

# *Rhodobacter capsulatus* Catalyzes Light-Dependent Fe(II) Oxidation under Anaerobic Conditions as a Potential Detoxification Mechanism<sup>∇</sup>

Alexandre J. Poulain<sup>1†</sup> and Dianne K. Newman<sup>1,2,3\*</sup>

Biology Department, Massachusetts Institute of Technology, 68-380, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139<sup>1</sup>; Earth, Atmospheric and Planetary Sciences Department, Massachusetts Institute of Technology, 68-380, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139<sup>2</sup>; and Howard Hughes Medical Institute, 68-380, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139<sup>3</sup>

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Diverse bacteria are known to oxidize millimolar concentrations of ferrous iron [Fe(II)] under anaerobic conditions, both phototrophically and chemotrophically. Yet whether they can do this under conditions that are relevant to natural systems is understood less well. In this study, we tested how light, Fe(II) speciation, pH, and salinity affected the rate of Fe(II) oxidation by *Rhodobacter capsulatus* SB1003. Although *R. capsulatus* cannot grow photoautotrophically on Fe(II), it oxidizes Fe(II) at rates comparable to those of bacteria that do grow photoautotrophically on Fe(II) as soon as it is exposed to light, provided it has a functional photosystem. Chelation of Fe(II) by diverse organic ligands promotes Fe(II) oxidation, and as the pH increases, so does the oxidation rate, except in the presence of nitrilotriacetate; nonchelated forms of Fe(II) are also more rapidly oxidized at higher pH. Salt concentrations typical of marine environments inhibit Fe(II) oxidation. When growing photoheterotrophically on humic substances, *R. capsulatus* is highly sensitive to low concentrations of Fe(II); it is inhibited in the presence of concentrations as low as 5  $\mu$ M. The product of Fe(II) oxidation, ferric iron, does not hamper growth under these conditions. When other parameters, such as pH or the presence of chelators, are adjusted to promote Fe(II) oxidation, the growth inhibition effect of Fe(II) is alleviated. Together, these results suggest that Fe(II) is toxic to *R. capsulatus* growing under strictly anaerobic conditions and that Fe(II) oxidation alleviates this toxicity.

Iron is one of the most (photo)redox-active metals involved in biochemical functions, and it can affect the cycling of many other key elements (e.g., C, S, N, and P), trace metals (33), metalloids, and organic compounds (6). It is well appreciated that microorganisms contribute greatly to iron cycling in nature through a diversity of processes, including both oxidation and reduction reactions (16). In the past decade, much attention has been paid to how such reactions can be used to support cellular growth (1, 7, 15, 17, 19, 37, 44–46) and/or iron acquisition (2, 42) under both aerobic and anaerobic conditions, and for some organisms, these processes are understood at the molecular level (10).

Our lab has been particularly interested in one branch of the microbial Fe cycle: phototrophic Fe(II) oxidation under anaerobic conditions (9, 11, 12, 23–25). While most of the organisms we and others have studied can grow by coupling Fe(II) oxidation to CO<sub>2</sub> fixation (15, 23, 46), not all strains that oxidize Fe(II) can use it as an electron donor to support growth. An example of this is *Rhodobacter capsulatus*, which can benefit from Fe(II) oxidation only via an indirect pathway: it grows photoheterotrophically on low-molecular-weight organic compounds that form due to a photochemical reaction between

biogenic Fe(III) and organic compounds that it cannot otherwise use (citrate and nitrilotriacetate [NTA]) (4). This observation led us to hypothesize that microbial Fe(II) oxidation might be more broadly useful to microorganisms by making refractory organic compounds, such as humic substances, more bioavailable through photochemical degradation (4).

In this work, we set out to test this hypothesis using *R. capsulatus*. In addition, we sought to increase our understanding of Fe(II) oxidation by this organism by studying the effect of Fe(II) speciation and important environmental variables (e.g., light, pH, and [Cl<sup>-</sup>]) on the rate of Fe(II) oxidation. Along the way, we serendipitously discovered that low levels of Fe(II) are toxic to *R. capsulatus* when it is growing on humic substances under anaerobic conditions and that Fe(II) oxidation appears to alleviate this toxicity.

## MATERIALS AND METHODS

**Growth conditions.** We carried out growth and cell suspension assay experiments using both *R. capsulatus* SB1003 and *R. capsulatus* DW5 (a *puhA* deletion mutant deficient in subunit H of the reaction center) incapable of photosynthetic growth (47). Both strains were grown aerobically and chemoheterotrophically at 30°C in YP medium (0.3% yeast extract and 0.3% Bacto peptone [Difco]). SB1003 and DW5 were grown anaerobically and chemoheterotrophically at 30°C in freshwater medium buffered at pH 7.0 and amended with glucose (30 mM) and dimethyl sulfoxide (60 mM). Freshwater medium was prepared as previously described, buffered at pH 7.0 with bicarbonate, and used as the base medium for phototrophic growth (12, 15). To represent estuarine and coastal environments, we used a brackish medium in which the chloride concentration was increased from 10 mmol · liter<sup>-1</sup> to 500 mmol · liter<sup>-1</sup>. For photoheterotrophic growth, freshwater medium was supplemented with 5 mM acetate; the headspace was 80% N<sub>2</sub>–20% CO<sub>2</sub>. For photoautotrophic growth, H<sub>2</sub> was used as an electron donor with a headspace containing 80% H<sub>2</sub> and 20% CO<sub>2</sub>. Unless otherwise noted, cells were grown at 30°C with constant illumination equidistant (ca. 30

\* Corresponding author. Mailing address: Biology Department, Massachusetts Institute of Technology, 68-380, 77 Massachusetts Avenue, Cambridge, MA 02139. Phone: (617) 324-2770. Fax: (617) 324-3972. E-mail: dkn@mit.edu.

† Present address: Biology Department, Center for Advanced Research in Environmental Genomics, University of Ottawa, 160-30 Marie Curie, Ottawa, ON, Canada K1N 6N5.

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cm) from two 60.0-W incandescent light sources providing to a total irradiance of ca.  $30 \text{ W} \cdot \text{m}^{-2}$ .

**Light sources.** Different light sources were used during growth or cell suspension assay experiments. We used irradiance that is biologically relevant and characteristic of UV radiation (300 to 400 nm), visible radiation (400 to 700 nm), or infrared (IR) radiation (700 to 880 nm). The incandescent light source (60-W light bulbs) emitted 38% visible light and 63% IR radiation. The fluorescent light source (full spectrum; 6,500 K; Lumichrome) emitted 93% visible light and 3% IR radiation; an emission peak at 365 nm provided the remaining energy (4%). During humic substance photolysis experiments, we used an additional monochromatic UV source (8 W) emitting at a maximum wavelength of 365 nm. This additional UV source was coupled to the fluorescent setup, which then provided 19% UV radiation, 79% visible light, and 2% IR radiation. Irradiance was measured using an ILT 900 wide-band spectroradiometer from International Light Technologies (Peabody, MA). All the light sources used in this study provide wavelengths naturally found in the environment and likely to reach anoxic aquatic and terrestrial niches traditionally occupied by photosynthetic bacteria.

**Cell suspension assays.** Unless otherwise specified, all cell suspension assay mixtures were prepared and the assays were conducted at  $30^\circ\text{C}$  in an anaerobic chamber containing an atmosphere consisting of 5%  $\text{H}_2$ , 80%  $\text{N}_2$ , and 15%  $\text{CO}_2$ . Cells were typically pregrown until the mid-exponential to late exponential phase, using a 1% inoculum originating from an overnight culture grown photoheterotrophically on 5 mM acetate. Typically, 50 ml of culture was harvested, washed once, and resuspended in an equal volume of assay buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid], 20 mM  $\text{NaHCO}_3$ , 20 mM NaCl). The cell solution was dispensed to obtain the desired final assay volume. Typically, these assays were performed using an initial  $[\text{Fe(II)}]$  of  $100 \mu\text{mol} \cdot \text{liter}^{-1}$ . We also conducted cell suspension assays using an initial  $[\text{Fe(II)}]$  of  $500 \mu\text{mol} \cdot \text{liter}^{-1}$ . All glassware was left in the anaerobic chamber for at least 1 week prior to use to remove traces of oxygen. Cells were incubated ca. 15 cm from an incandescent light source providing a total irradiance of ca.  $25 \text{ W} \cdot \text{m}^{-2}$ . The dark controls were vessels kept in the chamber in a tightly closed box that prevented light from reaching the samples.

To assess the potential for *R. capsulatus* to reduce newly formed Fe(III), cell suspension assays were performed using an initial  $[\text{Fe(III)}]$  of  $100 \mu\text{mol} \cdot \text{liter}^{-1}$  [Fe(III) from a freshly prepared  $0.1\text{-mol} \cdot \text{liter}^{-1}$   $\text{FeCl}_3$  stock solution]. Fe(III) reduction was measured in the dark and in the presence of various ligands at different pHs. Fe(II) concentrations were measured as a function of time using the ferrozine assay (36) as previously described (4, 12, 23).

The effect of pH on Fe(II) oxidation was determined in short-term incubation experiments (<3 h) by preparing assay buffers (using HEPES) with pHs of 6, 7, and 8. When required, the pH was corrected using a sterile stock solution of HCl (5 M) or NaOH (5 M).

Fe speciation in solution was estimated using the MINEQL (v 4.6) chemical equilibrium program with updated stability constants from the NIST 46.6 database and references 28 and 43. To evaluate the role of pH in Fe binding by humic substances, we used the Visual MINTEQ (v 2.61) software with precomputed humic substance properties based on the NICA-donnan model. The predicted inorganic speciation of Fe(II) was comparable for the two models.

**Organic compounds tested.** Simple and complex types of organic matter were tested for the capacity to alter Fe(II) oxidation by *R. capsulatus*. Simple organic compounds were chosen based on their aliphatic or aromatic structure and their ability to efficiently chelate iron (32). The compounds tested were tricarballylate, nicotinate, NTA, picolinate, citrate, fumarate, and malate (Fig. 1). The humic substances tested were fulvic acids and humic acids, as well as natural organic matter obtained from the International Humic Substances Society and isolated from soil and water. Samples were chosen to represent humic substances ranging from mainly microbially to terrestrially derived. Humic substances referred to as "Elliott" humic substances are fulvic acids that originated from a fertile prairie soil in the United States (reference no. 2S102F). Humic substances referred to as "Pony Lake" humic substances are fulvic acids that originated from a eutrophic pond in Antarctica where organic matter is derived exclusively from microbes, is highly aliphatic, and does not contain lignin (reference no. 1R109F) (18). We also performed experiments with Suwannee River humic substances (fulvic acid, humic acid, and Suwannee natural organic matter fractions). The model structure of the humic substances presented here (Fig. 1) is based on a model developed by Buffle et al. for fulvic acids (3). This structure provides a general idea of the various moieties likely to be present in humic substances but does not faithfully represent the true structures of the humic substances used in this study.

**Apparent rates of Fe(II) oxidation and Fe(III) reduction.** For cell suspension assays, apparent rates were calculated by determining the slope of the linear part of the plot of  $[\text{Fe(II)}]$  versus time; apparent rates are expressed in

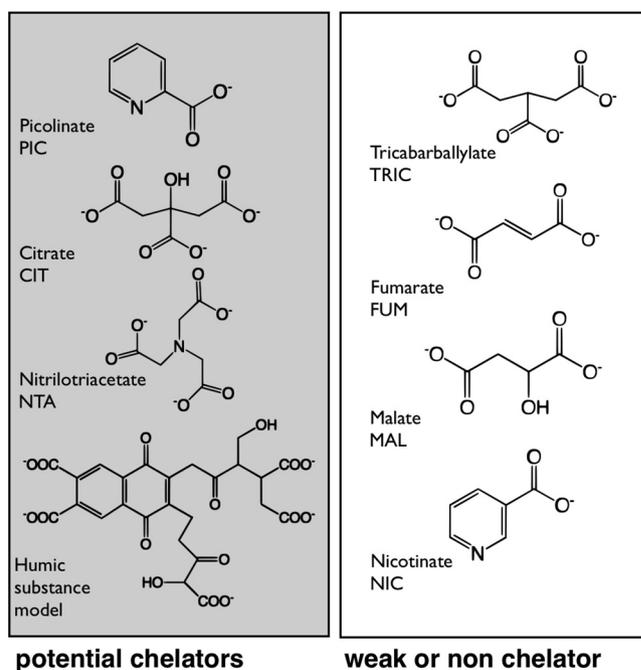


FIG. 1. Molecules used as organic partners in Fe(II) oxidation by *R. capsulatus*. Fe chelators are shown in the panel on the left. The structure shown for humic substances is a representative structure, modeled using the results of Buffle et al. (3).

$\text{nmol} \cdot \text{liter}^{-1} \cdot \text{s}^{-1}$ . Some rates were derived from growth experiments and are expressed in  $\text{nmol} \cdot \text{liter}^{-1} \cdot \text{min}^{-1}$ .

**Statistical tests.** Significant differences in Fe(II) oxidation rates among our various treatments were determined using the data analysis package from Microsoft Excel and JMP 8.0 software. To comply with the normality criterion, data were log transformed when required. *t* tests and one-way analysis of variance tests were used to determine whether there were significant differences between our treatments. A post hoc Tukey test was performed to determine which treatments were different from one another.

## RESULTS

**Fe(II) oxidation by *R. capsulatus* is light dependent and coupled to the photosynthetic machinery.** To gain a better understanding of the role of light in catalyzing microbial Fe(II) oxidation, we tested whether Fe(II) oxidation by *R. capsulatus* was (i) strictly carried out by phototrophically grown cells and (ii) coupled to the photosynthetic machinery. No Fe(II) oxidation was observed during chemoheterotrophic growth in the dark and at pH 7 for the first 7 days while *R. capsulatus* was growing slowly heterotrophically (Fig. 2). However, as soon as light was made available to the cells, rapid oxidation of Fe(II) occurred, depleting the initial pool of Fe(II) by ca. 60% (Fig. 2). We confirmed this using cell suspension assays (Fig. 3). Upon exposure to only light, the wild type rapidly oxidized Fe(II); the *puhA* mutant, deficient in a functional reaction center, did not oxidize Fe(II) (Fig. 3A). Because cells grown in the dark could immediately oxidize Fe(II) once light was present, we also tested whether this process was induced by light and could continue in the dark once it was initiated.  $[\text{Fe(II)}]$  was monitored during an initial period of exposure to light for 5, 10, 20, and 40 min, and cells were subsequently transferred to dark conditions. Fe(II) oxidation stopped as

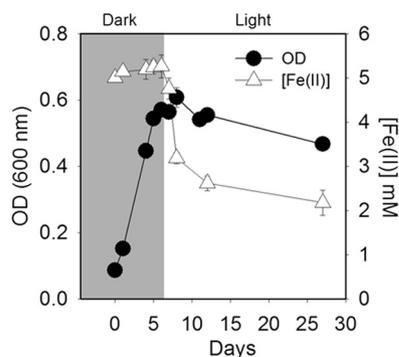


FIG. 2. Fe(II) oxidation is a light-dependent process. Fe(II) concentrations and chemoheterotrophic growth of *R. capsulatus* under anoxic conditions in the presence of 5 mM Fe(II) are indicated. Cells were first grown in the dark for 6 days (on glucose and dimethyl sulfoxide) until they reached stationary phase and were subsequently exposed to light from day 6 to day 27. The symbols show the averages of triplicate cultures, and the error bars represent the standard deviations. OD (600 nm), optical density at 600 nm.

soon as the light was switched off but resumed immediately upon reexposure to light (Fig. 3B and 3C). In the dark, no Fe(III) reduction was observed over the time course of these experiments.

Both incandescent and fluorescent light enabled Fe(II) oxidation by *R. capsulatus*, and the two types of light yielded similar oxidation rates. Chemoheterotrophically grown cells exhibited oxidation rates that were one-half those of cells grown photoheterotrophically (data not shown).

Previously, we showed that *Rhodospseudomonas palustris* TIE-1 oxidizes Fe(II) at higher rates when it is pregrown on Fe(II) (24). Although *R. capsulatus* cannot grow photoheterotrophically on Fe(II) like *R. palustris* TIE-1, we tested whether Fe(II) oxidation by *R. capsulatus* could be enhanced by preexposing cells to Fe(II) during growth on another substrate. Cells grown photoheterotrophically on acetate in the presence of Fe(II) exhibited oxidation rates that were  $\sim 3$  times higher than those of cells grown with only trace concentrations of Fe(II) in the growth medium (data not shown).

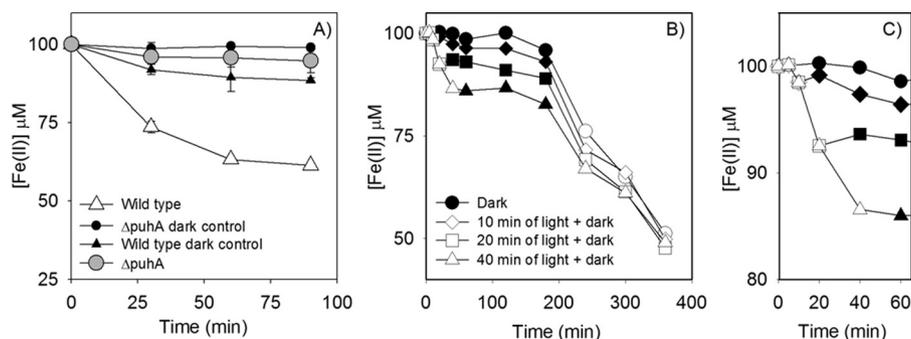


FIG. 3. Fe(II) oxidation is a light-dependent process coupled to the photosynthetic machinery. An Fe(II) oxidation assay was performed with cells grown chemoheterotrophically in the dark under anoxic conditions without Fe(II) added to the growth medium. (A) The wild type and the *puhA* mutant were exposed to light or kept in the dark in the presence of an [Fe(II)] of  $100 \mu\text{mol} \cdot \text{liter}^{-1}$ . The symbols show the averages of triplicate cultures, and the error bars represent the standard deviations. (B and C) Wild-type cells were assayed for their oxidation ability by exposing them for increasing periods of time to light and subsequently transferring them to dark conditions (indicated by filled symbols). After 200 min, cells were transferred to light conditions (indicated by open symbols). The [Fe(II)] was  $100 \mu\text{mol} \cdot \text{liter}^{-1}$ . Panel C is an enlargement of the first 60 min of panel B. The data are representative of duplicate experiments.

#### Humic substances facilitate Fe(II) oxidation by *R. capsulatus*.

To ascertain whether *R. capsulatus* could oxidize Fe(II) in the presence of humic substances as it oxidizes Fe(II) bound to citrate or NTA (4), we used samples that originated from different environments and comprised mainly terrestrially or microbially derived humic substances. Both sources of humic substances significantly enhanced the apparent net rates of Fe(II) oxidation by *R. capsulatus* SB1003 at pH 7 (Fig. 4A and B). Altogether, the apparent oxidation rates increased  $\sim 10$ -fold compared to control treatments.

To determine whether the facilitating role of the organic partner (citrate, NTA, or humic substances) could be due to its chelating properties, we chose a range of compounds that were aliphatic and aromatic, including some chelators of Fe(II) and Fe(III) (Fig. 1 and Table 1). The oxidation rates were significantly lower in the absence of an organic partner or when an organic compound that exhibited no or weak chelating properties was used (e.g., tricarballylate, fumarate, nicotinate, or malate) (Fig. 4A and B). Upon addition of known chelating compounds (e.g., picolinate, citrate, and NTA) at a ratio of [Fe(II)] to ligand concentration of 1:2, the oxidation rates increased 5- to 20-fold compared to the control treatments (Fig. 4A and B).

To test whether the oxidation rates were controlled by Fe speciation in solution, we used NTA as a representative chelator. We measured Fe(II) oxidation rates with increasing concentrations of NTA using a constant [Fe(II)] (Fig. 4C). The maximum oxidation rates were obtained for a ratio of [NTA] to [Fe] of  $\geq 1$ , corresponding to ca. 100% of the Fe present complexed to NTA (Fig. 4C and Table 2).

#### pH affects both Fe(II) oxidation and Fe(III) photoreduction.

Because the photoreduction of Fe(III) is pH dependent, with the rate increasing with decreasing pH (8, 31, 41), we tested whether Fe(II) oxidation by *R. capsulatus* was also pH dependent. We conducted cell suspension assays at pH 6, 7, and 8 using no ligand, NTA, citrate, or humic substances (Fig. 5). While the rates of Fe(II) oxidation in the presence of NTA decreased slightly from pH 6 to pH 8 (from  $36.40 \text{ nmol} \cdot \text{liter}^{-1} \cdot \text{s}^{-1}$  to  $30.12 \text{ nmol} \cdot \text{liter}^{-1} \cdot \text{s}^{-1}$ ), the rates of Fe(II) oxidation in the presence of humic substances, as well as

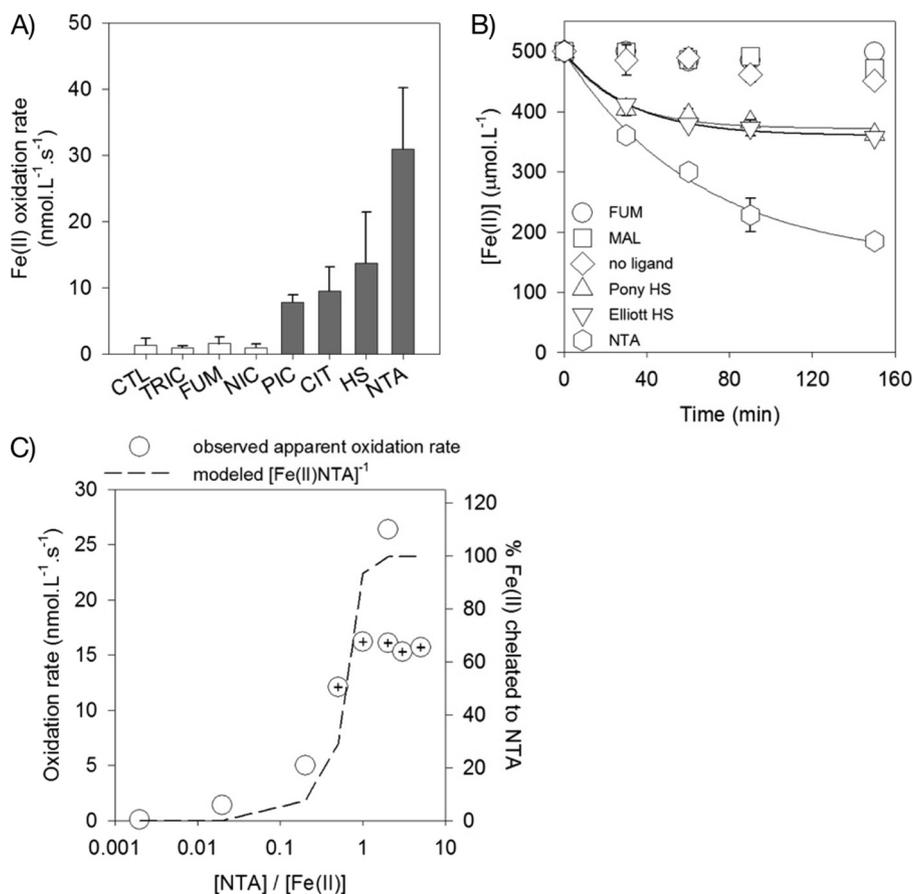


FIG. 4. Chelation enhances Fe(II) oxidation. (A) Fe(II) oxidation rates for various ligands. Each bar represents the average of 3 to 17 different observations, and the error bars represent the standard deviations. For the no-ligand control (CTL),  $n$  was 16; for tricarballylate (TRIC),  $n$  was 3; for fumarate (FUM),  $n$  was 3; for nicotinate (NIC),  $n$  was 3; for picolinate (PIC),  $n$  was 10; for all humic substance experiments pooled (HS),  $n$  was 17; and for NTA,  $n$  was 12. Experiments were carried out at an  $[\text{Fe(II)}]$  of  $100 \mu\text{mol} \cdot \text{liter}^{-1}$  under incandescent light. Humic substances were used at a final concentration of  $1 \text{ mg} \cdot \text{liter}^{-1}$ , and other ligands were used at a final concentration of  $200 \mu\text{mol} \cdot \text{liter}^{-1}$ . (B) Fe(II) oxidation assay with cells grown phototrophically without Fe(II) added to the growth medium. Cells were exposed to light in the absence of ligands or in the presence of fumarate ( $1 \text{ mM}$ ), malate ( $1 \text{ mM}$ ), Pony Lake humic substances (Pony HS) ( $1 \text{ mg} \cdot \text{liter}^{-1}$ ), Elliott soil humic substances (Elliott HS) ( $1 \text{ mg} \cdot \text{liter}^{-1}$ ), and NTA ( $1 \text{ mM}$ ) in the presence of  $500 \mu\text{mol} \cdot \text{liter}^{-1}$  Fe(II). Each symbol represents the average of duplicates, and the error bars represent the data ranges (most error bars are smaller than the symbols). (C) Fe(II) oxidation rates obtained with cells grown phototrophically without Fe(II) in the growth medium in the presence of various NTA-to-Fe(II) ratios. The Fe(II) concentration was kept constant at  $500 \mu\text{mol} \cdot \text{liter}^{-1}$  (open circles) or  $100 \mu\text{mol} \cdot \text{liter}^{-1}$  (circles with crosses). The dashed line shows the MINEQL model prediction for the relative abundance of Fe(II) bound to NTA.

in the absence of any ligand, increased significantly by an order of magnitude from pH 6 to pH 8 (Fig. 5). Results identical to those obtained in the presence of humic substances were obtained using citrate (data not shown).

TABLE 1. Thermodynamic constants and corresponding equations included in MineQL v4.6 and MINTEQA2 modeling

Reaction <sup>a</sup>	Log K <sup>b</sup>
$\text{Fe(II)} + \text{CIT}^{3-} = [\text{Fe-CIT}]^-$	5.89
$\text{Fe(II)} + \text{CIT}^{3-} + \text{H}^+ = [\text{Fe-HCIT}]$	9.91
$\text{Fe(II)} + 2\text{CIT}^{3-} = [\text{Fe-CIT}_2]^{4-}$	7.8
$\text{Fe(II)} + \text{PIC}^- = [\text{Fe-PIC}]^+$	4.90
$\text{Fe(II)} + 2\text{PIC}^- = [\text{Fe-PIC}_2]$	9.0
$\text{Fe(II)} + \text{NTA}^{3-} = [\text{Fe-NTA}]^-$	10.18
$\text{Fe(II)} + 2\text{NTA}^{3-} = [\text{Fe-NTA}_2]^{4-}$	11.9
$\text{Fe(II)} + \text{bacteria} = [\text{Fe-bacteria}]$	3.9

<sup>a</sup> CIT, citrate; PIC, picolinate.

<sup>b</sup> Log K values were updated using references 28, 32, and 43.

**Salt concentrations encountered in coastal and marine environments hamper Fe(II) oxidation.** To evaluate the potential for Fe(II) oxidation to occur in the presence of salt concentrations relevant for coastal and marine systems, we carried out cell suspension assays using Fe(II)-NTA and Fe(II) bound to humic substances as substrates. Salinity was altered for these experiments using both chloride concentrations ranging from  $0.1 \text{ mmol} \cdot \text{liter}^{-1}$  to  $500 \text{ mmol} \cdot \text{liter}^{-1}$  and sulfate concentrations ranging from 1 to  $500 \text{ mmol} \cdot \text{liter}^{-1}$ . Figure 6 shows the chloride data, which are also representative of what occurs in the presence of sulfate (data not shown). Fe(II)-NTA oxidation remained stable throughout the range of concentrations tested but sharply decreased at an  $[\text{NaCl}]$  of  $500 \text{ mmol} \cdot \text{liter}^{-1}$ . Consistent with the data shown in Fig. 4, the rate of Fe(II) oxidation in the presence of NTA and decreased sharply at an  $[\text{NaCl}^-]$  of  $500 \text{ mmol} \cdot \text{liter}^{-1}$ . The drop in the Fe(II) oxidation rate with  $500$

TABLE 2. Fe(II) inorganic and organic speciation at equilibrium

Ligand	Fe(II) species		% of total Fe(II)		
	Species <sup>a</sup>	Charge	pH 6	pH 7	pH 8
Citrate	Fe <sup>2+</sup> (aqueous)	2	9.1	5.9	1.5
	Fe-CIT (aqueous)	-1	88.9	92.3	38
	Fe-CIT <sub>2</sub> (aqueous)	-4	0	1.8	0
	Fe(II)-CO <sub>3</sub> (solid)	Solid	0	0	60.5
Picolinate	Fe <sup>2+</sup> (aqueous)	2	21.6	15.3	1.5
	Fe-PIC (aqueous)	1	52.3	49.8	9.8
	Fe-PIC <sub>2</sub> (aqueous)	0	26.1	34.9	14
	Fe(II)-CO <sub>3</sub> (solid)	Solid	0	0	74.7
NTA	Fe <sup>2+</sup> (aqueous)	2	5	0.5	0
	Fe-NTA (aqueous)	-1	95	99.5	100
	Fe(II)-CO <sub>3</sub> (solid)	Solid	0	0	0
None	Fe <sup>2+</sup> (aqueous)	2	95	16.6	2.5
	Fe-HCO <sub>3</sub> (aqueous)	1	5	1.8	0
	Fe(II)-CO <sub>3</sub> (solid)	Solid	0	81.4	97.5

<sup>a</sup> CIT, citrate; PIC, picolinate.

mmol · liter<sup>-1</sup> cannot be attributed to a physiological salt shock, as this drop was also observed with cells that had been pregrown with high salt concentrations (500 mmol · liter<sup>-1</sup>).

**Fe(II) is toxic to *R. capsulatus* under environmentally relevant conditions.** Given that humic substances promote Fe(II) oxidation by *R. capsulatus* in cell suspension assays, we next sought to determine whether the photochemical breakdown products resulting from the reaction of these substances with biogenic Fe(III) could enhance photoheterotrophic growth, as we previously observed for citrate and NTA (4). In the absence of Fe(II) under incandescent light, *R. capsulatus* was able to grow photoheterotrophically on 10 mg · liter<sup>-1</sup> humic substances, albeit poorly (to a maximum optical density at 600 nm of ~0.3); no growth on humic substances was observed in the dark (data not shown). Addition of 100 μmol · liter<sup>-1</sup> Fe(II) to

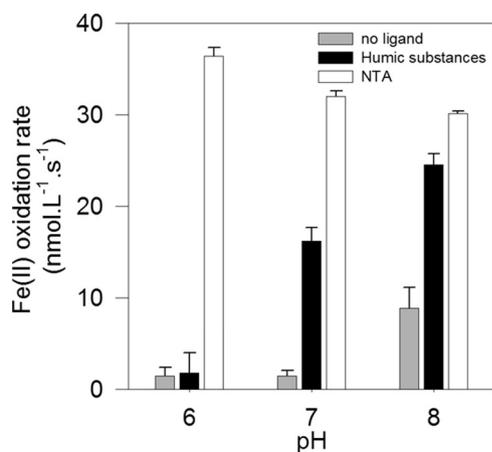


FIG. 5. Fe(II) oxidation rates are sensitive to pH. Fe(II) oxidation rates were obtained with cells grown in the absence of Fe(II). Cell suspension assays were performed in the presence of NTA (200 μmol · liter<sup>-1</sup>), Elliott humic substances (1 mg · liter<sup>-1</sup>), or no added ligand at pH 6, 7, and 8 using an [Fe(II)] of 100 μmol · liter<sup>-1</sup>. Each bar represents the average of triplicate cultures, and the error bars indicate the standard deviations.

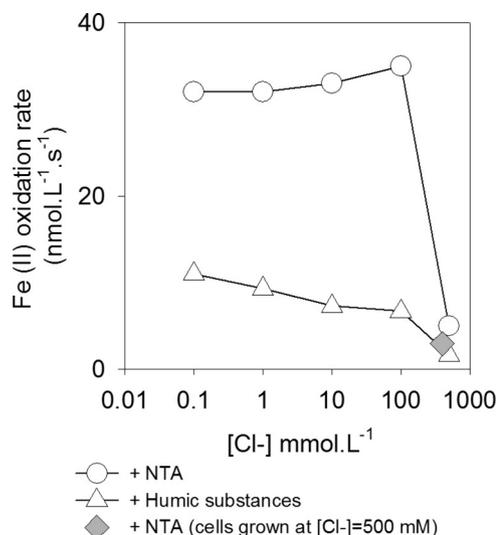


FIG. 6. Fe(II) oxidation is hampered at high salinity. Fe(II) oxidation rates were obtained with cells grown in the absence of Fe(II) in the growth medium. Cell suspension assays were performed in the presence of NTA (200 μmol · liter<sup>-1</sup>) or Elliott humic substances (1 mg · liter<sup>-1</sup>). Fe(II) oxidation rates were determined with an [Fe(II)] of 100 μmol · liter<sup>-1</sup> and [NaCl] ranging from 0.1 to 500 mmol · liter<sup>-1</sup>. The data are representative of triplicate experiments.

this medium did not enhance growth. On the contrary, we found that growth on humic substances was greatest in the absence of Fe(II) and that increasing the Fe(II) concentration had an increasingly deleterious effect on growth (Fig. 7). Surprisingly, this effect could be seen at concentrations as low as 5 μM. When *R. capsulatus* was grown on simpler carbon sources, such as glucose or acetate, that promote vigorous growth (optical densities typically ranging from 1 to 2), addition of 5 mM Fe(II) was required before growth was hampered (data not shown). Because of the sensitivity of *R. capsulatus* to Fe(II) in the presence of humic substances, we were unable to determine whether growth was enhanced by Fe(III)-mediated photochemical breakdown of humic substances.

To test the hypothesis that *R. capsulatus* is susceptible to Fe(II) toxicity under anaerobic conditions and that Fe(II) oxidation may be a mechanism to alleviate this toxicity, we performed a series of experiments to determine whether the ability to oxidize Fe(II) conferred a growth advantage. We grew cells photoheterotrophically on humic substances in the presence of 100 μmol · liter<sup>-1</sup> Fe(II) and altered variables that affect the Fe(II) oxidation rate (i.e., the presence of an iron chelator and/or pH). When cells were grown at pH 7 in the presence of Fe(II) with no ligands other than the humic substances, their doubling time was approximately sixfold greater than that of the control with no Fe(II). During this period, Fe(II) was slowly oxidized (Fig. 8A and B). In the presence of NTA, growth proceeded at rates comparable to those observed in the absence of Fe(II) (Fig. 8A and 8B). Fe(II) was readily oxidized during the first few days, as the cells were growing (Fig. 8B). When cells were grown at pH 8.25 in the presence of humic substances and Fe(II), growth was not significantly hampered and Fe(II) was readily oxidized (Fig. 8A). In general, growth was enhanced under conditions that favored Fe(II)

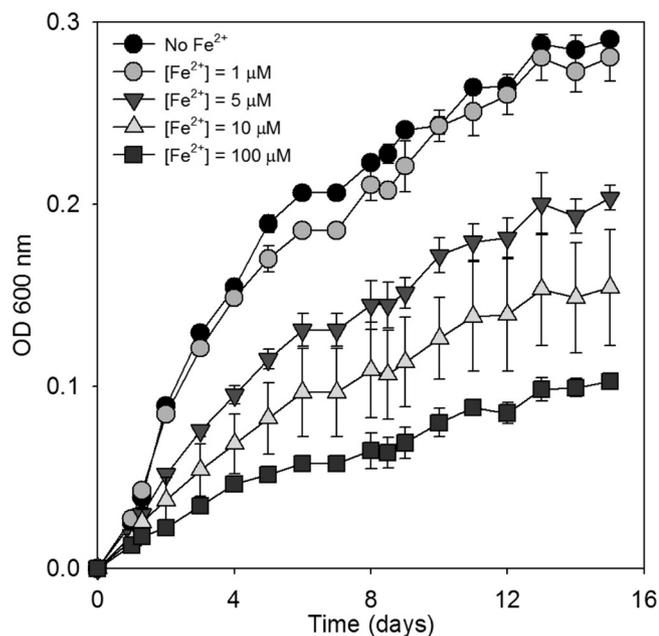


FIG. 7. Micromolar concentrations of Fe(II) are toxic. Photoheterotrophic growth on  $10 \text{ mg} \cdot \text{liter}^{-1}$  humic substances was determined in the presence of increasing concentrations of Fe(II). The symbols represent the averages of triplicate cultures, and the error bars show the standard deviations. OD 600 nm, optical density at 600 nm.

oxidation. Fe(II) was oxidized at low cell density and was completed as the cells entered stationary phase (Fig. 8B). When Fe(III) was provided alone or in the presence of a chelator, growth was unaffected (Fig. 8A). Light microscopic observation of the cells did not reveal cell encrustation under any of these conditions. In addition, we performed assays of

oxidation of  $100 \mu\text{mol} \cdot \text{liter}^{-1}$  Fe(II) using cell suspensions in the presence of other divalent metals ( $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$ ) at various concentrations (1, 10, 100, and  $1,000 \mu\text{M}$ ) and found that only  $\text{Cu}^{2+}$  inhibited Fe(II) oxidation at concentrations of  $\geq 10 \mu\text{M}$  (data not shown).

## DISCUSSION

In this study, we set out to determine how important environmental variables, such as light, the presence of iron chelators, pH, and salinity, affect the rate of Fe(II) oxidation by *R. capsulatus*. We also sought to test whether photochemical degradation of humic substances by biogenic Fe(III) could provide substrates to support photoheterotrophic growth of *R. capsulatus*, similar what Caiazza et al. (4) previously showed occurs with Fe(III)-mediated photochemical breakdown of citrate and NTA. Along the way, we discovered that Fe(II) could be toxic at low micromolar concentrations to *R. capsulatus* under environmentally relevant conditions and that Fe(II) oxidation appears to alleviate this toxicity.

The fact that reduced transition metals, such as Cu(I) and Fe(II), are toxic to microorganisms and other types of cells under aerobic conditions is well established (20). The Fenton reaction [e.g.,  $\text{H}_2\text{O}_2 + \text{Cu(I)} \rightarrow \text{Cu(II)} + \text{HO} \cdot + \text{OH}^-$  or  $\text{H}_2\text{O}_2 + \text{Fe(II)} \rightarrow \text{Fe(III)} + \text{HO} \cdot + \text{OH}^-$ ] generates hydroxyl radicals that contribute to DNA and protein damage (22, 27, 40). Far less is understood about the nature of Cu(I) or Fe(II) toxicity under anaerobic conditions. Recent work to elucidate the mechanism of Cu(I) toxicity indicates that, rather than targeting DNA as previously thought, Cu(I) targets proteins containing iron-sulfur clusters in the periplasm of *Escherichia coli* (29, 30) and that this occurs in the absence of oxygen (29). To our knowledge, there is only one previous report describing Fe(II) toxicity to bacteria under anaerobic

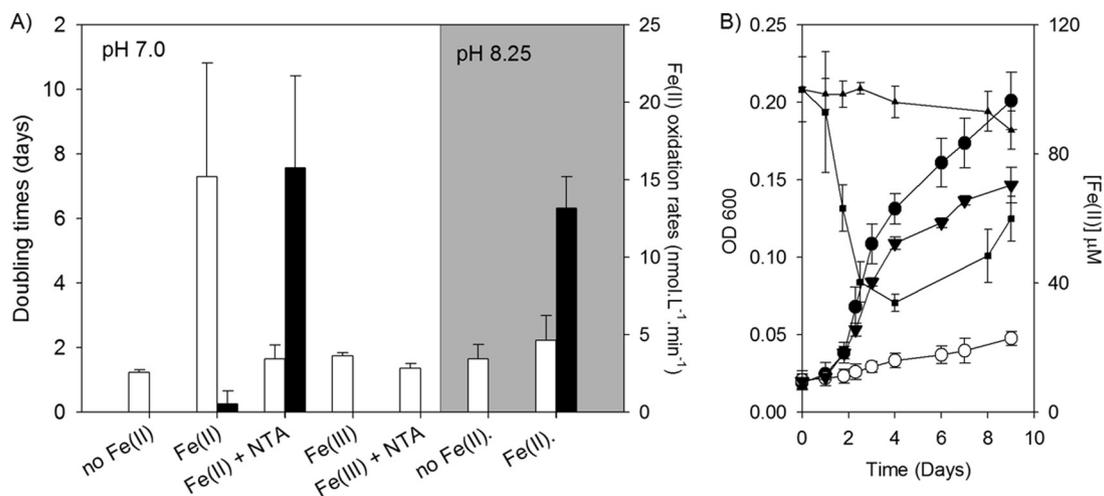


FIG. 8. Fe(II) oxidation can be a detoxification mechanism. (A) Doubling time for *R. capsulatus* cells grown photoheterotrophically on humic substances under different conditions promoting light-dependent iron oxidation. Open bars indicate the doubling time. Filled bars indicate the Fe(II) oxidation rate. The bars represent the averages of triplicate cultures, and the error bars show the standard deviations. The data in the gray portion of the graph were obtained at pH 8.25. (B) Optical density of *R. capsulatus* photoheterotrophically on humic substances (fulvic acids from Suwannee River) in the absence and presence of Fe(II) with or without NTA added as a chelator. [Fe(II)] are also plotted under these conditions. Open circles,  $10 \text{ mg} \cdot \text{liter}^{-1}$  humic substances plus  $100 \mu\text{M}$  Fe(II); filled circles,  $10 \text{ mg} \cdot \text{liter}^{-1}$  humic substances alone; inverted filled triangles,  $10 \text{ mg} \cdot \text{liter}^{-1}$  humic substances plus  $100 \mu\text{M}$  Fe(II) plus  $200 \mu\text{M}$  NTA; filled triangles, [Fe(II)] in the absence of a chelator; filled squares, [Fe(II)] in the presence of NTA. OD 600, optical density at 600 nm.

conditions (14). In that work, a variety of oral streptococci were found to be sensitive to millimolar levels of reduced transition metal cations, including Fe(II), under anaerobic conditions. While the complete set of cellular targets of these cations was not defined, Fe(II) appeared to reduce the acid tolerance of these organisms by inhibiting the F-ATPase (14). We do not yet know the cellular targets of Fe(II) in *R. capsulatus*.

Fe(II) oxidation by *R. capsulatus* proceeds at rates in cell suspension assays that are comparable to those achieved by bacteria that can grow on Fe(II) photoautotrophically (11, 21, 25). Moreover, the ability to oxidize Fe(II) appears to be constitutive and is dependent only on the presence of light and a functional photosystem in order to proceed. If Fe(II) oxidation alleviates Fe(II) toxicity, this “ready-to-rock” process would be advantageous. Moreover, the fact that *R. capsulatus* is insensitive to Fe(III) and the fact that it grows best when conditions are optimized to promote Fe(II) oxidation further suggest that Fe(II) oxidation has a detoxification function. A role for Fe(II) oxidation in detoxification may help explain previous observations for other Fe(II)-oxidizing bacteria, such as *Acidivorax* sp. strain BoFeN1, which oxidizes Fe(II) during mixotrophic growth on acetate and nitrate, where the amount of the organic cosubstrate controls the rate and extent of Fe(II) oxidation (26). The degree to which an organism is sensitive to Fe(II) may constrain its ability to utilize Fe(II) as an electron donor for growth. Consistent with this, true photoferrotrophs, such as *R. palustris* TIE-1, are impervious to millimolar levels of Fe(II), even when they are growing photoheterotrophically.

Regardless of whether Fe(II) oxidation has a detoxification function, *R. capsulatus* can rapidly oxidize Fe(II) under the appropriate conditions. At neutral pH, oxidation is slow in the absence of an organic partner or when a nonchelating agent is present, but adding micromolar concentrations of citrate, NTA, or picolinate significantly enhances Fe(II) oxidation. Similarly, the Fe(II) oxidation rate increases in the presence of humic substances provided at typical concentrations found in nature, which is likely related to their metal binding capacity (3, 39). As the pH increases, the Fe(II) oxidation rate of *R. capsulatus* increases regardless of whether Fe is in the presence of humic substances. In contrast, as the salinity increases, the Fe(II) oxidation rate decreases. This is consistent with a previous report focusing on aerobic Fe(II)-oxidizing bacteria that showed that Fe(II) oxidation was inhibited by chloride (5, 35). Salinity does not affect the speciation of Fe(II), so this inhibition must result from something else. pH and chelation, however, do affect Fe(II) speciation, and this is likely the reason why Fe(II) oxidation rates increase at high pH and in the presence of chelators.

The favorable effect of increasing the pH on biologically mediated Fe(II) oxidation might initially seem paradoxical from the cell's perspective. Indeed, increasing the pH should, in the absence of a chelator, favor Fe(III) hydrolysis and precipitation, especially at pH 8 (34), and hence be deleterious to the cells. Modeling calculations suggest that as the pH increases, a greater proportion of Fe(II) is bound to humic substances, which can be explained if Fe(II) and protons compete for carboxylic binding sites on humic substances (13, 39). However, over the range of pHs tested, protonation is not expected to significantly affect NTA speciation or its binding to

Fe(II), which is consistent with our observation that the Fe(II) oxidation rate is indifferent to the pH when Fe(II)-NTA is the substrate. In the absence of any ligands, Fe(II) oxidation is enhanced at pH 8. This may be explained by the following findings: (i) a higher pH favors the formation of hydrolyzed species [e.g., Fe(OH)<sup>+</sup>], which favors the thermodynamics of the oxidation reaction (38); (ii) at a higher pH, the cells are altered in such a way that Fe(II) oxidation is favored [e.g., changes in membrane composition or charge occur that permit Fe(II) to more readily access the oxidation site], and (iii) at a higher pH, the activity of free Fe(II) in solution is decreased by favoring the formation of an insoluble species (e.g., solid FeCO<sub>3</sub>). Assuming that soluble unchelated Fe(II) is toxic, then decreasing its concentration by contributing to the formation of insoluble species should alleviate this stress.

Because *R. capsulatus* is highly sensitive to environmentally relevant concentrations of Fe(II), it is not a good model organism to test the hypothesis that biogenic Fe(III)-mediated photodegradation of humic substances supports microbial (photo)heterotrophic growth. Given that in nature *R. capsulatus* lives in complex microbial communities, it is possible that such reactions might stimulate the growth of neighboring cells that can better tolerate Fe(II). Future work will address whether this is the case. Serendipitously, however, the sensitivity of *R. capsulatus* to Fe(II) allowed us to discover that detoxification is a potentially important biological function of Fe(II) oxidation under anaerobic conditions. It will be interesting to learn whether this is also the case for other Fe(II)-oxidizing microorganisms.

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