Iron Transport in Microalgae: the Isolation and Biological Activity of a Hydroxamate Siderophore from the Blue–Green Alga *Agmenellum quadruplicatum*

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Supernatants from iron-deficient cultures of the marine, coccoid blue–green alga *Agmenellum quadruplicatum* strain PR-6 contained a chloroform-soluble iron-binding hydroxamate which was stimulatory for the growth of *Arthrobacter flavescens* JG-9. Production of this material was maximal at $1\ \mu\text{M}$ -Fe, was temperature dependent, and was increased by adding NH_4^+ . At the absorption maximum of 430 nm the $A_{1\text{cm}}^{1\%}$ of the purified ferric complex was 32. The iron-free compound yielded nitroso dimers on periodate oxidation and was 60% as active on a weight basis for growth of *Arth. flavescens* as ferrioxamine B. At concentrations as low as $1\ \text{ng ml}^{-1}$ the purified hydroxamate stimulated growth of iron-depleted *Agm. quadruplicatum* in a chemically defined medium. Growth factor activity for *Arth. flavescens* JG-9 was also found in the supernatants and/or cell extracts of other blue–green algae and several diatoms grown under low-iron conditions.

INTRODUCTION

The uptake and transport of iron has long been recognized as a central problem in nutrition. In laboratory cultures of algae, the problem of iron availability has been alleviated by the use of synthetic chelators such as EDTA. For natural populations, however, very little information exists on possible modes of iron uptake and transport (Hutner, 1972). Harvey (1937) proposed that diatoms utilized colloidal particles of ferric hydroxides and phosphates, while Goldberg (1952) concluded that the iron in ferric chelates of citrate, ascorbate and artificial humate was unavailable to the marine diatom *Asterionella japonica*.

Over the past two decades it has been shown that many micro-organisms produce high-affinity ferric-iron transport compounds. There are two general classes of these microbial ferric-chelating compounds, secondary hydroxamates and catechols, collectively termed siderophores (Lankford, 1973; Neilands, 1973). It can be argued that microalgae, particularly at the alkaline pH and aerobic conditions in the photic zone, should possess a siderophore-mediated iron uptake system in order to render the insoluble colloidal iron into an available form (Hutner, 1972). However, this particular aspect of algal iron uptake and assimilation has been little investigated (Lankford, 1973).

Siderophore activity has been detected in soil, dung (Neilands, 1973) and various marine ecosystems (Estep *et al.*, 1975). Murphy *et al.* (1976) suggested that hydroxamate chelators produced by blue–green algae may antagonize growth of other microalgae and hence be a factor in blue–green algal blooms. Recently, Simpson & Neilands (1976) isolated schizokinen, the dihydroxamate siderophore produced by *Bacillus megaterium*, from the culture supernatant of iron-starved *Anabaena* sp.

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The purpose of our studies was to examine further the hypothesis that microalgae, in particular the blue-green algae, have a siderophore-mediated iron transport system.

METHODS

Organisms. The blue-green algae *Agmenellum quadruplicatum* strains PR-6 and BG-1, *Coccochloris elabens* strains 17A and Di, *Anacystis marina* strain WH-20, *Lyngbya lagerheimii* strain Mont, *Oscillatoria williamsii* strain Mev, *Oscillatoria* sp. strain JCM, *Microcoleus chthonoplastes* strain Ba-1, *Plectonema terebrans* strain Cal SP-31, *Schizothrix calcicola* strain Man and an unidentified filamentous form (strain A-2), and the diatoms *Amphora* sp. strain Amp and *Cylindrotheca* sp. strain N-1 are marine isolates of this laboratory (Van Baalen, 1962; Morgan, 1975). The green algae *Chlorella autotrophica* strain Indiana 580 and *Dunaliella tertiolecta* strain Dun were obtained from Dr R. R. L. Guillard of the Woods Hole Marine Laboratory, U.S.A.; and *Chlorella sorokiniana* strain Tx 7-11-05, *Chlorella ellipsoidea* and *Chlorella pyrenoidosa* were from Dr Jack Myers of the Laboratory of Algal Physiology, the University of Texas at Austin, U.S.A. The siderophore auxotroph *Arthrobacter flavescens* JG-9 was obtained from Dr C. E. Lankford, Department of Microbiology, The University of Texas at Austin, U.S.A.

Culture media and procedures. Marine isolates were grown in medium ASP-2 (Provasoli *et al.*, 1957) as modified by Van Baalen (1962), with various deletions or supplements as noted in the text. ASP-2 basal medium contained (g l⁻¹): NaCl, 18; MgSO₄·7H₂O, 5·0; KH₂PO₄, 0·05; CaCl₂·2H₂O, 0·37; KCl, 0·60; NaNO₃, 1·0; FeCl₃·6H₂O, 0·004; Na₂EDTA·2H₂O, 0·03; Tris, 1·0; and 10 ml P-1 trace metals stock solution containing (mg l⁻¹): H₃BO₃, 3426; MnCl₂·4H₂O, 432; ZnCl₂, 31·5; MoO₃ (85%), 3·0; CuSO₄·5H₂O, 0·3; CoCl₂·6H₂O, 1·2. Where indicated, vitamin B₁₂ was added at 4 µg l⁻¹ and vitamin B₁ at 1 mg l⁻¹. *Chlorella sorokiniana*, *Chl. ellipsoidea*, *Chl. pyrenoidosa* were cultured either in medium C of Kratz & Myers (1955), or in medium Cg-10 of Van Baalen (1967), or in medium Ct-10, i.e. medium Cg-10 with Tris buffer instead of glycylglycine buffer. Stock cultures were carried on the appropriate medium solidified with 1% agar (Difco 0140) and incubated under fluorescent or tungsten lamps at room temperature (22 to 25 °C).

The algae were grown in liquid culture at various temperatures using the test tube method of Myers (1950). Illumination was provided by four F24T12CW/HO or F48T12CW/XHO fluorescent lamps, two on each side of the water bath, 8 cm from the 22 × 175 mm Pyrex growth tubes. Air enriched to 1·0 ± 0·1 % (v/v) CO₂ was continuously bubbled through the cultures.

Contaminating iron in the ASP-2 medium without added iron was decreased by filtering the medium through Seitz filters (Arthur H. Thomas Co., Philadelphia, Pa, U.S.A.; No. 4580) previously washed with 3 M-HCl and rinsed with deionized distilled water (Kurowski & Pirt, 1971). These media are designated SF in the text and, unless otherwise indicated, EDTA and iron were not added.

Growth was occasionally monitored by chlorophyll *a* content [determined in 80% (v/v) acetone extracts using 82·04 cm² mg⁻¹ as the absorption coefficient at 663 nm (MacKinney, 1941)] but more usually by turbidimetry using a colorimeter with a coloured glass filter (660 nm). Specific growth rates (*k*) are reported in log₁₀ units d⁻¹; when *k* = 0·301 d⁻¹ the doubling time is 24 h. The packed cell volume of appropriate cell suspensions were determined after centrifuging for 2 h at 2800 *g* in cell volume tubes (Bellco Glass Co., Vineland, N.J., U.S.A.; no. T-442).

Isolation of *Agm. quadruplicatum* PR-6 siderophore. Cells grown through two transfers in complete ASP-2 + B₁₂ medium and washed three times with ASP-2 SF medium (minus Fe, minus EDTA) were inoculated at 2·5 × 10⁶ cells ml⁻¹ into the siderophore production medium [ASP-2 + B₁₂, minus Fe (0·5 µg residual Fe ml⁻¹), minus EDTA, one-tenth of the normal concentration of P-1 trace metals, plus 3 mM-NH₄Cl] and incubated for 48 h at 30 °C. (Careful washing of the inoculum was required for maximum siderophore production as any EDTA carried over repressed siderophore biosynthesis.) Cells were removed from the culture fluid by centrifugation; FeCl₃ in 0·1 M-HCl was added to the supernatant and the pH was adjusted to 4·5 with concentrated HCl. The iron-saturated supernatant was extracted three times with 0·5 vol. redistilled chloroform, which removed essentially all of the ferric-reactive material and *Arth. flavescens* JG-9 growth factor activity from the aqueous phase. The chloroform extract was evaporated to near dryness at 39 °C in a vacuum rotary evaporator. The residue was redissolved in 1·0 ml chloroform/ethanol (1:1, v/v), applied to a 1·5 × 15 cm column of Sephadex LH-20, and eluted with the chloroform/ethanol mixture. A brick-red band, containing the *Arth. flavescens* JG-9 growth factor activity was eluted first, followed by several bands detectable by their yellowish colour or their fluorescence under 365 nm light. None of the bands gave a positive reaction with Arnow's reagent for catechols.

Biological assays. *Arthrobacter flavescens* JG-9 basal medium and the bioassay for siderophore growth factor activity of algal supernatants and cell extracts were as reported previously (Estep *et al.*, 1975). Algal cells were extracted using a slight modification of the method of Byers *et al.* (1967). Frozen cells were sus-

pended in 95 % (v/v) ethanol, and the mixture was heated to near boiling point and allowed to stand for 2 to 3 h with periodic stirring. After cell debris had settled, the extract was filtered through 0.4 μm pore-size membrane filters. Fresh water algal strains were tested by streaking dense suspensions on *Arth. flavescens* JG-9 lawns.

Viability (colony-forming units) of iron-depleted *Agm. quadruplicatum* PR-6 was determined using the single cell plating techniques of Van Baalen (1965, 1967).

For preparation of the iron-depleted inoculum, a loopful from a 7 to 14 d slant culture was transferred into basal medium ASP-2 + B₁₂ and grown through two successive transfers to about 1×10^7 cells ml⁻¹. The cells were then harvested and washed with medium ASP-2 SF (minus Fe, minus EDTA, minus P-1 trace metals) by centrifuging and resuspension in three successive changes of the wash medium. The washed cells were resuspended in ASP-2 + B₁₂ SF (minus Fe, minus EDTA, one-tenth of the normal concentration of P-1 trace metals), diluted to 4.75×10^5 cells ml⁻¹ and incubated at 39 °C. After 24 h incubation, the cells (0.52×10^7 to 0.57×10^7 ml⁻¹) were harvested, washed three times with ASP-2 SF (minus Fe, minus EDTA, minus P-1 trace metals) and diluted to give 2000 cells ml⁻¹. The bioassay medium for recovery from iron deficiency, developed during the course of this work, was: ASP-2 + B₁₂ SF (minus EDTA, minus P-1 metals) with FeCl₂ · 6H₂O added to give 0.2 Fe μg ml⁻¹ and MnCl₂ · 4H₂O added at 1.73 μg ml⁻¹ prior to autoclaving for 15 min at 121 °C. The iron-depleted, washed inoculum was mixed in a large volume of the bioassay medium and dispensed in 20 ml samples to pre-sterilized growth tubes containing the siderophore isolated from *Agm. quadruplicatum* PR-6 (1 to 200 ng ml⁻¹) or other test compound.

Various compounds were assayed for growth stimulating activity on algal lawns (2000 cells ml⁻¹) prepared using iron-depleted, washed cells suspended in ASP-2 + B₁₂ SF (minus EDTA, 0.2 g Fe ml⁻¹, 0.4 μg MnCl₂ · 4H₂O ml⁻¹, 1 mM-NH₄Cl) solidified with 1 % (w/v) agar (Difco 0140) washed in distilled water. Aqueous solutions of the chelating compounds were filter-sterilized and absorbed into water-washed sterile 13 mm diam. filter paper discs (Schleicher & Schuell, no. 740-E) which were then placed on the algal lawns. After incubation for 4 to 5 d at 32 °C under tungsten lights, the plates were examined under a dissecting microscope.

All glassware was routinely soaked in 3 to 5 M-HCl for 12 to 14 h to remove contaminating iron deposits and rinsed with distilled water a minimum of seven times.

Chemical assays. Algal cellular iron content was assayed using ferrozine (Stookey, 1970) or bathophenanthroline sulphonate, sodium salt (Diehl *et al.*, 1965) as chromogenic reagents. Cell material was wet ashed in 2.0 ml of concentrated acid mixture (HNO₃/H₂SO₄/HClO₄; 10:1:4, by vol.) in 30 ml Corex centrifuge tubes heated on a modified Kjeldahl digestion apparatus. Blanks and iron standards (iron wire in 0.1 M-HNO₃) were similarly processed and the amount of iron in the blank (0.8 to 1.0 μg) was subtracted from the results for the test solutions. Absorbance was measured at 562 nm or 535 nm respectively, for the two reagents.

Bound hydroxylamine was assayed by the method of Csaky (1948) as modified by Gibson & Magrath (1969). The assay, however, is not quantitative and recovery of hydroxylamine may be variable (Byers *et al.*, 1967). Hydroxylamine (assayed without acid digestion), sodium nitrite, and ferrioxamine B hydrochloride (prepared by adding FeCl₃ · H₂O to Desferal in aqueous solutions) were used as standards. The absorbance of the diazo-coupled product was measured at 563 nm.

Cultures and culture supernatants were tested for dihydroxyphenolic compounds with Arnow's reagent (Arnow, 1937) using catechol and 2,3-dihydroxybenzoic acid as standards. The absorbancy of the test solutions was measured at 510 nm.

Chemicals. Samples of ferrichrome and rhodotorulic acid were generously supplied by Professor J. B. Neilands, Department of Biochemistry, University of California at Berkeley, U.S.A. Desferal (desferrioxamine B mesylate) was obtained from Ciba-Geigy Chemical Co., Summit, N.J., U.S.A.

RESULTS

Survey of microalgae for siderophore activity

In preliminary studies, culture supernatants and cells of *Agm. quadruplicatum* strains PR-6 and BG-1 and *Coccochloris elabens* strains Di and 17A after growth on iron-limited medium were assayed for hydroxamate siderophores. Growth factor activity for *Arth. flavescens* JG-9 was found but was variable and not dependent on the iron concentration of the medium. Csaky tests for bound hydroxylamine, performed on the algal supernatants which stimulated *Arth. flavescens* JG-9, were positive but very low. Subsequently, iron-sufficient cells of *Agm. quadruplicatum* PR-6 grown in medium ASP-2 + B₁₂ were extracted

Table 1. *Survey of microalgae for siderophore production: growth stimulation of Arthrobacter flavescens JG-9 and the Csaky reaction*

Microalgal strain*	JG-9 pad assay		Csaky reaction
	Culture supernatant	Ethanol cell extract	
Blue-green algae			
<i>Agmenellum quadruplicatum</i> PR-6	+	+	+
<i>Agmenellum quadruplicatum</i> BG-1	—	+	—
<i>Anacystis marina</i> WH-20	—	—	—
<i>Coccochloris elabens</i> 17A	+	+	+
<i>Coccochloris elabens</i> Di	—	+	—
<i>Microcoleus chthonoplastes</i> Ba-1	—	+	—
<i>Lyngbya lagerheimii</i> Mont	—	—	—
<i>Plectonema terebrans</i> Cal SP-31	—	—	—
<i>Oscillatoria williamsii</i> Mev	—	+	—
<i>Oscillatoria</i> sp. JCM	—	+	—
<i>Schizothrix calcicola</i> Man	—	+	—
Unidentified species A-2	—	+	—
Green algae			
<i>Dunaliella tertiolecta</i> Dun	—	—	—
<i>Chlorella autotrophica</i> Ind 580	—	—	—
Diatoms			
<i>Amphora</i> sp. Amp	—	+	—
<i>Cylindrotheca</i> sp. N-1	+†‡	+‡	—

* All strains were cultured at 30 °C in medium ASP-2+ vitamins B₁ and B₁₂ (minus Fe, minus EDTA, plus 50 mg NH₄Cl l⁻¹, one-tenth of the normal concentration of P-1 trace metals). For growth of the diatoms, the medium was supplemented with 125 mg Na₂SiO₃·5H₂O l⁻¹.

† Chloroform extract of culture supernatant concentrated 1000-fold.

‡ Zone of inhibition of background growth around pad immediately followed by a zone of stimulation.

with methanol, ethanol, acetone, 85% (w/v) phenol, chloroform and several combinations of these solvents in a Soxhlet apparatus. Siderophore growth factor activity was best recovered using chloroform extraction for 24 h. When the chloroform extract was evaporated to 5.0 ml and assayed for growth factor activity for *Arth. flavescens* JG-9, the extract gave a growth stimulatory effect equivalent to about 0.7 µg Desferal (g dry wt algae)⁻¹. This material gave a positive Csaky test for bound hydroxylamine. By comparison, Byers *et al.* (1967) found *Bacillus megaterium* contained 400 µg schizokinen (g dry wt)⁻¹ when grown in iron-sufficient medium without a synthetic metal chelator.

Siderophore activity was recovered from a variety of blue-green algae and diatoms when grown on low-iron medium (Table 1). Supernatants were tested directly, except that of *Cylindrotheca* sp. strain N-1 which was a 1000-fold concentrated chloroform extract. The freshwater algae *Chl. pyrenoidosa*, *Chl. ellipsoidea* and *Chl. sorokiniana* gave a positive response only when streaked on JG-9 lawns. Supernatants of *Agm. quadruplicatum* PR-6 yielded the highest siderophore activity, equivalent to 1 µg Desferal ml⁻¹.

In a survey of specific growth rates and yield of cells in low-iron media without added EDTA, *Agm. quadruplicatum* PR-6 was clearly superior to *Coccochloris elabens* strains Di and 17A and *Agm. quadruplicatum* strain BG-1. It grew reproducibly in medium ASP-2 + B₁₂ without EDTA at 80% of the rate in medium containing EDTA. The cell yield (dry wt ml⁻¹) for any given limiting amount of iron was nearly twice that of the other three organisms. Iron added at 0.1 µg Fe ml⁻¹ to ASP-2 medium minus EDTA gave a maximum growth rate and biomass production after 72 h. This alga was therefore selected for further study.

Fig. 1

Fig. 2

Fig. 1. *Arthrobacter flavescens* JG-9 growth factor activity in supernatants of iron-depleted *Agmenellum quadruplicatum* PR-6 as a function of the temperature and NH_4Cl concentration. The PR-6 inoculum was grown at 30 °C in complete ASP-2 + B_{12} medium, washed three times with ASP-2 SF (minus Fe), resuspended in ASP-2 + B_{12} (minus Fe, minus EDTA, one-tenth of the normal concentration of P-1 trace metals, plus NH_4Cl as indicated) at an initial cell density of about 0.25×10^7 cells ml^{-1} , and incubated at 26 °C (●), 30 °C (○) or 39 °C (□) for 48 h. Siderophore activities of algal supernatants are expressed as Desferal equivalents.

Fig. 2. Time course of *Agmenellum quadruplicatum* PR-6 growth and siderophore production. The PR-6 inoculum was grown and prepared as in Fig. 1. The initial cell density was about 0.5×10^7 cells ml^{-1} , cultures were incubated at 30 °C, and 1 mM- NH_4Cl was added to the basal medium. Cultures were taken at intervals for estimations of siderophore concentration (○, expressed as Desferal equivalents) and algal dry weight (●).

Isolation and properties of the siderophore from *Agm. quadruplicatum* PR-6

Hydroxamate production increased about 100-fold at 30 °C if 2 mM- NH_4Cl was added to the medium, but there was only a slight increase if cultures were incubated at 26 or 39 °C (Fig. 1). Accumulated siderophore activity was maximal about 48 h after the beginning of iron depletion (Fig. 2).

A sufficient quantity of the *Agm. quadruplicatum* PR-6 siderophore for chemical and biological assays was obtained by scaling up cultures to 250 ml in 50 × 500 mm culture tubes. The siderophore was isolated as described in Methods. The major fraction following elution from Sephadex LH-20 with chloroform/ethanol (1:1, v/v) was evaporated under nitrogen and dissolved in a minimum volume of 0.02 M-ammonium formate, pH 9.0. When this sample was passed through a 1.5 × 20 cm column of Sephadex G-25 and eluted with 0.02 M-ammonium formate, pH 9.0, it was separated into a major and a minor band. Both bands had qualitatively identical ultraviolet and visible absorption spectra and growth factor activity. The minor band was not investigated further. When the major band was rechromatographed on Sephadex G-25, it moved as a single band. Ammonium salts were removed by adjusting the eluate pH to 4.5 and extracting the ferric hydroxamate into chloroform. A typical yield was 2.0 to 2.5 mg of purified compound per litre of culture supernatant.

The ferric compound was soluble in chloroform, butan-2-ol, ethanol and methanol. It was only sparingly soluble in acetone and was insoluble in diethyl ether. The solubility in aqueous solutions was increased considerably above pH 8.0.

Fig. 3. Absorption spectra of the *Agmenellum quadruplicatum* PR-6 siderophore (○) and PR-6 desferrisiderophore (●) in chloroform. Shown for comparison are spectra of ferrioxamine B (□) and desferrioxamine B (■) in water.

The absorption spectrum of the purified compound in chloroform had a broad absorption peak at 430 nm, typical of ferric hydroxamates (Fig. 3). The $A_{1\text{cm}}^{1\%}$ in chloroform was 32, which is in the general range reported for ferric hydroxamates (Neilands, 1966). There were only small changes in absorbance with pH change (2.0 to 9.5) suggesting that the compound is a trihydroxamate, although a dihydroxamate with another strong complexing group cannot be ruled out (Byers *et al.*, 1967; Emery, 1965; Gibson & Magrath, 1969).

Iron was removed from the ferric complex by shaking the chloroform solution with 5 M-HCl followed by washing the chloroform twice with distilled water [this is essentially the method used by Snow (1965) to remove iron from mycobactin].

Periodate oxidation of 0.1 mg of the desferrihydroxamate by the method of Emery & Neilands (1960, 1962) gave a product with an absorption peak at 267 nm. The intact desferrisiderophore had no absorption peak near this wavelength at the same concentration. Oxidation of Desferal with periodate under the same conditions gave an apparent molar absorption of 8810. This value is less than the theoretical yield for nitrosoalkane dimers from Desferal but is in good agreement with values obtained with other polyhydroxamic acids (Atkin & Neilands, 1968; Gibson & Magrath, 1969).

The hydroxylamine content of the siderophore was estimated by the method of Csaky (1948) following the acid hydrolysis procedure of Gibson & Magrath (1969); 0.231 mg siderophore gave an absorbance of 0.66. This compares with 0.1 μmol sodium nitrite and hydroxylamine standards which gave an absorbance of 0.438 when assayed without acid and 0.055 mg ferrioxamine B, prepared by adding iron to Desferal, which gave an absorbance of 0.38.

Despite the lack of strict quantification, these chemical assays support the conclusion that the *Agm. quadruplicatum* PR-6 siderophore is probably a trihydroxamate.

The iron content of siderophore, estimated by extracting a chloroform solution (containing 11.82 mg) with 5 M-HCl (Snow, 1965) and quantifying the iron with ferrozine (Stokey, 1970), was about 4% (w/w). From this value a molecular weight can be calculated for the iron complex as 1300, assuming a 1:1 binding ratio. This then gives a molar absorption of about 4100 which accords with the previously recorded value of 3000 to 4000 for ferric polyhydroxamic acids (Neilands, 1966, 1973).

Fig. 4

Fig. 5

Fig. 4. Growth of *Agmenellum quadruplicatum* PR-6 and excretion of *Arthrobacter flavescens* JG-9 growth factor activity as a function of the initial iron concentration. The PR-6 inoculum was grown and prepared as in Table 1 and resuspended in ASP-2 + B₁₂ SF (Fe added as indicated, minus EDTA, one-tenth of the normal concentration of P-1 trace metals, plus 2 mM-NH₄Cl) at an initial cell density of about 0.3×10^6 cells ml⁻¹. Cultures were incubated at 30 °C for 48 h. Algal supernatant siderophore concentration (○) was estimated turbidimetrically (*A*₆₆₀) in liquid culture *Arth. flavescens* JG-9 assay and growth (●) was determined as chlorophyll *a* concentration.

Fig. 5. Effect of *Agmenellum quadruplicatum* PR-6 siderophore and trace metal concentration on the growth of iron-depleted, washed PR-6 cells. Iron-depleted, washed cells were incubated at 39 °C in medium containing 0.2 µg Fe ml⁻¹ and four-tenths of the normal concentration of Mn²⁺ (no other P-1 trace metals) with purified PR-6 siderophore added at 10 (○), 5 (□), 1 (△) and 0 (+) ng ml⁻¹ or four-tenths of the normal concentration of P-1 trace metals with PR-6 siderophore added at 10 (●), 5 (■), 1 (▲) and 0 (×) ng ml⁻¹. The iron and trace metals were added prior to autoclaving and no NH₄Cl was added to the bioassay medium.

Biological features of the *Agm. quadruplicatum* siderophore

The specific growth factor activity of the purified siderophore for *Arth. flavescens* JG-9 was estimated by plate and liquid culture bioassays to be about 60% of an equivalent weight of ferrioxamine B.

Iron added to *Agm. quadruplicatum* PR-6 cultures repressed siderophore production (Fig. 4). Excretion of siderophore activity was maximal with added iron at 1 µM and was completely repressed at 50 µM-iron. This behaviour is typical of siderophore repression in various microbial systems (Lankford, 1973; Neilands, 1973).

Agmenellum quadruplicatum PR-6 grown in basal medium ASP-2 + B₁₂, washed three times with ASP-2 SF (minus Fe, minus EDTA, minus P-1 metals), then depleted of iron for 24 h at 39 °C in medium ASP-2 + B₁₂ SF (minus Fe, minus EDTA, one-tenth of the normal concentration of P-1 trace metals), exhibited a lag of 10 to 12 h in recovering to the normal generation time of 4 h in medium minus EDTA, but containing 2 µM-Fe. Adding the purified siderophore at 0.10 µg ml⁻¹ decreased this lag period by 1.5 to 2.0 h but the effect was not dependent on the initial cell concentration over the range tested (1.6×10^5 to 40×10^5 cells ml⁻¹). A siderophore-dependent lag in growth initiation from small cell inocula seen in some bacterial systems (Lankford *et al.*, 1966) could not be demonstrated in *Agm. quadruplicatum* PR-6.

Two conditions which strongly inhibit the rate of recovery from iron deficiency were (i) vigorous washing via vortex mixing of the iron-deficient cells used as inocula, and (ii) autoclaving added iron with the medium. Under these conditions recovery from iron deficiency

was largely dependent on added siderophore (Fig. 5). Although maximum siderophore stimulation varied, sometimes occurring at 10, 5 or even 1 ng ml⁻¹, the results were qualitatively similar: adding the purified siderophore to iron-deficient cells always resulted in more rapid recovery. Concentrations of the isolated siderophore higher than 10 ng ml⁻¹ gave no greater stimulation and appeared to inhibit recovery in a few cases. Iron-deficient, washed cells did not grow unless FeCl₃ was added to the bioassay medium. Manganese was also required for recovery from iron deficiency as no growth occurred if manganese was omitted from the bioassay medium. Trace metal interactions may be an explanation for some of the difficulty in experimental control.

Other compounds were assayed for growth stimulating activity using algal lawns of *Agm. quadruplicatum* PR-6. Rhodotorulic acid, 2,3-dihydroxybenzoic acid, acetylacetone and 8-hydroxyquinoline were inhibitory with no stimulation beyond the zone of complete inhibition. Growth was stimulated by acetohydroxamic acid, sodium citrate, oxalic acid, lactic acid, salicylic acid, kojic acid, ferrichrome Desferal and the purified *Agm. quadruplicatum* PR-6 siderophore as estimated by visual and microscopic comparison of colony size and colony colour against the control pads.

DISCUSSION

Iron repression of biosynthesis, grown factor activity for *Arth. flavescens* JG-9, and a positive Csaky test for bound hydroxylamine may be considered sufficient evidence that *Agm. quadruplicatum* PR-6 produces a hydroxamate siderophore and thus has an iron transport system of the type common to most aerobic micro-organisms. Detection of growth factor activity for *Arth. flavescens* JG-9 in some of the blue-green algae, diatoms and green algae surveyed suggests that microalgae as a whole may produce iron-repressible ferric-complexing compounds under the appropriate conditions of low-iron availability (Simpson & Neilands, 1976; Murphy *et al.*, 1976).

The purified siderophore from *Agm. quadruplicatum* PR-6 is unusual in that it is extractable from aqueous media into chloroform, a property in common with the recently discovered exochelin excreted by *Mycobacterium bovis* (Macham & Ratledge, 1975). A trihydroxamate siderophore is possibly indicated by the small spectral changes of the iron complex at different pH values. Nitroso dimer generation via periodate oxidation indicates the compound contains secondary hydroxamate groups.

Evidence that the hydroxamate functions as a siderophore is the derepression of biosynthesis by iron depletion and the stimulatory activity in recovery of *Agm. quadruplicatum* PR-6 from iron deficiency. Iron-deficient cells exhibited a growth response to the isolated siderophore at concentrations of 1 ng ml⁻¹, and 5 ng ml⁻¹ was sufficient to saturate growth. Phytoplankton samples have been found to excrete 7 to 50% of photoassimilated carbon (Fogg *et al.*, 1965) and excretion of nitrogenous substances, chiefly amino acids and small peptides, is reported to be common in algal cultures (Walsby, 1974; Hellebust, 1974). Such excretion was, in general, thought to be the result of an imbalance between metabolic pathways. Hood *et al.* (1969) suggested that release of extracellular peptides and amino acids by *Anabaena variabilis* was due to lack of metabolic control over amino acid biosynthesis. Siderophore excretion and recovery from iron deficiency by *Agm. quadruplicatum* PR-6 indicates a high degree of metabolic control over iron-assimilatory mechanisms and iron-dependent metabolic pathways. Excretion of organic compounds, especially nitrogenous substances, by phytoplankton may be a reflection of a similar iron-uptake mechanism. The subtle influence that siderophores, having both growth stimulatory and inhibitory activity, may play in microalgal species succession and diversity has been pointed out (Hutner, 1972).

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