until their weights were constant at room temperature with a silica gel desiccator.

Samples (0.5 ml) that were used for protein determinations were collected in acid-washed glass tubes and were stored at −20°C until the experiment was completed. Prior to the assay the samples were freeze-dried. Protein contents were estimated by using the Lowry method (5). Standard solutions of bovine serum albumin were used for calibration purposes.

Carbohydrate concentrations were determined by the anthrone method (5); the assay was calibrated by using standard solutions of D-glucose.

Iron uptake. The experiments in which we examined uptake of iron were performed with toxic and nontoxic cultures which had been iron starved for 3 days. The optical density at 740 nm of each of these cultures was adjusted to 0.2. Samples (10 ml) were added to 25-ml Erlenmyer flasks, which were placed on a shaker at room temperature at different distances from the light source to obtain the desired light intensities. The preparations were continuously illuminated from one side with Philips type TL AK 40 W/33 fluorescent tubes. The uptake experiments were started by adding 100 μl of the standard Fe solution used for 02 medium (58 μg of Fe per ml), in which the EDTA concentration was increased 10-fold to avoid iron precipitation, and 50 μl of a ^{59}Fe solution (14 μg/ml; 500 μCi/ml; Amersham) to each sample. After the iron mixture was added, 1-ml samples were removed and filtered on Super-450 membrane filters (pore size, 0.45 μm; diameter, 25 mm; Gelman) over a period of 2 h. After the filters were placed in glass tubes, they were counted with Minaxi series 5000 γ counter (Pack-

TABLE 1. Effect of nitrogen-limited growth on the ratio of toxin content to dry weight, the ratio of toxin content to dry weight minus carbohydrate content, and the ratio of toxin content to protein content for *M. aeruginosa* grown in continuous cultures at a dilution rate of 0.013 h^{-1} and a light intensity of 40 microeinsteins m^{-2} s^{-1}

$NaNO_3$ concn (mM)	Microcystin RR content/dry wt (ng/μg)	Microcystin RR content/dry wt minus carbohydrate content (ng/μg)	Microcystin RR content/protein content (ng/μg)
0.35	0.73 ± 0.07	1.62 ± 0.30	4.48 ± 0.06
0.60	1.05 ± 0.01	2.03 ± 0.01	4.73 ± 0.56
5.80	2.17 ± 0.27	2.87 ± 0.40	4.49 ± 0.25

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TABLE 2. Effect of phosphate-limited growth on the ratio of toxin content to dry weight, the ratio of toxin content to dry weight minus carbohydrate content, and the ratio of toxin content to protein content for *M. aeruginosa* grown in continuous cultures at a dilution rate of 0.013 h^{-1} and a light intensity of 75 microeinsteins m^{-2} s^{-1}

K_2HPO_4 concn (μM)	Microcystin RR content/dry wt (ng/μg)	Microcystin RR content/dry wt minus carbohydrate content (ng/μg)	Microcystin RR content/protein content (ng/μg)
14.4	0.46 ± 0.40	1.35 ± 0.69	2.36 ± 0.03
143.5	1.07 ± 0.01	1.35 ± 0.04	2.10 ± 0.01

ard). Total iron uptake was calculated and was expressed as nanograms of Fe per milligram of protein.

Cellular iron contents were determined by a photometric method (Norwegian standard method for iron determination NS 4741), using water samples. A 25-ml portion of a culture was centrifuged at 12,000 × *g* for 10 min in a Sorvall type RC-5B Superspeed apparatus. The resulting pellet was washed three times with 70 mM HNO_3 and then resuspended in 25 ml of distilled water and acidified with 0.25 ml of 7 M HNO_3. Then 0.25 g of $K_2S_2O_8$ was added, and the preparation was autoclaved for 30 min at 120°C. After cooling, 3 drops of an ascorbic acid solution (0.5 g of ascorbic acid in 25 ml of water) was added in order to keep the iron in solution. Then we added 2.0 ml of a hydroxyl-ammonium chloride solution (10 g of $HONH_3Cl$ in 100 ml of water), 2.0 ml of a tripyridyl-triazine solution [78 mg of $C_3N_3(C_5H_4N)_3$ in 250 ml of water], and 2.0 ml of a sodium acetate solution (102 g of $CH_3COONa \cdot 3H_2O$ in 250 ml of water). After 5 min the A_{593} of the sample was determined.

TABLE 3. Effect of iron-limited growth on the ratio of toxin content to dry weight and the ratio of toxin content to protein content for *M. aeruginosa* grown in continuous cultures at a dilution rate of 0.009 h^{-1} and a light intensity of 16 microeinsteins $^{-2}$ s^{-1}

$FeCl_3$ concn (μM)	Microcystin RR content/dry wt (ng/μg)	Microcystin RR content/protein content (ng/μg)
0.3	2.15 ± 0.08	3.54 ± 0.34
0.8	2.48 ± 0.07	5.73 ± 0.06
10.0	3.30 ± 0.31	7.07 ± 0.38

Toxin content determination. Samples (50 to 300 ml) were collected and frozen at −20°C. After the samples were thawed, glacial acetic acid was added to a final concentration of 5%. The samples were then extracted for 90 min with stirring at 4°C. After centrifugation at 12,000 × *g* for 10 min in a Sorvall type RC-5B Superspeed apparatus, the pellets were extracted with 5% acetic acid, and the pooled supernatants were applied to activated C_{18} cartridges. The cartridges were rinsed with water and eluted with 5 or 10 ml of methanol depending on the biomass. The toxin content was determined by high-performance liquid chromatography performed with an internal surface reverse-phase column (9). The mobile phase was 12% acetonitrile–88% 0.1 M KH_2PO_4 (pH 6.8), and the flow rate was 1 ml/h. The detector was set at 238 nm. All peaks were tested for toxin activity by using a standard mouse bioassay (10). The toxin contents were quantified by using purified toxin standards for *M. aeruginosa*.

When we began this study, we were able to determine only microcystin RR contents. Therefore, this toxin was the toxin quantified when we examined continuous cultures. Later, improvements in the method and the availability of new stan-

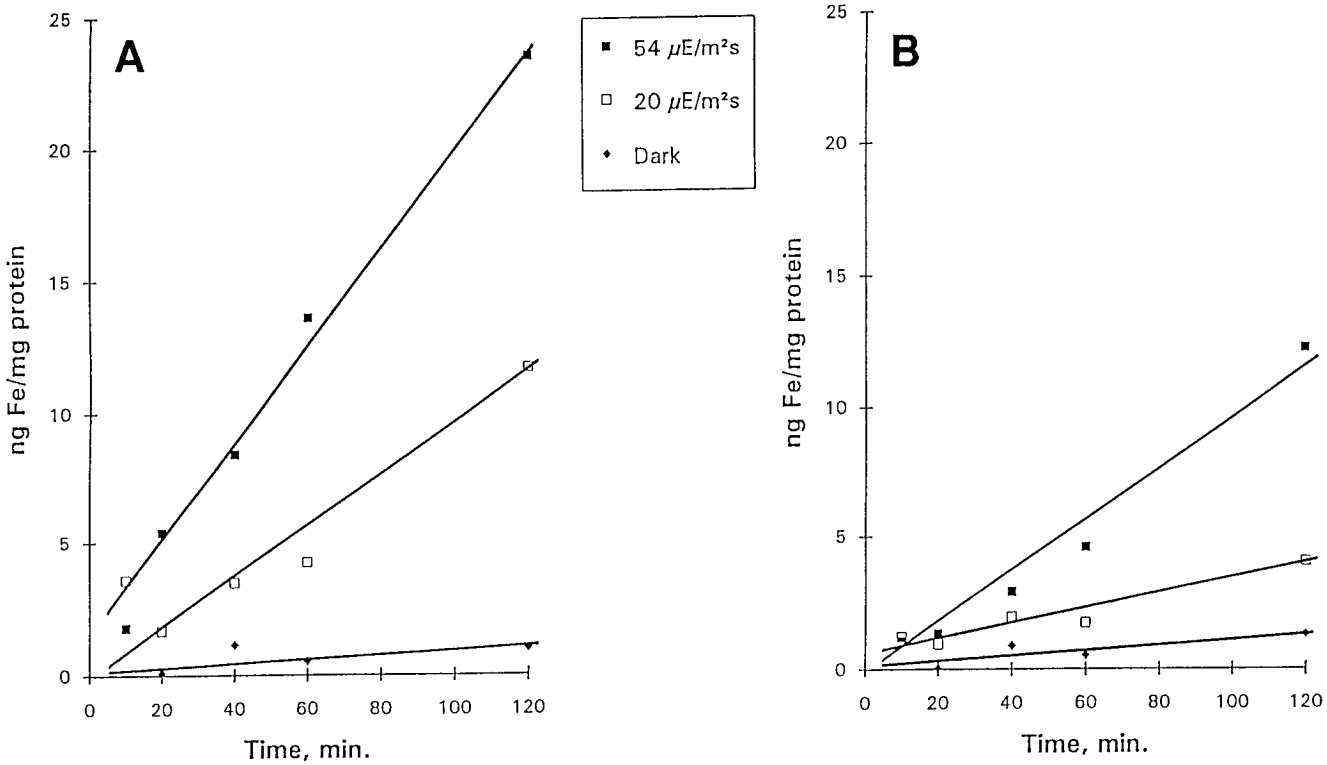

FIG. 1. Iron uptake as function of light intensity in Toxic (A) and nontoxic (B) *M. aeruginosa* cultures. μE, microeinsteins.

TABLE 4. Effect of different EDTA concentrations on the ratio of toxin content to protein and the ratio of iron content to protein content in *M. aeruginosa*

EDTA concn[a]	Microcystin RR content/protein content (ng/μg)	Microcystin LR content/protein content (ng/μg)	Fe content/protein content (ng/μg)
1×	11.48 ± 0.11	8.48 ± 0.04	4.5 ± 0.2
5×	10.42 ± 0.65	8.50 ± 0.95	3.0 ± 1.1
10×	8.56 ± 0.47	7.30 ± 0.07	2.1 ± 1.1

[a] 1× EDTA is the concentration of EDTA used in 02 medium. In all cultures the Fe concentration was the concentration used in 02 medium.

dards allowed us to detect other microcystins, and in the batch culture study of EDTA-inhibited iron uptake both microcystin RR and mycrocystin LR contents were determined.

Phosphate and nitrate are important factors in the eutrophication of lakes and also seem to affect toxin production in cyanobacteria (13, 19). Therefore, we began this investigation by examining the effects that limited availability of these components had on growth of and toxin production by *M. aeruginosa* grown in continuous cultures. Tables 1 and 2 show that in *M. aeruginosa* the ratio of toxin content to dry weight decreased during nitrogen- or phosphate-limited growth. The same effect of nitrogen-limited growth has been described previously for *M. aeruginosa* in batch cultures (19) and for nitrogen- and phosphate-limited growth of *Oscillatoria* strains (13), which produce a similar toxin. We found that in *M. aeruginosa* the decrease in the ratio of toxin content to dry weight was not due to a decrease in toxin production, since the ratio of toxin content (the toxin is a small peptide) to protein content remained nearly constant (Tables 1 and 2). We found that the carbohydrate content increased at both low NO_3 concentrations and low PO_4 concentrations (data not shown). Subtracting the carbohydrate content from the dry weight revealed that the ratio of toxin content to corrected dry weight remained nearly constant (Tables 1 and 2). Therefore, the decrease in the ratio of toxin content to dry weight under nitrogen- and phosphate-limited conditions was mainly caused by an increase in cellular weight due to carbohydrate accumulation. These results show that the ratio of toxin content to dry weight or LD_{50} should not be used to express changes in toxin production. Our results revealed that NO_3 and PO_4 have no influence on toxin production in *M. aeruginosa* and that the ratio toxin content to protein content, which we have described previously (14), is a much better way to express changes in toxin production than the

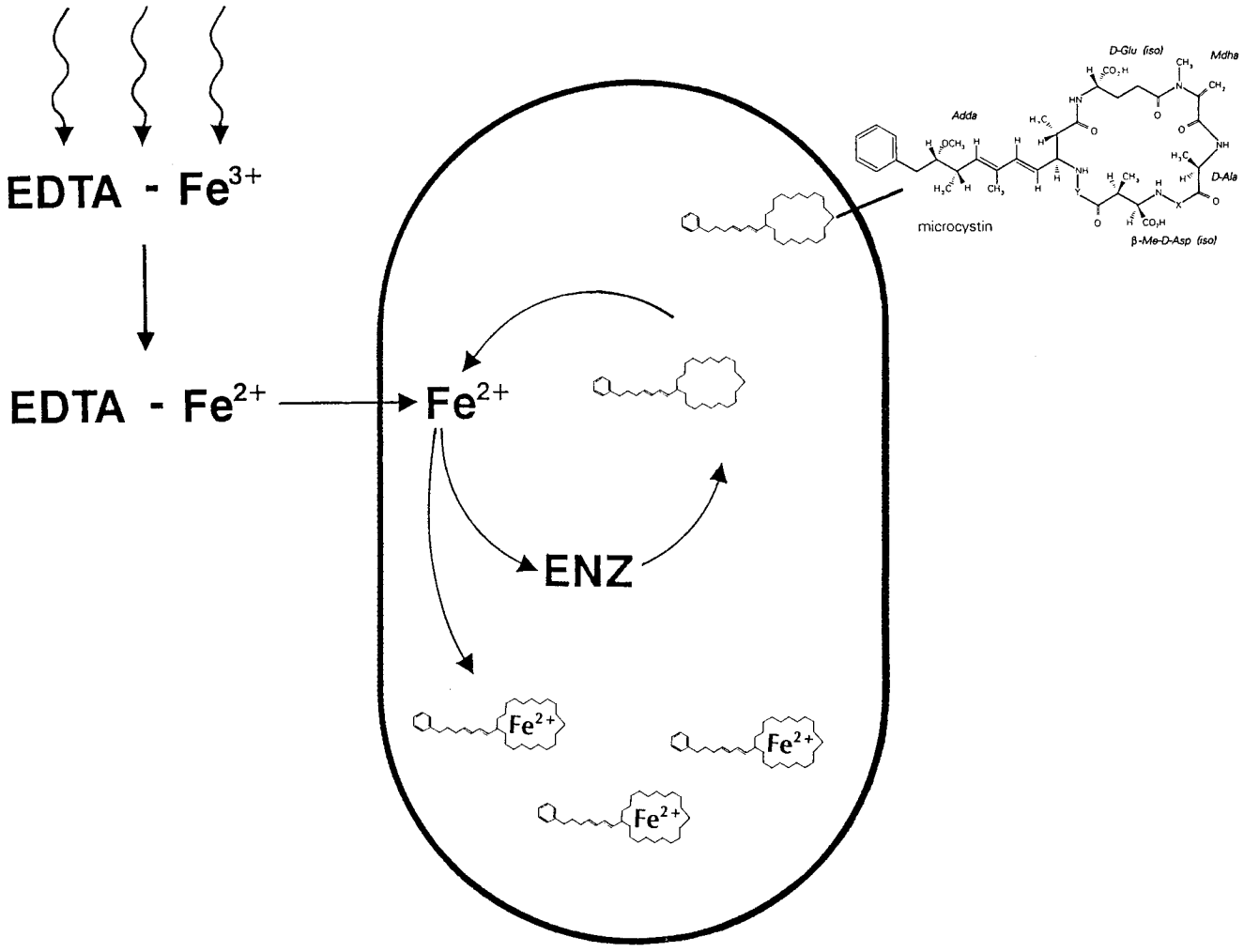

FIG. 2. Working model for the possible mechanism for toxin production in *M. aeruginosa* CYA 228/1. See text for explanation. ENZ, enzyme.

ratio of toxin content to dry weight. Using the latter parameter to determine changes in toxin production might in fact be misleading.

It has been found previously that the peptide toxin from *M. aeruginosa* binds iron and other cations (7). It has also been found that the toxin inhibits iron uptake by rabbit reticulocytes (11). A decrease in the iron concentration in the medium resulted (Table 3) in decreases in both the ratio of toxin content to dry weight and the ratio of toxin content to protein content. These results are similar to the results obtained when the light intensity was decreased (14). Therefore, we examined the relationship between light intensity and iron uptake and found that iron uptake increased as light intensity increased (Fig. 1). On the basis of these findings we propose that the well-documented effect of light intensity on toxicity (13–15, 19) in *M. aeruginosa* could be due to the effect of light intensity on iron uptake.

When Lukac and Aegerter examined the influence of trace metals on growth of and toxin production by *M. aeruginosa* (8), they found that iron had an effect on toxin production. However, in contrast to our findings, these authors found that the toxin content increased as the iron concentration decreased. Therefore, we grew *M. aeruginosa* in batch cultures with 02 medium containing different EDTA concentrations, since iron uptake is reduced as the EDTA concentration increases. Our results (Table 4) show that both the ratio of cellular Fe content to protein content and the ratio of toxin content to protein content are reduced by increasing the EDTA concentration. Although the differences are not very large, the tendency is clear. The discrepancy between our findings and those of Lukac and Aegerter (8) could be due to the different strains used. However, toxin production in *Microcystis* strains seems to be influenced in some way by iron.

We also found that the toxin-producing *M. aeruginosa* strain has a more efficient iron uptake system than the strain that does not produce toxin (Fig. 1). The toxin could be an intracellular chelator which keeps the cellular level of free Fe^{2+} low. Fe^{3+} in the medium seems to be converted to Fe^{2+} by light before it is transported into algal cells (3, 6). The nontoxin-producing *Microcystis* strain does not have this intracellular chelator (toxin) and must therefore have a lower cellular Fe^{2+} concentration; this is obtained by an iron uptake system less efficient than that in the toxin-producing *Microcystis* strain.

The presence of D-amino acids in microcystin strongly suggests that toxin production in the genus *Microcystis* is mediated by a nonribosomal mechanism (2). Bacitracin is a peptide that has a structure similar to the structure of the toxic peptide produced by *Microcystis* strains. Therefore, the mechanism of toxin production in *Microcystis* strains may be similar to the nonribosomal mechanism of bacitracin production in *Bacillus* strains (4). There is some evidence that a peptide synthetase occurs in cyanobacteria (12).

On the basis of the information described above, we propose a working hypothesis for peptide toxin production in *M. aeruginosa*; this hypothesis is shown in Fig. 2. We assume that the peptide toxin is an intracellular chelator which inactivates free cellular Fe^{2+} and that microcystin is produced by an enzyme (synthetase) whose activity is controlled by the amount of free Fe^{2+} present.

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