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Induction of Siderophore Activity in *Anabaena* spp. and Its Moderation of Copper Toxicity

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Growth of *Anabaena* sp. strain 7120 (in the absence of chelators or added iron) was inhibited by the addition of 2.1 to 6.5 μM copper and was abolished by copper concentrations of 10 μM or higher. When the copper was chelated to schizokinen (the siderophore produced by this organism in response to iron starvation), the toxic effects were eliminated. Analysis of culture filtrates showed that the cupric schizokinen remains in the medium, thereby lowering the amount of copper taken up by the cells. Although this organism actively transports ferric schizokinen, it apparently does not recognize the cupric complex. Thus, *Anabaena* sp. is protected from copper toxicity under conditions in which siderophore is being produced. For cells grown in low iron, the accumulation of extracellular schizokinen was observed to parallel cell growth and continue well into stationary phase. The actual iron status of the organism was monitored by using iron uptake velocity as an assay. Cultures grown on 0.1 μM added iron were found to be severely iron limited upon reaching stationary phase, thus explaining the continued production of schizokinen. These data show that the siderophore system in *Anabaena* spp. has developed primarily as a response to iron starvation and that additional functions such as alleviation of copper toxicity or allelopathic inhibition of other algal species are merely secondary benefits.

Many microorganisms respond to iron limitation by secreting low-molecular-weight iron chelators known as siderophores. Solubilized iron can then be scavenged from these siderophore complexes through the use of specific membrane transport systems (35). A number of cyanobacteria have been found to produce hydroxamate-type siderophores (4, 29, 30, 34), but the only one which has been structurally identified is schizokinen (41). This siderophore coordinates a ferric ion via two hydroxamate groups and the α-hydroxycarboxylate group of a citrate moiety (39). Schizokinen is known to facilitate iron uptake in *Anabaena* sp. strains 6411 and 7120 (15, 23) and has also been observed as an extracellular product of *Anabaena flos-aquae* (J. Hering and F. M. M. Morel, personal communication) and of rice field cyanobacteria (1). It was first discovered as a siderophore in the gram-positive bacterium *Bacillus megaterium* (7, 32).

Although cyanobacterial siderophores are synthesized in response to iron starvation, these siderophores are capable of binding other metal ions such as copper (29, 30). This adventitious metal complexation could be an important factor in the well-established tendency of algal exudates to moderate copper toxicity (12, 13, 26). A number of studies have indicated that it is mainly free cupric ions which govern the toxic effect of copper towards phytoplankton and that copper ion activity is diminished in natural systems by materials such as humic acids (2, 17, 19). Accordingly, the cyanobacterium *Aphanizomenon flos-aquae* is considerably less sensitive to copper in the presence of EDTA or a sediment extract (43). However, there are cases in which binding to organic ligands appears to enhance metal toxicity (22). *Anabaena* sp. strain 7120, for example, is more inhibited by copper in the presence of nitrilotriacetic acid (26). Of even greater surprise, *B. megaterium* shows enhanced susceptibility to copper toxicity in the presence of two siderophores, schizokinen and ferrioxamine B (3). Both of these cupric siderophore complexes apparently utilize the corresponding ferric siderophore uptake systems and, thereby, increase the rate of copper entry into the cell. In contrast, the iron transport system of *Ustilago sphaerogena* does not recognize the copper complex of its siderophore, ferrichrome (11).

Copper sulfate has been the treatment of choice for controlling blooms of nuisance algae (particularly cyanobacteria) in lakes and reservoirs for over 80 years (28). The treatment procedure is complicated by the fact that there is considerable variability in copper sulfate sensitivity both among different algal species and as a function of the stage in the growth cycle at which the bloom is treated (28). Since some of this variability could be due to the copper-chelating properties of siderophores, we have undertaken a study of the interrelationship between siderophore production and copper sensitivity in *Anabaena* sp. strain 7120.

MATERIALS AND METHODS

Culture conditions. *Anabaena* sp. strain 7120 was obtained from R. Haselkorn and grown on a modified BG-11 medium lacking citrate, EDTA, and iron, as described previously (23). Iron levels were further depleted by passing the nitrate, phosphate, and carbonate salt solutions through a Chelex 100 column (Bio-Rad Laboratories). Stock liquid cultures were maintained at 33°C on 10^{-7} M added $FeCl_3$ and at a cell density of 100 to 150 Klett units, using continuous light at 2,500 lx. For the copper addition experiments, cells were collected on a 0.45-μm-pore-size membrane filter (Millipore Corp.), rinsed, and suspended in the above BG-11 medium further modified to exclude all trace metals. Copper was added as $CuCl_2$, $CuSO_4$, or a preincubated mixture with schizokinen. Growth was monitored in a Klett-Summerson colorimeter fitted with a green no. 54 filter (100 Klett units = 2×10^7 cells per ml). Cell densities were determined microscopically with a hemocytometer on cells stained with toluidine blue.

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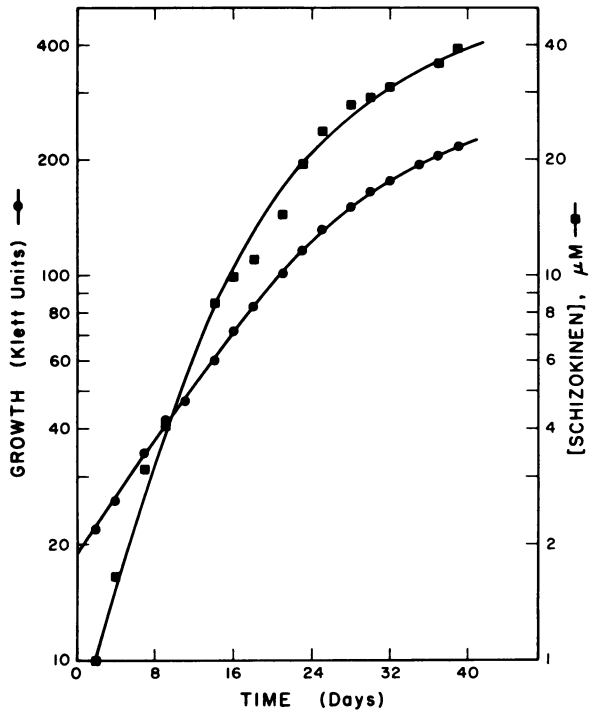

FIG. 1. Relationship between growth (●) and schizokinen production strain 7120. (■) in *Anabaena* sp. Cultures were grown without added iron but with the addition of 1 mM citrate. Slow rate of growth was due to the large culture volume of 500 ml as compared to the 25-ml volume used for Fig. 2 to 5. Schizokinen concentration was determined in extracellular filtrate.

Copper analyses. The concentration of copper in culture media was determined by atomic absorption spectroscopy in the flame mode (Instrumentation Laboratories model 551). Cells were removed by filtration prior to analysis of the medium. The standard curve was calculated by adding known concentrations of copper to freshly prepared growth medium.

Schizokinen assay. A 5- to 20-ml portion of culture filtrate was lyophilized and suspended in 1.5 ml of 5 mM $FeCl_3$ in 0.14 m $HClO_4$. Particulate material was removed by Millipore filtration, and the resulting ferric schizokinen solution was quantitated by its A_{490} (ϵ = 2,600 M^{-1} cm^{-1}) (15). For determinations of cell-associated schizokinen, cells were collected from 50 ml of culture by centrifugation. The resulting cell paste was suspended in 5 ml of water and the pH was adjusted to 1.5 with 0.1 M HNO_3. The schizokinen was extracted into 10 ml of chloroform-phenol (1:1, by weight); 40 ml of ethyl ether was then added and the schizokinen was back-extracted into 4 ml of distilled water. Residual phenol was removed by extraction with 8 ml of ether followed by bubbling with nitrogen. The water layer was adjusted to pH 7 with 0.1 M NaOH and then lyophilized and treated with $FeCl_3$ as above. Schizokinen concentrations were corrected for 65% recovery in the extraction procedure.

Iron uptake. Schizokinen was purified from culture supernatants of *B. megaterium* (23, 32). Uptake of [^{55}Fe]ferric schizokinen by *Anabaena* sp. strain 7120 was measured as described previously (15), with the following changes. All assays were performed on cells suspended to a density of 50 Klett units and exposed to 100 nM ^{55}Fe (7 mCi/mg) plus 150 nM schizokinen in uptake buffer containing 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.0, in addition to other salts. Filters containing ^{55}Fe-labeled cells were transferred to scintillation vials, partially solubilized by treatment with 0.3 ml of 0.1 N NaOH for 1 h, and then suspended in 5.5 ml of Liquiscint (National Diagnostics) overnight prior to scintillation counting. Uptake rates were calculated from the linear initial slopes obtained within the first 10 min of iron addition.

RESULTS

Induction of siderophore activity. Previous studies on *Anabaena* sp. strain 6411 showed that the rate of schizokinen production is controlled by iron availability. As the concentration of iron added to the growth medium is decreased, the concentration of schizokinen in the extracellular medium increases (23, 41). The release of schizokinen by *Anabaena* sp. strain 7120 has now been examined with respect to the growth cycle in iron-limited cells. The accumulation of schizokinen in the growth medium was found to be proportional to cell density throughout growth phase (Fig. 1) and to continue well into stationary phase. Such a secretion pattern is typical of hydroxamate siderophores in bacteria and fungi (36). A similar buildup of siderophore during stationary phase has been observed in the cyanobacteria *Anabaena flos-aquae* and *Anacystis nidulans*) (29).

A more detailed picture of the iron status of a culture throughout its growth cycle can be obtained by quantitating the rate of ferric siderophore uptake. Earlier studies with *Anabaena* sp. strain 6411 showed that this capability is also regulated by iron availability (35). The uptake system saturates at high concentrations of ferric schizokinen as is typical of a protein-mediated transport system, it requires metabolic energy input in the form of ATP, and its activity is inhibited when cells are exposed to iron concentrations of >0.1 μM (23). When the iron uptake activity of *Anabaena* sp. strain 7120 exposed to 0.1 μM iron was investigated throughout the growth cycle, an interesting pattern emerged (Fig. 2). The inoculum for this experiment was grown to high cell density in the absence of added iron and had a high rate of iron uptake. Upon dilution into 0.1 μM iron, the cells exhibited logarithmic growth for the first 100 h (Fig. 2A), but underwent a marked decrease in iron uptake activity during this same time period (Fig. 2B). This drop in activity reflects the satisfaction of cellular iron requirements by the 0.1 μM iron added to the medium and consequent repression of the iron uptake system. It should be noted that all cells were washed prior to iron uptake assays to remove any iron remaining in the growth medium, so that the assays would reflect only the physiological iron status of the cells themselves.

After 100 h of growth and approximately five cell doublings, the culture exposed to 0.1 μM iron again became iron limited, as evidenced by the decrease in the growth rate and by the increase in the iron uptake rate (Fig. 2). Addition of another dose of 0.1 μM iron at 500 h, when the cells were approaching stationary phase, again resulted in a marked repression of the iron uptake system as the cells became iron sufficient. This immediate response in a population which had essentially stopped dividing implies that the cells can control iron uptake activity by inactivation of the membrane transport system. A similar type of behavior was observed previously when strain 7120 cells were incubated with 0.5 μM iron, and uptake leveled off within 2 h after only 10% of the added iron had been incorporated (15).

The data in Fig. 2 show that the iron status of the culture varies considerably during the growth cycle and that cells in 0.1 μM iron generally become iron limited in stationary phase. Exposure to higher concentrations of iron (i.e., 0.4 to 1.1 μM) caused successively greater inhibition of iron uptake (Fig. 3A) with the result that cells in >0.7 μM iron remained iron sufficient during stationary phase (i.e., 200 to 300 h). A similar graded response of stress in 0.1 μM Fe and sufficiency in 1.0 μM has been observed in *Anabaena* sp. strain 7120 (W. Fish, M. Dalrymple, and J. Sanders-Loehr, unpublished results), using the concentration of intracellular flavodoxin as a measure of iron starvation (18). When cells adapted to 1.0 μM iron were placed in a low-iron medium, they rapidly ran out of iron as indicated by their increased iron uptake activity (Fig. 3B). These experiments suggests that *Anabaena* sp. does not build up significant iron reserves and, therefore, is geared toward responding to the amount of iron in the environment at any point in time.

Copper toxicity. A further indication of iron deficiency was obtained from the observation that, in *Anabaena* sp. strain 7120 grown without iron, addition of 15 μM schizokinen caused a twofold increase in the growth rate (Fig. 4). Such growth-stimulating effects are due to the ability of schizokinen to scavenge residual iron in the medium and make this iron available to the cells (7). In contrast, addition of 10 μM copper caused complete inhibition of growth (Fig. 4). However, when this same amount of copper was combined with 15 μM schizokinen, the deleterious effect was alleviated. The protective effect of schizokinen indicates that it forms a copper complex which is not taken up by the ferric schizokinen transport system. This conclusion is supported by an analysis of the copper content in the culture medium 40 h after exposure of the cells to 1 to 10 μM copper sulfate (Table 1). In the absence of added schizokinen approximately 50% of the copper was removed from the medium by the cells, while in the presence of schizokinen in the medium a considerably smaller fraction of the copper became cell associated.

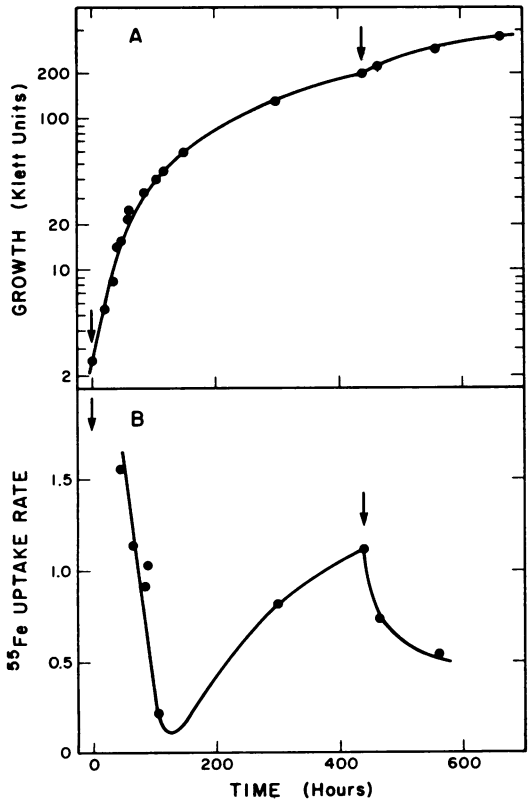

FIG. 2. Growth (A) and iron uptake activity (B) of *Anabaena* sp. in response to the addition of 0.1 μM $FeCl_3$ (indicated by arrows). Rate of uptake of ^{55}Fe from ferric schizokinen is given in nanomoles per liter of cell suspension per minute. To determine the rate of iron uptake, cells were suspended from the growth medium by filtration, rinsed with iron-free buffer, and then subjected to a standard uptake assay with 100 nM Fe plus 150 nM schizokinen.

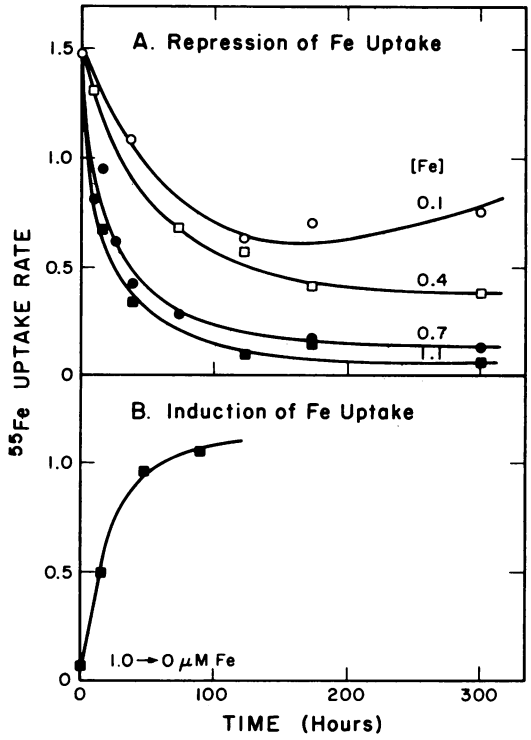

FIG. 3. Repression and induction of iron uptake system in response to iron availability. Iron uptake assay was as described in the legend to Fig. 2. (A) Inoculum grown in 0.1 μM iron and suspended at zero time in 0.1 (○), 0.4 (□), 0.7 (●), or 1.1 (■) μM iron at a cell density of 30 Klett units. Cells exhibited logarithmic growth for 120 h. (B) Inoculum treated with 1.0 μM iron for several days and then suspended at zero time in a medium with no added iron at a cell density of 15 Klett units. Cells underwent two divisions during the course of this experiment.

The response of iron-limited cells to various amounts of copper is shown in Fig. 5. Since copper is a nutrient, concentrations below 1 μM actually stimulated growth, whereas concentrations between 2 and 6.5 μM become progressively more inhibitory. It is of interest that the higher concentrations caused a lag in growth which was slowly overcome with time. The duration of the lag period was proportional to the amount of copper added. Thus, cells grown in 2.1 μM copper exhibited a 40-h lag, while those grown in 6.5 μM copper had a 100-h lag. An analysis of the copper concentration in the growth medium at 95 h (near the end of the lag period) showed a substantial increase in copper content compared to that at 45 h (Table 1), indicating that some of the cell-associated copper was released during the lag period.

It is likely that recovery from copper toxicity involves lysis of a fraction of the cell population, thereby releasing

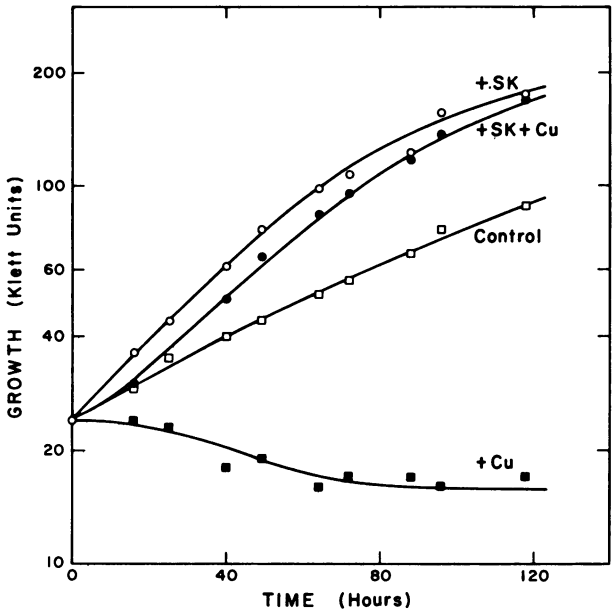

FIG. 4. Effect of added copper and schizokinen (SK) on growth of *Anabaena* sp. strain 7120. Inoculum was grown on 0.1 μM iron and suspended in a medium with no added iron (□) and with 15 μM schizokinen (○), 15 μM schizokinen plus 10 μM $CuCl_2$ (●), or 10 μM $CuCl_2$ (■).

intracellular components such as proteins into the medium which further bind copper and lower the copper ion activity (26). Our observation of a drop in cell density after the addition of inhibitory amounts of copper (Fig. 4 and 5) provides evidence that some cell lysis has occurred. Similar results were obtained in an earlier study of *Anabaena* sp. strain 7120 in which cell lysis was also observed and was followed by a decrease in cell-associated copper as the cells recovered from copper toxicity (26). The present study suggests that the presence of siderophores, both intracellular and extracellular, must also be considered a factor in the resistance to and recovery from copper toxicity.

An example of the importance of schizokinen is seen in the effect of the iron status of the organism on its response to copper. Culture grown in low (0.1 μM) and high (10 μM) iron were inoculated into media containing no added iron, but variable amounts of copper. The high-iron cells, which were iron repressed (Fig. 3) and thus not likely to be producing schizokinen at the time of exposure to copper, showed considerably greater sensitivity to copper. In contrast to the low-iron cells (Fig. 5), the growth of the high-iron cells was significantly inhibited by the addition of 1 μM copper and totally inhibited by the addition of 5 μM copper (with no subsequent recovery). Consequently, it would appear that schizokinen does play a role in lowering copper ion activity and thereby sparing other cellular components. Whether these protective effects are due to extracellular, secreted schizokinen or to intracellular schizokinen released during cell lysis remains a matter of conjecture. We have found, however, that for normal iron-limited cultures in growth phase the majority of the schizokinen is free in the medium, with only 10 to 20% being cell associated.

TABLE 1. Uptake of added copper by *Anabaena* sp.

$CuCl_2$ added (μM)	Cu concn in growth medium (μM)[a]		
	40 h	40 h (SK)[b]	95 h
0	<0.1	ND	<0.1
1	0.6	ND	1.0
2	0.8	ND	1.4
4	1.4	2.7	2.3
6	3.0	5.0	5.7

[a] Determined by atomic absorption spectroscopy. ND, Not determined.
[b] Excess schizokinen (SK; 1.8-fold) added at time of copper treatment.

DISCUSSION

Although the total iron concentration in lake water can be in excess of 10 μM, dissolved iron concentrations are typically below 1 μM, with levels close to 0.1 μM being fairly common (24, 31, 33, 44). In our laboratory experiments on *Anabaena* sp. strains 7120 (present work) and 6411 (23), using schizokinen production and ^{55}Fe uptake as indicators of iron stress, we found distinct signs of iron limitation during growth in 0.1 μM iron. Similar results have been reported for the cyanobacteria *Anabaena flos-aquae*, *Anacystis nidulans*, *Microcystis aeruginosa*, and *Gleocapsa alpicola*, in which high concentrations of strong metal-complexing agents (presumed sideophores) are produced by cells grown in 0.4 μM iron (29). Iron limitation has been observed in a number of natural cyanobacterial populations (8, 27, 38, 44), and extracellular siderophores have been detected (25, 33, 34). Furthermore, the amount of siderophore produced is generally far in excess of the available iron. *Anabaena* sp. strains 6411 and 7120, for example, when grown at iron concentrations of 0.1 μM or less, build up schizokinen concentrations which are 10- to 10^3-fold higher than the amount of iron in the medium (15, 41). This process results in significant concentrations of iron-free chelator which can then react with other metal ions in the environment such as copper.

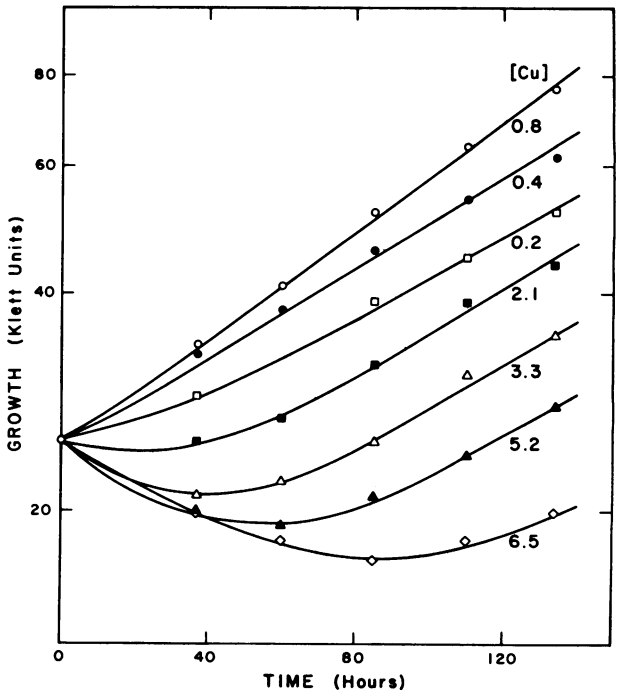

FIG. 5. Response of *Anabaena* sp. to variable amounts of copper. Inoculum was grown on 0.1 μM iron and suspended in a medium with no added iron, but with 0.2 to 6.5 μM $CuSO_4$.

The role played by siderophores in ecological systems has been the subject of much conjecture. Previous work on cyanobacteria has shown that siderophore levels are strongly regulated by iron (23, 30, 41). However, the continued production of siderophores by stationary-phase cultures has led to the question of whether they might also be having an allelopathic effect whereby they limit the growth of organisms which cannot utilize that particular ferric siderophore complex (5, 33, 34). This type of antibiotic activity has been suggested for several bacterial systems (21, 37). The present results on the iron status of *Anabaena* sp. show that the continued synthesis of siderophore in stationary phase is primarily the result of iron starvation. Thus, the observed allelopathic effects must be viewed as a coincidental side reaction. If allelopathy were the primary role of siderophores, their synthesis would not be so strictly controlled by iron availability.

A related question is whether siderophore biosynthesis is stimulated by high concentrations of metals such as copper (even though it is repressed by high concentrations of iron). The available data indicate that the role of siderophores in moderating copper toxicity is likely to be coincidental. Cultures of *Anabaena flos-aquae*, for example, accumulated ~15 μM siderophore (schizokinen) at stationary phase in response to low iron (0.1 μM), and no increase in siderophore concentration was observed in cells exposed to inhibitory amounts of copper (30). In our experiments with *Anabaena* sp. strain 7120, we observed that cultures grown on 10 μM iron had an increased sensitivity to copper, implying that the repression of siderophore synthesis by high iron cannot be overcome by added copper. Although a recent study of *Anabaena cylindrica* and *Plectonema boryanum* indicated enhanced levels of extracellular chelators being produced in copper-treated cells (20), it is unlikely that these chelators were siderophores. The cultures were grown in 1 μM iron (a level generally inhibitory to siderophore production) and at very low cell densities (equivalent to ~10 Klett units in our experiments), conditions in which siderophore concentrations are generally too low to be measured directly.

Even though induction of siderophore synthesis appears to be independent of environmental copper levels, the excretion of siderophores may have a marked effect on copper speciation and, hence, its toxicity. We have found that copper concentrations of >2 μM are inhibitory to *Anabaena* sp. strain 7120 in the absence of added iron or chelators. The inhibition is overcome by the addition of excess schizokinen which has a high affinity for Cu(II). Measurement of copper binding to schizokinen in culture filtrates of *Anabaena flos-aquae* has led to an estimated conditional formation constant of 10^8 (30). Our analyses of extracellular copper concentrations show that considerably less copper becomes cell associated when schizokinen is present in the culture medium.

The above results with *Anabaena* spp. are in marked contrast to the behavior of *B. megaterium*. The ferric schizokinen uptake system in that organism actually recognizes the copper chelate, and copper toxicity is dramatically enhanced by the presence of the siderophore (3). The *B. megaterium* results are surprising since the ferric complex is known to be 6-coordinate and octahedral (39), whereas the cupric complex is more likely to be 4-coordinate and square planar (9). Thus, the siderophore receptors of *Anabaena* sp. and *B. megaterium* must be responding to quite different features of the schizokinen complex. A similar conclusion was reached upon the observation that the *Anabaena* sp. transport system will also utilize the siderophore aerobactin (15) while the *B. megaterium* transport system will not (16).

Although copper sulfate is used extensively for the treatment of algal blooms, the effective dose varies considerably due to the influence of a number of environmental factors. Copper ion activity (and toxicity) is reduced by the formation of $CuCO_3(aq)$ and $Cu_2(OH)_2CO_3(S)$ at pH 7 and higher (28) and by the presence of organic chelators such as humic substances (40) or materials released by cell lysis (26). Increased concentrations of copper-binding ligands have been observed, for example, following the decline of natural blooms of *Aphanizomenon*, *Oscillatoria*, and *Planctonema* spp. (10). In addition, significant amounts of copper can be adsorbed onto suspended solids such as the hydroxides of iron and aluminum (6, 42). The physiological state of the resident phytoplankton is important as well, since populations at higher cell densities tend to be more resistant to copper toxicity (14, 26). In this particular case, the accumulation of siderophores in the extracellular medium may well be an important factor in explaining the greater resistance of older populations. Cyanobacteria frequently become more iron limited as a bloom progresses (10, 24, 44) and, thus, increase their production of siderophores. These considerations argue for the early application of copper sulfate, prior to the establishment of substantial cell densities.

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