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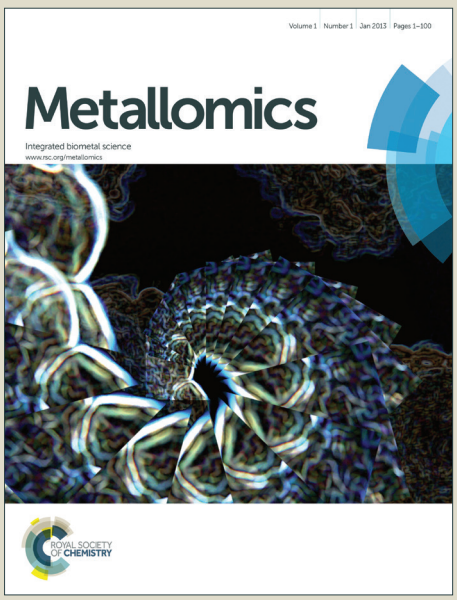

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Uptake mechanisms for inorganic iron and ferric citrate in *Trichodesmium erythraeum* IMS101

Kelly L. Roe[1*] and Katherine A. Barbeau[1]
[1]Geoscience Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla CA 92093

[*]Currently at: Department of Chemistry, Colorado School of Mines, Golden CO 90401

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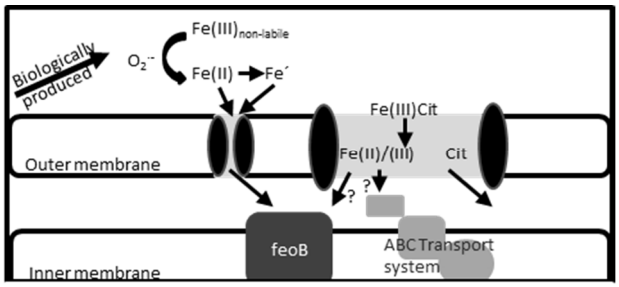

Trichodesmium erythraeum IMS101 utilizes two different iron uptake systems to acquire iron from $FeCl_3$ and Ferric Citrate, potentially allowing *Trichodesmium* to have a competitive advantage in its bacteria-rich colony environment.

Abstract

Growth of the prevalent marine organism *Trichodesmium* can be limited by iron in natural and laboratory settings. This study investigated the iron uptake mechanisms that the model organism *T. erythraeum* IMS101 uses to acquire iron from inorganic iron and iron associated with the weak ligand complex, ferric citrate. IMS101 was observed to employ two different iron uptake mechanisms: superoxide-mediated reduction of inorganic iron in the surrounding milieu and a superoxide-independent uptake system for ferric citrate complexes. While the detailed pathway of ferric citrate utilization remains to be elucidated, transport of iron from this complex appears to involve reduction and/or exchange of the iron out of the complex prior to uptake, either at the outer membrane of the cell or within the periplasmic space. Varied iron uptake strategies may allow *Trichodesmium* to effectively scavenge iron in oligotrophic ocean environments.

Introduction

Iron can be a limiting nutrient to marine microorganisms in some areas of the world's oceans. *Trichodesmium*, a nitrogen fixing cyanobacterium found in the tropical

and subtropical ocean, has a high iron quota (1, 2) due largely to the energy and iron-expensive processes of nitrogen fixation and photosynthesis. Iron limitation decreases growth, photosynthesis and nitrogen fixation in cultures of the model N_2-fixing cyanobacterium *Trichodesmium erythraeum* IMS101 (2-6) and similar effects are believed to occur in nature (7-9). In the ocean, *Trichodesmium* can be found in colonial forms which provide an enriched microenvironment that supports a diverse microbial community (10-13). These *Trichodesmium*-associated microbes include marine bacteria that produce siderophores, iron-binding ligands, under iron limiting conditions (14, 15). Recent studies investigating iron uptake strategies in *Trichodesmium* have indicated some variability in the bioavailability of ferri-siderophore complexes to *Trichodesmium*(5, 9). However, it is likely that *Trichodesmium* must acquire iron through pathways other than the traditional siderophore uptake systems since strain IMS101 lacks any readily identifiable complete TonB-dependent transport system as required for conventional Fe(III)-siderophore acquisition (16, 17).

Diverse iron acquisition systems that are not mediated by ferri-siderophore complexes have been reported for various microorganisms (18-22). These include the existence of transporters for inorganic ferric (Fe(III)) iron, inorganic ferrous (Fe(II)) iron, or ferric iron complexes with weak ligands like citrate. Another possibility is the reduction of inorganic or organically complexed ferric iron followed by either 1) uptake by ferrous iron transporters such as FeoB or 2) re-oxidation of the iron and interaction of the resulting ferric iron with ferric ion transporters such as the classic ABC system (18).

Several recent models for iron acquisition by planktonic organisms have emphasized iron reduction, in particular the FeL model for some eukaryotes (23), the

Fe(II)s model for certain prokaryotes and eukaryotes (24, 25) and a model for *Synechocystis* (26). In these systems, Fe(III) is either reduced at or near the cell surface by a reductase (24, 27-30), reduced in the periplasmic space (26) or reduced in the surrounding milieu by an excreted reducing agent such as superoxide (23) prior to uptake. Superoxide released into the medium can thermodynamically reduce a range of organically complexed iron species and has been shown to reduce these complexes under oceanographically relevant conditions (31). Since the production of reactive oxygen species, which includes superoxide, has been observed in numerous marine phytoplankton species (27, 32-36), superoxide could be available to react with and reduce ferric iron, thus creating a more bioavailable iron pool for marine organisms.

Trichodesmium strain IMS101 has been shown to take up iron from ferric citrate and inorganic iron in laboratory culture conditions (5). Although the precise uptake pathway is not known IMS101 has the potential to utilize inorganic or organic forms of Fe(III) and Fe(II) because its genome contains homologs to the ferric ABC transporter gene *futABC*, a ferrous iron transporter *feoB*, a putative ferric reductase with homology to the gene for the yeast ferric reductase *fre1* and a partial TonB complex (5, 17). Strain IMS101 has been shown to produce superoxide (a known reductant of ferric iron) in culture (36) but it is unknown if this production has any impact on the pool of iron that may be available for uptake by strain IMS101.

In this study, the uptake mechanisms utilized by IMS101 to acquire iron from inorganic iron species and ferric citrate chelates were investigated via the use of an Fe(II) binding ligand, superoxide dismutase (SOD), ascorbate and kinetically inert chromium complexes. To determine whether iron reduction was occurring extracellularly prior to

transport three treatments were used (additions of an Fe(II) binding ligand, SOD and ascorbate) to probe different aspects of the reduction process. The Fe(II) binding ligand, bathophenanthrolinedisulfonic acid (BPDS) (37), traps any Fe(II) formed extracellularly and inhibits reduced iron from entering the cell, similar to what is known for ferrozine, another commonly used Fe(II) binding ligand (24, 27, 35, 38, 39). SOD was used to investigate iron reduction by superoxide in the bulk medium. SOD is specific to superoxide (40) and catalyzes the diproportionation of superoxide to hydrogen peroxide and oxygen making superoxide unavailable for iron reduction. Ascorbate can reduce Fe(III) (41) and can be used to increase Fe(II) in solutions and potentially increase uptake if reduced iron is important in the uptake pathway. The final probe used in this study was a radiolabelled Cr(III)-citrate complex. Chromium is known to form nearly identical complexes with siderophores as iron, but chromium is kinetically inert to ligand substitutions and reduction (42, 43). Detection of intracellular Cr will only be seen if the entire metal-ligand complex is taken up. The combination of approaches used in this work reveals fundamentally different iron acquisition pathways in *Trichodesmium* IMS101 for inorganic Fe vs ferric citrate iron sources.

Experimental

Growth Conditions. Non-axenic *Trichodesmium erythraeum* IMS101 cultures (obtained from J. Waterbury) were grown on a platform shaker (104 RPM) at 24°C with a 12 hour light/dark cycle (75 μE m^{-2} sec^{-1}) and maintained in R medium (5). Briefly, R medium was composed of 75% offshore Pacific seawater (collected ~400 miles off the coast of California) and 25% Milli-Q water, 2.5×10^{-6} M Na_2EDTA, 1.0×10^{-7} M ferric citrate,

8.0×10^{-6} M KH_2PO_4, 1.0×10^{-7} M $MnCl_2$, 1.0×10^{-8} M Na_2MoO_4, 1.0×10^{-8} M $ZnSO_4$, 1.0×10^{-10} M $CoCl_2$, 1.0×10^{-10} M $NiSO_4$ and 1.0×10^{-10} M Na_2SeO_3. For Fe limited cultures, an Fe-replete culture was gravity filtered onto a 3.0 µm-pore size filter, rinsed twice and resuspended in Low-Fe R medium (ferric citrate omitted). Fe limited cultures used for iron acquisition experiments were acclimated to low Fe conditions by maintaining the cells in the initial Low-Fe R medium for one week, followed by an additional two weeks of growth in fresh Low-Fe R medium. The medium for radiolabelled ^{55}Fe experiments was similar to the R medium, but was passed through a chelex column and amended with a trace metal mix that omitted the EDTA and ferric citrate (R-FeEDTA) at pH 8. All glassware, filter holders, filters and bottles were acid cleaned and microwave sterilized.

Experimental Solutions.

^{55}Fe solutions - Radioactive solutions were made fresh prior to each experiment and stored in the dark at 25°C. Radioactive stocks were prepared from a stock solution of $^{55}FeCl_3$ (Perkin Elmer 82.68 mCi mg^{-1}) or a stock solution of ^{14}Citric acid (Perkin Elmer 116.4 mCi $mmol^{-1}$). Fresh working stocks of $^{55}FeCl_3$ in 0.007 M HCl were used to make ^{55}Fe solutions and a fresh non-radiolabelled working stock of $FeCl_3$ in 0.007 M HCl was used to make solutions with ^{14}Citrate. The ^{55}Fe-Citrate and Fe-^{14}Citrate solutions were made by mixing $FeCl_3$ with sodium citrate or ^{14}C-citric acid (made in Milli-Q and stored at -20°C) in 0.4 µm filtered UV irradiated seawater and allowed to equilibrate overnight in the dark at pH 6.6. After the equilibration period all FeCit stocks were diluted 1,000 times to their final experimental concentrations of 10 nM Fe with 2 µM, 20 µM or 100 µM citrate. An iron free ^{14}C-citrate solution was made by diluting citric acid in 0.4 µm

filtered UV irradiated seawater and equilibrating the solution overnight in the dark. The final concentration of ^{14}Citrate used in experiments was 30 nM or 2 µM. The inorganic iron $^{55}FeCl_3$ solution was diluted into UV irradiated seawater immediately prior to the start of each experiment to minimize colloid formation at the start of the experiment. The final concentration of $^{55}FeCl_3$ in experiments was 10 nM. It should be noted that the $FeCl_3$ added to the uptake experiments likely partitioned into a spectrum of poorly-defined colloidal and soluble forms of varying lability over the time course of the experiments.

^{51}Cr Solutions - Radioactive solutions were made fresh prior to each experiment and stored in the dark at 25°C. Radioactive stocks were prepared from a stock solution of $^{51}CrCl_3$ (Perkin Elmer 961.46 mCi mg^{-1}). Fresh working stocks of $^{51}CrCl_3$ or non-radiolabelled $CrCl_3$ in 0.007 M HCl were used to make ^{51}CrCit solutions or Cr^{14}Cit solutions. The Cr-Citrate solutions were made following the procedure of Hamada et al. (44,45) by mixing $CrCl_3$ with sodium citrate (made in Milli-Q and stored at -20°C) or ^{14}C-citric acid in 0.4 µm filtered UV irradiated seawater and allowed to equilibrate overnight in the dark at pH 6.6. During the equilibration period the Cr-Cit solution changed color from green to purple. The formation of the kinetically inert Cr-Citrate complex was validated by UV-VIS spectroscopy with peaks at 400 and 580 nM (44, 45). After the equilibration period all CrCit stocks were diluted 1,000 times to their final experimental concentration of 10 nM Cr with 2 µM citrate. It should be noted that the synthesized "CrCit complex" was probably actually a mixture of 1:1 and 1:2 Cr:Cit complexes, however at the 1:200 Cr:Cit ratio the 1:2 complex was likely the dominant species.

Fe reduction probe solutions - A 100 mM bathophenanthrolinedisulfonic acid (BPDS) (Sigma) stock, an Fe(II) chelator, was made in Milli-Q and stored at 4ºC. Superoxide dismutase (SOD) (Sigma) stocks at 8108 U ml^{-1} and an 81021 U ml^{-1} were made in Milli-Q water and stored at -20ºC. An 81021 U ml^{-1} denatured SOD stock was made in Milli-Q, boiled for 10 min and cooled to room temperature the day of the experiment. A stock of 1 M sodium ascorbate (Sigma) in Milli-Q was made fresh the day of the experiment. The final concentrations of BPDS, SOD and ascorbate used in experiments were 100 – 300 µM, 6.66 – 66.6 U ml^{-1}, and 1 mM, respectively. The addition of ascorbate to the uptake medium changed the pH from 8 to7 similar to what was observed by Maldonado and Price in similar experiments (30).

^{55}Fe uptake inhibition experiments. Strain IMS101 cultures were grown to late exponential phase (14 days) in Low-Fe R medium before being harvested for uptake experiments as described in detail in Roe et al., 2012. Bacterial abundance in the cultures was reduced by gently rinsing *Trichodesmium erythraeum* IMS101 filaments three times with R-FeEDTA on a 3.0 µm polycarbonate filter and resuspending with R-FeEDTA (5). For the killed controls a final concentration of 0.01% glutaraldehyde was added to the culture and allowed to equilibrate for one hour at room temperature. All of the uptake experiments in this study were conducted during the light portion of the diel cycle but kept in dark conditions.

For the Fe uptake inhibition experiments iron was supplied as $^{55}FeCl_3$ or ^{55}FeCit (1:200, Fe:Citrate) and incubated with BPDS, SOD, or denatured SOD. ^{55}FeCit was also

incubated with ascorbate. The BPDS, SOD, and denatured SOD were added to each treatment 15 min prior to the addition of Fe. Each treatment had 3 live replicates and 2 killed controls. After addition of the radiolabelled ^{55}Fe, all of the bottles were placed in an incubator on a platform shaker at 24^{o}C in a dark box for 3 hours. The sampling procedure is explained in detail in Roe et al., 2012. Briefly, the culture was size fractioned into a *Trichodesmium* fraction (>3 μm) or bacterial fraction (<3 μm - >0.4 μm). A 5 ml sample was taken for the bacterial fraction and a 10 ml sample was taken for the *Trichodesmium* fraction. The cells were collected onto the filter (<12.7 cm Hg), rinsed with 5 ml seawater, 2 ml of Ti-Citrate wash (46) for 2 min to remove extracellular iron and a final rinse with 5 ml seawater. The filters were then placed in a scintillation vial with the scintillation cocktail Ecolite (MP), stored overnight, and read on a Beckman LS 6000IC scintillation counter. To determine *Trichodesmium* specific Fe uptake (described in detail in Roe et al., 2012) the 3.0 μm bacterial Fe uptake (determined from microscopy cell counts and the <3.0 μm to >0.4 μm bacterial fraction Fe uptake) and the 3.0 μm glutaraldehyde killed control was subtracted from the *Trichodesmium* fraction.

Fe(II) production experiments. The Fe(II) production experiments were set up in triplicate for each treatment. In these experiments $^{55}FeCl_3$ was added 15 min prior to the addition of the *Trichodesmium* culture to minimize any Fe(II) signal and allow for oxidation of any Fe(II) in the $FeCl_3$ stock. After 15 min the *Trichodesmium* and BPDS were added to each bottle. 6.66 U ml^{-1} SOD was added at this time to the SOD treatment bottles. To create killed *Trichodesmium* controls, the cells were exposed to 0.01% glutaraldehyde for 1 hour and then rinsed on a 3.0 μm filter and resuspended in uptake

medium to minimize the amount of glutaraldehyde in the experimental culture. The experimental bottles were then placed in an incubator on a platform shaker at 24°C in illuminated or dark conditions for 3 hours. The Fe(II)-BPDS was collected on a Sep-Pak column, packed with C_{18} resin (Waters Association), similar to the procedure of (39). A 10 ml sample was filtered through a 3.0 µm filter, the filtrate was loaded on to the Sep-Pak column, rinsed with 10 ml of 0.5 M NaCl buffered with 0.005 M $NaHCO_3^-$ (pH 8), 20 ml of 0.5 M NaCl buffered with 0.005 M HCl (pH 3), and eluted in 5 ml methanol. The methanol was evaporated from the scintillation vial, Ecolite was added, and stored overnight before being counted on a Beckman LS 6000IC scintillation counter. The Sep-Pak was conditioned with 20 ml methanol followed by 10 ml Milli-Q. The same Sep-Pak was used for each Fe treatment and rinsed between each use with 10 ml methanol and 10 ml Milli-Q. A series of abiotic controls with BPDS and the two iron sources were set up identical to the Fe(II) production experiments except, IMS101 was omitted from the medium.

Citrate complex uptake experiments. A series of uptake experiments with varying citrate concentrations were conducted, in which ^{55}FeCit was supplied at a ratio of 1:200, 1:2,000, and 1:10,000 Fe:Cit. Additional experiments designed to examine the FeCit uptake mechanism in *Trichodesmium* were conducted with ^{51}CrCit (1:200 Cr:Cit), Fe^{14}Cit (1:200 Fe:Cit), and ^{14}Cit . These experiments were set up and conducted similarly to the ^{55}Fe uptake inhibition experiments described above.

***Trichodesmium* cell counts.** For the Fe inhibition experiments, an aliquot from each live radioactive bottle was preserved in 1% glutaraldehyde. The bacterial fraction was filtered onto a 0.4 µm filter and the *Trichodesmium* fraction was filtered onto a 3.0 µm filter (discussed above) and stained with DAPI (stain with 5 µg/ml, for 5 min). *Trichodesmium* cell counts were obtained from counting the trichomes ml^{-1} and cells per trichome to calculate cells ml^{-1}. The bacteria on all of the filters were counted to obtain bacteria ml^{-1}.

For the Fe(II) production experiment, one non-radioactive culture was set up identical to radioactive cultures and preserved in 1% glutaraldehyde. The sample was filtered onto a 3.0 µm filter where *Trichodesmium* cell counts were obtained from counting the trichomes ml^{-1} and cells per trichome to calculate cells ml^{-1}. All slides were made within 3 days of being collected and were counted on an Olympus AX70 EPI fluorescence microscope.

Data analysis. A Students two tailed t-test was used to compare the radiolabelled $^{55}FeCl_3$ and ^{55}FeCit uptake between the control, BPDS and SOD treatments. A two tailed t-test was used to compare the ^{55}FeCit uptake between the different citrate ligand concentration treatments. A set of two tailed t-tests were used to compare the differences in the amount of Fe(II) produced by live *Trichodesmium* and the SOD and killed control treatments. The t-tests were completed with commercially available software in Excel. For all t-tests the 95% confidence level was used to determine statistical differences.

Results and Discussion

$^{55}FeCl_3$ uptake inhibition experiments

Incubation experiments with the Fe(II) trapping ligand, BPDS, and the superoxide scavenging enzyme superoxide dismutase, SOD, were conducted with $^{55}FeCl_3$ to determine if an extracellular reductive step is utilized by *Trichodesmium erythraeum* IMS101 for iron acquisition. Strain IMS101's iron uptake from inorganic iron, $^{55}FeCl_3$, was similar to previously published values (5) after three hours of incubation in the dark (Table 1). Complete inhibition (100%) of iron uptake from $^{55}FeCl_3$ by strain IMS101 was observed in the presence of 100 μM BPDS (Table 1), indicating that reduction of iron to Fe(II) prior to uptake may be an important step for utilizing inorganic iron (ie. BPDS inhibits IMS101 iron uptake from $^{55}FeCl_3$ by trapping an Fe(II) intermediate before it can be utilized by the organism) . Some of the inhibition could, however, be attributed to BPDS instead binding highly labile ferric iron (non-colloidal) in solution. BPDS is known to reduce some Fe(III) in solution ((39) and references within) and BPDS bound similar amounts of iron in fresh cell-free medium (~0.30 nM) as total strain IMS101 iron uptake after three hours of incubation (~0.15 nM). However, we have shown that uptake of iron from $FeCl_3$ by strain IMS101 increases by about a factor of six over 9 hours (5) while the amount of Fe-BPDS formed without cells present remained constant over this same time span, suggesting that strain IMS101 can access more than the most highly labile iron available to BPDS.

To further investigate the potential for Fe(III) reduction prior to uptake, strain IMS101 was incubated with SOD to determine what role superoxide may play as a reductant of Fe(III) during iron acquisition. A decrease in iron uptake by IMS101 was observed with increasing SOD concentrations, up to ~80% at 66.6 U ml^{-1} SOD (Table 1),

suggesting that superoxide influences iron acquisition, possibly by acting as a reductant of iron. The addition of denatured SOD to the uptake medium had no effect on iron uptake, indicating that the effect of SOD on iron uptake is due to its reaction with superoxide and not a detrimental effect on strain IMS101 or a non-specific interaction with $FeCl_3$ (Table 1).

The utilization of superoxide as a reductant for Fe (Table 1) is consistent with what has been observed in another marine cyanobacterium, *Lyngbya majuscula* (35). Although superoxide was not measured in this study, strain IMS101 is known to produce superoxide at a rate of ~20 fmole $cell^{-1}$ hr^{-1} in culture (36). The results of this study suggest that biologically produced superoxide is reducing inorganic Fe in the bulk medium and that the reduced Fe is then available for uptake by strain IMS101. Based on the published superoxide production rate (36), we predict that the superoxide produced should be capable of reducing iron well in excess of the amount of iron taken up by strain IMS101 (Table 1), if Fe is the only entity reacting with superoxide.

^{55}Fe(II) Production Experiments – role of superoxide

The net production of Fe(II) by strain IMS101 from $^{55}FeCl_3$ was examined after three hours of incubation in the dark. Total Fe(II) produced by strain IMS101 was 10.91 $\pm$ 1.25 $\times$ 10^{-18} mole Fe $cell^{-1}$ (Figure 1A). The net amount of Fe(II) produced by strain IMS101 in the presence of SOD decreased by ~80% and was similar to the amount of Fe(II) produced in the glutaraldehyde killed control (Figure 1A). Both the SOD treatment and the glutaraldehyde killed control are statistically different from the live strain IMS101 iron only treatment ($P < 0.05$). When IMS101 filtrate (experimental culture

incubated for 3 hrs in the dark before removal of IMS101 by filtration) was incubated with $FeCl_3$ in the dark for 3 hrs, a detectable signal of Fe(II) above the SOD treatment could be observed (Figure 1C) and the Fe(II) formed was ~50% lower than the Fe(II) observed in the presence of strain IMS101. All of the Fe(II) production results indicate that strain IMS101 was likely producing a reductant (e.g. superoxide) that could be excreted into the bulk medium and may persist long enough (superoxide half-life ranges from seconds to minutes in seawater (47-50)) to reduce iron.

Under the tested conditions strain IMS101 was shown to produce Fe(II) from inorganic iron (Figure 1) at a rate that was approximately double the iron uptake rate once the glutaraldehyde killed control was accounted for, suggesting that reduction of iron is not the rate limiting step in iron acquisition by strain IMS101. The rate of diffusion of the ferrous iron to the cell vs the rate of re-oxidation of Fe(II) and precipitation as Fe(III) may be a bigger factor limiting uptake in strain IMS101 cultures since reduction does not appear to be localized at the cell surface.

The generation of superoxide by strain IMS101 and in turn, the amount of Fe(II) produced, would likely be affected by the metabolic activity within the cells. Increased extracellular superoxide production has been observed in *Chattonella marina* (51, 52) and in *Cochlodinium polykrikoides* (53) in illuminated conditions and has been shown to be related to photosynthetic activity. During illuminated conditions strain IMS101 was observed to produce more Fe(II), ~20% ($P<0.05$) (Figure 1B), which may be due to strain IMS101 being more metabolically/photosynthetically active, allowing for greater production of superoxide that could react with and reduce iron. Previous iron uptake experiments with strain IMS101 showed a slight increase in $FeCl_3$ uptake during

illuminated vs. dark conditions (5) which may be related to the differences in the amount of Fe(II) produced in these conditions.

Potential uptake mechanism(s) that could be used for $FeCl_3$ acquisition

Based on the experimental results with strain IMS101 a working model for uptake of inorganic Fe(III) is proposed in which an extracellular reduction step prior to uptake is necessary. The reduction of Fe(III), which is likely partitioned into a spectrum of poorly-defined colloidal and soluble forms of varying lability under our experimental conditions, appears to occur in the bulk medium by superoxide. Extracellular superoxide production by *Trichodesmium* has been shown (36) and superoxide can persist long enough (54) to diffuse away from the cell surface and interact with iron in the bulk medium. (It should be noted that other potential reductive pathways which utilize additional reductants (i.e., extracellular proteins, reactive metabolite - quinones) or the putative cell surface Fre1-like reductase cannot be ruled out by these experimental results.). Once the iron is reduced, the Fe(II) could be transported inside the cell by *feoB*. It may also be possible that the Fe(II) generated by reduction forms Fe′ which could then be the species transported inside of the cell. A third possibility is that IMS101 utilizes the Fe stress-induced protein (IdiA) or an analog of it, which is typically a part of an ABC transporter, to transport iron (whether it is reduced Fe(II) or Fe′) from the periplasmic space to the inside of the cell (55).

^{55}Fe-citrate complex uptake inhibition experiments

The inhibition by BPDS and SOD of ^{55}Fe uptake from Fe(III)-citrate complexes ($^{55}FeCit$) showed different patterns than those with $^{55}FeCl_3$. A 1:200 Fe:Citrate ratio was used to ensure complete formation of the ferric dicitrate complex (56, 57). Iron uptake from FeCit by strain IMS101 (Table 1) was ~ 2 times greater than uptake from $FeCl_3$ after three hours of incubation in the dark.

Iron uptake from FeCit decreased when strain IMS101 was incubated with BPDS (Table 1). However, the inhibitory effect of BPDS on iron uptake from FeCit was smaller than that observed for $FeCl_3$ uptake at comparable BPDS concentrations (Table 1). Due to the potential for competition between BPDS and citrate for Fe binding, the results with BPDS are a bit ambiguous. At high (300 μM) BPDS concentrations, BPDS may be able to reduce and bind iron thus decreasing the ferric citrate concentration, while at lower (100 μM) BPDS concentrations less of the Fe(II)-BPDS complex may form, reducing the inhibitory effect. Nonetheless, it is possible that the observed reduction in iron uptake with increased BPDS addition is due to some interaction between strain IMS101, ferric citrate, and BPDS as control experiments in cell free medium with BPDS added to FeCit (10 nM Fe:200 μM citrate, previously equilibrated) showed similar amounts of Fe(II)-BPDS formation (about 0.8 nM) regardless of the BPDS concentration used (100 μM or 300 μM) .

An inhibition experiment with SOD was conducted to see if superoxide was necessary for FeCit uptake. Superoxide has been shown to reduce organically complexed iron, including FeCit (31). No inhibitory uptake effect was seen when strain IMS101 was incubated with varying SOD concentrations (Table 1) suggesting reduction by superoxide is not important for FeCit uptake.

Uptake experiments with ascorbate, a known reductant of Fe(III) (41, 58, 59), showed ~65% reduction in FeCit iron uptake (Table 1). This is somewhat surprising, given that the presence of ascorbate might be expected to increase the amount of Fe(II) in the system. Although it is not known if the reduced iron stays as an intact Fe(II)Cit complex or dissociates, it is unlikely that the Fe(II) dissociates from citrate after reduction by ascorbate since Fe(II)Cit complexes can persist under our experimental conditions (60). Additionally, uncomplexed Fe(II) could presumably be taken up by the same mechanism as we observed for inorganic iron uptake, arguing against the presence of significant uncomplexed Fe(II) in these experiments where iron uptake was significantly reduced in the presence of ascorbate. Thus, the observed decrease in iron uptake with the addition of ascorbate may indicate that the Fe(II)Citrate complex is not utilized/recognized by the cell. Alternatively, ascorbate may act as an inhibitor to some component of the *Trichodesmium* iron uptake system. It should be noted that, due to the change in pH with the addition of ascorbate (from pH 8 to 7), it is possible that the observed reduction in iron uptake could be a result of a change in IMS101 cell physiology. However, microscopic examination of the experimental cultures did not show any obvious differences between the control and ascorbate treatments, and previous studies have not identified adverse impacts of ocean acidification on *Trichodesmium* physiology (6). In any case, from the combined results with BPDS, ascorbate and SOD it does not appear that FeCit is reduced in the extracellular medium in a manner that enhances the acquisition of iron from this complex for *Trichodesmium.*

Metal-Citrate complex uptake experiments

Iron uptake from ^{55}FeCit by strain IMS101 was examined at 200, 2,000 and 10,000 fold excesses of citrate to iron. Iron uptake decreased with increasing ligand concentration (Figure 2). Relative to uptake at 1:200 Fe:citrate, a ~25% decrease in iron uptake was observed at 1:2,000 ($P < 0.05$) and ~60% decrease in iron uptake was observed at 1:10,000 ($P < 0.05$) Fe:citrate (Figure 2). As the ligand concentration increased, the amount of unchelated iron (FeIII′) decreased by a factor of 10 (1:2,000 Fe:citrate)) to 50 (1:10,000 Fe:citrate)) (calculated from equations in Garg et al., 2007 and rates from Rose and Waite, 2003). The calculated decrease in Fe′ with greater excess citrate is much larger than the relative decrease observed in iron uptake. This result suggests that Fe′ is not the important species taken up by strain IMS101 when using FeCit as an iron source. The apo citrate ligand also does not appear to compete efficiently with ferric citrate for the uptake site. Both the varying Fe:citrate ratio results and the lack of significant inhibition from Fe(II) probes suggest that ferric citrate is the important species utilized by strain IMS101. The decrease in uptake with increasing citrate concentrations suggests that the mono ligand complex (1:1 Fe:citrate), which would decrease with increasing citrate concentration, may be the species most actively involved in uptake.

To further investigate the pathway of iron acquisition from ferric citrate, experiments were performed in which strain IMS101 was incubated with Fe^{14}Cit or ^{55}FeCit (both 1:200) (Figure 3A). Strain IMS101 was observed to accumulate approximately three times more ^{14}C than ^{55}Fe intracellularly, which is slightly higher than the 2:1 ratio of the complex (61). These results suggest that FeCit is taken up as a whole complex. However, uptake of ^{14}C from uncomplexed labelled citrate added at high

concentrations (2,000 nM) was similar to the amount of ^{14}C taken up from the $Fe^{14}Cit$ complex (9.4 ± 3.5 mole cell^{-1} $\times$ 10^{-18}). At low added citrate concentrations (30 nM) no ^{14}C uptake was observed. These results suggest that uncomplexed citrate may be incorporated intracellularly in a concentration-dependent fashion, possibly through porins, which makes the results with $Fe^{14}Cit$ difficult to interpret.

Since iron is kinetically labile and citrate may have more than one uptake pathway into the cell it is difficult to determine how the iron in FeCit is taken up from experiments with $Fe^{14}Cit$ or $^{55}FeCit$. Fe could be acquired as the entire Fe(III)Cit complex by a potential outer membrane receptor or through a porin which ferric dicitrate can pass through (porin cut-off <1500 daltons), with subsequent transport across the periplasmic membrane. Alternatively, the FeCit complex could be altered by reduction or a ligand exchange mechanism prior to uptake. Radiolabelled ^{51}Cr(III) citrate complexes (supplied as a mixture of 1:1 and 1:2 Cr:Cit complexes) were employed to distinguish between these two possibilities (intact or altered complex transport) as the kinetically inert chromium complex will only be detected intracellularly if the entire complex is taken up. When $^{55}FeCit$ and $^{51}CrCit$ (200:1) were supplied to the cultures, only the ^{55}Fe could be detected inside of the cell (Figure 3B). Since CrCit could not be detected intracellularly (regardless of which species, 1:1 or 1:2), the mechanism of transport of FeCit is unlikely to involve uptake of the intact complex, since the CrCit complex would also be able to be transported in this same fashion. The results with $^{51}CrCit$ suggest that while the metal-ligand complex is bioavailable, an additional dissociation step, potentially iron reduction or ligand exchange, needs to occur prior to uptake. Ligand exchange seems most likely as citrate can bind Fe(II).

Discussion of possible mechanism for acquisition of Fe from ferric citrate

Based on our experimental results, a working model for the acquisition of Fe from Fe(III)Cit by strain IMS101 is proposed in which the Fe(III)Cit complex undergoes some kind of iron removal in the local environment of the cell (ie. at a cell surface receptor or within the periplasmic space) rather than in the bulk medium. Results from the experiments performed in this study indicate that thermodynamic dissociation of Fe(III)Cit to form Fe′, or extracellular reduction of the Fe(III)Cit complex by superoxide or another excreted reductant prior to uptake does not enhance the ability of IMS101to acquire Fe from ferric citrate. Although the ferric citrate complex may be important for recognition by strain IMS101, it is not clear how it is recognized since strain IMS101 does not have any identifiable outer membrane receptors. The recognition and transport of iron in ferric citrate complexes is typically accomplished by a TonB-dependent outer membrane receptor uptake system, *fecABCDE* (e.g., (62)), where the iron dissociates from citrate and is transported to the cytoplasm (63). Since strain IMS101 does not have a Fec uptake system, it is possible that strain IMS101 utilizes a non-classical ferric citrate uptake system for acquisition of this iron source. Numerous alternative ferric citrate uptake systems have been described in bacteria (21, 64-71).

Results with Cr(III)-citrate suggest that the iron in the Fe(III)-citrate complex is transported inside of the cell only after a reductive step with dissociation from citrate and/or a non-reductive ligand exchange mechanism. The reduction/exchange step could happen at the cell surface in association with a specific receptor, or the intact Fe(III)-citrate complex could diffuse into the periplasmic space via a porin. Once in the

periplasm the iron could be internalized by an ABC transporter, *futABC* or *Idia* homolog, or by the Fe(II) transporter, *feoB*. At the moment, we cannot distinguish between these possibilities.

Conclusion

It appears that *Trichodesmium erythraeum* IMS101 utilizes two different iron uptake mechanisms to acquire bioavailable iron, a superoxide-mediated reductive step prior to uptake of inorganic $FeCl_3$ from the bulk medium and a superoxide-independent transport system to acquire iron from the ferric citrate complex (Figure 4).

The use of superoxide as a reductant for inorganic iron may be beneficial for IMS101 as superoxide has been shown to reduce labile iron in dust particles (72) and can be used as an antimicrobial agent. Although superoxide is capable of reducing iron in dust, it is generally believed not to be an important source of iron in surface waters (72). However, it may be important in a *Trichodesmium* colony environment where dust can be collected and kept in close proximity to *Trichodesmium* where the amount of bioavailable iron could be enhanced by superoxide and the reducing environment of the colony interior. IMS101's ability to produce superoxide may give IMS101 an additional advantage since IMS101 has antioxidant defenses (73) and could use superoxide to help control the bacterial population on *Trichodesmium* colonies in an effort to compete with the bacteria for iron (5) or other resources. Our results are consistent with the recent findings of Rubin and Shaked (2011) that *Trichodesmium* is able to mobilize iron from inorganic mineral sources and suggest superoxide reduction of Fe(III) as a possible

mechanism. It should be noted, however, that the $FeCl_3$ added here as an iron source represents a much more labile iron phase than the synthesized iron oxides and desert dust used in the previous study (74).

The elucidation of *Trichodesmium erythraeum* IMS101 iron acquisition strategies in this study contributes to our overall understanding of how marine organisms acquire iron. The data obtained in this study for inorganic iron uptake agree with the Fe(II)s model (24, 25) and the idea that unchelated iron can be a source of reduced iron, and the concentration of the reduced iron at the cell surface is important for iron uptake. However, it is clear from the uptake results with ferric citrate that neither of the models, Fe(II)s or FeL (23), can completely explain strain IMS101 ferric citrate uptake even though both models are designed with organisms that do not possess any classical siderophore transport systems, similar to strain IMS101. Although reduction of ferric citrate may be a factor in strain IMS101 iron uptake this reduction is not mediated by superoxide and the reduced species in the bulk medium do not appear to be important for uptake, which does not agree with the FeL model where Fe(II)L is important for uptake. The observed strain IMS101 ferric citrate utilization also does not agree with the Fe(II)s model as uptake does not appear to be dependent on the unchelated iron concentration in the medium. This suggests that more information about iron uptake pathways in marine organisms is needed to advance our knowledge of iron bioavailability in the ocean environment and the development of more robust models of iron biogeochemistry in marine systems.

Acknowledgements

We would like to thank J. B. Waterbury for the initial *Trichodesmium* culture. D. Parker for helpful discussions. This work was funded by NSF grant OCE-0327070 to Kathy Barbeau and Margo G. Haygood and NSF grant OCE-1061068 to Kathy Barbeau and Bianca Brahamsha. This manuscript was improved by the comments of two anonymous reviewers.

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Table 1. ^{55}Fe % uptake from $^{55}FeCl_3$ and $^{55}FeCitrate$ per *Trichodesmium erythraeum* IMS101 cell. Average mole ^{55}Fe uptake for each iron source is shown for control treatments from 3 experiments. % uptake for different treatments measured in triplicate is shown relative to the Fe source control treatment. Treatments include adding BPDS (bathophenanthrolinedisulfonic acid) - 100 µM, 3X BPDS - 300 µM, SOD (superoxide dismutase) - 6.66 U ml^{-1}, 10X SOD - 66.6 U ml^{-1}, DSOD (denatured SOD) - 66.6 U ml^{-1}, and Asc (ascorbate) - 1 mM at their respective final concentrations.* Indicates that the treatment is statistically different from the control ($P<0.05$).

Source	Treatment	Ave mole ^{55}Fe cell^{-1} × 10^{-18}	% Uptake	Experiment
$FeCl_3$	Control	2.75 ± 1.06	100	
	BPDS		0.3*	A
	SOD		67.3	B
	10X SOD		20.0*	B
	DSOD		111.8	C
FeCit	Control	8.49 ± 3.81	100	
	BPDS		69.1*	D
	3X BPDS		23.4*	D
	SOD		106.7	E
	10X SOD		100.4	E
	Asc		35.1*	F

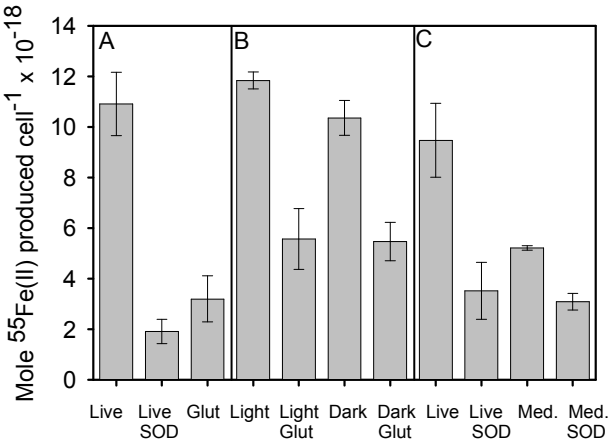

Figure 1. ^{55}Fe(II) trapping/production experiments with $^{55}FeCl_3$. A) The Live treatment contained 10 nM $FeCl_3$, 100 µM BPDS and live *Trichodesmium erythraeum* IMS101. Live SOD treatment contained 10 nM $FeCl_3$, 100 µM BPDS, IMS101, and 6.66 U ml^{-1} SOD. The Glut treatment contained $FeCl_3$, 100 µM BPDS and glutaraldehyde killed *Trichodesmium*. B) Strain IMS101 was incubated with $FeCl_3$ in light or dark conditions. Each treatment contained 10 nM $FeCl_3$, 100 µM BPDS and strain IMS101.. C) The Live treatment contained 10 nM $FeCl_3$, 100 µM BPDS and strain IMS101. Live SOD treatment contained 10 nM $FeCl_3$, 100 µM BPDS, strain IMS101, and 6.66 U ml^{-1} SOD. The medium treatment (Med.) had strain IMS101 incubated in the medium for 3 hrs and then removed immediately prior to the addition of 10 nM $FeCl_3$ and 100 µM BPDS. The medium (Med.) SOD treatment had strain IMS101 incubated in the medium for 3 hrs and then removed immediately prior to the addition of 10 nM $FeCl_3$, 100 µM BPDS and 6.66 U ml^{-1} SOD. All treatments were incubated with $FeCl_3$ for 3 hrs. Data are averages of 3 replicates and error bars are standard deviation of the mean. All experiments were conducted with 1.3 – 1.5 $\times 10^4$ cells ml^{-1}.

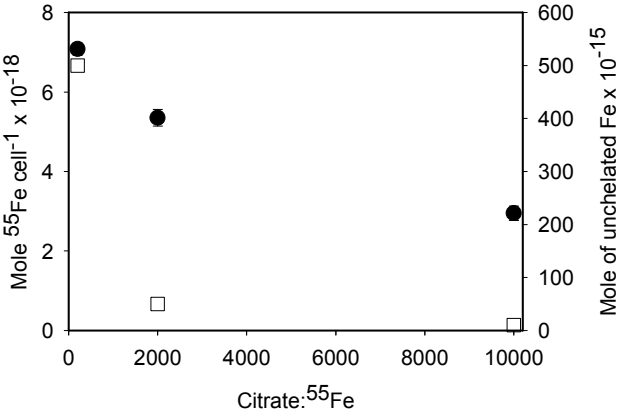

Figure 2.
Trichodesmium erythraeum IMS101 ^{55}Fe uptake per cell (filled circles) from FeCit at varying ligand concentrations after a 3 hour incubation. FeCit was supplied at a ratio of 200:1, 2,000:1 and 10,000:1 Cit:Fe at 10 nM Fe. Data are averages of 3 replicates and error bars are standard deviation of the mean. The calculated unchelated Fe (Fe′) concentration is shown as white squares. Experiment was conducted with 2.8×10^4 cells ml^{-1}.

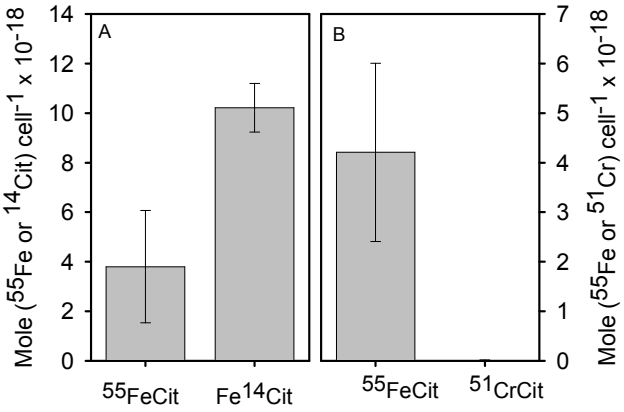

Figure 3. *Trichodesmium erythraeum* IMS101 FeCit uptake experiments with ^{55}Fe, ^{51}Cr and ^{14}Citrate. A) Uptake experiments were conducted with a 1:200 Fe:Cit ratio at 10 nM Fe. B) Uptake experiments were conducted at a 1:200 metal:Cit ratio and metal concentrations of 10 nM. Data are averages of 3 replicates and error bars are standard deviation of the mean. Experiment was conducted with 2.8 – 5.5 $\times 10^4$ cells ml^{-1}. Note that all axis are different ranges.

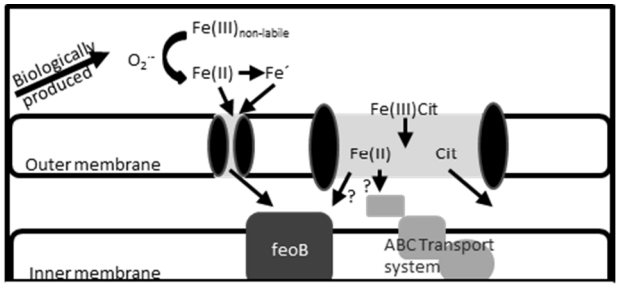

Figure 4. Schematic diagram of iron transport mechanisms in *Trichodesmium erythraeum* IMS101. Iron acquisition is accomplished by either extracellular reduction of inorganic Fe by superoxide and the recognition of FeCit at the outer membrane followed by reduction and/or removal of Fe from the complex prior to transport.