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Use of Superoxide as an Electron Shuttle for Iron Acquisition by the Marine Cyanobacterium *Lyngbya majuscula*

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Reduction of iron from the ferric state to the ferrous state is one strategy employed by microorganisms in near-neutral environments to increase its biological availability. In recent years, the existence of mobile reducing agents produced by microorganisms to promote iron reduction, known as electron shuttles, has been demonstrated. Production of electron shuttles has been shown for several organisms, employing a variety of mostly organic molecules as the electron carrier. Here we show that the coastal cyanobacterium *Lyngbya majuscula* produces iron-reducing superoxide radicals ($O_2^{\bullet -}$) and that this facilitates increased iron uptake. We suggest that superoxide is a useful electron shuttle because it reacts rapidly and almost indiscriminately with Fe(III)−organic complexes and its precursor, dissolved oxygen, is ubiquitous in the photic zone. We further suggest that, for these reasons, the generation of superoxide by marine oxygenic photosynthetic microorganisms and its use in facilitating iron uptake may be a reasonably widespread process.

Introduction

Iron is an essential micronutrient for nearly all living organisms (*1*) and is required by some microorganisms in relatively large quantities (*2*). This is problematic for those that inhabit marine environments, where iron is highly insoluble (*3*) and the dissolved fraction is predominantly strongly bound to organic compounds (*4*). Consequently, marine microalgae have been forced to develop specialized mechanisms for iron acquisition. Within nature, the two main chemical mechanisms that increase iron solubility at near-neutral pH are formation of organic complexes and reduction of ferric iron to the more soluble ferrous state. Both pathways have been exploited by microorganisms inhabiting such environments, in the former case by release of cellular products that form strong complexes with iron specifically to aid iron uptake (siderophores) and in the latter case by reduction of iron from Fe(III) to Fe(II) using either surface-bound iron reductases (*5*) or by release of iron-reducing compounds (so-called electron shuttles) into the surrounding milieu (*6*).

While production of siderophores has been shown to occur in some marine microorganisms (*7*), doubts have been raised about the feasibility of this strategy as a primary iron acquisition mechanism (*8*). Siderophore production typically occurs only when organisms are under stress due to iron limitation and requires considerable expenditure of both energy and materials by organisms. In addition, although iron−siderophore complexes are thermodynamically stable, the kinetics of ligand exchange with existing complexes or iron oxyhydroxides is slow (*8*). The second major strategy, iron reduction, is commonly used by plants, yeast, and other microorganisms (*1*, *9*−*11*). The production of electron shuttles extends the iron-reducing ability of the organism beyond direct contact between the cell surface and the substrate. Reported electron shuttles are typically organic compounds and include both purpose-built endogenous molecules and exogenous molecules, such as humic substances (*6*). However, endogenous electron shuttles, like siderophores, also require considerable expenditure of energy and materials by the organism.

In oxygenated marine waters, the most stable chemical forms of iron are insoluble Fe(III) oxyhydroxides. However organic compounds are able to form complexes with iron that can maintain it in a soluble form for many hours to days as a result of the slow dissociation of these complexes (*12*, *13*) or in some cases to maintain iron as soluble complexes at equilibrium (*4*). In particular, terrestrial inputs of natural organic matter (NOM) in combination with iron can result in dissolved iron concentrations of up to 10 μM in coastal waters (*14*), several orders of magnitude greater than in open ocean conditions. Due to the complicated and highly variable forms of naturally occurring iron-binding compounds, it is unlikely that microorganisms could internalize the resulting complexes directly and thus would first need to convert iron to a suitable form for uptake. Reducing iron from Fe(III) to Fe(II) would be an appropriate strategy, as reduction results in much weaker complexes (*13*), leading to a substantial amount of iron being present in an uncomplexed form that may be more readily internalized.

In natural waters, dissolved oxygen has the ability to act as a diffusible redox-active agent through formation of the superoxide radical. In this work, we have investigated the role of superoxide as an electron shuttle in the iron acquisition strategies of *Lyngbya majuscula*. *L. majuscula* is a nonheterocystous, nitrogen-fixing, filamentous cyanobacterium inhabiting warm coastal waters worldwide, including those of Hawaii and Florida; Zanzibar, Tanzania; Cook Islands, New Zealand; and Queensland, Australia. Nuisance blooms of *L. majuscula*, which are often toxic to humans and aquatic fauna, have been occurring with increasing frequency in Moreton Bay near Brisbane, Australia (*15*). Evidence of increased inputs of iron and NOM to Moreton Bay has led to speculation that iron may be involved in stimulating *L. majuscula* blooms, prompting us to further investigate its iron metabolism.

Experimental Section

Reagents. Clean seawater was obtained from Sydney Offshore Reference Station, filtered through 0.22 μM Millipore membranes and stored in the dark at 4 °C when not in use. Stock solutions of 3 mM $FeCl_3 \cdot 6H_2O$ (Ajax chemicals) in 0.1 M HCl (Fluka puriss p.a. plus), 0.2 M ethylenediaminetetraacetic acid (EDTA, Ajax chemicals), and 0.2 M diethylenetriaminepentaacetic acid (DTPA, Fluka) were prepared in 18 MΩ cm

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Milli-Q water. Secondary stocks of concentrated Fe(III)−EDTA were prepared by mixing appropriate volumes of 3 mM $FeCl_3$ and 0.2 M EDTA and then diluted 1000 times into clean seawater to the appropriate final concentration immediately prior to each experimental run.

A radiolabeled iron stock was prepared by mixing 3 mM $FeCl_3$ stock with an appropriate volume of ^{55}Fe (New England Nuclear), such that 1−10% of the iron was ^{55}Fe. Fe(III)−EDTA solutions for use in iron uptake studies were prepared as above using the radiolabeled iron stock. Stock solutions of 0.1 M ascorbate (Sigma), 0.1 M ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid, monosodium salt hydrate; Fluka], 2.6 g mL^{-1} SOD (Cu−Zn form, from bovine erythrocytes, 2000−10 000 units mg^{-1}; Sigma), 134 mM xanthine at pH 9.8 (Sigma), and 1 unit mL^{-1} xanthine oxidase (XO) (Sigma) were prepared in 18 MΩ cm Milli-Q water. SOD and XO were stored at −86 °C when not in use. bovine serum albumin (BSA, 1 mg mL^{-1}) was obtained from Fisher Biotec. Diphenyleneiodonium chloride (DPI, 6 mM; Sigma) was dissolved in 10% v/v ethanol. Reagent grade chemicals were used in all cases.

Collection, Treatment, and Characterization of *L. majuscula*. *L. majuscula* was harvested from Moreton Bay. Populations were maintained in clean, filtered seawater under natural room lighting at room temperature, without additional treatment, for up to 1 week prior to use. No changes in metabolic activity (photosynthetic rate and nitrogen-fixation rate) were detected in the organism during this period (personal communication, S. Albert, The University of Queensland). Scanning electron micrographs of *L. majuscula* were obtained using an Hitachi S-360 microscope. Associated organisms were removed by pulse sonication at 47 kHz for 3 min. *L. majuscula* was then rinsed three times in clean, filtered seawater, and allowed to recover from the sublethal treatment for approximately 12 h prior to use in further experiments. As an independent means of removing associated organisms, a small amount of *L. majuscula* was also grown in clean, filtered seawater plus 10 μg mL^{-1} cycloheximide, 1 μg mL^{-1} tetracycline, and 20 μg mL^{-1} nalidixic acid under natural laboratory lighting at room temperature for approximately 1 month prior to use in experiments.

As field-harvested populations exhibited variability in terms of absolute magnitudes of some parameters (e.g. superoxide production), *L. majuscula* from the same batch was used in all experiments in which absolute parameters were measured. When relative parameters were measured (e.g. iron uptake experiments), a control from the same batch of *L. majuscula* was included with each experiment and results were normalized to the control.

Measurement of Fe(II) and Superoxide. Fe(II) and superoxide were determined using chemiluminescence-based methods with an FeLume chemiluminescence system (Waterville Analytical) (*16*). Samples were generated in-line by passing the solution of interest through approximately 15−25 mg of *L. majuscula* cells housed on a 26 mm GF/F filter (Millipore) in an opaque Sartorius filter holder. Samples and chemiluminescence reagent were continuously delivered to a spiral glass flow cell via separate tubes using a peristaltic pump at a flow rate of 3 mL min^{-1}. There was a lag time of 10−20 s between in-line generation of the sample and mixing with the chemiluminescence reagent in the flow cell. Mixing of sample and reagent in the flow cell resulted in light emission that was detected with a Hamamatsu photomultiplier tube (PMT) operating at an applied voltage of 1200 V. Fe(II) production rates were then calculated from the calibrated data by multiplying the measured Fe(II) concentration by the solution flow rate.

The reagent used for Fe(II) determination was 0.5 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) in 0.5 M NH_4OH (Sigma) adjusted to pH 10.3. The system was calibrated by standard additions of Fe(II) from a stock of 2 μM $Fe(NH_4SO_4)_2 \cdot 6H_2O$ (Ajax chemicals) at pH 3.5 to the same solution that was used for each experimental run such that the lag time for the added iron to reach the flow cell was the same as that in an experimental run (*17*). Working stocks were prepared weekly by dilution of 4.0 mM $Fe(NH_4SO_4)_2 \cdot 6H_2O$ (Ajax chemicals) in 0.2 M HCl (Fluka puriss p.a. plus). Because Fe(II) rapidly oxidizes at seawater pH, PMT signals corresponding to particular Fe(II) concentrations were determined by extrapolation of signals to the time of addition of Fe(II) on a semilog plot. The calibration procedure therefore accounts for the oxidation of iron in the samples, as the added iron will oxidize at the same rate when added to the same solution used in the experimental runs, and thus reported Fe(II) concentrations correspond to those at the point at which the Fe(II) is generated. Calibration curves were constructed using logarithmic plots.

The reagent used for superoxide determination was 1 μM methyl *Cypridina* luciferin analogue (MCLA, Fluka) in 0.05 M acetate buffer at pH 6.0, which is highly selective for superoxide (*18*). The system was calibrated by standard addition of superoxide stock solution, generated photochemically immediately prior to use as follows. A solution of 41 mM acetone (BDH) and 12 M absolute ethanol (Fluka) in 1 mM borate buffer at pH 12 was irradiated using a 150-W Xe lamp (Oriel) for 3−5 min. The superoxide concentration in the stock was quantified by UV spectrophotometry at 240 nm using an Ocean Optics fiber optic spectrophotometry system (*19*). Standard additions were made immediately after extinguishing the lamp into the same solution that was used for each experimental run such that the lag time for the added superoxide to reach the flow cell was the same as that in an experimental run. As superoxide can disproportionate during the lag between its addition and reaching the flow cell, PMT signals corresponding to particular superoxide concentrations were determined by extrapolation of signals to the time of addition of superoxide on a plot of 1/signal versus time. The calibration procedure therefore accounts for the disproportionation of superoxide in the samples, and thus, reported superoxide concentrations correspond to those at the point at which the superoxide is generated. Calibration curves were constructed using linear plots. Superoxide production rates were then calculated from the calibrated data by multiplying the measured superoxide concentration by the solution flow rate.

For experiments in which superoxide was measured, all solutions also contained 15 μM DTPA to prevent unwanted disproportionation of superoxide by binding of copper and iron (*20*).

Iron Uptake Experiments. Iron uptake was determined by incubating approximately 15−25 mg of *L. majuscula* in 150 mL of seawater containing 100 nM radiolabeled Fe(III) and 1 μM EDTA, during daylight hours under natural room lighting. Further additions of 1 mM ascorbate, 1 unit L^{-1} XO plus 134 μM xanthine, 134 μM xanthine only, 10 mg L^{-1} SOD, 10 mg L^{-1} BSA, 60 μM DPI, 1 mM ferrozine, or 1 mM ascorbate plus 1 mM ferrozine were made to respective treatments; no further additions were made to the control treatment. Each treatment was incubated for 4 h and then subsampled in quadruplicate. Subsamples were rinsed in titanium−citrate−EDTA prepared according to the method of Hudson and Morel (*21*) ($TiCl_3$ from Riedel de Hahn and trisodium citrate and EDTA from Sigma) to remove any extracellular iron. Subsamples were dried at 65 °C for at least 8 h, and then 2.0−5.0 mg was weighed and placed in a glass scintillation vial with 5 mL Beckman ReadyScint scintillation cocktail. Sample activity was determined using a Packard TriCarb Liquid Scintillation Counter.

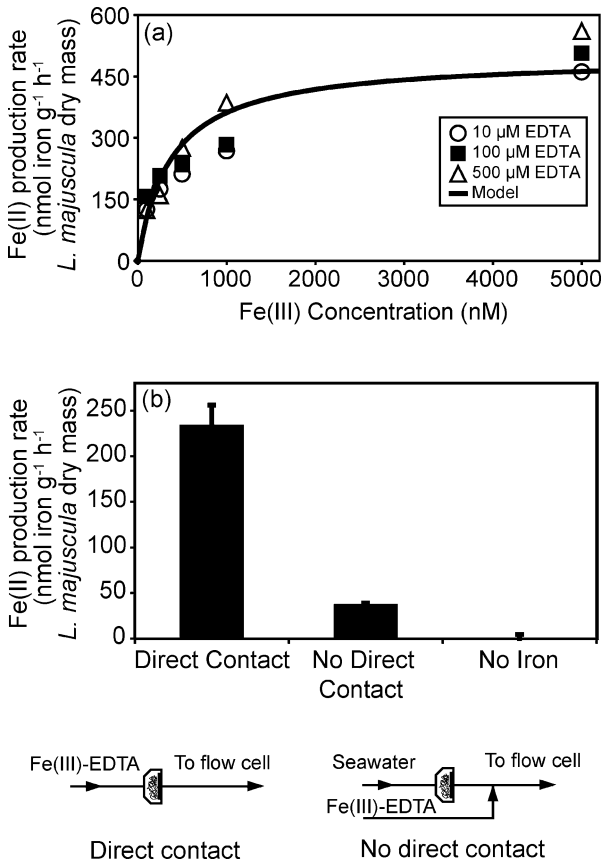

FIGURE 1. Production of Fe(II) by *L. majuscula* using a flow-through technique. (a) Fe(II) production in the presence of varying concentrations of Fe(III) bound to EDTA. A Monod-type kinetic model has been fitted to the data by linear regression of reduction rate versus reduction rate/substrate concentration, yielding a maximum specific reduction rate of 497 nmol of Fe g^{-1} h^{-1} *L. majuscula* dry mass. (b) Fe(II) production in the presence of Fe(III)−EDTA supplied as 1 μM Fe(III) complexed with 10 μM EDTA mixed with seawater that has passed through *L. majuscula*, illustrating different experimental configurations. Error bars represent the standard deviation from Fe(II) measurements in a single sample of *L. majuscula* (same sample for all cases).

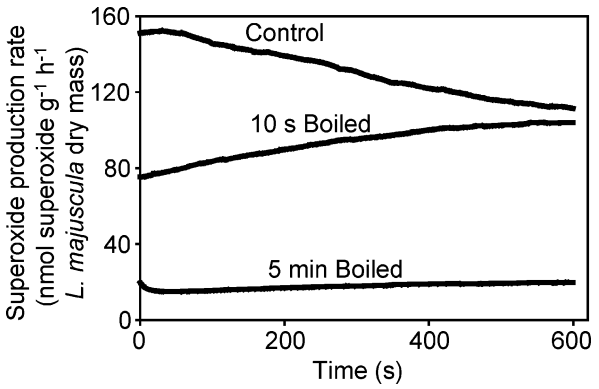

FIGURE 2. Production of superoxide by *L. majuscula* using a flow-through technique. The superoxide production rate in the control increased suddenly to a maximum of about 155 nmol g^{-1} h^{-1} *L. majuscula* dry mass before decreasing gradually over time, suggesting close regulation of superoxide production by the organism. Heat shock resulted in inhibition of superoxide production.

Statistical Analysis. Results were evaluated for statistical signficance using the unpaired Students' *t*-test function assuming equal variances in Microsoft Excel.

Results and Discussion

Iron Reduction and Superoxide Production by *L. majuscula*. Using Fe(III)−EDTA as a model iron−organic complex, we passed seawater containing Fe(III)−EDTA at a range of naturally occurring iron concentrations through a small amount of *L. majuscula* in an in-line filter holder and measured Fe(II) in the effluent, as shown in Figure 1a. Varying the concentration of EDTA did not affect Fe(II) generation, suggesting that uncomplexed EDTA was not participating in the electron transfer between the organism and iron. In addition, this indicated that there was no competition between the reductant and the ligand for iron, implying that complexed Fe(III) is reduced without dissociation of Fe(III) from the complex. To test whether the reducing agent was bound to the cell surface, we then passed seawater without Fe(III)−EDTA through the filter holder containing the *L. majuscula* and then mixed the effluent with a solution of Fe(III)−EDTA downstream of the filter holder as shown in Figure 1b. Measurable quantities of Fe(II) were still generated, indicating that the reductant was mobile and stable in seawater for at least several seconds, i.e., that the reductant was an electron shuttle of some kind. Previously reported electron shuttles have generally been organic molecules either synthesized by the organism or of extracellular origin, such as NOM (*6*, *22*, *23*). However, dissolved oxygen can also participate in electron transport, prompting us to consider its one-electron-reduced form, superoxide, as a potential electron shuttle. The decrease in Fe(II) associated with the change from direct contact between the organism and Fe(III)−EDTA to no direct contact would be consistent with superoxide as the reducing agent, since in the ~5 s lag between generation by *L. majuscula* and mixing with the Fe(III)−EDTA, superoxide would decay by a factor of around 40%−99% due to the presence of reactive copper in seawater (at an estimated concentration of 0.1−1 nM (*24*), reacting with a rate constant of about 10^9 M^{-1} s^{-1} (*25*, *26*)). Extracellular production of superoxide by marine microalgae has previously been observed (*27*, *28*), and superoxide reacts quickly and efficiently with a range of forms of dissolved iron at seawater pH (*29*).

Again passing seawater through *L. majuscula* on an in-line filter, this time in the absence of reactive iron and copper (which catalyze the rapid destruction of superoxide), we directly measured production of superoxide using MCLA chemiluminescence (*18*). Production of superoxide by *L. majuscula* rapidly reached a maximum rate and then gradually decreased over time, as illustrated in Figure 2. This decline is probably due to continuous flushing of superoxide away from the organism faster than its reducing capacity can be replenished. Although it is unclear whether the decrease is a deliberate response from the organism or simply due to depletion of a substrate, the existence of a finely tuned control system would be expected to be quite important, as superoxide production would result in considerable export of electrons and energy by the organism. A similar decline in superoxide production over time has recently been reported for another superoxide producing microalga, *Chatonella marina* (*30*).

Since it is possible to produce superoxide abiotically, we also confirmed that the organism was actively producing superoxide by inactivating proteins using heat. Immersion of *L. majuscula* in boiling water for 10 s resulted in a substantial decrease in superoxide production followed by a slow recovery, most likely due to a temporary specific disruption of the enzymes regulating superoxide production. After a longer immersion time of 5 min, superoxide production was almost completely inhibited. Microscopic examination of heat-shocked *L. majuscula* cells revealed negligible lysis, indicating that the inhibition was due to interruption

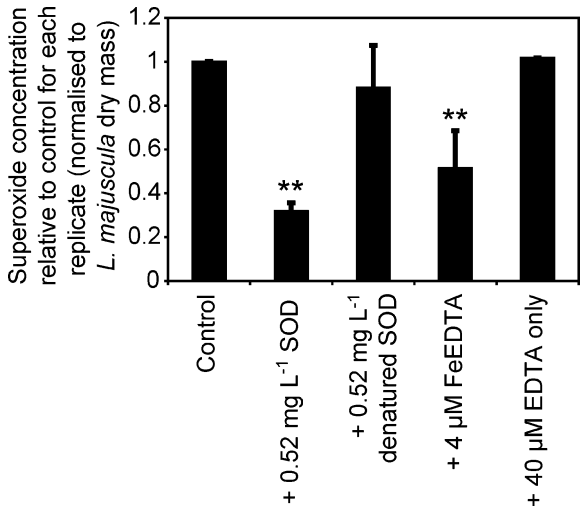

FIGURE 3. Effect on superoxide production of additions of 0.52 mg L^{-1} SOD, 0.52 mg L^{-1} SOD irreversibly denatured by boiling for 5 min, Fe(III)−EDTA supplied as 4 μM Fe(III) complexed with 40 μM EDTA, and 40 μM EDTA only. Superoxide concentrations were determined as the average of the initial 100−200 s of reliable data, during which time superoxide concentrations were relatively constant. Error bars represent the standard deviation from measurements of duplicate samples. Two asterisks indicate that the treatment was statistically different from the control at the 0.01 significance level.

of metabolic activities, rather than destruction of the cell structure. Replicate experiments with *L. majuscula* collected from Moreton Bay at different times showed similarly large decreases in superoxide production when boiled for several minutes. Almost complete inhibition of iron reduction was also observed when Fe(III)−EDTA was passed through *L. majuscula* boiled for 5 min (data not shown).

Addition of SOD (which rapidly and specifically catalyses the destruction of superoxide) to the seawater resulted in a significant ($p < 0.01$) decrease in signal by 68 ± 4%, confirming that we were indeed measuring superoxide, as shown in Figure 3. Addition of SOD that had been irreversibly denatured by boiling for five minutes had no significant effect, indicating that the signal reduction was due to specific catalysis of superoxide destruction by SOD, rather than nonspecific scavenging. The complete inactivation of SOD by heat additionally indicated that the decrease in output signal observed when *L. majuscula* was immersed in boiling water could not be simply due to release of SOD from lysed *L. majuscula* cells. A significant ($p < 0.01$) decrease in output signal by 49 ± 17% was also observed when 4 μM Fe(III) complexed with 40 μM EDTA was added to seawater, but not upon addition of EDTA alone, confirming that the Fe(III)−EDTA complex was reacting with superoxide produced by *L. majuscula*.

To verify that *L. majuscula* was responsible for superoxide production, rather than the various other microorganisms that inhabit the sheath of the field-harvested populations used in this work (including bacteria and diatoms), we briefly sonicated a sample of *L. majuscula* at a sublethal dose and then rinsed it several times in clean seawater in order to remove these other organisms. Electron micrographs illustrating the effect of sonication are shown in Figure 4. After allowing the sample to recover for approximately 12 h, we observed very little decrease in superoxide production compared with the control. Substantial superoxide production was also observed in a sample of field-harvested *L. majuscula* that was maintained in the laboratory for several weeks in the presence of eukaryote-specific and heterotrophic bacteria-specific antibiotic compounds, also shown in Figure

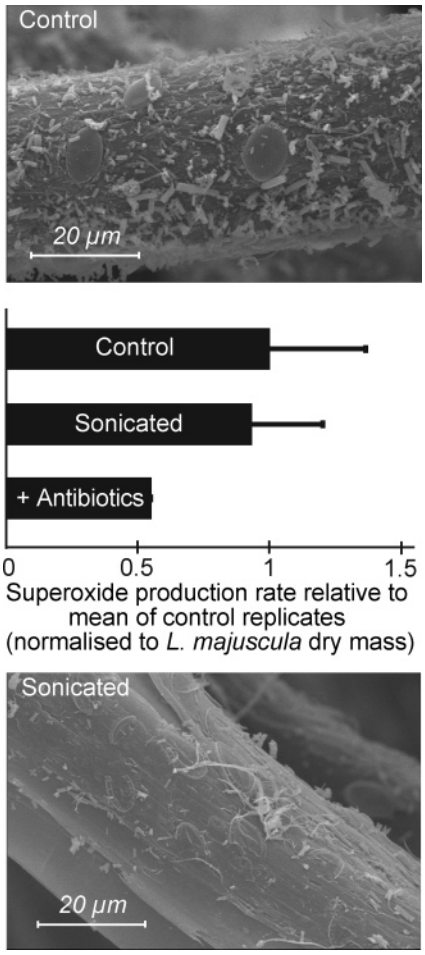

FIGURE 4. Superoxide production rates in *L. majuscula* treated to remove associated organisms such as bacteria and diatoms. Superoxide production rates decreased only slightly in *L. majuscula* that had been pulse sonicated to remove associated organisms when compared with the control. Scanning electron micrographs clearly indicate the removal of almost all associated organisms by sonication. Some damage to *L. majuscula* cells is also evident, which may account for the slight decrease. Substantial (although decreased) superoxide production was also observed in *L. majuscula* cells grown in the presence of eukaryote-specific and heterotrophic bacteria-specific antibiotics. Light microscopy confirmed the absence of associated microorganisms. Production rates were calculated from the average of 10 s of data measured 10 min after starting the pump, after which time superoxide concentrations were relatively constant in all samples. Error bars represent the standard deviation from duplicate determinations.

4, although superoxide production was less than that in the control and sonicated samples. This decrease could be due to a variety of reasons, including a negative effect of the antibiotics on *L. majuscula*, differences in general physiological status between the antibiotic-treated and control samples, or possibly due to some contribution to superoxide production by the associated organisms (either directly or synergistically). Although it appears that the majority of superoxide measured is produced by *L. majuscula*, it still may be the case that in the environment the entire consortium of organisms is interdependent for processes related to iron acquisition.

Uptake of Iron by *L. majuscula*. Using ^{55}Fe as a tracer, we observed that *L. majuscula* is able to internalize iron initially provided in the form of Fe(III)−EDTA, as shown in Figure 5. Although absolute values for iron uptake varied by several orders of magnitude between different field-harvested populations of *L. majuscula*, uptake rates were typically 10−

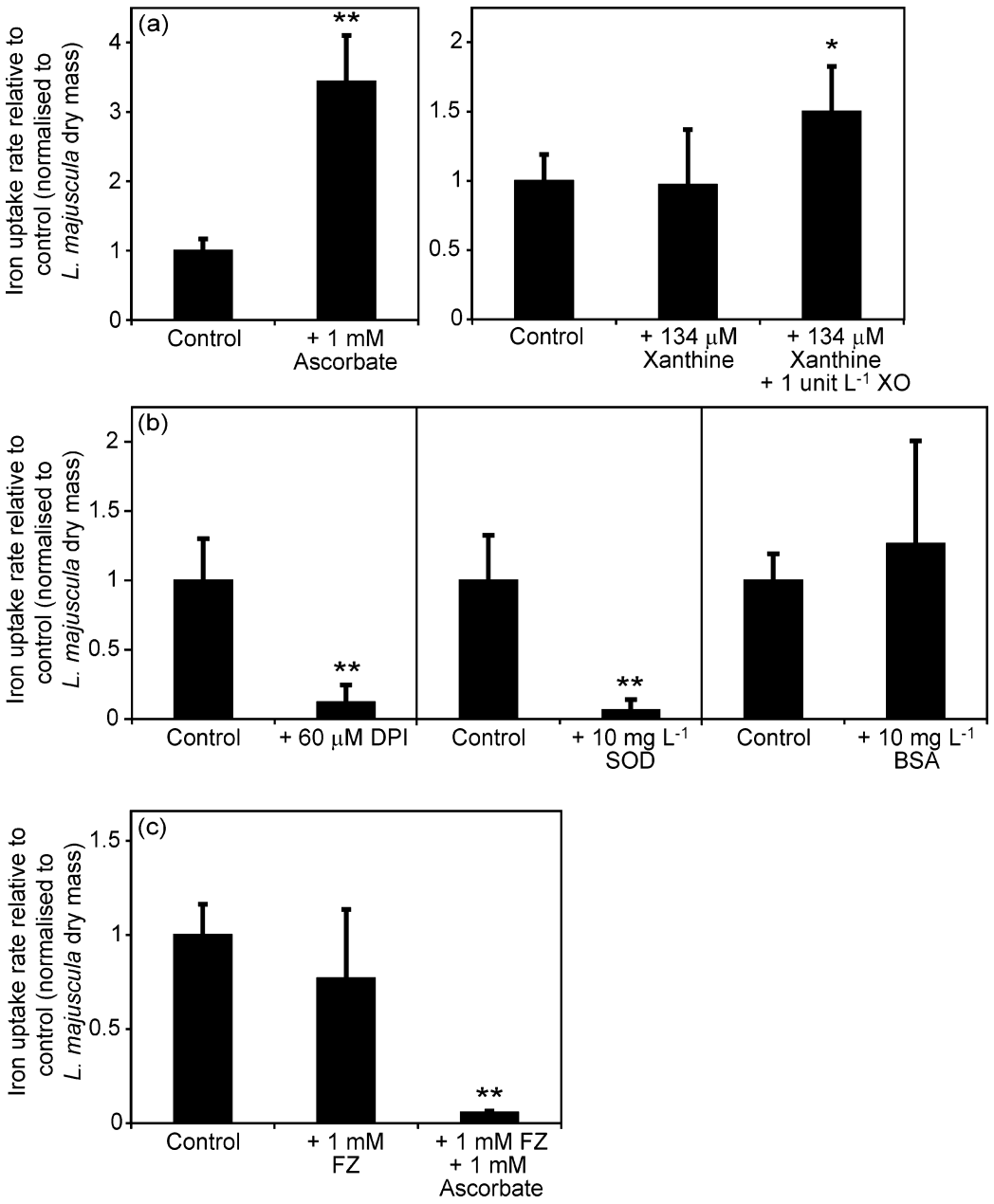

FIGURE 5. Uptake of iron by *L. majuscula* when iron was supplied as Fe(III)−EDTA (100 nM Fe(III), 1 μM EDTA) in the presence of (a) 1 mM ascorbate, 134 μM xanthine only, and 1 unit L^{-1} xanthine oxidase (XO) + 134 μM xanthine; (b) 60 μM diphenyleneiodonium chloride (DPI), 10 mg L^{-1} superoxide dismutase (SOD), and 10 mg L^{-1} bovine serum albumin (BSA); and (c) 1 mM ferrozine (FZ), and 1 mM FZ + 1 mM ascorbate. Xanthine without XO was included as a control for the addition of XO, and BSA was included as a control for the addition of SOD (denatured SOD was unsuitable as the denatured protein precipitated and subsequently interfered with iron uptake). Error bars represent the standard deviation from quadruplicate determinations. One asterisk indicates that the treatment was statistically different from the control at the 0.05 significance level; two asterisks indicate that the treatment was statistically different from the control at the 0.01 significance level.

100 pmol of Fe g^{-1} h^{-1} *L. majuscula* dry mass, such that iron reduction rates were easily sufficient to meet measured uptake demands.

To investigate the mechanism of iron uptake in more detail, we conducted a series of experiments in which we added various chemicals to influence the uptake system (see Figure 5). Addition of ascorbate to the solution, which increases the rate of reduction of Fe(III) to Fe(II) (*5*), strongly promoted uptake of iron ($p < 0.01$), thus demonstrating that reduction of iron increases its availability to *L. majuscula*. Iron uptake was also significantly ($p < 0.05$) stimulated by the addition of xanthine in the presence of xanthine oxidase, which catalyses the oxidation of xanthine by dissolved oxygen to produce superoxide (*31*). Thus, it is clear that reduction of iron by superoxide, however it may be produced, will increase iron availability to *L. majuscula*.

Next, we sought to establish whether reduction of iron was essential for its uptake by *L. majuscula* or merely a fortuitous occurrence. Iron uptake was significantly ($p < 0.01$) inhibited by 88 ± 13% upon addition of 60 μM diphenyleneiodonium chloride (DPI), a compound that inhibits the action of flavoproteins, such as NADPH oxidases, by forming adducts with reduced flavin groups (*32*). NADPH oxidases are known to generate extracellular superoxide in mammalian immune cells and have been shown to be responsible for extracellular superoxide production in *C. marina* (*27*). The *FRE1* ferric reductase system of the yeast *Saccharomyces cerevisiae* also involves a flavoprotein with strong similarity

to the NADPH oxidase enzyme in mammalian cells (*33*). Reduction of iron by *S. cerevisiae* is an essential step in its iron acquisition mechanism (*11*).

Iron uptake by *L. majuscula* was also significantly ($p < 0.01$) inhibited by 94 ± 8% upon addition of 10 mg L^{-1} SOD to the extracellular solution, indicating that superoxide is involved in iron uptake by the organism. SOD catalyses the disproportionation of superoxide, thus preventing the superoxide from reducing iron to the ferrous state. The high concentration of SOD is necessary to compete effectively for superoxide with iron−EDTA, which is also a highly efficient superoxide dismutase (*34*). As SOD could potentially affect iron uptake by binding iron, in addition to disproportionation of superoxide, we also examined the effect of 10 mg L^{-1} BSA. Iron uptake in the presence of BSA was not significantly different to iron uptake in the control, indicating that nonspecific protein effects were unlikely to be responsible for the decrease in iron uptake in the presence of SOD. Furthermore, the ligands responsible in active SOD that are responsible for metal binding are already bound to metals (copper and zinc in this case); therefore, it is highly unlikely that unbound ligands would be available in the SOD to complex iron. Given the large decrease in iron uptake caused by the additions of DPI and SOD, it thus appears that reduction of iron by superoxide is a requisite step for uptake of at least 80−90% of iron acquisition by the samples of *L. majuscula* used in this work.

Finally, we added ferrozine as a trap for ferrous iron produced by iron reduction. Ferrozine forms a strong complex with ferrous iron, thus preventing its uptake by the organism. However, addition of 1 mM ferrozine resulted in no significant decrease in iron uptake by *L. majuscula*. We checked the possibility that *L. majuscula* is able to internalize the whole ferrous−ferrozine complex by also adding ascorbate, which reduces iron to Fe(II) that can be subsequently trapped by ferrozine. As indicated in Figure 5c, iron uptake is almost completely inhibited (by 94 ± 1%) upon addition of both ascorbate and ferrozine, demonstrating that *L. majuscula* cannot internalize the whole complex. This leaves two possible explanations for the effect of ferrozine: (1) the organism does not take up ferrous iron, despite the fact that reduction of iron to the ferrous state is involved in the uptake mechanism at some level, or (2) the organism is able to take up iron in the ferrous state, which is not accessible to ferrozine. If the organism does not take up ferrous iron, then the only way in which reduction could promote iron uptake would be by reducing ferric complexes to weaker ferrous complexes, which would then dissociate, and the free (dissolved inorganic) Fe(II) oxidizes to free (dissolved inorganic) Fe(III), thus increasing the amount of free Fe(III) available to the organism. However, in this case free Fe(II), which is formed in the process, would still be trapped by ferrozine, leading to a decrease in iron uptake in its presence. Thus, we conclude that *L. majuscula* is able to access ferrous iron that cannot be complexed by ferrozine. Although the mechanism by which this ferrous iron is internalized is not clear from our work, it probably involves some interaction between the organism and organically complexed ferrous iron, which is the immediate product of superoxide-mediated reduction of organically complexed ferric iron (*29*), since ferrozine rapidly complexes free ferrous iron.

The interaction between the organism and reduced iron also explains why the presence of both ascorbate and ferrozine inhibits uptake almost completely. When iron is reduced only by superoxide produced by the organism, there will be a strong concentration gradient such that the majority of iron reduction occurs close to the organism's surface. In this case, proteins involved in iron uptake will be able to readily access the reduced iron and evidently are able to out-compete ferrozine for the majority of this iron, in whichever form it may occur. However when ascorbate is added, reduction of iron occurs predominantly far from the organism's surface. In this case, the proteins involved in iron uptake cannot access most of the reduced iron. Instead, the reduced iron may either reoxidize to the original ferric−organic complex or dissociate to give free Fe(II), which is then bound by ferrozine. Due to the high concentration of ascorbate, the process of iron reduction and subsequent binding by ferrozine in the bulk solution will lead, probably quite rapidly, to iron being sequestered from the original pool that is available to *L. majuscula* to the unavailable ferrous−ferrozine complex, thus inhibiting iron uptake by the organism. The efficiency of this process was apparent from the formation of an obvious pink coloration associated with the ferrous−ferrozine complex within the first hour of incubations.

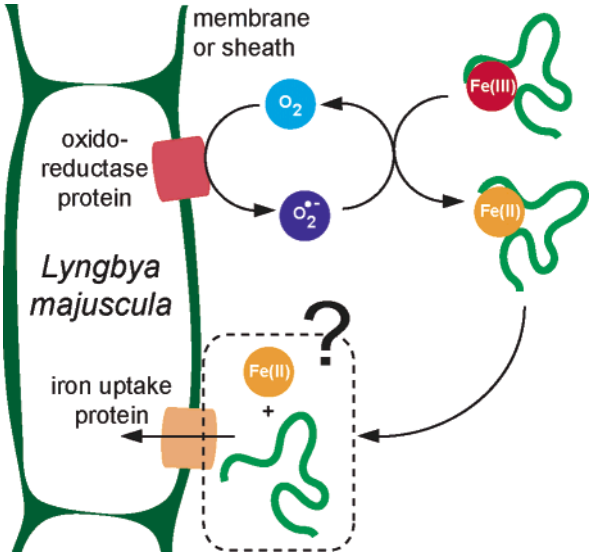

FIGURE 6. Conceptual model for superoxide-mediated iron uptake in *L. majuscula*, indicating uncertainty about how ferrous iron is internalized by the organism.

The reduction of Fe(III) to Fe(II) by superoxide thus appears to be a critical process in the iron acquisition mechanism of *L. majuscula*. However, it is not clear how the organism internalizes the ferrous iron. It may be possible that the reduction and uptake mechanisms are quite closely coupled near the cell surface and/or that there is some compartmentalization of chemical reactions facilitated by the organisms sheath and membrane layers. A conceptual model illustrating the process of superoxide-mediated iron uptake by *L. majuscula* is shown in Figure 6.

Implications of Findings. Superoxide is implicated in a variety of activities related to marine phytoplankton, including defense (*35*), photosynthesis, and oxygen removal for nitrogen fixation (*36*, *37*), which is inhibited by O_2. Our results show that superoxide produced by *L. majuscula* can also serve as an electron shuttle that increases iron availability to the organism. In contrast to siderophore production, in this process the organism does not expend energy by release of purpose-built molecules into the environment. Iron binding by siderophores is also slow and inefficient compared to iron reduction (*8*). Although *L. majuscula* must expend an electron for each superoxide molecule produced, uptake of Fe(II) results in retrieval of the electron. The likelihood of retrieval is increased by the relatively rapid and nonspecific reaction of superoxide with a wide range of Fe(III) complexes (*38*), particularly in the presence of relatively high concentrations of suitably reactive Fe(III) complexes such as may be found in coastal environments. Even in the open ocean, where much lower iron concentrations will result in lower

efficiency, reduction of iron by superoxide at biologically important rates has been shown to occur (*39*).

Although *L. majuscula* could feasibly use natural organic matter as exogenous organic shuttle compounds, there are several reasons why this strategy seems inferior to use of superoxide in oxygenated coastal waters. First, redox reactions require the formation of an encounter complex prior to electron transfer. Because of the wide range of humic substances present in coastal waters, the probability of efficient formation of an encounter complex at redox active sites on the organism's surface would be far less than with a small, uncharged molecule such as dioxygen. Reduced quinone-type moieties, which are the most likely electron carriers within NOM (*6*), also react rapidly with dissolved oxygen to give superoxide anyway (*40*−*42*), resulting in a chemical pathway that would be less efficient than direct electron transfer to dissolved oxygen. Finally, dissolved oxygen is ubiquitous in the photic zone of most natural waters, whereas concentrations of NOM can vary considerably depending on environmental conditions such as the amount of terrestrial runoff in coastal regions. While these arguments support the efficiency of superoxide in oxygenated coastal waters, clearly there are circumstances in which such a strategy will not succeed and alternatives such as organic electron shuttles will prevail, with perhaps the most obvious being anoxia.

In addition to its involvement in iron acquisition by *L. majuscula*, reported here, superoxide has recently been suggested to participate in iron acquisition by the marine diatom *Thallasiosira weissflogii* (*39*). Extracellular superoxide production has also been observed in several other marine organisms, including *C. marina* and other raphidophytes, as well as the dinoflagellate *Cochlodinium polykrikoides* (*43*). Superoxide should assist in promoting iron availability to these organisms, regardless of any other purpose for which it may be produced, given its rapid reaction with a range of ferric−organic complexes (*29*). Although further work is required to determine its full extent, it thus appears that the use of superoxide as an electron shuttle to reduce iron could feasibly be an important mechanism for iron acquisition by a wide range of marine microorganisms.

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