Iron Uptake by Toxic and Nontoxic Strains of *Microcystis aeruginosa*[†]

Manabu Fujii,^{1*} Andrew L. Rose,^{2,3} and T. David Waite³

*Department of Civil Engineering, Tokyo Institute of Technology, 2-12-1-M1-4 Ookayama, Tokyo 152-8552, Japan¹;
 Southern Cross GeoScience, Southern Cross University, Lismore, New South Wales 2480, Australia²; and School of
 Civil and Environmental Engineering, The University of New South Wales, Sydney, New South Wales 2032, Australia³*

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Iron uptake by microcystin-producing and non-microcystin-producing strains of *Microcystis aeruginosa* was investigated through short-term uptake assays. Although strain-specific differences were observed, the siderophore-independent Fe uptake kinetics were essentially similar (e.g., maximum uptake rates of 2.0 to 3.3 amol · cell⁻¹ · h⁻¹) for the wild-type toxic strain PCC7806 and a genetically engineered mutant unable to produce microcystin.

The occurrence of *Microcystis aeruginosa*, a freshwater bloom-forming cyanobacterium, in water bodies used for drinking water supplies represents a serious health concern, as some strains can produce a potent hepatotoxin, microcystin. A number of studies over the last few decades have shown that environmental and nutritional factors regulate microcystin synthesis by this organism, including photosynthetically active radiation (7), temperature, and macro- and micronutrients such as nitrate (8), phosphate (14), and iron (Fe) (9, 10, 21). However, the physiological function of this secondary metabolite is still unclear (1, 19–21). Incubation of *M. aeruginosa* cells under Fe limitation results in increasing production of microcystin, as well as upregulated transcription of genes involved in cyclic heptapeptide synthesis (1, 9, 10, 20), possibly as a result of dissociation of the Fe homeostatic protein (Fur) bound to the promoter region of the microcystin gene cluster (11). Additionally, the Fe uptake rate by microcystin-producing strains

has been shown to be significantly higher than that in non-microcystin-producing strains (21). This type of evidence combined with a possible function of microcystin as a moderate metal chelator (18) and pore-forming peptide embedded in the outer membrane (16) led some researchers to speculate that microcystin may play a role in intracellular storage or as a transport peptide in Fe metabolism (21). Despite its potential relevance to toxin production, however, the mode of Fe uptake by toxic and nontoxic strains of *M. aeruginosa* and any link of microcystin to Fe metabolism are poorly understood.

In this work, the kinetics of ferrous iron [Fe(II)] and ferric iron [Fe(III)] uptake by toxic and nontoxic strains of *M. aeruginosa* have been investigated by measuring the uptake rates of radiolabeled ⁵⁵Fe by cultures of these strains grown in chemically well-defined medium. Batch cultures of microcystin-producing and non-microcystin-producing strains of *M. aeruginosa* (PCC7806 and PCC7005, respectively) were incubated in Fe-

TABLE 1. Kinetic model for Fe chemistry used in this study

System	Reaction	Rate constant ^a			Reference
		Symbol	Value	Unit	
Fe(III)	Fe(III)' + Cit → Fe(III)-Cit	k_{f-Cit}	2.1×10^5	$M^{-1} s^{-1}$	5
	Fe(III)-Cit → Fe(III)' + Cit	k_{d-Cit}	2.7×10^{-4} – 2.8×10^{-3b}	s^{-1}	3
Fe(II)	Fe(II)' + Cit → Fe(II)-Cit	k_{f-Cit}^*	5.0×10^2	$M^{-1} s^{-1}$	17
	Fe(II)-Cit → Fe(II)' + Cit	k_{d-Cit}^*	2.0×10^{-3}	s^{-1}	17
	Fe(II)' + EDTA → Fe(II)-EDTA	k_{f-EDTA}^*	1.4×10^3	$M^{-1} s^{-1}$	6
	Fe(II)-EDTA → Fe(II)' + EDTA	k_{d-EDTA}^*	2.0×10^{-4}	s^{-1}	6

^a Definitions for rate constants are as follows: k_{f-Cit} , complexation rate constant for ferric citrate complex; k_{d-Cit} , dissociation rate constant for ferric citrate; k_{f-Cit}^* , complexation rate constant for ferrous citrate complex; k_{d-Cit}^* , dissociation rate constant for ferrous citrate; k_{f-EDTA}^* , complexation rate constant for ferrous EDTA complex; and k_{d-EDTA}^* , dissociation rate constant for ferrous EDTA complex.

^b Dissociation of ferric citrate (k_{d-Cit}) is a function of citrate concentration ([Cit]) due to the formation of two mononuclear ferric citrate complexes [Fe(III)Cit and Fe(II)Cit₂], as described by $k_{d-Cit} = k_{d-FeCit1}/1 + K_{FeCit2}[Cit]$ (3), where $k_{d-FeCit1}$ represents the dissociation rate constant for ferric mononuclear complex [i.e., Fe(III)Cit] and K_{FeCit2} is the conditional stability constant for Fe(III)Cit and Fe(III)Cit₂ at a pH of ~8. Values for $k_{d-FeCit1}$ ($3.7 \times 10^{-3} s^{-1}$) and K_{FeCit2} ($1.3 \times 10^4 M^{-1}$) were previously reported (3).

* Corresponding author. Mailing address: Department of Civil Engineering, Tokyo Institute of Technology, 2-12-1-M1-4 Ookayama, Tokyo 152-8552, Japan. Phone: 81-3-5734-2597. Fax: 81-3-5734-3577. E-mail: fujii.m.ah@m.titech.ac.jp.

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FIG. 1. Uptake of ^{55}Fe by the toxic strain PCC7806 (A and D), the nontoxic strain PCC7005 (B and E), and the microcystin-deficient $\Delta mcyH$ mutant (C and F) of *M. aeruginosa* as a function of unchelated Fe(III) (A to C) or Fe(II) (D to F) concentration. Cells were precultured in the medium fraquil*, where the initial Fe(III) and EDTA concentrations were adjusted to 0.1 μM and 26 μM , respectively, to acclimate to moderate Fe limitation. The short-term Fe uptake assays were then undertaken by resuspending the cells in Fe- and ligand-free fraquil*, adding either equilibrated $^{55}\text{Fe(III)}$ -EDTA or $^{55}\text{Fe(III)}$ -citrate complex and incubating for 3 h in the absence and presence of ascorbate. Symbols and error bars are averages and plus-minus standard deviations of the data when EDTA ($\times$) or citrate ($\diamond$) was used as the Fe-binding ligand. Solid and dotted lines represent the model fit to the uptake rates using parameters from linear and nonlinear regression analysis, respectively.

limited fraquil* medium (2) and sacrificed for Fe uptake assays (4). Although the similarity (99%) between the 16S rRNA sequences of strains PCC7005 and PCC7806 is recognized (12), a microcystin-deficient ($\Delta mcyH$) mutant of PCC7806 developed by insertional inactivation of the *mcyH* gene (15) was also used to examine the strain-specific difference in Fe uptake. The extent of microcystin production (or lack of production) was previously confirmed for the strains used by use of a protein phosphatase 2A inhibition assay (1).

The methodology and conditions used for cell incubations and uptake experiments were identical to those documented earlier (4). Cells were incubated in fraquil* medium with moderate Fe limitation (containing, in addition to a well-defined suite of major and minor nutrients, 0.1 μM Fe and 26 μM EDTA) and harvested during the daytime in late exponential growth phase (at cell densities between 3×10^6 and 20×10^6 cell ml^{-1} as measured by a hemocytometer) on 0.65- μm -pore-size polyvinylidene difluoride (PVDF) membrane filters. Subsequently, the cells were resuspended into Fe- and ligand-free fraquil* medium and short-term Fe uptake experiments were initiated by adding a solution of radiolabeled $^{55}\text{Fe(III)}$ pre-equilibrated with either EDTA or citrate at different ligand/Fe ratios (14 to 370 parts EDTA and 9.6 to 260 parts citrate) (see Table S1 in the supplemental material) in order to provide a

range of concentrations of biologically available unchelated Fe, commonly denoted as Fe'. In experiments in which Fe(II) uptake was examined, the assay was performed with the additional presence of 1 mM ascorbate in order to reduce all Fe(III) to Fe(II), as well as to prevent reoxygenation of the generated Fe(II). To determine whether cellular exudates influence Fe acquisition, uptake experiments were also conducted in the presence of cellular exudates excreted under Fe starvation conditions. For this purpose, the spent medium was prepared by incubating the harvested cells in Fe-free fraquil* ([Fe] = 0 μM and [EDTA] = 26 μM) for 1 day at cell numbers of 4×10^6 to 9×10^6 cell ml^{-1} , followed by removal of cells from the culture medium by filtration. The filtered cells were then split into two aliquots and resuspended either in the exudate solution after the addition of $^{55}\text{Fe(III)}$ -EDTA stock at final concentrations of 0.7 μM ^{55}Fe and 52 μM EDTA or in fresh fraquil* medium with a nutrient composition identical to that of the spent medium. All incubations were performed at 27°C for 3 to 4 h (over which time linear Fe uptake was confirmed). Cultures were then vacuum filtered onto the PVDF membrane filter, and cells on the filter rinsed with an EDTA-oxalate solution (50 mM EDTA and 100 mM oxalate at pH 7) to remove extracellularly adsorbed Fe. The radioactivity of the sample was measured using a liquid scintillation counter

TABLE 2. Half-saturation constants and maximum Fe uptake rates for the toxic and nontoxic strains of *M. aeruginosa* acclimated under moderate Fe limitation^a

Strain	Primary substrate	Linear regression ^b		Nonlinear regression ^c	
		K_S (pM)	$\rho_S^{\max}$ [mean amol cell ⁻¹ h ⁻¹ ($\pm$ SD)]	K_S (pM)	$\rho_S^{\max}$ [mean amol cell ⁻¹ h ⁻¹ ($\pm$ SD)]
PCC7806	Fe(III)'	33 ($\pm$ 3.4)	3.3 ($\pm$ 0.16)	33 ($\pm$ 4.5)***	3.3 ($\pm$ 0.13)***
PCC7005	Fe(III)'	41 ($\pm$ 8.2)	13 ($\pm$ 1.2)	34 ($\pm$ 5.5)***	13 ($\pm$ 0.54)***
$\Delta mcyH$	Fe(III)'	51 ($\pm$ 11)	3.9 ($\pm$ 0.44)	49 ($\pm$ 19)*	3.9 ($\pm$ 0.44)***
PCC7806	Fe(II)'	970 ($\pm$ 380)	2.0 ($\pm$ 0.28)	1,200 ($\pm$ 700)	2.2 ($\pm$ 0.30)***
PCC7005	Fe(II)'	2,000 ($\pm$ 1,100)	4.5 ($\pm$ 0.91)	2,900 ($\pm$ 940)*	5.5 ($\pm$ 0.55)***
$\Delta mcyH$ mutant	Fe(II)'	840 ($\pm$ 170)	2.4 ($\pm$ 0.15)	650 ($\pm$ 220)*	2.3 ($\pm$ 0.14)***

^a The toxic strain was PCC7806, and the nontoxic strains were PCC7005 and the $\Delta mcyH$ mutant. The Fe limitation conditions were 0.1 μ M Fe.

^b $K_{Fe'}$ and $\rho_{Fe'}^{\max}$ were determined from linear regression analysis in the Eadie-Hofstee plots (see Figure S1 in the supplemental material).

^c Nonlinear regression analysis was undertaken by use of R version 2.13.0, a free software for statistical computation. Asterisks represent statistically significant levels as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

with concurrent counts of an ⁵⁵Fe standard. A kinetic model developed in our previous work (Table 1) that describes the chemical reactions involving Fe species in the system was used to calculate steady-state concentrations of unchelated Fe(II) and Fe(III) [i.e., Fe(II)' and Fe(III)']. To avoid the complexity of photochemically mediated Fe transformations in the calculation of Fe' concentrations, all Fe uptake experiments were performed under dark conditions.

The uptake rates of extracellular ⁵⁵Fe for the three strains were measured over a range of Fe(II)' and Fe(III)' concentrations (Fig. 1). Michaelis-Menten-type saturation theory was assumed to describe uptake rates (ρ_s) as follows: $\rho_s = \rho_s^{\max}[S]/K_S + [S]$, where $[S]$ indicates the concentration of biologically available Fe, K_S represents the half-saturation constant, and $\rho_s^{\max}$ represents the maximum uptake rate under the conditions examined. The Fe uptake parameters for the various systems investigated were determined from linear and nonlinear regression analyses, which essentially provided similar results (Table 2). The saturation theory adequately described the measured uptake rates in both the Fe(II)- and Fe(III)-dominated systems (Fig. 1). Furthermore, by incubating cells in spent medium amended with ⁵⁵Fe, we confirmed that cellular exudates produced under Fe-deficient growth conditions did not facilitate ⁵⁵Fe uptake for any of the strains examined (Fig. 2). While siderophore-mediated Fe uptake has been identified for a range of pathogenic bacteria and for a few cyanobacteria, such as *Anabaena* and *Synechocystis* spp. (13), these results suggest that the production of extracellular exudates such as siderophores and extracellular polysaccharides under the conditions examined here are not effective in facilitating short-term Fe uptake by *M. aeruginosa*, in accord with findings in previous studies (4).

The microcystin-producing strain of *M. aeruginosa* (CYA 228/1) has previously been suggested to acquire Fe at a higher rate ($\sim$ 2-fold) than the non-microcystin-producing strain (CYA 43) (21). In contrast, our findings indicate that the rates of Fe uptake by PCC7806 and the nontoxic variant were essentially identical. Inconsistency between the findings of the current study and those of previous investigators may be due to strain-specific differences in the Fe uptake rate. Indeed, the $\rho_s^{\max}$ for PCC7005 was determined to be greater than the $\rho_s^{\max}$ for the other two strains by 2- to 4-fold, with the extent of exceedance dependent on the redox state of iron (Table 2).

If microcystin was responsible for intracellular transport or extracellular acquisition of Fe as previously proposed (21), the artificial mutation of genes involved in microcystin synthesis might result in a decline in Fe uptake compared to that in the wild-type strain. It is unlikely that an alternative peptide with a function similar to that of microcystin can be solely expressed in the mutant strain to maintain the Fe uptake system. Recent proteomic studies (22; R. Alexova, T. C. Dang, M. Fujii, M. J. Raftery, T. D. Waite, B. D. Ferrari, and B. A. Nailan, unpublished data) consistently indicated that multiple proteins involved in photosynthesis and carbon and nitrogen metabolism were differentially expressed between toxic and nontoxic cells. The regulation of a redox-sensitive protein (thioredoxin) was

FIG. 2. ⁵⁵Fe uptake rates for the toxic (PCC7806), nontoxic (PCC7005), and microcystin-deficient mutant of PCC7806 ($\Delta mcyH$ mutant) strains of *M. aeruginosa* in the presence of cellular exudates. The spent medium was prepared by incubating cells in Fe-free fraquil* followed by removal of the cells by filtration of the culture medium. Cells on the filter were split into two aliquots and incubated either in the filtrate solution after the addition of ⁵⁵Fe(III)-EDTA stock (in the presence of exudates) or in fresh fraquil* with the same nutrient composition as the spent medium (in the absence of exudates, i.e., control). In either case, final concentrations were adjusted to 0.7 μ M for ⁵⁵Fe and 52 μ M for EDTA. The data and error bars represent the means and standard deviations from triplicate experiments. The asterisk indicates that Fe uptake in the presence of exudate was significantly different from that in the control at a P value of <0.01 using a single-tailed heteroscedastic t test.

TABLE 3. Specific growth rates for the toxic and nontoxic strains of *M. aeruginosa* in fraquil* with different initial Fe concentrations^a

Strain	Initial Fe concn (μM)	Specific growth rate (day ⁻¹) ^b		
		Early exponential growth phase (days 1–5)	Late exponential growth phase (days 6–10)	Exponential growth phase (days 1–10)
PCC7806	0.01	0.81 (±0.02)	0.23 (±0.08)	
	0.1	0.77 (±0.05)	0.53 (±0.01)	
	1	0.78 (±0.08)	0.87 (±0.02)	
	10	0.74 (±0.01)	0.83 (±0.03)	
PCC7005	0.01	0.73 (±0.02)	0.15 (±0.03)	
	0.1	0.80 (±0.001)	0.76 (±0.01)	
	1	0.89 (±0.004)	0.69 (±0.03)	
	10	0.85 (±0.02)	0.70 (±0.03)	
Δ mcvH mutant	0.01			0.29 (±0.07)
	0.1			0.56 (±0.02)
	1			0.43 (±0.14)

^a The toxic strain was PCC 7806, and the nontoxic strains were PCC7005 and the Δ mcvH mutant. The initial Fe concentrations were from 0.01 μM to 10 μM. In all incubations, concentrations of nutrients and trace metals other than Fe were constant. The concentration of EDTA was adjusted to 26 μM. Incubational conditions were also invariant among incubations.

^b Data shown are means with plus-minus standard deviations from duplicate runs.

found to be altered by the presence of microcystin, possibly via binding to cysteine residues of phycobilisomes and carbon metabolism proteins, implying a potential role in oxidative stress regulation (22). As shown in Table 3, the growth rates measured for the microcystin-deficient mutant were lower than those of the wild-type strain, presumably due to the absence of microcystin even under conditions where similar Fe uptake rates are expected. Furthermore, dependency of microcystin synthesis on environmental and nutritional factors other than Fe is now widely reported (7–10, 14, 21), supporting a more global role of microcystin.

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