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IRON ACQUISITION BY CYANOBACTERIA:
SIDEROPHORE PRODUCTION AND IRON TRANSPORT BY ANABAENA

By
PETER JAMES LAMMERS

A dissertation presented in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY
ENVIRONMENTAL SCIENCES AND RESOURCES - CHEMISTRY

Portland State University

1982

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AN ABSTRACT OF THE DISSERTATION of Peter James Lammers for
the Doctor of Philosophy in Environmental Sciences and
Resources - Chemistry, presented June 4, 1982.

Title: Iron Acquisition by Cyanobacteria: Siderophore
Production and Iron Transport by Anabaena

APPROVED BY MEMBERS OF THE DISSERTATION COMMITTEE:

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The cyanobacterium Anabaena sp. (PCC #6411) is known to produce the siderophore, schizokinen, in response to iron limitation. Environmental factors which influence schizokinen production have been examined utilizing a bioassay method based upon the stimulation of growth of the siderophore-requiring bacterium Arthrobacter flavescens JG-9. Schizokinen production by Anabaena increased in low-iron media and was stimulated approximately five-fold in media containing one millimolar citric acid. Growth of Anabaena under nitrogen-fixing conditions reduced extracellular schizokinen concentrations 3-4 fold, in late growth phase, compared to cells grown on nitrate. Cells grown in ammonium-containing medium yielded intermediate concentrations of schizokinen. The Csaky assay, which is often used to detect hydroxamate siderophores, was found to be subject to interferences that can yield erroneously high values. The problems associated with detection and quantitation of cyanobacterial siderophores are discussed.

Anabaena was found to utilize schizokinen to accumulate >90% of the [^{55}Fe]-ferric iron added to the medium. Iron transport capability was increased in iron-starved cells. The transport system appears to be fairly specific for schizokinen, in that an acetylated derivative of schizokinen also supported iron transport, but the structurally related siderophore, aerobactin, and the trihydroxamate siderophore, ferrioxamine B, did not support iron uptake by Anabaena. The uptake of ferric schizokinen

displayed saturation-type kinetics with an apparent K_M of 35 nM, and required the input of metabolic energy. Light-driven transport was blocked by uncouplers and ATPase inhibitors. Transport in dark-adapted cells was additionally blocked by inhibitors of respiration. We conclude that ATP serves as an energy source for the cellular uptake of schizokinen.

Two other kinds of Anabaena were examined for siderophore production. Anabaena sp. (PCC #7120) was found to produce a substance which stimulates the growth of the Arthrobacter JG-9. This substance binds iron and has a similar absorbance maximum to schizokinen in ferric-perchlorate solutions. Anabaena 7120 also utilized ferric schizokinen and ferric acetyl-schizokinen for iron uptake, but not aerobactin or ferrioxamine B. These results suggest that the iron transports systems of Anabaena 6411 and 7120 may be the same. Anabaena cylindrica Lemm. apparently does not produce or utilize a schizokinen-type siderophore. No Arthrobacter JG-9 stimulating material could be detected in low-iron filtrates, even if concentrated 25-fold.

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TABLE OF CONTENTS

	PAGE
ACKNOWLEDGMENTS.....	iii
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
SECTION	
I. INTRODUCTION	
STATEMENT OF PROBLEM.....	1
BIOAVAILABILITY OF IRON.....	5
STRUCTURES AND CHARACTERISTICS OF MICROBIAL SIDEROPHORES.....	9
SIDEROPHORE-DEPENDENT IRON TRANSPORT SYSTEMS....	22
II. EXPERIMENTAL	
GROWTH MEDIA AND CULTURE CONDITIONS.....	30
SCHIZOKINEN PRODUCTION FROM <u>Bacillus megaterium</u> .	42
Characterization of schizokinen.....	51
Modifications of schizokinen.....	55
AEROBACTIN FROM <u>Aerobacter aerogenes</u>	58
SIDEROPHORE ASSAYS.....	61
Csaky assay for hydroxamic acids.....	61
<u>Arthrobacter</u> JG-9 bioassay for siderophores.....	68
Iron binding assay for siderophores.....	71
IRON UPTAKE ASSAYS.....	73

III.	RESULTS	
	EFFECTS OF IRON AND CHELATORS ON GROWTH OF <u>Anabaena</u> 6411.....	79
	SIDEROPHORE PRODUCTION BY <u>Anabaena</u> 6411.....	87
	TRANSPORT OF FERRIC SCHIZOKINEN BY <u>Anabaena</u> 6411.....	91
	KINETICS AND ENERGY DEPENDENCE OF FERRIC SCHIZOKINEN TRANSPORT.....	95
	SPECIFICITY OF IRON UPTAKE IN <u>Anabaena</u> 6411.....	100
	SIDEROPHORE ACTIVITY IN DIFFERENT STRAINS OF <u>Anabaena</u>	104
	SIDEROPHORE ASSAYS WITH <u>Anabaena cylindrica</u>	108
IV.	DISCUSSION.....	109
	SIDEROPHORE ASSAYS.....	110
	IRON TRANSPORT.....	114
	RESPONSES TO IRON LIMITATION.....	119
	ENVIRONMENTAL IMPLICATIONS.....	125
	CONCLUSIONS.....	126
	FUTURE DIRECTIONS.....	127
V.	REFERENCES.....	130

LIST OF TABLES

TABLE		PAGE
I	Media for Cyanobacteria.....	31
II	Compounds that Satisfy Growth Requirement of <u>Arthrobacter flavescens</u> JG-9.....	39
III	Media for Bacterial Species.....	40
IV	Concentrations of Schizokinen in <u>Anabaena</u> Filtrates.....	67
V	Concentration of Schizokinen (μM) in <u>Anabaena</u> Filtrates.....	88
VI	Effect of Metabolic Inhibitors on Ferric Schizokinen Uptake in <u>Anabaena</u> 6411.....	99
VII	Iron Speciation in BG-11 Medium as Predicted by MINEQL.....	121

LIST OF FIGURES

FIGURE	PAGE
1 Structure of Ferrichrome-type Siderophores.....	11
2 Structures of Ferrioxamine B, Fusarinines, and Rhodotorulic Acid.....	15
3 Structures of Catechol-type Siderophores.....	18
4 Structures of Citrate-hydroxamate Siderophores Including Schizokinen.....	20
5 Summary of Membrane Uptake Systems for Specific Metal Complexes in <u>E. coli</u>	24
6 Growth of <u>Anabaena</u> 6411 in Uptake Medium and BG-11(mc).....	37
7 Anion Exchange Chromatography of Crude Schizokinen.....	46
8 Gel Permeation Chromatography of Schizokinen.....	48
9 NMR Spectra of Schizokinen.....	50
10 Visible Absorption Spectrum of Ferric Schizokinen as a Function of pH.....	53
11 Spectrophotometric Titration of Schizokinen with FeCl ₃	54
12 NMR Spectrum of Acid Hydrolyzed Schizokinen.....	57
13 NMR Spectrum of Acetyl-Schizokinen.....	60
14 NMR Spectrum of Aerobactin.....	63
15 Standard Curve for Csaky Assay.....	65
16 Growth Response of <u>Arthrobacter flavescens</u> to Various Concentrations of Purified Schizokinen.....	69
17 Visible Absorption Spectra for <u>Anabaena</u> Filtrates in 5 mM Fe(III), 0.14M HClO ₄	72
18 Growth of <u>Anabaena</u> 6411 in BG-11 Medium as a Function of Added Iron.....	81
19 Growth of <u>Anabaena</u> 6411 With and Without Chelators.....	85

FIGURE	PAGE
20 Growth of <u>Anabaena</u> 6411 in BG-11(mc) Without Combined Nitrogen.....	86
21 Uptake of [⁵⁵ Fe] Ferric Schizokinen.....	93
22 Rate of Uptake of [⁵⁵ Fe] Ferric Schizokinen as a Function of Iron Concentration in the Growth Medium.....	94
23 Kinetics of Uptake of Ferric Schizokinen.....	96
24 Effect of CCCP and DCCD on the Uptake of Ferric Schizokinen in <u>Anabaena</u> 6411.....	98
25 Iron Uptake Activity by <u>Anabaena</u> for Several Ferric Siderophore Complexes.....	103
26 Iron Uptake Activity by <u>Anabaena</u> 7120.....	107

INTRODUCTION

Cyanobacteria, also known as blue-green algae, are the most plant-like of all procaryotic organisms. They are widely distributed from polar seas to thermal springs as well as in soils and fresh water. Cyanobacteria derive their metabolic energy from sunlight, abstracting hydrogen atoms from water and liberating oxygen gas. Reducing power thus generated is used to fix carbon dioxide gas as carbohydrate. For most cyanobacteria, CO_2 is the sole source of cell carbon. Many of these organisms are also capable of reducing molecular nitrogen (N_2) to ammonia for amino acid biosynthesis. As a result, cyanobacteria have exceedingly simple nutritional requirements; consisting mainly of phosphorus, magnesium, calcium, and trace metals including iron and molybdenum. Cyanobacteria have long been studied as model systems for photosynthesis and nitrogen fixation. Genetic analysis of these processes, made possible by recent advances in recombinant DNA technology, holds promise for eventually producing more efficient and possibly new nitrogen-fixing agricultural crops(1).

In the natural environment, cyanobacteria have something of a Jekyll and Hyde reputation. On the positive

side, some blue-greens form productive symbiotic associations with other organisms. The most notable of these is the Anabaena-Azolla symbiosis that is currently being used in southeast Asia to fertilize rice paddies instead of expensive chemical fertilizers (2). On the negative side, however, are the many species that form "blooms" in lakes that can totally dominate other phytoplankton and adversely affect water quality(3). Toxins are produced by many species and floating scums associated with blooms are particularly objectionable (4).

The factors that govern phytoplankton succession and dominance in aquatic ecosystems are poorly understood. Competition for available nutrients is certainly a factor along with seasonal variations in temperature and light intensity. In addition, a potentially important parameter is the availability of the micronutrient iron. Iron is a component of many essential biological catalysts including the cytochromes and iron-sulfur proteins involved in the photosynthetic electron transport chains of all phytoplankton. Iron containing proteins are also involved in the metabolism of N_2 and H_2 , in the reduction of ribotides to deoxyribotides (precursors of DNA), in oxidation and hydroxylation of a host of inorganic and organic metabolites and finally in the decomposition or utilization of hydrogen peroxide (5). The availability of iron for biological use is not a simple matter however. Ferric ions, as $Fe(H_2O)_6^{3+}$, have a great tendency to form

polymeric ferric hydroxide that is extremely insoluble. The presence of natural chelators such as humic acids can keep ferric ions in solution, but whether this increases or decreases iron bioavailability is an open question. Many microorganisms have evolved the capability to produce powerful iron chelators, known as siderophores, to complex insoluble iron which can then be transported inside the producing cells via specific, siderophore-dependent uptake systems (6). The production of a hydroxamate-type siderophore during a cyanobacterial bloom of Anabaena flos-aqua in a eutrophic bay has been documented (7). Furthermore, partially purified preparations of this compound were found to greatly inhibit the growth of Scenedesmus, a green alga which had disappeared during the bloom. The growth inhibition caused on addition of the hydroxamate was reversed by the addition of excess iron, suggesting that siderophore-induced iron limitation for competing species may well be important in establishing bloom conditions.

Despite the potential importance of iron limitation on phytoplankton competition in aquatic ecosystems, very little is known about the subject. Only one cyanobacterial siderophore has been structurally characterized (8), and the extent of cyanobacterial siderophore production is not well understood. No investigations of siderophore-dependent iron transport by cyanobacteria have been reported to date. As a result, it is very difficult to

evaluate the potential significance of iron limitation and competition for iron as a determinant of aquatic ecosystem composition and stability.

This dissertation will deal with the dual questions of cyanobacterial siderophore production and siderophore-dependent iron transport across cyanobacterial membranes. The organism chosen for study is Anabaena sp. (ATCC #27898, Pasteur Culture Collection #6411) which is known to produce the dihydroxamate siderophore, schizokinen (8). Techniques for measuring siderophore production have been evaluated in addition to factors that quantitatively affect siderophore production. The iron transport system has been examined with respect to induction, substrate specificity, substrate affinity, and energy requirements.

In addition, the production of siderophores by two other species of Anabaena, strain 7120 (Pasteur Culture Collection) and Anabaena cylindrica Lemm., has been examined along with their ability to take up iron complexed by several siderophores.

A brief review of the factors influencing the bio-availability of iron and a description of the properties of known siderophores and their associated uptake systems will follow to provide a frame of reference for the results of the present study.

Bioavailability of Iron

Despite the fact that iron comprises nearly five percent of the earth's crust, soluble iron in oxygenated waters is often extremely scarce. Under these conditions, iron will exist primarily in the +3 oxidation state which readily hydrolyzes to form polymeric ferric hydroxide species. The solubility product constant of $\text{Fe}(\text{OH})_3$ is $10^{-38.7}$ M at 25°C (9). This value leads to a concentration of $\text{Fe}(\text{III})(\text{aq})$ of only 10^{-18} M at pH 7. However, this ignores the contribution of soluble $\text{Fe}(\text{III})$ species such as $\text{Fe}(\text{OH})^{2+}$, $\text{Fe}(\text{OH})_2^+$, and $\text{Fe}(\text{OH})_3$. The formation constant for $\text{Fe}(\text{OH})_3(\text{aq})$ is very difficult to measure experimentally. An upper limit on the solubility of ferric hydroxide of 10^{-8}M has been calculated by taking advantage of the nearly linear plots of the free energy of formation of each successive hydrolysis product versus the number of coordinated hydroxides (10) to estimate the formation constant for $\text{Fe}(\text{OH})_3(\text{aq})$. Even this higher value is considerably below the optimal values for cyanobacterial growth. For example, Nostoc muscorum requires 3×10^{-6} M iron for maximal growth on nitrate and 2×10^{-5} M iron for maximal growth under nitrogen-fixing conditions (11). In this case the solubility of iron in the growth medium was increased above 10^{-8} M by the presence of phosphate and EDTA; such complexes are excellent sources of iron for organisms which make siderophores. In natural systems

where the level of chelators is much lower, iron tends to become a limiting nutrient due to its removal from solution as a ferric hydroxide precipitate (12, 13).

The fact that iron can be a limiting nutrient to natural algal populations has been known for over twenty years. Enrichment experiments conducted with water samples from Lake Erie, The Sargasso Sea, and the Indian Ocean have demonstrated increased algal productivity with added iron (14-16). The formation of ferric hydroxide is not the only mechanism reducing the bioavailability of iron. The precipitation of iron-phosphorous complexes is thought to be an important pathway for the loss of these nutrients to sediment (17). Furthermore, while the phosphorous can be returned to the water column following reduction of iron (III) to iron (II) in anoxic sediments (18), Murphy and Lean have shown that a large fraction of iron returning from sediments to open water is not available for biological uptake (19). This iron was associated with magnesium and had a molecular weight between 1000 and 5000 daltons. To further complicate the picture, the effect of potential chelators present in sediments such as humic acids on iron bioavailability is not well understood. Fulvic acid, a fraction of humic acids, has been shown to be an effective trace metal chelator for some cyanobacteria that require EDTA and citrate in order to grow in a synthetic medium (20). This effect required that the fulvic acid be used in a proper ratio with the essential nutrients, a condition

that is hard to predict in natural systems. The effect of the other fractions of humic acids upon iron speciation and the resulting influence on cyanobacterial growth has not been established.

Potentially the most important factor controlling the availability of iron to phytoplankton is the production of biological chelators. Among the first efforts to study this possibility was the report by Fogg and Westlake on extracellular pigment-polypeptide complexes produced by Anabaena cylindrica Lemm. (21). The extracellular concentration of this substance was shown to be quantitatively affected by iron (22), and it was later demonstrated that up to 12% of the iron supplied to the medium was bound by a large peptide (23). The specific biological function of this pigmentpolypeptide complex has never been established however. It is doubtful that this material represents a true siderophore (see below), however a role in iron metabolism cannot be ruled out.

Only recently have efforts to detect true cyanobacterial siderophores been undertaken. Van Baalen and co-workers have detected siderophore activity from cyanobacterial mats collected from nearshore marine environments (24), and also in axenic culture of the marine cyanobacterium Agmenellum quadruplicatum (25). The structure of these compounds is not yet known. Simpson and Neilands have isolated the dihydroxamate siderophore, schizokinen, from Anabaena sp. (PCC #6411). This siderophore is also

produced by the Gram positive bacterium Bacillus megaterium and is the only structurally characterized cyanobacterial siderophore known to date (7).

Other studies have given indications that siderophore production by cyanobacteria may not be uncommon. Several different Anabaena strains have been studied by McKnight and Morel with respect to their production of strong extracellular copper chelators that are probably siderophores (26). Here again, the identity of these compounds is not known, since their identification as siderophores is based on the fact that iron limitation greatly increases the extracellular concentration of the strong copper-complexing compound. Lange has proposed that the ability to grow on synthetic medium without added chelators can be used as an assay for the production of biological chelators by cyanobacteria (27). Six of the ten species he examined grew well without synthetic chelators present. Furthermore, sterile filtrates from cultures that grew normally were able to overcome the chelator requirements of the other four species. In order to prove that these presumptive chelators are actually siderophores, three criteria need to be demonstrated. A "true" siderophore must display high affinity for iron, accumulate in response to iron limitation, and promote the uptake of iron by the siderophore-producing cells. These questions have not yet been addressed.

Structures and Characteristics of Microbial Siderophores

Since the evolution of an oxidizing atmosphere on this planet by photosynthetic O_2 production from water, the availability of iron in most aquatic systems has been limited by formation of iron(III) minerals of low solubility and perhaps by the formation of high molecular weight hydroxybridged ferric polymers. This condition has encouraged biological systems to evolve specific chelating agents for ferric iron, called siderophores (6). Since the first isolation of a siderophore (ferrichrome) by Neilands in 1952 (28), scores of siderophores have been identified from bacteria, fungi, yeast, and cyanobacteria. There are now seven recognized families of siderophores: ferrichromes, ferrioxamines, fusarinines, rhodotorulic acids, enterobactins, mycobactins, and aerobactins. Hydroxamate ligands prevail in all of the above families except the enterobactins, which contain catecholate ligands. Both form stable five membered ring complexes with iron.

The iron atom in these complexes is d^5 , high spin (29,30), and as a result of a lack of crystal field stabilization energy, is readily exchangeable (31). The siderophores as a group are characterized by tremendous formation constants with Fe(III) that range from 10^{23} to 10^{52} (6). The hydromamate containing siderophores are much more numerous than the tri-catecholate ligands which are limited to the true bacteria (6).

The ferrichrome family of siderophores are characterized by three hydroxamate groups linked to a cyclic hexapeptide (Figure 1, source ref. 6). Ferrichrome itself is produced by Ustilago sphaerogena, a fungus, but it is also utilized for iron transport by several bacteria that do not produce it, including Escherichia coli, Salmonella, and Arthrobacter flavescens (32). Other members of this family include ferrichrome A, ferrichrysin, ferrirubin, ferrirhodin, sake colorant A, and ferrichrome C. The antibiotic albomycins are also ferrichrome derivatives produced by Actinomyces grisens, and they are effective against bacteria which utilize ferrichrome for iron transport. Ferrichrome-type siderophores are commonly produced by Aspergillus, Penicillium, Neurospora, Ustilago, and other smut fungi, and one yeast species Cryptococcus which produces ferrichrome C (6).

Ferrichrome: $R = R' = R'' = H$; $R''' = CH_3-$

Ferrichrome A: $R = R' = HOCH_2-$;

Ferrichrysin: $R = R' = HOCH_2-$; $R'' = H$; $R''' = CH_3-$

Ferricrocin: $R = R'' = H$; $R' = HOCH_2-$; $R''' = CH_3-$ *

Ferrirubin: $R = R' = HOCH_2-$;

Ferrirhodin: $R = R' = HOCH_2-$;

Ferrichrome C: $R = R'' = H$; $R' = R''' = CH_3-$ *

Sake Colorant A: $R = HOCH_2-$; $R' = R''' = CH_3-$; $R'' = H$ *

Figure 1. Structure of ferrichrome-type siderophores.

Ferrichrome-type siderophores will form stable complexes with six coordinate metal ions which prefer octahedral geometry, with iron (III) being the most efficiently complexed metal ($K_f=10^{29.1}$ for ferrichrome). Ferrichrome complexes with chromic ions have been prepared in addition to aluminum and gallium complexes (33, 34). The chromic complex is kinetically inert because of the d^3 electronic configuration which leads to sizeable crystal field stabilization energy and slow ligand substitution (35). These chromic-desferri-ferrichrome derivatives have been useful in determining the absolute configuration of the possible coordination and optical isomers and the fate of the siderophore-metal complex during transport into cells (to be described below). In the case of the ferrichromes, the trans geometric isomer is unlikely to form due to steric hindrance, while both the cis Δ and Λ cis diastereomers are possible. Both solution methods (circular dichroism) and crystal structure data indicate that the Δ cis form predominates (35).

Two ferrichrome-family members are of special interest in that the ferric complexes of ferrichrome A, produced by Ustilago sphaerogena (36), and malonichrome, produced by Fusarium roseum (37), are not active as iron transport agents for the producing organism or for the test organism Arthrobater flavescens JG-9 which responds to nearly all siderophores. The biological function of these compounds

is still in question. The formation constant for ferri-chrome A is 10^{32} which is higher than most other natural hydroxamates (6). One possibility is that these compounds are designed to solubilize iron which can thereafter exchange with the functional siderophore and enter the cell. Also, both compounds are more acidic than the functional siderophores produced by the same organism. It has been suggested that this quality may be helpful in solubilizing iron minerals (37).

The ferrioxamine family of siderophores contains three hydroxamate groups inserted into a linear backbone as shown in Figure 2A which presents the structure of ferrioxamine B (38). The ferrioxamines are produced by a number of species of Streptomyces and Nocardia (39). Two members of this class, ferrioxamines E and D₂, undergo cyclization via amide bond formation between a terminal succinyl group and a terminal amino group (6). Certain ferrioxamine derivatives display antibiotic activity and are thus termed ferrimycins. As with the albomycins, the toxic agent is an extra group attached to a complete and functional iron transport agent (38). The ferrioxamines are devoid of optical activity and several have been prepared by chemical synthesis (40). One of these, the mesylate salt of ferrioxamine B (Desferal), is used in the treatment of iron toxicity and the iron overload associated with the transfusion treatment of Cooley's anemia and thalassemia (35).

The fusarinines are also trihydroxamate siderophores produced by Fusarium species (6). The structure of fusarinine B is shown in Figure 2B. Note the unusual aminoacyl ester linkages, and also the free amino groups which cause these compounds to be basic in contrast to the neutral ferrichromes. The amino acyl ester linkage is quite rare (amino acyl-tRNA being the notable exception) and is rapidly hydrolyzed under mild conditions (39).

The rhodotorulic acid (RA) group of siderophores are all dihydroxamate compounds that combine with two iron atoms to form a complex of the formula Fe_2RA_3 at pH values between four and eleven (41). The structure of rhodotorulic acid is shown in Figure 2C. These siderophores are produced by at least 52 different strains of yeast among the genera Aessosporoa, Cryptococcus, Leucosporidium, Rhodosporidium, Rhodoturula, Sporidiobolus, and Sporobolomyces (6). Perhaps the most striking feature common to this group are the prodigious yields of siderophore that can be isolated. While some siderophores are isolated with yields of only 1-10 milligrams per liter of culture fluid, and sometimes less, yields of 3-4 grams per liter are often obtained from RA producing cultures (42). Rhodotorulic acid is a potent growth factor for Arthobacter flavescens JG-9 and is also being tested for use in treatment of iron overload (35).

The family of catecholate siderophores is typified by enterobactin, also called enterochelin (Figure 3A).

A.

B.

XIII Fusarinines

XIIIa Fusarinine: $n = 1$ XIIIb Fusarinine A: $n = 2$ XIIIc Fusarinine B: $n = 3$ XIIId Fusarinine C (Deferrifusigen): $n = 3$, cyclo

C.

Rhodotorulic acid

Figure 2. Structure of ferrioxamine B, fusarinines, and rhodotorulic acid.

This siderophore is produced by Escherchia coli, Salmonella typhimurium, and Aerobacter aerogenes (now Klebsiella pneumoniae) (32). Enterobactin is sparingly soluble in water above pH 7 to 8 where the 2-hydroxyl group begins to ionize, the pKs of the catechol group being near 8.4 and 12.1 (23). At less than neutral pH, the molecule's ester linkages hydrolyse to form the monomer dihydroxybenzoyl serine.

The stability constant for ferric enterobactin has been obtained spectrophotometrically at pH 5, where a measurable distribution of iron between enterobactin and EDTA can be obtained (23). The stability constant is

$$K^* = \frac{[\text{Fe}(\text{ent})^{3-}] [\text{H}^+]^6}{[\text{Fe}^{3+}] [\text{H}_6 \text{ ent.}]} = 10^{-9.7}$$

Using the above values for the protonation constant and substituting for the hydrogen ion concentration, the formation constant (K_f) can be estimated as

$$K_f = \frac{[\text{Fe}(\text{ent})^{3-}]}{[\text{Fe}^{3+}] [\text{ent}^{6-}]} = 10^{52}$$

This is the largest formation constant of any known iron chelate, and is 7-12 log units larger than the log ₃ values of the bidentate catecholates. This "chelate effect" is much greater than the relatively small difference between the trihydroxamate siderophore chelates and

the $\log$ of triacetohydroxamate, which in the case of ferrichrome is 0.8 and for ferrioxamine B is 2.2 log units (6).

The enterobactin molecule is a trimer of 2,3-dehydroxybenzoyl serine joined through labile ester linkages between the serine hydroxyl and the carboxylate group of the adjacent serine (Figure 3). The monomers are often found in culture fluid together with the cyclic parent compound. A related compound 2,3 dihydroxybenzoyl glycine (also called itoic acid) has been isolated from low iron cultures of Bacillus subtilis (44). The compound facilitates iron transport in B. subtilis and is an example of a "monomeric" siderophore (6). Other phenolate siderophores are 2-N, 6-N-Di- (2,3-dihydroxybenzoyl)-L-lysine produced by Azotobacter vinelandii (25), and two linear tricatechols, parabactin produced by Paracoccus denitrificans (46) and agrobactin which is produced by Agrobacterium tumefaciens, the agent of crown gall disease in plants (47). These last two are also referred to as "spermidine" siderophores due to the presence of a residue of spermidine providing the linear backbone. Interestingly, the ferric ion is coordinated to the two distal catechols and the 2-(2-hydroxyphenyl) oxazoline residue (Figure 3), with an affinity very similar to enterobactin (48a).

Recently, experiments designed to determine if agrobactin is important to Agrobacterium tumefaciens invasion

Enterobactin

B.

1

Parabactin (R=H)

Agrobactin (R=OH)

C.

Fe(III)-Mycobactin P

Figure 3. Structures of catechol-type siderophores.

of plant tissue through chelation of iron present in the plant, were conducted. Surprisingly, no agrobactin could be detected in the diseased plant tissue and the bacteria may have been utilizing citrate coordinated iron provided by the plant (48a).

The mycobactin family of siderophores is unique in that all are lipophilic molecules that are strictly cell membrane associated and, thus, may behave as Fe(III) ionophores (49). The structure of mycobactin P is shown in Figure 3. All members of this group are dihydroxamate molecules with the fifth and sixth ligands provided by the phenolic hydroxyl and the nitrogen of the oxazoline ring. Estimates of the binding affinity of mycobactin P suggest that it is higher than that of ferrioxamine B (49). The chromium, aluminum, gallium, vanadium, and copper complexes with mycobactin P have all been prepared and the chromium complex has been found to reverse the growth promoting effect of mycobactin for M.paratuberculosis (50). Seven distinct groups of mycobacteria are known to produce mycobactins peculiar to that group. This property has been utilized for taxonomic purposes whereby thin-layer chromatography of cell extracts from low iron media is used to identify the mycobactin produced (51).

The last family of siderophores to be considered is the aerobactin group. This family includes the siderophore under investigation in this study, schizokinen, which is produced by the cyanobacterium Anabaena (strain ATCC

Figure 4. Structures of citrate-hydroxamate siderophores including schizokinen.

#27898) and also the Gram positive bacterium, Bacillus megaterium (8, 52). Arthrobactin (also called terregens factor) produced by Arthrobacter terregens (53), and aerobactin produced by Aerobactin aerogenes 62-1 complete this group (54). All of these compounds possess two hydroxamate-containing sidearms connected through amide bonds to the terminal carboxyls of a citric acid residue (Figure 4). The coordination of the ferric ion is provided by the four hydroxamate oxygens and the carboxyl and hydroxyl oxygens from the citrate moiety (6, 45-46). These compounds all display a pH dependent spectral shift to the red below pH 7.0, due to the protonation of the citrate hydroxyl and carboxyl groups. The absorbance maxima for aerobactin and schizokinen at pH 7 are 398nm and 395nm respectively, with aerobactin existing as a trivalent anion at physiological pH while schizokinen is a monovalent anion in its biologically active form. These siderophores are effective chelators of iron over a broad pH range, with only 3.5% of the total iron in the form of the free hexaaquo-ferric ion at pH 2.3 for the aerobactin species (and essentially no free iron above pH 2.7) (45). Above pH 4-5, the additional coordination by the citrate hydroxyl provides extra stability to the complex throughout the neutral and alkaline pH regions. Even so, the formation constant for ferri-aerobactin ($\log K = 22.9$) is 6-9 orders of magnitude smaller than the trihydroxamate siderophores discussed above.

Recently, several iron binding compounds have been isolated that contain sulfur ligands. Their synthesis is repressed by iron which strongly suggests that they are true siderophores. The thiohydroxamate group has been found in Pseudomonas fluorescens filtrates attached to the parent compound thioformin (57). These compounds form very stable copper(II) and iron(III) complexes and also display potent antibiotic activity. Another sulfur containing growth promoter that chelates iron has been isolated from Pseudomonas aeruginosa, and named pyochelin (58).

The literature on siderophores from higher plants and animals is very sparse. However, at least one siderophore/ionophore-like substance able to bind iron has been isolated from mammalian cells transformed by the SV 40 virus. When tissue cultures are given picolinic acid their growth is interrupted due to interference with their iron metabolism. Mutant cells that can overcome the interference of picolinic acid produce a heat stable, trypsin-insensitive peptide capable of binding iron (59). A provocative possibility is that cancer cells may secrete siderophore-like substances in vivo which satisfy the metal requirements of the neoplasm (60).

Siderophore-Dependent Iron Transport Systems

Iron transport via siderophore-dependent uptake systems has been the subject of a great deal of research in the last decade and several reviews have been published to

cover this material (32, 61-63). In general these systems are highly specific for a given siderophore and require the input of metabolic energy to operate. In many cases an organism will maintain, in addition to an uptake system for its native siderophore, one or more alternative transport systems for siderophores it doesn't produce but may encounter in the environment. Other organisms, notably the soil bacterium Arthrobacter flavescens JG-9, do not produce any siderophores of their own but utilize a great variety of naturally occurring siderophores for iron uptake (see Table 2 below). At least two siderophore systems have been located on genes carried by extrachromosomal elements (plasmids), which may help explain how such a great variety of organisms have come to utilize common siderophores (64, 65).

By far the most well characterized iron transport systems are those of the enteric bacterium Escherichia coli. This organism utilizes at least four separate and genetically distinct systems that recognize ferrienterobactin, ferrichrome, ferric citrate, and a low affinity system for which no ligand is known (62). [The recently discovered aerobactin system coded by the Col V plasmid (65) may constitute a fifth system or ferric aerobactin may enter via one of the other transport systems (66)]. A model for these uptake systems is shown in Figure 5.

Figure 5. Summary of membrane uptake systems for specific metal complexes in *E. coli*. Membrane receptor genes have been identified by uptake-defective mutants which have lost particular membrane proteins on SDS polyacrylamide gels. Infective or inhibitory agents are ones which have evolved to take advantage of pre-existing membrane receptors. Compilation from references 32, 62-70.

The enterobactin system is the most well characterized of the four transport systems in E. coli. At least thirteen genes are known to be involved in the enterobactin cycle and genetic mapping studies are well under way (62). The cycle begins with the formation of enterobactin and its release from the interior of the cell. These functions are controlled by the seven ent genes (A-G). Transport of ferrienterobactin across the outer membrane requires the presence of inducible outer membrane proteins, in particular the fep B gene product and a protein coded by the ton B gene, which is required for all except the low affinity iron uptake system. The function of the ton B protein remains an open question, as does its location. A putative ton B gene product has been identified in E. coli minicells (which do not have a chromosome) containing a ton B hybrid plasmid (67), and may be located on the cytoplasmic rather than the outer membrane (68). Three other outer membrane mutants affecting iron-siderophore transport have been isolated on the basis of resistance to phages or colicins which utilize those proteins for receptors (62). Little is known about how these proteins facilitate passage across the outer membrane, although a porin-type structure may be involved. Ferrienterobactin transport across the inner membrane occurs via a specific energy-requiring permease controlled by the fep gene (69). This process requires an energized membrane as evidenced by the sensitivity of the process to uncouplers, azide, and cyanide. Transport

activity is also sensitive to arsenate and iodoacetic acid, indicating an ATP requirement for transport. Once inside the cell, the enterobactin molecule is hydrolyzed to three residues of dihydroxybenzoyl serine (DBS). This reduces the "chelate effect" and reduces the redox potential of the Fe(III) atom into the range of physiological reductants, making possible the release of the iron for cellular proteins (70). These DBS monomers are not reutilized for enterobactin synthesis, but are released to the medium.

The ferrichrome transport system in E. coli, like the enterobactin system, requires an outer membrane receptor (fhu A) and the ton B gene product. It is also thought the entire complex is transported inside the cell since [⁵⁵Fe] ferrichrome and the kinetically inert Δ -cis-chromic [³H] deferriferrichrome enter the cell at identical rates (71). Evidence for a single hydroxamate permease in the cytoplasmic membrane, necessary for transport of all hydroxamate siderophores following their receptor mediated passage across the outer membrane, has been published (66). The transport of ferrichrome by inner membrane vesicles is known to require an energized membrane but not direct ATP bond energy. Uptake activity was inhibited by the ionophore valinomycin which collapses the electrical component of the electro-chemical membrane potential (72). A symport mechanism of transport has been proposed based on the presence of a binding site for divalent cations on the ferrichrome molecule (73). This

provides a net +2 charge to ferrichrome, enabling it to respond to the electrical component of the electrochemical gradient (interior negative).

Like ferrienterobactin, which is modified by ligand hydrolysis to help release the tightly bound iron atom, ferrichrome is modified during transport to a form which does not serve as an effective iron chelator. This occurs by the addition of one mole of acetate per mole ferri-chrome, via ester linkage to a hydroxamate -OH group (74). Because deferri-ferrichrome is also modified by cells with a functional uptake system, it is possible that the iron is released by reduction before modification takes place. This modification has also been demonstrated during ferri-chrome transport by Salmonella typhimurium LT-2 (75). The modified product can be converted back to ferrichrome by mild hydrolysis in acid or base. The modification of both enterobactin and ferrichrome would seem to serve two purposes. It would facilitate removal of iron from the extremely stable ferrisiderophore complexes for cellular use, and also be a mechanism by which cells rid themselves of a potentially deleterious ligand for iron in the cytoplasm. (74)

The third iron transport system in E. coli is strictly inducible and utilizes citric acid as an iron chelator and transport intermediate (70, 76). The detection of this system was greatly aided by the availability of mutants incapable of synthesis of the native siderophore,

enterobactin. The addition of purified enterobactin represses the citrate system, which is known to involve a new outer membrane receptor.

The low affinity iron transport system of E. coli is a non-energy-dependent event that is thought to involve a membrane carrier but has not been saturatable under normal assay conditions (62, 77). This activity can only be measured when the enterobactin, hydroxamate, and citrate systems are blocked due to mutation or lack of substrate. In addition, the presence of the iron chelator nitrilotriacetate (100 uM) which binds Fe (III) tightly, will block the low affinity system.

Another siderophore transport system that is relevant to the present study is the ferrischizokinen system in Bacillus megaterium, a Gram positive bacterium. This organism produces only schizokinen but also makes use of ferrioxamine B as an iron transport intermediate, while aerobactin, a close structural relative of schizokinen is not utilized (78). Ferrischizokinen uptake is an energy-dependent process, however, the exact site of energy coupling may involve iron incorporation into cellular acceptors rather than transport per se. Byers and co-workers have postulated a facilitated-diffusion type process with uptake being driven by energy-dependent incorporation of iron into cellular macromolecules (79). The ferrischizokinen complex is thought to enter the cell intact from studies of transport using [^{59}Fe , ^3H]-ferrischizokinen

(78). Competitive binding experiments with B. megaterium vesicle preparations that display no transport activity have shown that separate receptors for ferri-schizokinen and ferrioxamine B exist on the cell surface (80). Scatchard analyses revealed that the ferrioxamine B receptor had a higher affinity for its substrate than the schizokinen receptor, but a much smaller maximum binding capacity (one eighth as large as the schizokinen receptor). It is not clear whether these receptors led to separate transport systems or converge to a single carrier protein.

At least one report of iron transport studies on a cyanobacterium has appeared in the literature. Iron assimilation in the marine cyanobacterium Gomphosphaeria aponina was studied because iron metabolism was postulated to be important in the proliferation of this organism, thought to be antagonistic towards the Florida red tide dinoflagellate Pytochodiscus brevis (81). Iron transport was biphasic (absorption and uptake) and first-order with respect to iron (added as $^{59}\text{FeEDTA}$). Hydroxamate-containing siderophores were not involved as revealed by the absence of a positive response to chemical (Csaky) or biological (Arthrobacter JG-9) assays.

EXPERIMENTAL

GROWTH MEDIA AND CULTURE CONDITIONS

Cyanobacteria

Anabaena sp (#27898) was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). This strain is also on deposit with the Pasteur culture collection (#6411) and the University of Texas Culture Collection (UTEX #1597) where it is named as Nostoc sp (82). It will be referred to in this report by the PCC number 6411. The strain was originally isolated from California pond water by M. M. Allen (82). Another species used in this study previously identified as a Nostoc species is Anabaena 7120 which was kindly provided by R. Haselkorn. This strain is also on deposit with the Pasteur Culture Collection (#7120) and ATCC (#17893); its source is unknown. Anabaena cylindrica Lemm., obtained from ATCC (#27899), was isolated by S. P. Chu from pond water, Cambridge, England in 1939 (83).

For routine maintenance of the Anabaena species, medium BG-11 was used (84). The composition of this medium is listed in Table I. The medium was normally made up in advance and stored in acid washed polyethylene bottles at 4°C. The Mg and Ca salts were prepared as a separate 10X

TABLE I
MEDIA FOR CYANOBACTERIA

<u>Complete BG-11 (pH 7.1)</u> (per liter, distilled-deionized water)		<u>A5 Trace Metal Mix</u> (per liter, distilled-deionized water)	
NaNO ₃	1.5 gram	H ₃ BO ₃	2.86 grams
K ₂ HPO ₄	0.04 g.	MnCl ₂ ·4H ₂ O	1.81 g.
MgSO ₄ ·7H ₂ O	0.075 g.	ZnSO ₄ ·7H ₂ O	0.222 g.
CaCl ₂ ·2H ₂ O	0.036 g.	Na ₂ MoO ₄ ·2H ₂ O	0.390 g.
Citric acid	6.0 mg.	CuSO ₄	0.051 g.
Ferric ammonium citrate	6.0 mg.	Co(NO ₃) ₂ ·6H ₂ O	0.049 g.
EDTA	1 mg.		
Na ₂ CO ₃	0.02 mg.		
A5 Trace Metal Mix	1.0 ml.		
<u>Uptake Medium* (pH 7.0)</u> (per liter, distilled-deionized water)			
NaNO ₃	1.10 gram		
NH ₄ Cl	0.05 g.		
K ₂ HPO ₄	0.25 g.		
MgSO ₄ ·7H ₂ O	0.531 g.		
CaCl ₂ ·2H ₂ O	0.058 g.		

*Uptake medium was prepared as two stock solutions. A 100X solution of NaNO₃, NH₄Cl, and K₂HPO₄ was treated with Chelex 100 (Bio Rad) to remove endogenous iron. A 10X solution of Mg + Ca was prepared separately and added to the Chelex-treated nutrients just prior to filter sterilization as a complete 1X solution.

solution. The other reagents were dissolved at 10X concentrations in the order listed in approximately 800 ml of glass-distilled, demineralized water (Milli-Q system, Millipore Corporation). It was important to solubilize each reagent before the addition of the next to insure complete dissolution of all components. The media was then titrated to pH 7.1 and diluted to 1 liter. The A5 trace metal mix was stored as a 1000X solution. The media was

sterilized immediately prior to use by passage through 0.45 μm filters (Millex, Millipore Corporation).

For growth on solid media, BG-11 was supplemented with 1.5% agar (Difco) separately sterilized by autoclaving, and cooled to 45° C before mixing with complete filter-sterilized BG-11 also at 45°C (85). Slants or plates thus prepared, when inoculated with a growing culture of Anabaena 6411, showed visible growth within three to four days at 34° C and continuous light (2500 lux). Maintenance slants were shifted from these conditions after one week and left on a bench top at room temperature and out of direct sunlight. These slant cultures remained viable for up to one year although stocks were routinely transferred every six months.

A further modification of the standard BG-11 media was made for studies of iron metabolism. The chelators, EDTA and citric acid, were omitted unless otherwise noted, as was the ferric ammonium citrate. A stock solution of 0.50 mM FeCl_3 in 10mM HNO_3 was used to make iron additions to the modified BG-11 immediately prior to filter sterilization. This modified BG-11 will be referred to as BG-11(mc) (minus chelators). The residual iron concentration of BG-11 media (minus Fe) was estimated using atomic absorption spectroscopy on concentrated stock solutions at 1×10^{-8} M. While the residual iron in BG-11(mc) was not determined it is probably equal to or less than the value for complete BG-11.

In order to avoid iron introduced by glassware, all vessels used for growth, iron uptake assays, or media storage and transfer were rinsed in 6N HCl, followed by extensive rinsing with distilled-demineralized (Milli-Q) water.

All Anabaena species were routinely grown in continuous light provided by two cool white fluorescent bulbs (Sylvania) approximately 12 inches from the surface of the culture medium. The light intensity was measured at 2500 lux with a light meter (Westinghouse) at the same distance from the light source as the media surface. Reflective white paper was placed underneath the cultures to increase reflectivity. The incubator was maintained at 34° C.

To prepare an inoculum for an Anabaena culture, filaments gathered from the surface of an agar slant were added aseptically to BG-11(mc) supplemented with 0.1 μ M Fe(III) and grown until the absorbance was between 100 and 200 Klett units (see below). An aliquot of this inoculum was then added to a 25 ml test culture to achieve 10 to 20 Klett units. The inoculum was not normally washed since the presence of buoyant gas vacuoles inside the cells makes it difficult to obtain a good pellet of Anabaena filaments by centrifugation.

Turbidity, as determined in a Klett-Summerson colorimeter, was chosen as a convenient measure of growth for Anabaena 6411 and 7120. Both of these strains grow as

an even suspension in liquid culture (82), although a tendency for clumping was noted in the second transfer of 6411 in media free of combined nitrogen. Most cultures were grown in 125 ml sidearm flasks that enabled turbidity measurements without opening the flask and risking contamination. The colorimeter was fitted with a green (#54) filter, unless otherwise noted, so that changes in chlorophyll pigmentation observed in strain 6411 as a function of added iron would have a minimal effect on the turbidity measurements. Several alternative methods for growth measurements were considered but rejected due to lower accuracy. For example, dry weight determinations had only a small linear region between 30 to 90 Klett units, and were also variable in absolute terms from culture to culture, presumably due to differences in extracellular polysaccharide. Viable counts are difficult to determine reproducibly with filamentous organisms since it is necessary to sonically disrupt the filaments to form single cells which can give rise to countable isolated colonies on agar plates. This sonication can disrupt or break some cells and leave others as doubles or triples which reduces the accuracy of the procedure. Direct microscopic counting was considered excessively tedious and a Coulter counter was not available on a routine basis.

Two different tests were used to examine for bacterial contamination of Anabaena strains. The most important test was careful microscopic examination of aging cultures

in which the cyanobacteria have begun to lyse, as the presence of contaminating bacteria is very evident under these conditions (84). It was noted that very small granules were liberated by lysed cells. These granules move very rapidly via brownian motion but can be distinguished from bacteria by their smaller size and their similarity to granules present inside intact cells. It is possible that these are cyanophycin granules known to be present in many cyanobacteria (86). The second test used to determine algal purity was incubation of 0.1 ml of an aged culture on a standard nutrient agar plate at 37° C (84). The lack of bacterial colonies on complex media should be considered as necessary but not sufficient criteria of an axenic culture of Anabaena, due to the great variety of possible contaminants.

One contaminating bacterium was isolated from Anabaena 6411, a small gram positive rod-shaped organism discovered approximately six months after work with Anabaena 6411 had begun. This organism was detected as small pink colonies on a spread plate of Anabaena 6411 on complete BG-11 agar. This organism grew well on BG-11 plates with and without associated Anabaena, and has not been identified to date. The organism gave negative Csaky assays for hydroxamate siderophores and did not take up [⁵⁵ Fe] ferrischizokinen. Treatment of the contaminated culture in the dark with phenol using the procedure of Gorham and Chermichael (87), and plating on BG-11 agar

produced agar cores containing axenic filaments of Anabaena. However, due to fears that the phenol treatment may have damaged the Anabaena, a new culture of 6411 was obtained from the American Type Culture Collection. All work presented in this report concerning Anabaena 6411 was performed with this new culture.

Growth experiments with another growth medium for cyanobacteria were performed before deciding to use BG-11(mc) as a standard growth medium. Simpson and Neilands (8) used the uptake medium listed in Table I for growing Anabaena 6411. We found that while 6411 did grow on this medium, several modifications were required before growth would equal that demonstrated by 6411 with BG-11(mc). As shown in Figure 6, it was necessary to bubble this medium with 1% CO₂, 99% nitrogen to achieve maximum growth rates. However, bubbling BG-11 medium with this mixture prevented cells from growing at all. The uptake medium was also supplemented with the A5 trace metal mixture (1.0 ml/liter). Temperature and light conditions were as used for BG-11(mc) culture. While both of these media have the advantage of no chelators present, the overall ease of working with BG11(mc), and the higher growth yields routinely obtained with BG-11(mc) resulted in our choosing that medium for routine growth studies. With the exception of the experiments designed to determine the K_m for ⁵⁵Fe-schizokinen uptake (see below), all work

Figure 6. Growth of *Anabaena* 6411 in uptake medium and BG-11(mc). Iron ($1.0 \mu\text{M}$) was added to each culture immediately prior to filter sterilization. ($\square$ — $\square$) uptake medium without 1% CO_2 , 99% N_2 bubbling. (Δ — Δ) uptake medium with 1% CO_2 , 99% N_2 bubbling (approximately 10 ml/minute). ($\circ$ — $\circ$) standard BG-11(mc) medium.

reported here was performed with BG-11(mc) grown cells. The Km experiments were performed on cells grown in both uptake medium and BG-11(mc) with no detectable difference in the results.

Culture Conditions for *Arthrobacter flavescens* JG-9

Arthrobacter flavescens JG-9 is a siderophore-requiring (auxotrophic) strain that can be used as an assay for a variety of siderophores (32). A partial list of siderophores with growth factor activity for JG-9 appears in Table II.

The organism was grown at 30° C on slants or streak plates of maintenance medium (Table III) containing 20 micrograms Desferal per liter. Desferal is the methane sulfonate salt of ferrioxamine B (Ciba Pharmaceuticals) and yields free ferrioxamine B upon dissolution in water. Liquid cultures were grown at 30° C in JG-9 assay medium (Table III) in 15x180 mm culture tubes in a rotary shaker set at 200 rpm. The details of the assay for siderophore activity will be discussed below.

TABLE II

COMPOUNDS THAT SATISFY GROWTH REQUIREMENT
OF ARTHROBACTER FLAVESCENS JG-9 ^a

Compound	Concentrations ($\mu\text{g/ml}$) for half maximal growth
<u>Ferrichromes</u>	
ferrichrome	0.0003
ferrirubin	0.02
albomycin	3.0
<u>Aerobactins</u>	
arthrobactin	0.004
schizokinen	0.05
<u>Ferrioxamines</u>	
ferrioxamine B	0.0005
<u>Mycobactins</u>	
mycobactin	0.008
<u>Rhodotorulic acids</u>	
rhodotorulic acid	0.0002
coprogen	0.007
<u>Heme compounds</u>	
protohemin IX	0.08
coprohemin III	0.1
<u>Catechols</u>	
dihydroxybenzoylserine	10.0

^a Taken from Lankford (32).

TABLE III
MEDIA FOR BACTERIAL SPECIES

JG-9 Maintenance Medium (pH 7.4 before autoclaving)
(per liter distilled-deionized water)

Peptone	10 grams
Yeast extract	10 g.
K ₂ HPO ₄	2 g.
Desferal	20 micrograms
agar	30 g.

JG-9 Assay Medium (pH 7.6 before autoclaving)
(per liter distilled deionized water)

K ₂ HPO ₄	2.0 grams
(NH ₄) ₂ HPO ₄	0.5 g.
Yeast extract	4.0 g.
MgSO ₄ ·7H ₂ O	0.10 g.
Casamino acids	1.0 g.
Sucrose	10 g. (autoclave separately)

Bacillus megaterium SK Production Medium (pH 7.0)
(per liter distilled-deionized water)

K ₂ SO ₄	1.0 gram
K ₂ HPO ₄	3.0 g.
NH ₄ Acetate	3.0 g.
Sucrose	10 g.
L-Arginine .HCl	1.5 g.
MgSO ₄ ·7H ₂ O	0.800 g.
ZnSO ₄ ·7H ₂ O	8.6 mg.
MnSO ₄ ·H ₂ O	0.11 mg.

Culture Conditions for SK Production by Bacillus Megaterium

Bacillus megaterium (ATCC #19213) was used as a source of schizokinen instead of Anabaena 6411 due to its faster growth rate and ease of handling large scale cultures. B. megaterium was grown in the medium of Mullis et.al. (52) with a decreased sucrose concentration to minimize caramelization during Csaky assays. To prepare this medium with a minimal iron concentration, all components except the Mg, Zn, and Mn were dissolved at 10X concentrations in distilled-deionized water and run through a column of Chelex 100 resin (Bio Rad) in the sodium form to remove endogenous Fe. The metals were added to the deferrated medium and the pH adjusted to 7.0 prior to filter sterilization (0.22 μ m, Millipore). The residual iron concentration after this treatment was estimated to be less than $0.5 \times 10^{-8} \text{M}$ according to the growth response at different concentrations of added iron. On several occasions the medium was autoclaved in which case the sucrose was treated with Chelex 100 separately and autoclaved separately from the other non-metal salts before addition of the filter sterilized metals.

Large batch cultures of B. megaterium were grown to produce 7 to 10 liters of culture medium for a schizokinen preparation. Usually 500 ml cultures in 2.0 liter acid-washed erlenmeyer flasks were employed. The incubator was set at 37° C and highest shaker speed available since

heavy aeration is necessary to maximize schizokinen yields (52). An eight liter batch culture in a 10 liter carboy was grown once with agitation by a three inch magnetic stirring bar and a stream of forced sterile air. However, problems associated with carboy cultures including sterility control and inadequate aeration discouraged the use of this approach.

SCHIZOKINEN PRODUCTION FROM Bacillus megaterium

Bacillus megaterium was grown with several iron concentrations to determine the optimum level of added iron to maximize schizokinen yields. This experiment was necessary because higher schizokinen production levels per cell at the lower iron concentrations were offset by low cell numbers. The optimum level of iron was found to be about 0.5 μM Fe(III) added. At this concentration approximately 95 $\mu\text{g/ml}$ schizokinen was detected via the Csaky assay after 72 hours (below).

The batch cultures were harvested by centrifugation in acid washed 250 ml plastic centrifuge bottles using a GSA rotor and a Sorval RC2-B centrifuge set at 6,000 rpm for twenty minutes. The supernatants were pooled and stored frozen in acid washed polyethylene bottles. The combined supernatants were concentrated by rotary evaporation to approximately 200 ml. This procedure was greatly accelerated by running a polyethylene glycol solution at

2-4° C through the condensing coils of the Rotovap, and heating the bulk solution to 37° C.

Organic Extraction of Concentrated Supernatants

The first step in the purification of schizokinen is an extraction at low pH into a 50/50 (wt/wt) mixture of chloroform and phenol (52). The concentrated supernatant was first adjusted to pH 2.0 with concentrated HCl. Care must be taken to avoid large local HCl concentrations by vigorous stirring while adding the acid because of the tendency of schizokinen to break down at low pH. For this reason, it is also important to immediately begin the extraction after pH adjustment, and to readjust the pH after extraction to pH 4 or 5.

For 200 ml of concentrated medium at pH 2.0, 500 ml of CHCl_3 /phenol should be used in three batches of approximately 170 ml each for the complete removal of iron-reactive material (as judged by color formation with FeCl_3). The addition of two liters of ethyl ether to the combined organic layers will make them sufficiently non-polar for the schizokinen to be reextracted into distilled-deionized water (3 X 100 ml). The crude schizokinen solution is then concentrated by rotary-evaporation to about 50 ml.

Ferrischizokinen was found to be extractable by the above procedure as it also carries a net charge of zero at pH 2. Thus, it would be possible to purify schizokinen as

the ferric-complex, and then remove the iron by treatment with 8-hydroxyquinoline (see below). The primary advantage would be direct visualization of the red-colored complex during purification. However, all schizokinen preparations to date have been purified as iron-free schizokinen.

Purification of Schizokinen by Column Chromotography

Crude extracts of schizokinen from B. megaterium were partially purified on a column of the anion exchanger AG-2-X10 (Bio Rad). The resin was first converted to the acetate counter-ion from the chloride form as follows. A 22 X 2.5 cm column of AG-2-X10 in the chloride form was treated with two volumes of 1N NaOH to convert the resin to hydroxide counter-ions followed by a four volume distilled-deionized water rinse. The hydroxide ions were then neutralized by the addition of 4 volumes of 1 M acetic acid which completes the conversion. The column must be rinsed with 4 volumes of water and further treated with 100 ml of 0.1 M Tris buffer [tri-(hydroxymethyl) amino-methane] at pH 8.0 and a 2 volume water rinse. This is to insure that the resin is above pH 5.0. Without this final pH adjustment a significant fraction of schizokinen elutes with the void volume. This is likely due to protonation of the citrate carboxyl group of SK which produces a neutral molecule that will not bind to the column.

The crude schizokinen was loaded on to the column at pH 7.0 and rinsed in with 100 ml of distilled-deionized

water. Sample volumes of 50 to 100 ml have been used successfully. The bound SK was eluted with a linear gradient of NH_4Cl prepared with a gradient maker (Pharmacia) containing 250 ml of 0.1 M NH_4Cl in the mixing chamber and 250 ml of 1.0 M NH_4Cl in the second chamber. An elution profile for such a column is shown in Figure 7.

Several methods for detecting eluted schizokinen have been used. Two methods were based on the formation of a colored product upon addition of iron to an aliquot from each fraction. In early work a 0.10 ml aliquot was added to 0.90 ml of water containing 25 microliters of 0.1% FeCl_3 in dilute HCl . Under these conditions the absorbance maximum was at 440 nm. However, the variability of the final pH and light scattering caused by precipitated excess iron sometimes resulted in confusing elution profiles. A better procedure is to add a 0.1 ml aliquot of each fraction to 0.9 ml of 5 mM FeCl_3 in 0.14 M HClO_4 . This results in a constant low pH and an absorbance maximum at 490 nm without any precipitation problems (90). The Csaky assay (see below for experimental details) was also used to detect hydroxamate groups in column effluents. In all cases tested the hydroxamate peak identified by Csaky coincided with the ferric-positive peak. As a result the $\text{Fe(III)}\text{-HClO}_4$ reagent became the method of choice for schizokinen detection.

Figure 7. Anion exchange chromatography of crude schizokinen on BioRad AG-2-X10 resin (acetate form) pH 5.0 in 2.5 X 25 cm column. 100 ml sample followed by 100 ml distilled water rinse. Anionic material was then eluted with a linear gradient of 0.1-1.0 M NH₄Cl. Fractions were analyzed for hydroxamic acid using Csaky assay (A₅₄₃). Fraction volume = 7.2 ml.

A hydroxamate positive peak that elutes with the void volume on AG-2-X10 columns was always noted from B. megaterium filtrates and was also reported by Simpson and Neilands from Anabaena filtrates (8). This material is thought to be a neutral decomposition product of schizokinen, the structure of which is unknown (52).

The anionic ferric positive material (schizokinen) was desalted by either repeating the chloroform phenol extraction or passage through a gel filtration column of BioGel P2 (Bio Rad) or Sephadex G-10 (Pharmacia).

Purified schizokinen was prepared by careful fractionation of the eluant from a BioGel P2 column (run subsequent to the desalting procedure used after the AG-2-X10 column). Figure 8 presents an elution profile from a P-2 column analyzed by iron binding (A_{440}) or with the Csaky assay (A_{543}). Pure schizokinen was obtained from fractions 31-40 as determined by proton NMR spectroscopy. The NMR spectrum of these fractions together with a published spectrum of authentic schizokinen is shown in Figure 9. It has been our experience that the late fractions from the ferric-positive peak are the most likely to be contaminated. It is usually necessary to obtain NMR spectra on each of the trailing fractions prior to pooling to avoid including contaminated material. The elution volume of pure schizokinen was between 46 and 54 percent of the bed volume of the P-2 column.

Figure 8. Gel permeation chromatography of schizokinen AG-2-X10 column. Bio Gel P2 column (1.5 X 80 cm) eluted with water. (●—●) hydroxamates by Csaky assay (A₅₄₃) and (▲—▲) iron binding assay (A₄₄₀). [0.10 ml each fraction, 0.9 ml H₂O, and 25 μ l 0.5% FeCl₃ in 0.1N HCl]. Fraction volume 2.3 ml. Fractions 31-40 contained pure schizokinen.

Figure 9.

NMR spectra of schizokinen. Peak assignments:
a, methylene protons adjacent to hydroxamates.
b, methylene protons adjacent to amides.
c, methylene protons of citric acid residue.
d, methyl protons of terminal acetyl groups.
e, inner methylene protons of n-propyl chains.

SCHIZOKINEN

NMR SPECTRUM OF SK FROM
ANABAENA SP. SIMPSON AND NEILANDS
J. PHYCOL. 12:48 (1976)
 STRAIN: ATCC# 27898, PCC# 6411

NMR SPECTRUM OF SK USED IN
 UPTAKE ASSAYS. SOURCE:
BACILLUS MEGATERIUM ATCC# 19213

Characterization of Schizokinen

Purified iron-schizokinen was characterized spectroscopically to demonstrate that our isolate displayed a shift toward the red in the wavelength of maximum absorbance with decreasing pH as expected of citrate-type siderophores (35). As shown in Figure 10, Fe-SK produces a broad visible absorbance peak centered about 390 nm at pH 7, which shifts to 450 nm at pH 2.3. By contrast, the aerobactin complex with iron has a maximal absorbance at 399 nm at pH 7.0 (55).

The relative stability of the absorbance peak position near pH 7 was utilized for quantitation of purified schizokinen preparations. Spectrophotometric titrations with standard iron (III) solutions (Harleco Chemicals, 1000 parts per million) were carried out by adding small aliquots of iron (17.9 mM) in dilute HCl to a known volume of the schizokinen stock solution. The pH was then readjusted with 0.1N NaOH to pH 6.8-7.2 and the spectrum from 350-450 nm recorded after each addition. A typical plot of the absorbance at 390 nm versus added iron is shown in Figure 11. Using the volume corrected absorbance at the equivalence point, a molar extinction coefficient of 2050 liter mol⁻¹cm⁻¹ was calculated. The comparable value for ferric aerobactin is 2170 liter mol⁻¹cm⁻¹ (55). Using the standardized schizokinen solution we also calculated an extinction coefficient for ferric schizokinen in the 5mM Fe(III)-HClO₄ reagent used to detect schizokinen

Figure 10.

Visible absorption spectrum of ferric schizokinen as a function of pH. Concentration of pH 6.7 sample is 0.2 mM. Absorbances are not corrected for small changes in concentration due to the addition of 0.1 or 1.0 M HCl.

Figure 11. Spectrophotometric titration of schizokinen with FeCl₃ (17.9 mM) in dilute HCl. Sample was titrated to pH 6.8-7.2 after each addition and the spectrum from 500-350 nm recorded five minutes after each addition. Total volume initially 2.50 ml. Change in volume at equivalence point + 0.280 ml.

in the chromatography work. At 490 nm an extinction of 6.2 mg/ml or 2600 liter mol⁻¹cm⁻¹ was calculated. This latter value is actually more useful for routine work because of the problems associated with trying to saturate a schizokinen-containing solution with iron at pH 7 without creating a Fe(OH)₃ precipitate.

It has been consistently noted that schizokinen is not stable at low pH values (less than 2.5). An NMR spectrum of schizokinen left near pH 2 for three days at room temperature is included for reference (Figure 12).

Modifications of Schizokinen

Iron was removed from ferric schizokinen using the general procedure described by Lucky et.al. (94). Ferric schizokinen was lyophilized then dissolved in 2.5 ml of methanol containing a fifty-fold molar excess of 8-hydroxyquinoline. The mixture was left at 25° C for one hour, then the methanol was evaporated in a gentle (cotton-filtered) air stream. The residue was taken up in 5.0 ml H₂O and extracted with 2 X 5 ml CHCl₃. The colorless water layer contained pure schizokinen as judged by NMR spectroscopy. Recovery according to an iron binding assay was 30%.

Acetyl-schizokinen was prepared by dissolving deferri-schizokinen in pyridine (2.0 ml) and adding a 2-fold molar excess of acetic anhydride with stirring. The mixture was left overnight before the addition of concentrated NH₄OH to a final concentration of 3N. After three hours, the

Figure 12

NMR spectrum of acid-hydrolyzed schizokinen in D_2O . The solution was left for three days at pH 2, then adjusted to pH 7.8 prior to lyophilization. The residue was dissolved in 0.4 ml D_2O for spectrum. Hydrolysis of the terminal acetyl group is evident from the decrease in the 2.1 ppm terminal methyl proton resonance and the presence of a new resonance at 1.8 ppm attributable to free acetate. A further change is the decreased intensity of the 3.6 ppm triplet and appearance of a new multiplet at 2.9 ppm for the methylene protons adjacent to the hydroxamate.

mixture was evaporated to dryness under a stream of nitrogen to volatilize most of the pyridine and ammonium acetate. The material was then dissolved in distilled water, applied to a Sephadex G-10 column, and eluted with distilled water. The product was identified by NMR spectroscopy (Figure 13).

Several attempts were made to remove the terminal acetyl groups of the hydroxamate-containing arms of the schizokinen molecule by acid hydrolysis in hopes of replacing the acetyl moieties with radiolabelled acetic anhydride. However, even under mild conditions (20 minutes at 60° C in 0.1N HCl) the hydrolysis resulted in additional modifications which could be detected in the NMR spectrum. Reacetylation with acetic anhydride (as above) yielded a mixture of products which were not separable by gel filtration chromatography.

AEROBACTIN FROM *Aerobacter aerogenes*

A sample of aerobactin (Figure 4) was generously provided by Dr. J. B. Neillands of the University of California. A portion of the sample was tested with the Fe(III)-HClO₄ reagent and using the extinction coefficient for schizokinen it was calculated that approximately 1.5 mg of aerobactin was present.

The sample was then run through a Sephadex G-10 column to remove any salts or impurities present. The aerobactin eluted in a peak centered at 57.6 ml from a

Figure 13

NMR spectrum of acetyl-schizokinen in D₂O. The spectrum shows a new acetyl methyl resonance at 2.05 ppm which is in a slightly less electron-withdrawing environment than the 2.1 ppm methyl groups of the terminal acetates. The expected 1:2 intensity ratio for the methyl protons at 2.05 and 2.1 ppm is observed. Furthermore, the citrate methylene protons have shifted from 2.6 to 2.9 ppm as expected for the addition of an electron-withdrawing group onto the citrate hydroxyl group.

column where schizokinen eluted at 63.8 ml. The peak fractions were pooled and dissolved in D₂O for the NMR spectrum shown in Figure 14 along with a spectrum of authentic aerobactin for comparison.

The aerobactin was quantitated by spectrophotometric titration at 399 nm (pH 7), as described for schizokinen.

SIDEROPHORE ASSAYS

Csaky Assay for Hydroxamic Acids

The Csaky assay for the detection of hydroxamates is based on the hydrolytic release of hydroxylamine which is then analyzed by colorimetry after reduction to nitrite (91). Because many siderophores contain the hydroxamic acid functional group, this assay has been widely used to detect siderophores. However, it is not very sensitive [detection limit about 10 μ M NH₂OH (92)] and not considered to be good quantitative tool (J. B. Neilands personal communication) due to variable release of the hydroxylamine group.

We utilized this assay for the detection of schizokinen during column chromatography and also to detect hydroxamic acids in Anabaena culture fluids. The procedure begins with hydrolysis of the sample (1.0 ml) in an equal volume of 6 N H₂SO₄ to release the hydroxylamine residue. The samples were then prepared as advised by Strickland and Parsons (93). The addition of 2.0 ml

Figure 14

NMR spectrum of aerobactin used in iron transport studies along with spectrum of authentic aerobactin (insert) taken from reference 54. The peak at 2.7 ppm is possibly due to a splitting of the 2.9 ppm peak (arising from multiple conformations of aerobactin) or to a contaminant in the preparation.

sulfanilic acid (10 grams in 1 liter 30% HOAC) and sodium acetate (35%) followed by a five minute oxidation with 1.0 ml I_2 (1.3 grams in 100 ml glacial acetic acid) produces nitrous acid from hydroxylamine. The excess iodine is neutralized with 2.0 ml sodium arsenite (2 grams in 100 ml H_2O), and the nitrous acid is diazotized with 2.0 ml N-(1-naphthyl)-ethylenediamine (0.1 gram in 100 ml H_2O) to form a colored product with maximal absorbance at 543 nm. Samples were then diluted to 25 ml in volumetric flasks and the absorbances determined in 10 cm cells. Standards used were hydroxylamine or schizokinen (Figure 15).

A recent paper has shown that quantitative release (85%) of hydroxylamine from aerobactin required hydrolysis in 3N H_2SO_4 at 120°C for 4 hours (92). Simple hydroxamic acids including acetohydroxamic and benzohydroxamic acid were released within 30 minutes under these conditions (>95%). We had previously chosen a 15 minute hydrolysis based on an experiment testing the effect of hydrolysis time at 95°C on color formation from an actual Anabaena filtrate.

<u>Hydrolysis Time (Minutes)</u>	<u>A543</u>
15	0.192
30	0.190
45	0.170
90	0.122

We observed maximum release by 15 minutes. Later experiments indicated that the discrepancy in optimum hydrolysis

Figure 15. Standard curve for Csaky assay using purified schizokinen hydrolyzed four hours at 120° C in 3N H₂SO₄. Recovery of hydroxylamine nitrogen from schizokinen was approximately 65% based on non-hydrolyzed hydroxylamine standards.

times was due to the presence of non-hydrolyzing, Csaky-positive material in our filtrates. This material accounts for as much as 30% of the Csaky response obtained with 15 minute hydrolysis and its relative contribution decreases with time of hydrolysis. Subsequently, all Csaky assays were performed after 4 hour hydrolysis at 120°C in 3N H₂SO₄. Although lower values were obtained under these conditions, they were still considerably higher than those from the Arthrobacter JG-9 bioassay (Table IV, extracted filtrates) or the iron-binding assay (see below). The lack of agreement was greatest for Anabaena filtrates from cells grown in NaNO₃. Correction of Csaky data for non-hydrolyzable or non-extractable Csaky-positive material does not substantially improve the agreement with the other assays. We conclude that a variety of substances such as additional hydroxamates (schizokinen degradation products?), nitrite, or hydroxylamine present in Anabaena filtrates interfere with the Csaky assay and make it unsuitable as a measure of schizokinen concentration.

It is recommended that investigators using the Csaky procedure to detect hydroxamates from cyanobacterial sources attempt an initial purification and concentration step prior to trying this assay as a quantitative tool. For example, adsorption to an XAD resin (Gilliam, personal communication) or an anion exchange resin for SK may allow separation of bound hydroxylamines from potential interferences. A further check on Csaky tests for siderophore

CONCENTRATIONS OF SCHIZOKINEN (μM) IN
ANABAENA FILTRATES

Nitrogen Source ^a	Assay ^b	Iron (μM) added to <u>Anabaena</u> cultures			
		<u>unextracted filtrates</u>		<u>extracted filtrates^c</u>	
		1.0	0	1.0	0
N ₂	Bioassay	0.2	0.9	0.8	2.1
	Csaky	5.4	41.3	n.d.	n.d.
NH ₄ ⁺	Bioassay	0.1	3.6	0.3	5.1
	Csaky	4.3	19.6	n.d.	n.d.
NO ₃ ⁻	Bioassay	0.1	0.5	2.2	6.6
	Csaky	74	28	3	22

^aAnabaena grown on BG-11(mc) [without nitrate] with N₂, 1mM NH₄Cl, or 13mM NaNO₃ as a nitrogen source.

^bArthrobacter JG-9 bioassays performed on late log phase Anabaena filtrates. Values calculated from a standard curve obtained with purified schizokinen. (n.d. means not determined) Csaky assays performed on filtrates of 11 day Anabaena filtrates. Values calculated from a standard curve obtained with purified schizokinen subjected to identical hydrolysis conditions as the samples.

^cAnabaena filtrates extracted as described below (page 69).

production is the repression of hydroxamate synthesis when iron is added to the growth medium being tested. Without this important control, data generated with this assay must be considered questionable. In a previous study of Anabaena 6411, Simpson and Neillands (8) reported that the amount of Csaky-positive material was proportional to the degree of iron starvation when cells were grown in a medium containing 13 mM NaNO_3 and 1 mM NH_4Cl . Although we failed to observe this relationship in unextracted filtrates of cells grown only on NaNO_3 , it was observed for cells grown on NH_4Cl (Table IV) or on a mixture of NaNO_3 and NH_4Cl .

Arthrobacter JG-9 Bioassay for Siderophores

A semi-quantitative assay for schizokinen was developed using the JG-9 strain grown in the assay medium of Estep et.al. (24), supplemented with standard schizokinen solutions. A typical growth response curve to purified schizokinen is shown in Figure 16. A fresh standard curve was run with each assay, and new standard solutions were prepared from a concentrated stock solution (100 $\mu\text{g}/\text{ml}$) periodically. (Loss of SK to container walls was noted with standards greater than three weeks old.) Tubes containing *Arthrobacter* assay media were preincubated overnight at 34° without shaking to monitor sterility.

Figure 16. Growth response of Arthrobacter flavescens JG-9 to various concentrations of purified schizokinen. Cultures grown for 24 hours at 30° C as described above. Schizokinen concentration refers to the concentration of the 0.50 ml test volume rather than the assay mixture which is ten-fold more dilute.

The general procedure was to filter sterilize (0.45 μ m) culture fluid from the Anabaena species and add 0.50 ml of the filtrate to 4.5 ml of assay medium in 15 X 180 mm culture tubes. The assay is linear over only a very small concentration range (0.05 - 1.0 μ g/ml) so that occasionally it was necessary to add less than the 0.50 ml test volume, in which case sterile water was added to make up the volume difference. The standards and test solutions were then inoculated with 0.1 ml from an overnight culture of the JG-9 strain grown in assay medium supplemented with 0.05 μ g/ml schizokinen. The cultures were incubated for 24-36 hours at 30° C on a rotary shaker set at 200 rpm. Growth was measured in a Klett-Sumerson colorimeter fitted with a red (#66) filter. Both distilled water and media blanks were determined and subtracted from experimental values.

The JG-9 bioassay was also performed on CHCl₃/phenol extracts of Anabaena filtrates. The extraction was scaled down such that 1.0-2.0 ml of filtrate (pH 2) was extracted three times with 5.0 ml of CHCl₃/phenol (1:1 wt/wt). Ethyl ether (80 ml) was added to the organic layer which was then extracted with 2 X 5 ml of distilled-deionized water. A known volume of this extract (pH near 7) was then lyophilized to remove traces of the organics that might interfere with growth of the test organism. The residue was taken up in water, filter sterilized, and an appropriate dilution added to the test culture.

A good control on the assay is the concentration of siderophore that gives a half-maximal growth response. In the case of schizokinen, our findings of 0.04-0.07 $\mu\text{g/ml}$ (Figure 16) are in good agreement with the literature value of 0.05 $\mu\text{g/ml}$ (Table II).

Iron-Binding Assay for Siderophores

Given the discrepancies between data generated with the Csaky assay and the JG-9 bioassay, we felt it necessary to confirm our confidence in the bioassay by using an independent technique to see if similar values could be obtained. The assay chosen was iron-binding in 0.14 M perchloric acid (see section on characterization of schizokinen). Because of the relatively low sensitivity of this procedure, 25 ml Anabaena filtrate were concentrated by freeze-drying and then dissolved in a small volume (1.0 ml) of 5mM Fe(III) in 0.14 M HClO_4 . The residue always contained insoluble material that was removed by filtration (0.22 μm Millipore) or by centrifugation at 17,000 x g for 20 minutes. The visible spectrum was then determined on a Cary-14 spectrophotometer between 400 and 600 nm.

Spectra for Anabaena 6411 filtrates grown without combined nitrogen and 0.1, 1.0 or 10 μM added iron are shown in Figure 17. As can be seen, the residual turbidity makes it difficult to determine with precision the true absorbance at the absorbance maximum for shizokinen (490 nm). However, because the 10 μM Fe culture appeared to

Figure 17. Visible absorption spectra for concentrated *Anabaena* 6411 filtrates dissolved in 5mM Fe(III) in 0.14M HClO₄. Cultures were grown with 0.1 μM iron (top), 1.0 μM iron (middle), or 10 μM iron (bottom).

have no ferric ion-reactive material, this curve provided a useful baseline. Using our extinction coefficient for SK in this reagent ($2600 \text{ lit M}^{-1}\text{cm}^{-1}$), it can be calculated that approximately 5.5 and 6.4 μM schizokinen are present in the Anabaena filtrates containing 1.0 and 0.1 μM added iron, respectively. These values are in general agreement with values obtained with the JG-9 bioassay (Table IV, see also RESULTS below). We conclude that the JG-9 bioassay is the most sensitive and accurate assay for schizokinen available.

IRON UPTAKE ASSAYS

Radioactive Chelates and Counting Procedure

In order to measure the small changes in concentration associated with iron uptake, it was necessary to utilize radioactive iron for these experiments. The isotope, $^{55}\text{FeCl}_3$ in 0.1 N HCl was purchased from New England Nuclear at a specific activity of 25.9 millicuries per milligram of iron. This isotope decays via electron capture to ^{55}Mn , a stable non-radioactive isotope. In the process a weak X-ray is emitted which can be detected by scintillation counting. The Mn(III) thus formed is readily reduced to Mn(II) in solution (94). Because Mn(II) is similar to iron (II) which does not form a stable complex with schizokinen, it is not likely that Mn-SK is a

significant species. Furthermore, only 1% of the iron in the sample was ^{55}Fe .

^{55}Fe -containing samples of 0.1 to 0.2 ml were dispersed in 10 ml of scintillation cocktail (Ready-Solv HP, Beckman Instruments). A counting "window" of ^3H was programmed on a Beckman LS-9000 scintillation counter which corresponded to the experimentally determined energy distribution for ^{55}Fe decay. The accumulation time for each sample was adjusted so that the sample counting error was less than 5%. For the concentration of ^{55}Fe present at the beginning of a typical assay (20nM), five minutes accumulation of counts per sample yielded a sample counting error of 2.0%. The overall counting efficiency, determined by comparison of the experimental counting rate (CPM) with the calculated disintegration rate (DPM), was 52%.

The half-life of ^{55}Fe is 2.6 years and the fraction remaining was calculated using a decay constant of 0.021 per month. The concentration of iron in a given sample was determined from the CPM. The CPM per ml was first divided by the counting efficiency to yield DPM per ml; then divided by 2.2×10^6 DPM per μCi to yield μCi per ml; then divided by the specific activity (corrected for decay) to yield $\mu\text{g Fe}$ per ml; and finally divided by the molecular weight to obtain $\mu\text{moles Fe}$ per ml.

The ^{55}Fe complexes with schizokinen, Desferal, aerobactin, and acetyl-schizokinen were prepared by mixing $^{55}\text{FeCl}_3$ in 0.1 N HCl with a two-fold molar excess of the

ligand. The pH was adjusted to 7 with 0.1 N NaOH, and the solutions left overnight before use to insure complete chelation.

Procedure for Iron Uptake Assay

Anabaena filaments were harvested from cultures grown to 50-100 Klett units by collection on 0.45 μ m or 0.22 μ m filters (Millipore). The collected filaments were washed with uptake medium (Table I), and resuspended to the same cell density (unless otherwise noted) in uptake medium. The suspension was then incubated for 25 minutes at 34° C with light from a 100 watt incandescent light located approximately twelve inches from the solution. Both polyethylene and glass test tubes were used, each having previously soaked for at least one hour in 6N HCl and extensively rinsed in deionized-distilled water.

Uptake assays were initiated by the addition of ^{55}Fe -siderophore (2:1 excess ligand). At timed intervals 0.40 ml aliquots were removed and rapidly filtered through 0.45 μ m filters (HA, Millipore) pre-wetted with uptake medium. Filters were then washed with one volume of ice cold uptake medium. (Better results were obtained when the aliquot was carefully spotted in the center of the filter to avoid contact with the walls of the vacuum manifold chamber.) The filtrate from each time point (0.20 ml in 10 ml Ready-Solv HP scintillation cocktail) was counted using the parameters described above. Duplicate blanks

were determined with ^{55}Fe -siderophore added to uptake medium without Anabaena filaments present then filtered and counted in the same manner as the experimental samples. This value represents the total initial ^{55}Fe -siderophore minus binding to the filter. Uptake of iron by cells was calculated by subtracting the experimental value for each filtrate from the blank value. For example, a blank for 20nM ^{55}Fe , 40nM schizokinen gave 3,600 cpm per 0.1 ml filtrate. When cellular uptake was allowed to continue to completion the filtrate gave less than 100 cpm per 0.1 ml (i.e. background). For determination of initial rates, only values below 40% total uptake (i.e. 2160 cpm per 0.1 ml filtrate) were utilized. The extrapolated zero-time intercept represented less than 10% of the total uptake (i.e. was greater than 3,200 cpm per 0.1 ml filtrate).

This assay procedure minimized the extrapolated zero time values (ascribed to specific and/or non-specific binding of ^{55}Fe -siderophore). Alternative procedures such as pre-washing of filters or rinsing filtered cells with the reductant, thioglycolate, yielded no significant improvements. The zero-time values were fairly reproducible and, thus, did not interfere with our desired kinetic measurements. Furthermore, a control experiment on heat-killed cells yielded iron-binding levels similar to extrapolated zero-time values on normal cells.

For experiments measuring the rate of iron uptake as a function of ^{55}Fe -schizokinen concentration, non-radioactive ^{56}Fe -SK (2:1 excess ligand) was added to a smaller concentration of ^{55}Fe -SK (usually 10nM). This avoided the very large counting rates associated with the higher ^{55}Fe concentrations which causes the difference between experimental and blank values to be small with respect to the total counting rate, introducing a large error. The uptake rates measured with carrier ^{56}Fe -SK present were corrected for specific activity to give the appropriate total iron uptake rate.

Direct scintillation counting of ^{55}Fe associated with Anabaena filaments trapped on filters was examined as an alternative to filtrate counting. However, the presence of a filter in the cocktail mixture decreased the overall ^{55}Fe counting efficiency by at least 50%. The Anabaena filaments also introduced an additional quenching that complicated the calculation of iron uptake. A procedure to bleach the Anabaena cells and help dissolve the membrane filter was employed in an attempt to overcome these difficulties. Treatment of the filters with 0.1 ml 70% HClO_4 and 0.2 ml H_2O_2 (30%) for two hours at room temperature bleached the cells and partially dissolved the filters as well. This procedure improved the counting efficiency by 4-28% but introduced yet another source of variability. Due to these difficulties and the overall acceptability of the filtrate counting method, this procedure was used

exclusively for the work reported in this thesis. Recent studies by S. J. Goldman (personal communication) have shown that it is possible to obtain reproducible filter counts using Dynagel-SBS xylene-based scintillation cocktail (J. T. Baker) which completely dissolved the filters. Uptake rates and extrapolated zero-time intercepts are similar to those obtained from counting filtrates.

Inhibitors of Iron Uptake

Uptake experiments with inhibitors of energy production were carried out to test the energy dependence of iron uptake. Two inhibitors, N, N¹-dicyclohexylcarbodiimide (DCCD) and carbonylcyanide-m-chlorophenylhydrazone (CCCP) are only sparingly soluble in water and so were dissolved in 95% ethanol. Potassium cyanide and sodium arsenate were dissolved in water and the pH of the sodium arsenate solution adjusted to pH 7.0 prior to use. For controls, an equivalent volume of ethanol or water was used.

Anabaena filaments were harvested and resuspended in uptake medium as described previously. The resuspended cells were exposed to inhibitor or control solvent for 20 minutes (DCCD and CCCP) or 50 minutes (KCN and Na₂AsO₄) before addition of ⁵⁵Fe-SK to initiate the assay. For dark uptake experiments the resuspended filaments were first dark adapted for ten minutes then treated with inhibitors or control solvent as above.

RESULTS

The Effects of Iron and Chelators on Growth of Anabaena 6411

The growth response of cyanobacteria to variable amounts of iron should fall into three categories. First are those concentrations that are less than adequate, which result in reduced growth. This nutrient limitation might manifest itself as an extended lag phase, slower growth rate, lower growth yields, or a combination of these effects. In the second group are those iron concentrations which give a maximal stimulation of growth, while the final category consists of those concentrations of iron that are high enough to be inhibitory due to toxic effects. The growth response of Anabaena 6411 to a wide range of iron concentrations is shown in Figure 18. In the cultures receiving no added iron (approximately 8.5×10^{-8} M by atomic absorption) or 1.0 μ M added iron an initial period of reduced growth was observed indicating an iron-limited state. However, once growth commenced, the rates approximate those observed with the 10 and 100 μ M iron cultures. These latter cultures displayed no lag in growth and maximal initial growth rates. Only the 1.0 mM iron culture exhibited lack of growth due to unknown toxic effects at millimolar total iron. Metal toxicity to algal culture will be greatly affected by the solution chemistry in the growth medium. For example, copper

Figure 18.

Growth of Anabaena 6411 in complete BG-11 medium as a function of added iron. Iron was added from a freshly prepared solution of filter-sterilized FeSO_4 to insure that all iron was initially present in a soluble state. 1×10^{-3} M iron ($\blacktriangle$ — $\blacktriangle$), 1×10^{-4} M iron ($\triangle$ — $\triangle$), 1×10^{-5} M iron ($\blacksquare$ — $\blacksquare$), 1×10^{-6} M iron ($\circ$ — $\circ$), no added iron ($\bullet$ — $\bullet$).

toxicity is known to be dependent on the free copper ion activity rather than the total amount of copper present (96). This is not likely to be the case for the observed toxicity of iron to Anabaena. Computer simulation of the solution chemistry predicts that the ferric ion activity and total soluble ferric hydroxides present in the cultures receiving 10^{-5} , 10^{-4} , and 10^{-3} molar iron will be identical, with the excess iron occurring as colloidal ferric hydroxide precipitate (see Table VII). It seems probable that the mechanism of iron toxicity relates most closely with the total amount of $\text{Fe}(\text{OH})_3$ precipitate formed. A more detailed examination of the solution chemistry in BG-11 growth medium as it relates to Anabaena growth and siderophore production will follow in the Discussion Section.

It is interesting to note that under these conditions the final yields are not proportional to the amount of iron added to the medium. The culture without added iron actually achieved the highest Klett values followed by the 1.0 μM added iron culture. These results suggest that iron limitation is not a significant threat to the growth of Anabaena 6411 in batch culture. Serial passage through at least three transfers in unsupplemented low iron medium had no serious effect on the growth of this strain, suggesting that stored iron was not responsible for the observed growth. Rather, the successful growth during iron limitation must be due to effective iron scavenging by Anabaena.

The above experiment was conducted in complete BG-11 medium containing the chelators EDTA and citric acid. Because we felt it desirable to omit these chelators from cultures where siderophore production and iron uptake were to be studied, their effect on the growth of Anabaena 6411 was tested (Figure 19). In this experiment we have focused on those concentrations where iron limited growth is observed. It is apparent that cultures supplemented with the chelators grew slightly better than the unsupplemented cultures at every iron concentration. This behavior may reflect some copper toxicity since removal of the chelators should increase the copper ion activity from $10^{-11.1}$ M up to $10^{-7.2}$ M (see Discussion). The differences in growth are slight however, and the overall pattern of growth is quite similar in this range of iron concentrations.

Anabaena 6411 also grew without added chelators in media free of combined nitrogen (Figure 20). This strain demonstrates an increased requirement for iron when fixing nitrogen. Evidence for this stems from the fact that when nitrogen-fixing cells growing in BG-11 medium (minus nitrate) without added iron are serially passed in the identical medium without added iron, the cells quickly become chlorotic and fail to grow well after the first transfer. Cells grown in the same medium on nitrate grow at nearly the same rate through at least three transfers without added iron. A similar lack of growth in Anabaena 6411 under iron-limited, nitrogen-fixing conditions was

Figure 19.

Growth of *Anabaena* 6411 in BG-11 medium with the chelators citric acid (6 mg/liter) and EDTA (1 mg/liter) (closed symbols) and without these chelators (open symbols). Iron concentration added: 1.0 μM (squares), 0.1 μM (triangles), no added iron (circles).

Figure 20. Growth of *Anabaena 6411* in BG-11(mc) without combined nitrogen. ($\blacktriangle$ — $\blacktriangle$) 0.5 μ M added iron, ($\bullet$ — $\bullet$) 0.1 μ M added iron.

also observed by Simpson and Neillands (8). A possible explanation is that cells under these conditions are less capable of producing siderophore (Tables IV and V).

A final observation on the growth of Anabaena 6411 is that true log phase growth is limited to the first three to four days of growth. Afterwards, cultures grow at a reduced rate that is not usually linear with time. This pattern is observed regardless of the concentration or oxidation state of the iron added to the medium. Furthermore, it does not seem to be due to the induction of nitrogen-fixing metabolism since cultures pre-adapted to growth on N_2 also show similar behavior (Figure 20). The most probable explanation for this effect is CO_2 limitation induced as the added carbonate species are fixed into cellular carbon. Another possible factor is self-shading effects which would reduce the light intensity striking a portion of each culture, and thus slowing overall growth.

Siderophore Production by Anabaena 6411

It has been previously demonstrated by Simpson and Neillands (8), that Anabaena 6411, when grown under iron-limiting conditions, produces Csaky-detectable, extracellular hydroxamates and that schizokinen is present in the supernatant of iron-limited cultures. In order to measure schizokinen production in response to iron availability, we have employed a more sensitive assay system utilizing the siderophore auxotroph, Arthrobacter flavescens JG-9, as a

CONCENTRATIONS OF SCHIZOKINEN (μM) IN
ANABAENA FILTRATES ^a

Nitrogen source ^b	Iron added to <u>Anabaena</u> culture			
	None		1.0 μM	
	Filtrate	Extract	Filtrate	Extract
N ₂	0.9	2.1	0.2	0.8
NH ₄ Cl, 1 mM	3.6	5.1	0.1	0.3
NaNO ₃ , 13 mM	0.5	6.6	0.1	2.2

^a Schizokinen concentration determined by growth factor activity for Arthrobacter flavescens JG-9, relative to a standard curve obtained with purified schizokinen. Errors were + 30% for values above 3 and + 70% for values below 2. Schizokinen concentrations were measured on untreated cell filtrates and on filtrates subjected to chloroform/phenol extraction as described above (p. 69).

^b Anabaena grown to late log phase on BG-11 medium (minus citrate, EDTA, NaNO₃) with nitrogen added as indicated. NaNO₃ and NH₄Cl concentrations are those used in uptake medium (8). NaNO₃ in standard BG-11 is somewhat higher, 18 mM.

test organism. In time-course experiments on Anabaena 6411 filtrates, we have found that siderophore concentrations (based on a schizokinen standard curve) increased steadily during the first five days of growth in standard BG-11(mc) medium. The amount of siderophore produced was inversely proportional to the amount of iron in the growth medium as expected. (See Table VII in the discussion.) After five days of growth all of the cultures showed an apparent drop in the Arthrobacter assay response with a complete loss of activity by fourteen days. In contrast, similar time course assays on Anabaena 6411 filtrates growing in BG-11(mc) without combined nitrogen did not drop off, but rather continued to increase for at least twelve days. One likely explanation for the observed differences was that growth of Anabaena on nitrate-containing media produces a substance which interferes with the Arthrobacter growth response and that this interfering substance is produced particularly in late growth phase. Therefore, we decided to determine whether organic extraction of schizokinen might separate it from interfering substances.

The effect of an organic extraction of filtrates prior to the Arthrobacter assay is shown in Table 5. The extraction was performed on late Anabaena filtrates from BG-11(mc) containing NaNO_3 (13mM) or NH_4Cl (1mM) as a nitrogen source and cultures free of combined nitrogen. In the case of the nitrate-containing Anabaena filtrates, we found that the growth factor activity could be restored

by extraction of the siderophore as in the standard purification procedure for schizokinen. Thus, it appears that siderophore levels continue to increase throughout the growth phase of Anabaena cultures in nitrate-containing medium and that the drop in growth factor activity noted in the time course experiments is due to the production of a substance which is inhibitory to the Arthrobacter test organism. The identity of this inhibitory substance remains a mystery. However, because extracted filtrates from nearly all cultures showed a slight stimulation of Arthrobacter growth response, it is not likely that production of the inhibitor is strictly limited to nitrate-containing medium.

Regardless of nitrogen source, all extracted Anabaena filtrates showed increased schizokinen production for cells grown in 0 added iron, relative to 1 uM added iron. Thus, this assay exhibits the expected response to iron limitation and can be used as a semi-quantitative measure of schizokinen production. Similar schizokinen levels were observed for cells grown in either NaNO_3 or NH_4Cl . Two- to three-fold lower levels of schizokinen were found for cells utilizing N_2 . Apparently cells make less schizokinen when nitrogen supplies are limited, even though they have an increased requirement for iron in the production of nitrogen-fixing enzymes. Presumably under these conditions nitrogen is preferentially incorporated into other molecules (e.g. proteins and nucleic acids).

The Arthrobacter bioassay also has been utilized to measure the effect of adding 1mM citrate to the BG-11(mc) growth medium on siderophore production. A thirteen day filtrate (155 Klett units) from a culture containing 1mM citrate and 0.1 μ M iron was extracted for Arthrobacter analysis. Prior to extraction 2.0 μ M schizokinen was measured, while after extraction 42 ± 4 μ M schizokinen was obtained. This represents a dramatic increase in siderophore production relative to standard BG-11(mc). The effect of 1mM citrate on Arthrobacter JG-9 growth was negligible. Because citric acid is a component of the schizokinen molecule, it is tempting to suggest that the observed stimulation in siderophore production is due to high intracellular concentrations of a biosynthetic precursor. If citrate were merely behaving as a siderophore to increase iron uptake, schizokinen production would have decreased. The alternative explanation that citrate increases iron starvation by limiting iron availability seems unlikely in view of the growth-promoting effect of 0.03 mM citrate (Figure 19) and the fact that cells can take up iron when it is added as ferric citrate (see iron uptake below).

Transport of Ferric Schizokinen by Anabaena 6411

The ability of Anabaena 6411 to utilize radiolabeled iron complexed with a two-fold excess of schizokinen is

shown in Figure 21. The removal of [^{55}Fe] ferric schizokinen from the uptake medium was linear with time until approximately fifty percent of the label was taken up for every concentration tested. Accumulation of ferric schizokinen continued at decreasing rates until nearly all the added iron was removed from the medium. Since the cellular volume represents less than 0.1% of the solution volume, the complete uptake of 90 nM iron from the medium has resulted in a cellular iron concentration greater than 0.09 mM. Since it is highly unlikely that this amount of iron would be merely associated with the cell surface (where the concentration would be greater than 1 mM), the cells appear to be actively accumulating iron.

A closer examination of the initial stage of ferric schizokinen uptake was conducted on Anabaena cultures grown with variable amounts of added iron (Figure 22). The iron uptake system in this organism appears to be under some type of metabolic control as evidenced by the inverse relationship between initial rate of [^{55}Fe] ferric schizokinen uptake and the amount of iron added to the growth medium. During the initial one minute period between addition of [^{55}Fe] ferric schizokinen to initiate the assay and our first data point, rapid binding to the cells occurs probably in both a specific and non-specific fashion. Following this a period of linear uptake was noted similar to that observed in Figure 21. Subsequent studies have concentrated on this linear phase of iron transport, the

Figure 21. Uptake of [^{55}Fe] ferric schizokinen; 0.9 nM (■—■), 9.0 nM (●—●), and 90 nM (○—○), by *Anabaena* 6411. Cells were grown in uptake medium (1% CO_2 bubbling) supplemented with 1.0 μM iron(III) and trace metals to 83 Klett units. Schizokinen was present in a two-fold molar excess to ^{55}Fe .

Figure 22. Rate of uptake of ^{55}Fe -schizokinen as a function of the iron concentration in the growth medium. *Anabaena* grown in zero ($\circ$ — $\circ$), 0.1 μM ($\square$ — $\square$), and 1.0 μM ($\triangle$ — $\triangle$) added iron. Cells resuspended to 52 Klett units in uptake medium containing 14 nM ^{55}Fe and 28 nM schizokinen.

duration of which is dependent on the concentration of radiolabeled iron added to the assay medium and the cell density. Washed cells and a two-fold excess of schizokinen have been utilized to insure that iron is siderophore-bound during assays. The presence of an equimolar amount of free schizokinen in the assay had no significant effect on iron uptake from ferric schizokinen because the cellular affinity for schizokinen is 50 times lower (i.e. assuming competitive inhibition, the K_I for schizokinen is approximately $1.5 \mu\text{M}$).

Kinetics and Energy Dependence of Ferric Schizokinen Transport

If the observed removal of iron from the assay medium is due to specific biological transport, the uptake system should be saturatable at high solute concentrations. In addition, if the cellular accumulation of ferric schizokinen seen in Figure 21 is due to biological transport, it should require the input of metabolic energy.

Evidence for the involvement of specific carriers in the transport of [^{55}Fe]-ferric schizokinen was obtained by studying the kinetics of iron uptake as a function of solute concentration. Results presented in Figure 23 (insert) demonstrate that [^{55}Fe]-ferric schizokinen uptake occurs via a saturatable system. Double reciprocal plots yield an apparent K_M of 35 nM. A V_{max} of approxi-

Figure 23. Kinetics of uptake of ferric schizokinen. *Anabaena* 6411 grown in uptake medium containing 0.1 μM added iron and resuspended to 47 Klett units in uptake medium. Graphs show calculated curves for $K_M = 36 \text{ nM}$ and $V_{\text{max}} = 2.7 \text{ pmol/min/ml}$.

mately 2.5 picomoles/min/ml was calculated for the conditions tested (Anabaena grown with 0.1 μ M Fe at a cell density corresponding to 47 Klett units). The apparent K_M value is at least three times lower than reported values for siderophore transport by E. coli (72, 76) and gives an indication of the extremely high affinity of the ferric schizokinen transport system for its substrate.

After showing that Anabaena 6411 is capable of accumulating ferric schizokinen against a concentration gradient, it was of interest to determine the energy requirements of the process. Since Anabaena 6411 is an obligate photoautotroph, light is the ultimate source of all metabolic energy. In order to determine whether Fe-SK uptake is energized directly by light-associated processes, uptake activity in the light and dark was measured. We found that uptake activity was only slightly decreased in the dark, ranging from 80-95% of light controls; dark adaptation periods from 15 minutes to two hours had little effect.

In order to discriminate between possible sources of energy for iron uptake, the effect of various inhibitors on iron transport was tested. Representative uptake data are shown in Figure 24 and a summary of the inhibitor results is given in Table VI. Both light and dark uptake were sensitive to the uncoupler carbonylcyanide-m-chlorophenylhydrazone (CCCP) which dissipates the trans-membrane electrochemical hydrogen-ion gradient. In addition, N,

Figure 24. Effect of CCCP and DCCD on the uptake of ferric schizokinen in *Anabaena* 6411 in the light. Cells were exposed to 8 μM CCCP (Δ - Δ), 5 μM DCCD ($\square$ - $\square$), or no inhibitor (O-O) for 20 minutes prior to the addition of 15 nM ^{55}Fe , 30 nM schizokinen.

TABLE VI
EFFECT OF METABOLIC INHIBITORS
ON FERRIC SCHIZOKINEN UPTAKE
IN ANABAENA

Inhibitor	Concentration	Uptake activity (%) ^a	
		Light	Dark
CCCP	8 μ M	10	0
DCCD	40 μ M	5	5
Arsenate	20 mM	50	15
Cyanide	100 μ M	90	20

^a Uptake activity relative to light or dark control in the absence of inhibitor. Values are $\pm$ 5% for CCCP, DCCD, and Na_2HAsO_4 and $\pm$ 10% for KCN. For dark uptake, cells were dark-adapted 10 minutes before adding inhibitor. Total time of exposure to inhibitor prior to addition of Fe-SK was 20 minutes for CCCP and DCCD, 50 minutes for Na_2HAsO_4 and KCN. Anabaena cultures were grown in uptake medium containing 0.1 μ M added iron.

N¹-dicyclohexylcarbodiimide (DCCD), an inhibitor of H⁺-translocating ATPases in bacteria, mitochondria, and chloroplasts (96), caused almost complete inhibition of iron uptake both in the light and dark. The DCCD-sensitivity of iron uptake implies that ATP is required for this process. The inhibition of iron uptake by arsenate, which inhibits ATP synthesis by acting as a substrate analog, supports this conclusion. The fact that [⁵⁵Fe]-ferric schizokinen uptake in the light is less sensitive to arsenate inhibition than dark uptake may possibly be due to a higher overall rate of ATP formation in the light.

The inhibition of [⁵⁵Fe]-ferric schizokinen uptake by potassium cyanide in the dark strongly suggests that dark iron uptake in Anabaena 6411 is driven by respiratory electron transport. Cyanide is a well known inhibitor of electron transport in bacteria and mitochondria due to strong binding of CN⁻ to the oxygen binding site in the terminal cytochrome oxidase of the electron transport chain. The lack of significant cyanide inhibition in the light indicates that Anabaena 6411 depends on photophosphorylation for light-driven iron uptake and oxidative phosphorylation for dark uptake.

Specificity of Iron Uptake in Anabaena 6411

We have examined the ability of Anabaena 6411 to utilize iron chelated by a structurally similar siderophore (aerobactin), a dissimilar siderophore (ferrioximine B or

Desferal) and also a derivative of schizokinen (acetyl-schizokinen). The initial rates of uptake of ^{55}Fe -siderophore (2 to 1 molar excess of ligand) are shown in Figure 26.

The rates of iron uptake from the schizokinen and acetylschizokinen complexes appear to be identical, although the y-intercepts, representing specific and non-specific binding, are different. Thus, iron transport is not significantly altered when the citrate hydroxyl group of schizokinen is modified by acetylation. In contrast, Arthrobacter JG-9 which generally shows much less siderophore specificity than Anabaena exhibited only 1/3 as much growth activity with acetyl-schizokinen as it did with schizokinen. No iron uptake activity is observed when ^{55}Fe is chelated by excess Desferal, in concentrations up to 55 nM ^{55}Fe . [^{55}Fe]-ferric aerobactin also did not support iron transport by Anabaena 6411. This is consistent with results from other schizokinen-utilizing organisms like Bacillus megaterium which cannot assimilate ferric aerobacter despite its structural similarity to schizokinen (80). The increased negative charge of this complex may be at least partially responsible.

When ^{55}Fe citrate (100X excess citrate: Fe) was used as an iron source, uptake rates approaching those with ferric schizokinen were observed. However, due to the fact that schizokinen has a much greater iron affinity than

Figure 25.

Iron uptake activity by *Anabaena* 6411 for several ferric siderophore complexes. [^{55}Fe]-ferric schizokinen (O—O), [^{55}Fe]-ferric acetyl schizokinen (●—●), and [^{55}Fe]-ferric Desferal (■—■). Cells were grown in BG-11(mc) medium without added iron for six days, washed, and resuspended to 60 Klett units in uptake medium. [^{55}Fe]-siderophores were added to a final concentration of 30nM iron (40 nM siderophore) except [^{55}Fe]-ferric Desferal (18 nM iron, 36 nM Desferal).

citrate (K_f of approximately 10^{23} versus K_f of approximately 10^{11}), it is difficult to rule out the possibility that the observed uptake was due to removal of iron from citrate by endogenous schizokinen. Accurate determination of ferric citrate utilization can only be performed with a mutant which produces no high-affinity siderophores.

Siderophore Activity in Different Strains of Anabaena

The genus Anabaena has been divided into two separate groups (82). The first group is characterized by a lack of mobility, a lack of akinetes (spores), lysis by the cyanophage N-1, and cultures grow as an even suspension in liquid culture. This group includes Anabaena 6411 and another species we have tested for siderophore activity, Anabaena 7120. The second group produces akinetes, is motile (gliding), is not lysed by the cyanophage N-1, and does not grow as an even suspension in liquid culture. We also chose an organism from this group, Anabaena cylindrica, to test for siderophore activity.

Anabaena 7120 was tested for siderophore production using the Arthrobacter bioassay to examine filtrates from cultures grown without added iron. No activity at all was seen in filtrates unless they were first extracted with chloroform/phenol as described for schizokinen (see Experimental). After extraction 0.9-2.5 μ M siderophore (as schizokinen) was detected (range of three independent assays), for an 11 day sample grown without added iron (110

Klett units). We have also examined siderophore production by Anabaena 7120 using the iron-binding assay. A broad peak was observed centered near 490 nm, similar to the absorbance maximum of schizokinen in this test (5mM Fe(III), 0.14 M HClO₄). Using the extinction coefficient for ferric schizokinen (2600 liter mol⁻¹ cm⁻¹) a siderophore concentration of 1.4-1.9 μ M was calculated for a 16 day filtrate grown without added iron to 125 Klett units. The close agreement between the two assays is what would be expected if the majority of the siderophore activity from Anabaena 7121 is a hydroxamate (λ max = 490 nm in acid) with growth factor activity similar to schizokinen.

The ability of Anabaena 7120 to utilize several iron-siderophore complexes as substrates for iron transport was examined using the same methods developed for Anabaena 6411. Results presented in Figure 25 are very similar to what was found in strain 6411. Both schizokinen and acetyl-schizokinen support iron uptake at similar rates. The rate of iron transport in strain 7120 was 50% of the rate demonstrated by strain 6411 with schizokinen and 75% of the rate with acetyl-schizokinen. Aerobacter and Desferal did not support iron uptake by Anabaena 7120 (data not shown). The similar pattern of iron uptake in the two strains of Anabaena in response to different siderophores is further indication that schizokinen is the siderophore produced by Anabaena 7120.

Figure 26.

Iron uptake activity by Anabaena 7120 for [^{55}Fe]-ferric schizokinen (● ●), and [^{55}Fe]-ferric acetylschizokinen (○—○). Cells were grown in BG-11(mc) medium without added iron for six days, washed, and resuspended to 60 Klett units in uptake medium. Labelled iron was added to a final concentration of 30nM iron, (40 nM siderophore).

Siderophore Assays with *Anabaena cylindrica*

Anabaena cylindrica has also been examined for siderophore production as a representative species of the second grouping of the genus *Anabaena*. Cultures of *A. cylindrica* were grown without added iron in BG-11(mc) medium under the same conditions as *Anabaena* 6411. Visual growth continued in the absence of added iron although at a slightly reduced level relative to 1.0 μM Fe control cultures. The iron-free culture was harvested after two weeks and the supernatant concentrated twenty-five fold for *Arthrobacter* siderophore bioassays. No siderophore activity was found from these cultures when the concentrated culture fluid was subjected to the extraction procedure used with strains 6411 and 7120. Simpson and Neilands (8) have reported a finite level of hydroxamate positive material in extracellular fluids from *A. cylindrica*. However, this material was not repressed by added iron in a reproducible manner. These authors suggested that this hydroxamate was not likely to be a siderophore. However, because our extraction procedure may not work for all potential siderophores, and also because inhibition of the bioassay is known to occur with other *Anabaena* strains, it would be unwise to conclude that *Anabaena cylindrica* does not produce a siderophore. Iron uptake assays failed to show any marked uptake in the presence of schizokinen. Thus, it is unlikely that this species produces or utilizes schizokinen as a siderophore.

DISCUSSION

The work presented in this dissertation was motivated, in part, by the findings of Murphy, et.al., who showed that the green alga Scenedesmus was inhibited by a cyanobacterial siderophore, presumably because it could not utilize siderophore-bound iron (7). More recent results have demonstrated that another green alga, Chlamydomonas reinhartii, is inhibited by schizokinen and also by desferrioxamine B mesylate. A third, Chlorella vulgaris, is not affected by either siderophore regardless of the ratio of ligand to iron (98). Thus, two of three eucaryotic algae tested have proven to be susceptible to inhibition by hydroxamate siderophores. The implications of these findings with respect to phytoplankton succession and diversity clearly warrant further research. In order to provide a better data base for evaluating the ecological significance of phytoplankton competition for iron, we have focused our efforts on learning the details of siderophore-dependent iron acquisition by cyanobacteria. This work represents the first investigation of siderophore-mediated iron transport in this environmentally-significant group of organisms.

Siderophore Assays

In order to evaluate the effect of siderophores upon aquatic ecology, one must be able to determine the amount of siderophore being produced by a cyanobacterium and the factors which influence this production capability.

Several methods for detection and quantification of cyanobacterial siderophores have been evaluated; limitations to their use due to chemical interferences have been found.

By far the most sensitive and useful method is the Arthrobacter JG-9 bioassay system. Using this method it is possible to detect as little as 10^{-7} M siderophore (as schizokinen). However, use of this assay is not without its problems. We have shown that Anabaena produces a substance which is inhibitory to the test organism. This inhibition can be overcome by an organic extraction procedure that separates the siderophore from the inhibitory agent. The presence of the inhibitor is indicated by time-course experiments in which siderophore activity begins to drop in mid-log phase, and eventually declines to zero. Such behavior was also seen in the time-course data of Armstrong and Van Baalen on siderophore produced by Agmenellum quadruplicatum, a marine cyanobacterium (25). The identity of the inhibitory agent is not known at present. It is heat-stable and not extractable in chloroform/phenol. The presence of such an inhibitor in cyanobacterial filtrates is especially problematical for

situations in which the bioassay is to be used to screen for the production of siderophores by cyanobacteria. Not all siderophores will be extractable in chloroform/phenol and it is impossible to know a priori how to separate the presumptive siderophore from the potential interference. Thus, several solvent systems would have to be tried in order to completely rule out the possibility of siderophore production by any given strain (see reference 25). Alternative procedures such as ion-exchange chromatography or absorption resins like XAD may be useful to both concentrate and partially purify the preparation.

The most widely used assay has been the Csaky method for detection of bound hydroxylamine. The major problem with this assay is its lack of sensitivity. The detection limit is approximately $10^{-5}M$ or two orders of magnitude higher than the Arthrobacter assay (92). Furthermore, Anabaena produces substances that give a Csaky response, but are apparently not siderophores (i.e. production is unrelated to [Fe] in the growth medium). The consistently higher values obtained with this assay relative to the bioassay procedure or the ferric perchlorate iron-binding assay suggest that this method does not give a true indication of the concentration of siderophore present in a given preparation. The added Csaky-positive material may be degradation products from intact siderophores or hydroxylamine or nitrite-containing compounds, since any of these will give a Csaky response. A preliminary

purification and concentration of a test filtrate might make it possible to utilize the Csaky assay to detect cyanobacterial siderophores. However, our experience suggests that this method is not a useful tool for determining the concentration of siderophores in culture fluids.

Our results show that Anabaena 6411, in batch culture, produces schizokinen throughout its growth cycle, and that the concentration of this compound reaches 5-10 micromolar in cultures nearing stationary phase. Anabaena 7120 cultures achieve siderophore concentrations between 1 and 2.5 micromolar (as schizokinen) in late growth phase. These values are quite similar to the few literature values available for siderophore production by other cyanobacteria. Agmenellum quadruplicatum produces approximately 1.4 micromolar siderophore (relative to ferrioxamine B mesylate) as measured with the Arthrobacter bioassay on untreated filtrates (25). This value corresponds to the maximum level prior to the decline in activity observed after three days growth. Thus it seems likely that the concentration could go higher if inhibitory substances are responsible for the reduction in siderophore activity in the Armstrong and Van Baalen time-course data. The only other values available for comparison are from the data published by McKnight and Morel on strong copper chelators produced by Anabaena flos-aquae (26). Using a copper-sensitive electrode for titrations, these investigators found approximately 5-15 micromolar chelator in stationary

phase low-iron cultures. The compound contained hydroxamate liganding groups and its synthesis was repressed by added iron in the culture medium, strongly suggesting that it is a true siderophore. The conditional formation constant for the copper complex was indistinguishable from that with copper Desferal ($C_{K_f} = 10^{8.4}$). The in vivo function of this ligand would be primarily for iron binding, as bound copper is readily displaced by added iron. The excellent agreement between this data and our results using independent methods provides further support for the accuracy of the Arthrobacter data.

The marked stimulation of schizokinen production by Anabaena 6411 when grown in medium containing millimolar citrate provides a useful method for increasing schizokinen production from this organism on a preparative basis. Since the value of 42 micromolar schizokinen was determined with cells supplemented with 0.1 micromolar iron, it is possible that cultures unsupplemented with iron could produce even more schizokinen with citrate in the medium. This procedure may also be useful with Anabaena 7120 in order to produce enough siderophore for structural determination. In contrast to the cyanobacterial results, addition of 5 mM citrate had no stimulatory effect on schizokinen production in Bacillus megaterium (99). However, the organisms differ in that one uses carbon derived from photosynthesis, while the other uses carbon derived from glycolysis and respiration. In addition,

cyanobacteria do not possess a complete citric acid cycle, which may limit biosynthesis of this compound.

Our negative results concerning siderophore production by Anabaena cylindrica Lemm. should not be taken as definitive. Even though no activity was found with extracted or unextracted low-iron filtrates, this organism grows reasonably well through repeated transfers in low-iron media. McKnight and Morel (26) found approximately 5 micromolar strong copper chelator from A. cylindrica which had a higher conditional formation constant with copper than did the Anabaena flos-aquae siderophore ($^cK_f = 10^{10.2}$), presumably reflecting a different structure and, thus, possibly different solubility properties. This copper chelator also contained bound hydroxamate and was repressed by added iron.

Iron Transport

In general, the transport of iron siderophore chelates across cell membranes occurs via a specific transport system and requires metabolic energy (32). There are three common mechanisms for energy-mediated transport of nutrients across bacterial cell membranes (100). Membrane proteins (permeases) can behave as nutrient carriers or channels through the membrane. These systems are often driven by the electrochemical potential gradient of hydrogen ions ($\Delta\bar{\mu}H^+$). Binding protein systems are characterized by periplasmic proteins that utilize ATP hydrolysis

to increase their affinity for a nutrient or to facilitate passage of the nutrient through the membrane. These systems are sensitive to osmotic shock due to the loss of the periplasmic proteins. Finally, group translocation systems make use of intracellular modification of the nutrient by ATP or other high energy donors to drive the accumulation of the substrate.

Results presented here have shown that Anabaena 6411 accumulates [^{55}Fe]-ferric schizokinen and that this process requires the input of metabolic energy. The complete inhibition of both light and dark [^{55}Fe]-ferric schizokinen uptake by the ATPase inhibitor DCCD, which is not expected to affect electron transport or the development of a membrane potential (101), clearly shows that ATP is required and that the energized membrane state alone is not sufficient to drive the transport of iron. Similar results were obtained with arsenate which blocks ATP formation. Because membrane level phosphorylation is the only mechanism for ATP synthesis in cyanobacteria (102), it has not been possible to determine whether the inhibition caused by uncouplers is due only to interference with ATP formation or if a membrane potential is also required for iron transport.

In several other iron transports systems there has been a clear indication of a requirement for an electrochemical gradient. Ferrichrome transport in E. coli has been shown to occur in inner membrane vesicles via a

permease system which responds to the electrical component ($\Delta\psi$) of the membrane potential (72), possibly via a symport process with Ca^{2+} or Mg^{2+} bound to the ferrichrome molecule to produce a divalent cation (73). Ferric enterobactin uptake also requires an energized membrane state (69). Ferric enterobactin receptors with possible permease activity are present in both the outer and inner membranes (62), with the inner membrane system presumably accounting for the $\Delta\bar{\mu}_{\text{H}^+}$ dependence. In addition, there appears to be an ATP requirement for ferric enterobactin uptake in E. coli. However, in this heterotrophic organism, it is difficult to rule out the possibility that the primary target of ATP inhibitors is glucose uptake rather than iron uptake (69).

It is perhaps significant that ferric schizokinen and ferric enterobactin are monovalent and trivalent anions, respectively, at physiological pH. As a result both must be transported against the electrical component of the membrane potential (interior negative). The ATP requirement of both systems suggests that transport is more complicated than a simple permease-type system, yet whether either system will fit the binding protein or group translocation models (100) or some combination of both remains to be determined. These questions would be more easily answered if it were possible to follow the intracellular localization of iron with time. However, such experiments are fraught with difficulties due to the fact that cell

fractionation procedures tend to disturb iron distribution patterns within cells (103). As stated by Christensen (104): "Unfortunately, measurement of activities for cell compartments is at best difficult. Solutes are frequently shown to be dialyzable and free after cytolysis, but conceivably cytolysis could release them from bound forms existing in vivo." In gram negative bacteria such as cyanobacteria such studies are further complicated by the existence of a periplasmic fluid between the inner and outer cell membranes; i.e. it is difficult to determine whether soluble iron has been derived from the cytoplasm or the periplasmic space.

The affinity of siderophore transport systems for their substrates is generally higher in bacterial than in eucaryotic systems. For comparison, Neurospora crassa accumulates iron via its siderophore, coprogen, with a K_M of 20 μM (105). The ferrichrome and ferric enterobactin systems in E.coli are characterized by K_M 's of 0.15 - 0.25 micromolar and 0.1 - 0.36 micromolar respectively (72,76, 106). The apparent K_M for ferric schizokinen uptake in Anabaena 6411 is 0.03 micromolar, approximately one third of the E.coli values. The fact that the system reaches half-saturation at such a low concentration suggests that the affinity of the Anabaena siderophore transport system is high even by bacterial standards. This adaptation may help to counteract the large potential for dilution of the

extracellular products of cyanobacteria in aquatic environments.

It is not clear what step in the transport of ferric schizokinen by Anabaena is being saturated, since the detailed molecular mechanism of transport for this siderophore (or any other siderophore transport system studied to date) is not known. Given the small size of ferric schizokinen (476 daltons) relative to the cutoff for the molecular sieving action of outer membrane porins of E.coli (about 600 daltons for oligosaccharides [107]), it is possible that rapid equilibration across the outer membrane of Anabaena may occur via passive diffusion, followed by a slower transport across the inner membrane into the cytoplasm. The reverse situation is just as possible however, and both ideas must be regarded as speculative at this point.

The specificity of the Anabaena siderophore transport system has been studied in two strains, 6411 and 7120. Virtually identical results have been found in that each strain will transport ferric schizokinen and ferric acetyl-schizokinen. However, the structurally similar siderophore, aerobactin, does not support iron transport by either strain. The same situation is found with other schizokinen-utilizing bacteria, Bacillus megaterium and Salmonella typhimurium, which also cannot assimilate ferric aerobactin (80,94). A structurally dissimilar siderophore, ferrioxamine B mesylate or Desferal, also did not support

iron transport by Anabaena 6411 or 7120. B. megaterium, in contrast, does assimilate ferric Desferal. However, competitive binding assays have shown that this is a separate function, distinct from the schizokinen-dependent transport system (80). We conclude that Anabaena 6411 and 7120 are somewhat specific in their requirement for schizokinen, but that modification of the citrate hydroxyl liganding group does not significantly alter the activity of the molecule as a iron transport cofactor. It would be of interest to determine the activity of ferric arthrobactin in iron transport studies with Anabaena. This siderophore differs from schizokinen only in the length of the side arms, has the same charge (a monovalent anion versus aerobactin's trivalent negative charge), and has a molecular weight only 56 daltons higher than schizokinen.

Responses to Iron Limitation

Biological responses to metals are often difficult to establish due to the fact that the chemical speciation, or form of the metal, must be considered as well as the total amount present. For some metals the most important parameter is the free ion activity. This has been established for copper toxicity (96), zinc limitation (108), and manganese nutrition (109). A relevant question then is whether the biological response of Anabaena to iron-limitation can be related to a particular solution species of iron or whether the total amount of iron present is the

important parameter. In order to shed some light on this issue, the computer program MINEQL has been utilized to simulate the non-biological solution chemistry of BG-11 medium with and without the chelators EDTA and citric acid (110). One can then compare the biological responses with the predicted iron speciation for the various growth and siderophore production experiments that have been conducted.

The fundamental response of Anabaena to iron limitation is the production of the high affinity chelator schizokinen. Determinations of schizokinen production by Anabaena have been performed on cultures grown in BG-11(mc) medium that does not contain the chelators EDTA or citrate. A comparison of measured schizokinen concentrations and the major computer-predicted iron species as a function of added iron is given in Table VII. In the absence of synthetic chelators the iron speciation in BG-11(mc) is fixed by the insolubility of $\text{Fe}(\text{OH})_3$. As a result, the concentration of the major solution species $\text{Fe}(\text{OH})_2^+$ is constant regardless of the amount of iron added to the medium. Thus, it seems likely that the activities of the soluble iron species $\text{Fe}(\text{OH})_2^+$, $\text{Fe}(\text{OH})^{2+}$, and Fe^{3+} are not the critical parameters that control the production of schizokinen. This is in contrast to the situation with copper toxicity mentioned above, and is most likely related to the presence of regulatory systems which respond to the concentration of soluble iron species inside of cells

TABLE VII

IRON SPECIATION IN BG-11 MEDIUM AS PREDICTED BY MINEQL.
(ALL CONCENTRATIONS IN MOLAR TERMS)

Experiments without added chelators

	<u>Estimated total [Fe] in medium</u>		
	<u>10⁻⁸</u>	<u>10⁻⁷</u>	<u>10⁻⁶</u>
[SK] 5 days growth	1.3x10 ⁻⁶	4.0x10 ⁻⁷	1.0x10 ⁻⁷
Fe ³⁺	2.3x10 ⁻¹⁸	2.3x10 ⁻¹⁸	2.3x10 ⁻¹⁸
Fe(OH) ²⁺	1.58x10 ⁻⁹	1.58x10 ⁻⁹	1.58x10 ⁻⁹
Fe(OH) ₃ (s)	8.3x10 ⁻⁹	1.09x10 ⁻⁷	1.0x10 ⁻⁶

Experiments with added chelators

	<u>Estimated total [Fe] in medium</u>					
	<u>10⁻⁸</u>	<u>10⁻⁷</u>	<u>10⁻⁶</u>	<u>10⁻⁵</u>	<u>10⁻⁴</u>	<u>10⁻³</u>
Fe _x (OH) _y	6.7x10 ⁻¹³	7.9x10 ⁻¹²	1.4x10 ⁻¹⁰	1.6x10 ⁻⁹	1.6x10 ⁻⁹	1.6x10 ⁻⁹
Fe-citrate	5.5x10 ⁻¹⁰	6.5x10 ⁻⁹	1.2x10 ⁻⁷	1.2x10 ⁻⁶	1.2x10 ⁻⁶	1.2x10 ⁻⁶
Fe-EDTA	9.5x10 ⁻⁹	1.0x10 ⁻⁷	8.9x10 ⁻⁷	1.6x10 ⁻⁶	1.6x10 ⁻⁶	1.6x10 ⁻⁶
Fe(OH) ₃	--	--	--	7.2x10 ⁻⁶	9.8x10 ⁻⁵	1.0x10 ⁻³

rather than in the medium. As long as the intracellular iron levels are limiting, schizokinen production would continue as would accumulation of ferric schizokinen complexes formed in the culture medium. In cultures with little iron available, essentially all the iron present is scavenged by the cells. For cultures receiving larger amounts of iron (more than 10^{-6} M) the total amount present can be considered to be available, if needed, through the chelating capacity of secreted schizokinen. Whether the solubilization occurs by chelation of the soluble iron species followed by dissolution of $\text{Fe}(\text{OH})_3$ to regain equilibrium, or by direct attack of the solid phase iron by schizokinen is not known.

The growth of Anabaena has been assessed in BG-11 medium both with and without the chelators EDTA and citrate. The predicted iron speciation in BG-11 medium containing chelators is also shown in Table VII. Comparison of the growth response (Figure 18) with the major iron species reveals that in the range 10^{-8} to 10^{-5} M iron the ferric citrate, ferric-EDTA, and soluble ferric hydroxide complexes all show some correlation with the growth lag. However, we know that schizokinen is being produced in BG-11 with EDTA and citrate present at levels similar to those in BG-11(mc) (Table V). Since these levels approximate those of citrate and EDTA, some of the iron must be chelated by schizokinen, especially in

cultures receiving less than 10^{-6} M added iron. Thus, equilibrium predictions from the computer model are not likely to be representative of speciation in the culture medium. Furthermore, attempts to model the effect of schizokinen release into the medium by incorporating this ligand into the computer program would require a greater knowledge of the kinetics of schizokinen release than is presently available.

Above 10^{-5} M iron the precipitation of ferric hydroxide fixes the iron speciation in BG-11 containing EDTA and citrate. The only difference then between 10^{-5} and 10^{-4} M iron where growth was normal, and 10^{-3} M iron where little growth occurred, is the amount of $\text{Fe}(\text{OH})_3(\text{s})$ present. One interpretation of the "toxic" effects of millimolar iron (III) is that adsorption of essential trace metals by the solid phase $\text{Fe}(\text{OH})_3$ prevents Anabaena from acquiring the trace metals required for growth. Another possibility involves physical effects associated with the cell surface where the presence of large amounts of precipitated iron might interfere with normal functioning of the cell surface components. At present it is not possible to discriminate between these or other possibilities.

A major difference between BG-11 medium with chelators versus without chelators involves the speciation of copper. As noted below, the free copper ion activity

increases dramatically in the absence of EDTA and citrate. The biological response of Anabaena at a given iron concentration is lowered growth without the chelators (Figure 19). The growth enhancement effect of EDTA and citrate occurs regardless of the amount of iron added to the medium. Thus, the most likely explanation of the growth enhancement of EDTA and citrate is lowered toxicity by complexation of free copper ions. This interpretation is consistent with the findings of Jackson and Morgan (111) who showed that the growth promoting effect of EDTA on algal populations in seawater was due to lowered free copper activity. In addition, free copper ion activities in the range of 10^{-7} molar are in the same range as those previously found to be toxic to green alga (96, 109).

Copper Speciation

	<u>BG-11 Medium</u>	
	<u>minus chelator</u>	<u>plus chelator</u>
Cu ²⁺	$6.16 \times 10^{-8} \text{ M}$	$3.5 \times 10^{-13} \text{ M}$
Cu EDTA	--	$1.2 \times 10^{-8} \text{ M}$
Cu-citrate	--	$3.1 \times 10^{-7} \text{ M}$
Cu _x (OH) _y	$5.9 \times 10^{-9} \text{ M}$	$3.4 \times 10^{-14} \text{ M}$

Environmental Implications

Experiments presented here have demonstrated that Anabaena is capable of very efficient iron scavenging, to the extent that growth is not significantly limited by iron availability in our batch cultures. This finding supports the notion that siderophore-producing cyanobacteria possess a competitive advantage over other phytoplankton when iron becomes limiting in natural systems. However, since the extent of cyanobacterial siderophore production has barely been explored, it is not clear how general this phenomenon will be. For example, the report by Morton and Lee (112) showing an increased green to blue-green ratio at low iron, and a lower (G/BG) ratio at high iron, has often been cited as evidence that low iron levels favor eucaryotic green algae. It may be significant, however, that two one of the three cyanobacteria used for their experiments, Anabaena circinalis (Wis 1038) and Gloeotrichia echinulata (Wis 1052), were found to be unable to grow in synthetic medium without chelators (27), suggesting that siderophores are not produced by these strains. What is clear at this point is that more information is needed relative to both cyanobacterial siderophore production and the mechanisms of iron acquisition by eucaryotic algae.

Finally, it should be pointed out that Anabaena 6411, at high cell densities such as what might be expected near the surface of shallow or stratified lakes during a bloom,

may produce 10 micromolar schizokinen in iron limited waters. Even if this gets diluted one thousand fold, it will still be enough to stimulate ferric-schizokinen uptake (at approximately one-third the apparent K_M for uptake). Thus, the combined action of the powerful iron-chelating capability of schizokinen towards colloidal ferric hydroxides, and the high-affinity of the uptake system for the ferric-siderophore, virtually assures Anabaena of a steady supply of iron.

CONCLUSIONS

We have shown that Anabaena 6411 produces schizokinen in response to iron limitation and that concentrations between five and ten micromolar siderophore are achieved in batch cultures without added iron. It is also possible to increase schizokinen production at least four-fold by the addition of citric acid to the medium. We have also been able to show that Anabaena 7120 produces a siderophore in low iron medium.

Iron-schizokinen uptake activity in Anabaena 6411 is also enhanced in response to iron limitation. This activity requires ATP bond energy as demonstrated by the use of metabolic inhibitors. Iron uptake displays saturation-type kinetics with an apparent K_M of 30 nM. The ferric-siderophore uptake system will respond to schizokinen and acetyl-schizokinen; but the structurally similar siderophore, aerobactin, and the trihydroxamate, Desferal, do

not support iron uptake by Anabaena 6411. The response of Anabaena 7120 to the above set of ferric-siderophores was virtually identical, suggesting that schizokinen may be the native siderophore for this strain.

Anabaena cylindrica (ATCC #27899) was tested for siderophore activity using the Arthrobacter bioassay system on concentrated culture fluids and chloroform/phenol extracts. No siderophore activity was detected in either case. This strain does not appear to produce or utilize schizokinen as a siderophore.

FUTURE DIRECTIONS

It would be of interest to test the question of whether iron limitation and the resulting production of siderophores does indeed favor the establishment of cyanobacterial "blooms" in natural waters. An opportunity to study this question and also to screen natural problem-causing cyanobacteria for siderophore production exists in Klamath Lake, Oregon. This lake supports a large annual bloom of Aphanizomenon flos-aquae. The appearance of this cyanobacterium each spring correlates with the cessation of flow of the Williamson River which carries large amounts of humic material into the lake (E. M. Perdue personal communication). This is an especially attractive system for study since it affords the opportunity to test the effect of humic acids on iron metabolism; in particular whether humics increase or decrease iron bioavailability in

natural waters and also potential siderophore-humic acid interactions. Techniques described in this paper should facilitate the search for any siderophore activity produced by Aphanizomenon in situ, and also once it is established in axenic culture.

There are many unanswered questions concerning the mechanism of iron transport by Anabaena. For example, does schizokinen serve as an iron "taxi" delivering iron only to the cell surface, or is it co-transported inside the cell? This question can be answered by preparing radio-labelled acetyl-schizokinen using [^3H]-acetic anhydride. One could then independently follow the fate of the ^3H and ^{55}Fe labels in simultaneous transport assays. It would also be of interest to know if new outer membrane proteins are produced by iron-limited cells which facilitate ferric-siderophore uptake across the outer membrane. Also, can the outer membrane be removed with lysozyme in osmotically stable buffer to produce spheroplasts that retain ferric-schizokinen transport activity? Should such preparations become available it would be possible to study the effects of the ionophores valinomycin and nigerium upon iron uptake by Anabaena. These experiments would make it possible to determine if an energized membrane is required only for ATP formation or whether the electrical or chemical components of the membrane potential are also required. It would be of great interest to measure the apparent K_M of transport for spheroplasts and compare this with that from whole

cells. This would give an indication of the location of the saturatable step(s) for ferric schizokinen transport. Spheroplast preparations would also facilitate experiments designed to determine the intracellular localization of iron following transport.

The isolation of a mutant incapable of schizokinen synthesis would make possible several interesting lines of investigation. With such a mutant it would be possible to unambiguously study any low affinity iron transport systems that might exist in Anabaena 6411. For example, is ferric citrate utilized directly for transport and is there a non-chelated low affinity system analogous to the E.coli low affinity iron transport system?

Another question relates to the mechanism of iron removal from the schizokinen chelate once the complex is transported inside the cell. Are specific ferric-siderophore reductases involved and, if so, where are they located? Is the schizokinen molecule modified to make it easier to remove the iron and protect the iron centers inside the cell from attack by intracellular schizokinen?

This research has raised many more questions than it has answered, some of which are more important and significant than others. I leave it to the curiosity and interests of any who might follow to make the determination of which, if any, of these questions are worth answering.

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