

# Effects of Fe(III) Chemical Speciation on Dissimilatory Fe(III) Reduction by *Shewanella putrefaciens*

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*Shewanella putrefaciens*, a heterotrophic member of the  $\gamma$ -proteobacteria is capable of respiring anaerobically on Fe(III) as the sole terminal electron acceptor (TEA). Recent genetic and biochemical studies have indicated that anaerobic Fe(III) respiration by *S. putrefaciens* requires outer-membrane targeted secretion of respiration-linked Fe(III) reductases. Thus, the availability of Fe(III) to *S. putrefaciens* may be governed by equilibrium chemical speciation both in the solution phase and at the bacterial cell–aqueous or cell–mineral interface. In the present study, effects of Fe(III) speciation on rates of bacterial Fe(III) reduction have been systematically examined by cultivating *S. putrefaciens* anaerobically on a suite of Fe(III)–organic complexes as the sole TEA. The suite of Fe(III)–organic complexes spans the range of stability constants normally encountered in natural water systems and includes Fe(III) complexed to citrate, 5-sulfosalicylate, NTA, salicylate, tiron, and EDTA. Rates of bacterial Fe(III) reduction in the presence of dissolved chelating agents correlate with the thermodynamic stability constants of the Fe(III)–organic complexes, implying that chemical speciation governs Fe(III) bioavailability. Equilibrium Fe(III) sorption experiments measured the reversible coordination of Fe(III) with *S. putrefaciens* as a function of cell/Fe(III) concentration, time, and activity of competing chelating agents. Results show that *S. putrefaciens* readily sorbs dissolved Fe(III) but that adsorption is restricted by the presence of strong Fe(III)-chelating agents. Our results indicate that dissimilatory Fe(III) reduction by *S. putrefaciens* is controlled by equilibrium competition for Fe(III) between dissolved organic ligands and strongly sorbing functional groups on the cell surface.

## Introduction

Dissimilatory Fe(III)-reducing bacteria (FRB) participate in the biogeochemical cycling of Fe in marine and sedimentary environments (1, 2). FRB such as *Shewanella* and *Geobacter* capture energy for anaerobic growth via oxidation of organic matter coupled to enzymatically catalyzed dissimilatory reduction of Fe(III) as terminal electron acceptor (TEA) (1–

4). A geochemical consequence of this microbial respiratory process is the production, in anoxic settings, of soluble Fe(II) from sparingly soluble Fe(III) minerals such as goethite (FeOOH), ferrihydrite (Fe(OH)<sub>3</sub>), and hematite (Fe<sub>2</sub>O<sub>3</sub>). Solubilized Fe(II) diffuses upward in the sediment or water column, where in the oxic zone it can be reoxidized (and reprecipitated) abiotically or enzymatically via the activity of Fe(II)-oxidizing bacteria such as *Gallionella*, *Leptothrix*, or *Thiobacillus* (2). In settings where sulfate-reducing bacteria (e.g., *Desulfovibrio*) are active, microbially generated H<sub>2</sub>S can combine abiotically with Fe(II) to form pyrite (5). A majority of sedimentary pyrite is thought to arise by this process, linking FRB activity with the occurrence of pyrite in anoxic sediments, peat, and eventually petroleum and coal (6). Despite the potential importance of FRB activity in mediating a variety of environmental processes, the molecular mechanism of microbial Fe(III) reduction remains unclear. In addition, the geochemical conditions that regulate FRB activity have not been well-documented.

Gram-negative FRB are faced with a unique physiological problem; Fe(III) is mostly present in a solid form and is therefore unable to contact respiratory chain-linked terminal reductases that are generally located in or associated with the inner (cytoplasmic) membrane. One potential solution to this problem is to locate the site of Fe(III) binding and reduction at the outside aspect of the cell outer membrane, thereby facilitating cellular contact with dissolved Fe(III), Fe(III) sorbed onto detrital particles, or surfaces of Fe(III) minerals. Several recent biochemical and genetic studies support this hypothesis (7–9). Gaspard et al. (7) examined the distribution of Fe(III) reductase activity in subcellular fractions of *Geobacter sulfurreducens*, an obligate anaerobic FRB, and found that 80% of Fe(III) reductase activity was localized in the outer membrane. Protein solubilization and extraction studies further revealed that the putative Fe(III) reductase of *G. sulfurreducens* is targeted to a peripheral region of the outer membrane and may contain a *c*-type cytochrome (7). Similar results were also reported (9) for *Shewanella putrefaciens*, a facultative anaerobic FRB. The apparent outer-membrane localization of Fe(III) reductase enzymes in both *Geobacter* and *Shewanella* support a model in which FRB activity is conditioned on the complexation of Fe(III) with the outer bacterial surface. Thus, bioavailability of Fe(III) to FRB should be governed by the equilibria and kinetics of Fe(III) microbial coordination.

The goal of this study is to determine the effect of Fe(III) chemical speciation on its bioavailability to FRB. *S. putrefaciens* is used as a model FRB in experiments measuring rates of enzymatic Fe(III) reduction as a function of the speciation of aqueous Fe(III), supplied as sole TEA under strict anaerobic conditions. The bioavailability of Fe(III) is varied by supplying Fe(III) in the form of different organic complexes that vary in thermodynamic stability, from Fe(III)–citrate (1:1 formation constant  $\log K = 11.5$ ,  $I_{\text{NaCl}} = 0.1$  M) to Fe(III)–EDTA (1:1  $\log K = 25.0$ ). A range of other chelating ligands (5-sulfosalicylate, salicylate, NTA, tiron) were chosen to span the range of stability defined by the citrate and EDTA complexes. Additional experiments measured the sorption of Fe(III) onto metabolically inactive *S. putrefaciens* cells and included tests of the rate of Fe(III) sorption, the reversibility of uptake, and the dependence of uptake on cation/bacteria concentration, competing chelators, and cell condition. These results are used to examine the link between surface speciation and Fe(III) bioavailability.

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## Materials and Methods

**Fe(III) Reduction Rate Experiments.** *S. putrefaciens* strain 200R was used in all experiments (10). *S. putrefaciens* cultures were grown aerobically in LB liquid medium (11) to mid log phase (18 h at 30 °C), amended with chloramphenicol (to 25 µg/mL) and transferred to a Coy anaerobic chamber where Fe(III) reduction experiments were carried out. The anaerobic chamber contained an atmosphere of 5% H<sub>2</sub>/10% CO<sub>2</sub>/85% N<sub>2</sub>. O<sub>2</sub> concentrations in the Coy chamber were generally <50 ppm within the first 10 min of a transfer of materials into the chamber and were generally undetectable (<1 ppm) throughout the duration of all experiments. The transferred culture was used to inoculate a series of flasks containing Westlake medium (12) amended with 5 mM Fe(III)-citrate as sole TEA, lactate (50 mM) as an electron donor, and differing concentrations of chelating organic acids. Before inoculation, pH values were adjusted to ~7.0 with trace metal grade 1.0 M NaOH or HCl. No additional buffers were used to avoid potential interferences due to complexation of buffer species with Fe(III). In all cases, pH drift during growth experiments was less than 0.3–0.5 log unit, monitored using a glass pH-combination electrode. To exclude dissolved O<sub>2</sub>, liquid media were sparged with filter-sterilized recirculated anaerobic chamber air and stirred magnetically for 48 h prior to experiments.

Two types of reduction rate experiment were performed: (i) experiments at constant concentration of complexing agent and (ii) experiments where the concentration of one strong complexing agent (tiron) was varied relative to a weaker one (citrate). Constant concentration experiments involved amendment of Westlake media with 50 mM citrate, 5-sulfosalicylate, salicylate, NTA, tiron, or EDTA. Varying concentration experiments were conducted at a constant 50 mM citrate, but tiron concentration varied from 500 µM to 10 mM. Trials were performed in 15-mL centrifuge tubes mounted on a tissue culture rotator within the Coy chamber. Tubes contained 10 mL of liquid media and were inoculated with 10 µL of aerobically grown 200R, yielding a cell density of ~10<sup>8</sup> cells/mL. Cell densities were measured via acridine orange direct counts (AODC) of stained cells under an epifluorescence microscope. Rates of Fe(III) reduction were monitored by periodically extracting 100-µL aliquots, filtering through a 0.2-µm filter, and analyzing Fe(II) and total dissolved Fe (Fe(III) = Fe<sub>t</sub> - Fe(II)) using a modified ferrozine colorimetric method (13). Addition of colorimetric reagents to supernatants was done in the Coy chamber, followed by spectrophotometric analysis at 562 nm using a Spectronic UV-Vis spectrophotometer. Absorbances of experimental solutions were calibrated against sterile standards of Fe(III) and Fe(II) at different concentrations in media solutions containing chelator amendments. No precipitation of Fe(III) was observed in sterile media containing the different chelating agents in a 2-month observation period during and after experiments.

The ligands chosen for reduction rate experiments display a range of stabilities for each aqueous Fe(III)-ligand complex. Table 1 lists Fe<sup>3+</sup> and values for the equilibrium constants of coordination reactions with selected ligands at an ionic strength of 0.1 M, which corresponds roughly to the ionic strength of the growth medium of 200R in our experiments. The Fe(III) complex of citrate was chosen as a "baseline" of stability because all other selected ligands had a stronger chelation affinity for Fe(III). In FRB growth experiments, Fe(III) is commonly supplied as TEA in the form of soluble ferric citrate. Inorganic salts of Fe(III) such as ferric chloride are not generally useful for such cultivation studies because of the limited solubility of unchelated Fe(III) at the circumneutral to slightly basic pH conditions required for growth. The chelating organic acids selected for this study are of low to moderate molecular weight (160.1–326.3 g/mol) and

**TABLE 1. Equilibrium Stability Constants of Aqueous Complexes of Fe(III) and Organic Ligands Considered in This Study<sup>a</sup>**

equilibrium	log K
Fe <sup>3+</sup> + citrate <sup>3-</sup> = Fe-citrate(aq)	11.5
Fe-citrate(aq) + citrate <sup>3-</sup> = Fe-(citrate) <sub>2</sub> <sup>3-</sup>	4.4 <sup>b</sup>
Fe <sup>3+</sup> + sulfosalicylate <sup>3-</sup> = Fe-sulfosalicylate(aq)	14.6 <sup>c</sup>
Fe-sulfosalicylate(aq) + sulfosalicylate <sup>3-</sup> = Fe-(sulfosalicylate) <sub>2</sub> <sup>3-</sup>	10.6
Fe <sup>3+</sup> + NTA <sup>3-</sup> = Fe-NTA(aq)	15.9
Fe-NTA(aq) + NTA <sup>3-</sup> = Fe-(NTA) <sub>2</sub> <sup>3-</sup>	8.07 <sup>d</sup>
Fe <sup>3+</sup> + salicylate <sup>2-</sup> = Fe-salicylate <sup>+</sup>	16.3
Fe-salicylate <sup>+</sup> + salicylate <sup>2-</sup> = Fe-(salicylate) <sub>2</sub> <sup>-</sup>	12.0
Fe-(salicylate) <sub>2</sub> <sup>-</sup> + salicylate <sup>2-</sup> = Fe-(salicylate) <sub>3</sub> <sup>3-</sup>	8.1
Fe <sup>3+</sup> + tiron <sup>2-</sup> = Fe-tiron <sup>+</sup>	20.4
Fe-tiron <sup>+</sup> + tiron <sup>2-</sup> = Fe-(tiron) <sub>2</sub> <sup>-</sup>	15.0
Fe-(tiron) <sub>2</sub> <sup>-</sup> + tiron <sup>2-</sup> = Fe-(tiron) <sub>3</sub> <sup>3-</sup>	10.4
Fe <sup>3+</sup> + EDTA <sup>4-</sup> = Fe-EDTA <sup>-</sup>	25.0 <sup>e</sup>

<sup>a</sup> Values are referenced to an ionic strength of 0.1 M. From ref 15. <sup>b</sup> From ref 17. <sup>c</sup> From ref 18. <sup>d</sup> From ref 19. <sup>e</sup> From ref 16.

represent a mix of aliphatic and aromatic structures. Citric acid, NTA, and EDTA are aliphatic polycarboxylic acids possessing three, three, and four carboxyl groups, respectively. Salicylic acid, 5-sulfosalicylic (2-hydroxy-5-sulfobenzoic) acid, and tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) all are based on a one-ring aromatic structure having (salicylic) one carboxyl and one hydroxyl; (5-sulfosalicylic) one carboxyl, one sulfonic, and one hydroxyl; and (tiron) two sulfonic and two hydroxyl groups. Other chelating agents having polyaromatic or higher carbon number aliphatic structures were excluded from this study to minimize steric effects on Fe(III)-chelate bioavailability.

**Fe(III) Sorption Experiments.** For sorption experiments, batch cultures of 200R were grown aerobically in 1 L of LB media (as described above), harvested, amended with 25 µg/L chloramphenicol to arrest growth and synthesis of new proteins, centrifuged, and concentrated to 50 mL final volume in 0.1 M NaCl (14). Fe(III) sorption trials were done in 1.5-mL centrifuge tubes to which 200R cells and stock solutions of 1.0 mM Fe(III)-citrate and 0.1 M NaCl were added. Adjustments to pH were made immediately after the start of each experiment, using NaOH or HCl, and equilibrium pH was measured in each tube with a semi-micro glass combination pH probe just before the end of each experiment. All sorption experiments were conducted in air to minimize the influence of biotic Fe(III) reduction. Tubes were allowed to equilibrate under gentle agitation at room temperature (25 ± 1 °C) for up to 4 h. At the end of each experiment, reaction vessels were centrifuged at 6000g for 30 min to separate bacteria from the supernatant. Fe<sub>total</sub>/Fe(II) concentrations in each supernatant were measured spectrophotometrically using a modified ferrozine method (13). Because both Fe(II) and Fe(III) (as total dissolved Fe minus Fe(II)) in the supernatant were measured, it was possible to determine whether significant biotic or abiotic Fe(III) reduction occurred during experiments; no significant biotic Fe(III) reduction was observed at neutral pH. Under acidic conditions (pH < 5) nearly all Fe(III) was reduced abiotically after 4 h.

Reversibility of Fe(III) sorption was measured by resuspending the centrifuged cell mass obtained in uptake experiments into 1 mL of 1.0 M HNO<sub>3</sub>. Acidified cell suspensions were allowed to equilibrate with the supernatant solutions under gentle agitation for 4 h, after which the cell suspensions were re-centrifuged and the supernatants were analyzed for total dissolved Fe spectrophotometrically.

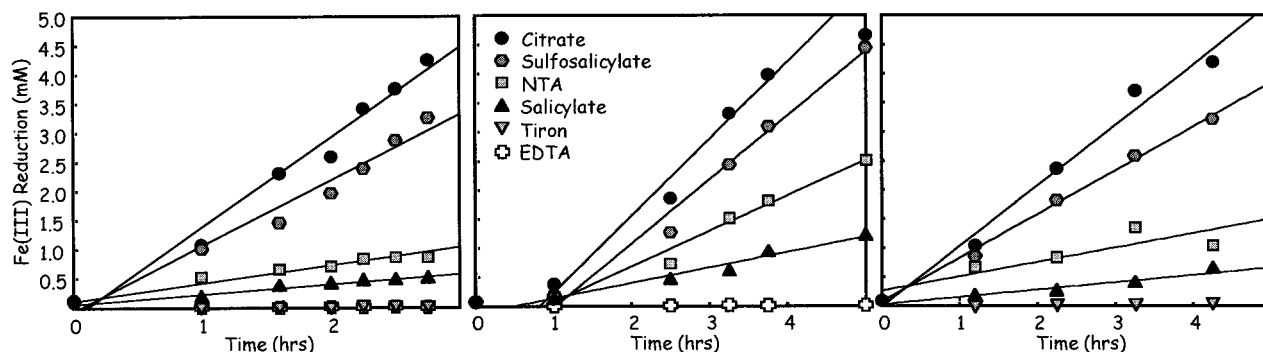


FIGURE 1. Results of replicate Fe(III) reduction rate experiments as a function of time and added chelator. Initial concentrations were Fe(III) 5 mM; citrate 5 mM; chelating agent 50 mM; pH 6.5–7.0. Lines are best-fit linear regressions of each data set.

TABLE 2. Rates (mM/h) of Fe(III) Reduction by *S. putrefaciens* 200R in the Presence of Different Organic Ligands<sup>a</sup>

	citrate	5-sulfosalicylate	salicylate	NTA	tiron	EDTA
trials	1.04	0.595	0.143	0.090	0	0
	1.54	1.13	0.183	0.307	0	0
	1.32	1.09	0.265	0.612	0	0
	1.04	0.748	0.125	0.246	0	0
mean	1.23	0.891	0.179	0.314	0	0
SD	0.245	0.261	0.062	0.219		

<sup>a</sup> Values are slopes of best-fit linear trends in mM Fe(III) reduced vs time. Experimental conditions are described in the text.

## Results and Discussion

### Fe(III) Reduction Rates versus Fe(III) Aqueous Speciation.

Fe(III) reduction rates varied systematically as a function of aqueous Fe(III) speciation. Figure 1 illustrates the results of three separate Fe(III) reduction rate experiments. The three data sets report the rate of Fe(III) reduction (initial Fe(III) concentration = 5 mM) by 200R in the presence of 50 mM citrate, 5-sulfosalicylate, salicylate, NTA, tiron, and EDTA (separately). Each data set shows that Fe(III) reduction proceeds, after an initial lag phase of varying length, according to a zero-order rate law. The Fe(III) reduction rate (mM/h) is defined as a linear increase in dissolved Fe(II) versus time (Figure 1). The Fe(III) reduction rates are linear through ~80–90% Fe(III) reduction, after which the rate of reduction diminishes. Fe(III) reduction rates varied systematically with the type of chelating ligand. The most rapid Fe(III) reduction was observed where Fe(III) was complexed by citrate. Reduction rates were progressively slower for other ligands, in the series 5-sulfosalicylate > NTA > salicylate > tiron = EDTA (Table 2). No measurable reduction was observed in the presence of tiron or EDTA, up to a maximum experimental duration of 72 h (data not shown).

The Fe(III) reduction experiments in this study were designed to minimize the influence of steric factors on Fe(III) bioavailability by selecting ligands that do not differ substantially in molecular mass or size. Potential steric effects that could inhibit Fe(III)–organic complex bioavailability include physical occlusion of surface-bound Fe(III) terminal reductases and cation binding sites by coordinating aqueous ligands. Steric effects do not appear to have been a primary determinant of Fe(III) bioavailability during experiments. Fe(III) reduction rates displayed a range of values in the presence of equimolar tiron, 5-sulfosalicylate, and salicylate despite the structural similarity of these ligands. Fe(III) bioavailability differed strongly in the presence of equimolar concentrations of citrate, 5-sulfosalicylate, and NTA despite these ligands having similar coordination properties with octahedral Fe(III). Salicylate and NTA are dissimilar structurally, and differ in their coordination number with respect

to Fe(III), yet these ligands resulted in similar Fe(III) reduction rates. Salicylate is a smaller ligand than either 5-sulfosalicylate or tiron, lacking the sulfonic groups of those ligands, but Fe(III) reduction in the presence of salicylate was slower than for any tested ligand other than tiron or EDTA. Conformational or steric factors alone cannot account for the results shown in Figure 1.

The selected ligands differ in the number of Fe(III) octahedral coordination sites that each may be expected to occlude in a 1:1 aqueous complex. Citrate, NTA, and 5-sulfosalicylate ligands each may occupy three of the available six octahedral positions about Fe(III). In contrast, salicylate and tiron in 1:1 arrangements with Fe(III) can each occupy two octahedral sites. Fe(III)–EDTA 1:1 complexes result in a conformation that blocks all six octahedral sites surrounding Fe(III). On the basis of these steric factors, it is expected that maximum stability with Fe(III) would be observed for the 1:1 complex with EDTA; 1:2 complexes with citrate, NTA, and 5-sulfosalicylate; and 1:3 complexes with salicylate and tiron, assuming availability of excess ligand. Calculations predicting the equilibrium aqueous speciation of Fe(III) and chelating agents in the growth media agree quantitatively with these expectations. Fe(III) speciation was estimated for each experimental solution using appropriate values for the concentrations of all added dissolved solutes in the growth media, published values of Fe(III) inorganic and organic aqueous complexation constants and ligand protonation constants referenced to infinite dilution (15–19), in concert with the equilibrium speciation algorithm EQ3NR (20). The dominant complexes of Fe(III) in media containing 50 mM citric acid, 5-sulfosalicylic acid, or NTA were estimated to be 1:2 metal:ligand species, while 1:3 species were predicted to dominate in media containing salicylic acid and tiron. The dominant predicted EDTA complex was a 1:1 species; higher-order EDTA equilibria are not expected based on coordination constraints and so were not included in calculations.

Experimentally derived Fe(III) reduction rates correlate strongly with conditional ( $I = 0.1$  M) stability constants of 1:1 aqueous complexes, as shown in Figure 2. These data define an *s*-shaped relationship comprising (i) rapid Fe(III) reduction under conditions where Fe(III) is weakly chelated in solution, (ii) no measurable microbial Fe(III) reduction when Fe(III) is chelated in organic complexes of relatively high stability, and (iii) an edge feature where microbial Fe(III) reduction rates abruptly accelerate as the stability of Fe(III)–chelate complexes is reduced through the log  $K$  interval of ~16–12. It is noteworthy that the 1:1 Fe(III)–ligand complex is not predicted to be the dominant Fe(III) species in any of the experimental media (excepting EDTA). However, no systematic relationship is found when Fe(III) reduction rates are compared with stability constants of conditionally dominant aqueous Fe(III)–organic complexes.

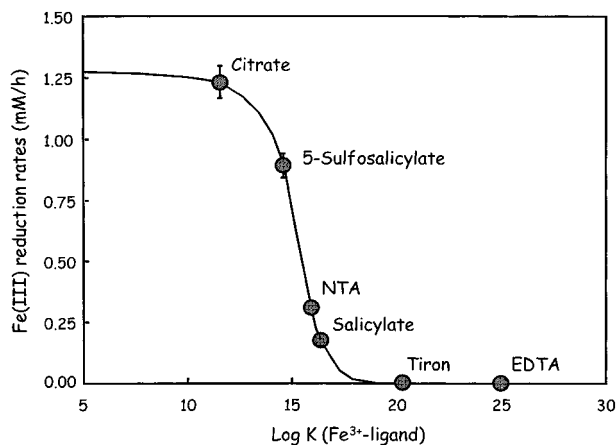


FIGURE 2. Experimental Fe(III) reduction rates (mM/h) vs 25 °C formation constants of Fe(III)–organic aqueous complexes, referenced to  $I = 0.1$  M. The line represents a smoothed interpolation connecting the data points. Error bars represent  $1\sigma$  standard deviations of mean reduction rates from replicate experiments.

This observation indicates that equilibrium thermodynamic factors may not alone constrain enzymatic Fe(III) reduction rates: if Fe(III) bioavailability were solely a function of competitive stability, a strong correlation should be observed between Fe(III) bioreduction rates and stability constants of dominant aqueous Fe(III) complexes.

One potential explanation for the trends observed in Figures 1 and 2 involves the equilibria and kinetics of ligand exchange between aqueous Fe(III)–organic complexes and surface-coordinated Fe(III)–cell complexes. Coordination of Fe(III) with the bacterial surface will depend both on the relative stabilities of the pertinent aqueous and surface complexes and the dissociation rates of differing aqueous Fe(III) complexes. It is likely that Fe(III) coordination with membrane-mounted terminal reductase enzymes occupies most (or all) cationic coordination sites; therefore, complete dissociation of the aqueous Fe(III)–organic complex is a likely prerequisite to enzymatic reduction. Dissociation of multiligand Fe(III)–organic complexes will normally proceed in a stepwise fashion with each dissociation step thermodynamically and kinetically governed by the stability constant of the dissociating aqueous complex and the stability constant of the competing complex, in this case a surface complex of Fe(III) at the bacterial cell wall.

Stepwise conditional stability constants ( $I = 0.1$  M ~ growth media) for aqueous complexes of Fe(III) and the selected ligands are shown in Table 1. For each ligand type, the largest positive stepwise formation constant is that of the 1:1 aqueous complex, followed by that of the 1:2 and 1:3 complexes, respectively. This trend of decreasing stepwise stability constants with increasing ligand number is characteristic for metal:ligand aqueous complexes, as reported elsewhere in detail (21, 22). As a result of this energetic and stoichiometric relationship among 1: $N$  metal:ligand complexes, in general the largest energetic obstacle that must be overcome in fully dissociating a 1: $N$  complex is the loss of the last complexed ligand. The thermodynamic stability of the 1:1 complex typically imposes the largest energetic obstacle in a competition among ligands for a cation (or vice versa). Where mixed-ligand complexes are prohibited thermodynamically, kinetically, or sterically, it is therefore expected that the stability of the 1:1 complex will effectively govern the distribution of cations among a set of competing ligands. Thus, the relationship observed in Figure 2 may be interpreted in terms of a competition for Fe(III) between cell-surface binding sites and aqueous ligands, where ternary surface Fe(III) complexes (i.e., ligand–Fe(III)–surface) are

largely prohibited or energetically disfavored. This model explains the observed correlation between Fe(III) reduction rates and 1:1 aqueous complex stability constants in terms of equilibrium thermodynamics, where surface Fe(III) complexes energetically outcompete, in terms of stability, 1:3 and 1:2 complexes of all selected ligands and all 1:1 complexes other than those involving tiron and EDTA. Thus, under the experimental conditions Fe(III) bioavailability for enzymatic reduction was maintained where stability constants (association  $\log K$ ) of competing aqueous Fe(III) complexes were  $<20$  (1:1 Fe(III):tiron).

An alternative explanation for the trend observed in Figure 2 involves the kinetics of ligand exchange reactions. Different rates of Fe(III) exchange between different aqueous ligands and the cell surface could potentially result in the observed variability in Fe(III) reduction rates. However, it is likely that any differences in ligand-exchange rates among the different selected ligands would be governed by the thermodynamic stabilities of the respective aqueous Fe(III)–organic complexes and would thus ultimately correlate with the stability constants of these complexes. Ligand exchange rates are governed by (i) the stability constant  $K_{OS}$  of a precursing outer-sphere complex between a fully hydrated cation ( $M(H_2O)_6^{n+}$  for octahedral coordination) and an aqueous ligand ( $L^{m-}$ ); (ii) the rate  $k_{-w}$  of water exclusion from the cationic hydration sphere occurring when the outer-sphere aquocation:ligand complex collapses to an inner-sphere species  $M(H_2O)_5L^{n-m}$ ; and (iii) the stability constant  $K_{ML}$  of the inner-sphere complex (23, 24). The overall rate expression for complex dissociation is then

$$-\frac{d[ML^{n-m}]}{dt} = \frac{K_{OS}k_{-w}}{K_{ML}}[ML^{n-m}] \quad (1)$$

Values for  $K_{OS}$  may be calculated using the approach of ref 25 and are principally dependent on the product of charges of the cation and anion. The rate-limiting variable in eq 1 is typically  $k_{-w}$ , which is cation-specific (26). In practice, when considering ligand exchange rates for a single cation such as Fe(III), eq 1 simplifies to a function of  $K_{ML}$ . Thus, the dissociation rate of an aqueous Fe(III)–organic complex varies as the inverse of the stability constant of that complex. For the complexes listed in Table 1, the rate-limiting step in complete species dissociation will be that disjoining the 1:1 complex because that species invariably possesses the largest positive  $\log K$  value in each stepwise 1: $N$  series. It is therefore possible that a combination of kinetic and thermodynamic factors governs the trend observed in Figure 2. However, in practical terms both kinetic and equilibrium constraints are controlled by the stability constants of the relevant aqueous complexes.

Although ligands forming less stable complexes with aqueous Fe(III) than citrate were not tested in this study, it is predicted that enzymatic Fe(III) reduction would proceed at only a slightly faster rate in the presence of such weakly complexing ligands. Naturally occurring low molecular weight organic acids such as acetate, oxalate, lactate, or succinate would be expected, based on the experimental results presented here, not to limit the bioavailability of Fe(III) to FRB. Most naturally occurring low molecular weight organic acids complex Fe(III) relatively weakly. For example, infinite dilution equilibrium formation constants for the 1:1 Fe(III)–acetate and –oxalate complexes are  $\sim 3.5$  and  $\sim 7.7$ , respectively, at 25 °C and 1 bar (15). In contrast, strongly chelating organic ligands such as (extraspecies) siderophores or contaminants such as EDTA might exert a limiting influence on the growth of FRB in sediments where the concentrations of these organic species are high.

A second set of Fe(III) reduction experiments was conducted in which two chelating agents were added to

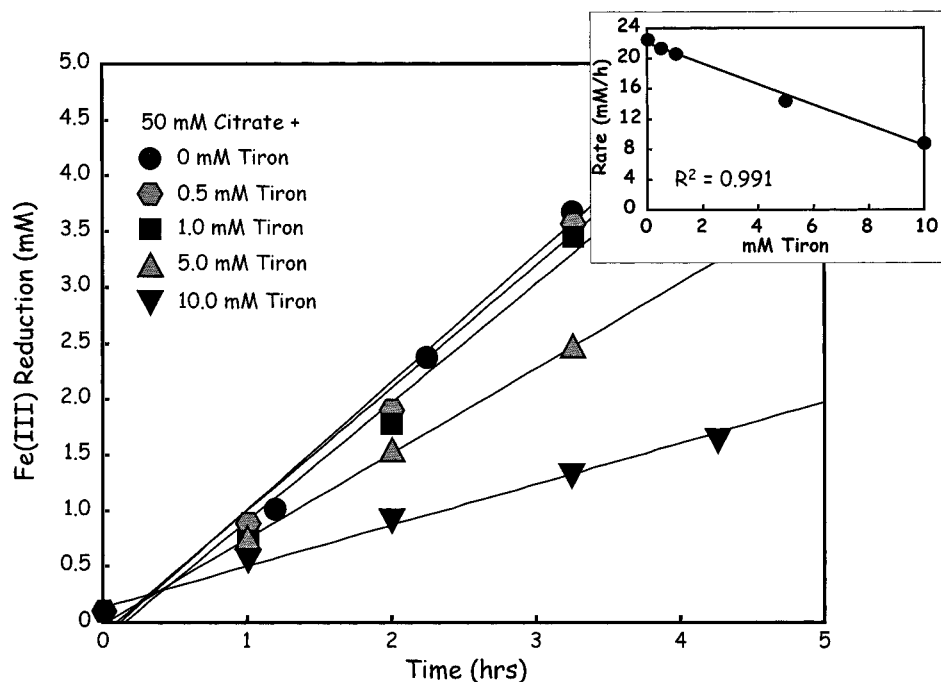


FIGURE 3. Results of Fe(III) reduction rate experiments as a function of citrate/tiron concentration and time. Inset diagram depicts correlation between reduction rates (mM Fe(III)/h) and concentration of tiron in mixed-ligand experiments.

solutions and their concentrations varied relative to each other. These experiments tested whether the effect of chelation on Fe(III) bioavailability is consistent with a mechanism of equilibrium competition for Fe(III) among aqueous chelating ligands or whether secondary effects such as toxicity, ternary interactions between ligands, or steric effects are manifested in mixed-ligand solutions vis-à-vis Fe(III) bioavailability to FRB. Fe(III) reduction rate experiments were performed under the same conditions as described previously, except that 50 mM citrate were present in all trials. Tiron concentrations were 0 mM, 500  $\mu$ M, 1 mM, 5 mM, and 10 mM. Rates of Fe(III) reduction by 200R in the presence of citrate and tiron are linear with respect to tiron concentration ( $r^2 = 0.991$ ) (Figure 3). Up to a concentration of 1 mM tiron (+50 mM citrate), Fe(III) reduction rates are only marginally slower than in the absence of this ligand. When tiron concentrations are increased to 5 mM Fe(III), reduction rates diminish significantly, dropping to approximately 60% of the rate observed in the presence of citrate alone. At tiron concentrations of 10 mM Fe(III), reduction proceeds at approximately 40% of the rate observed in citrate-only experiments.

Increasing tiron concentrations in the presence of 50 mM citrate also correspond to a systematic increase in the proportion of Fe(III) complexed with tiron and a decrease in the fraction of Fe(III) complexed with citrate. Fe(III) aqueous speciation during the experiments was calculated using the algorithm EQ3NR (20), stability constants for aqueous Fe(III)-organic complexes as tabulated in Table 1, protonation constants for all considered organic ligands, and stability constants for aqueous Fe(III)-inorganic complexes in the EQ3NR database. At 500  $\mu$ M tiron and 50 mM citrate at pH 7.0 in a model solution similar to the experimental media, approximately 5% of Fe(III) (calculated) is bound to tiron. At 1, 5, and 10 mM tiron (all else equal), approximately 10%, 50%, and 94% of Fe(III), respectively, is bound to tiron. It is noteworthy that, although increasing chelation with tiron diminishes the rate at which Fe(III) can be reduced by 200R, ~40% of the citrate-only Fe(III) reduction rate is maintained under conditions at which only 6% of Fe(III) or 250  $\mu$ M Fe(III) remains uncomplexed by tiron. At 50 mM tiron, the

fraction of Fe(III) unchelated by tiron is approximately 3 orders of magnitude lower, and under those conditions no discernible Fe(III) reduction occurred.

Successful enzymatic Fe(III) reduction under conditions where the majority of Fe(III) ions are strongly chelated suggests that competition for Fe(III) between the aqueous phase and the cell surface is predicated on the binding strength of a small number of strongly adsorbing surface sites. A high concentration of weakly binding surface sites would offer more locations at which Fe(III) sorption would occur but would not compete successfully for Fe(III) with many common organic ligands. In contrast, a low concentration of strongly binding surface functionalities, perhaps corresponding to active sites on Fe(III) reductase enzymes, could compete effectively with most natural ligands and weak chelating agents and also sustain activity in the presence of low concentrations of strong chelating agents such as siderophores.

The stability of Fe(III)-tiron complexation is similar to that exhibited by organic ligands common in marine systems, including bacterial siderophores and ligands released by cell lysis and grazing, for which reported conditional stability constants for Fe(III) complexation are  $\sim 21.6$ – $24$  (27) at nanomolar levels of Fe(III) and ligands. The vast majority ( $\sim 99.97\%$ ) of dissolved Fe(III) in seawater is coordinated with strongly chelating organic ligands, yielding  $\sim 0.07$  pM unchelated dissolved Fe(III) out of  $\sim 0.2$  nM available (28). Our experimental data support the view that strong chelation of Fe(III) in natural settings limits bioavailability but suggests that chelation does not preclude the growth of FRB unless the activity of dissolved chelating agents increases to a level sufficient to coordinate essentially all dissolved Fe(III). In marine settings the concentration of strong Fe(III)-chelating organic ligands is on the order of  $\sim 2$  nM (28), in excess of the total concentration of  $\text{Fe}^{3+}$  but not sufficiently high to completely nullify Fe(III) bioavailability to FRB. Under our experimental conditions where  $\sim 96\%$  of dissolved Fe(III) was chelated by tiron, dissimilatory Fe(III) reduction proceeded at a rate  $\sim 40\%$  of optimal. This observation supports a view that FRB are adapted to the presence of natural chelating agents and can maintain dissimilatory Fe(III) reduction at

low chelator concentrations via the formation of highly stable Fe(III)–bacteria complexes. High concentrations of chelators that bind Fe(III) more strongly than the cell surface could effectively remove Fe(III) from availability, whereas weaker chelators might not compete as effectively for Fe(III) with the cell surface.

Under the experimental conditions used in the present study, *S. putrefaciens* cells appear to compete successfully for Fe(III) with moderately to strongly chelating ligands (citrate, 5-sulfosalicylate), implying a highly stable bacteria cell surface–Fe(III) complex. Bacterial cell densities during Fe(III) reduction experiments were approximately  $10^8$  cells/mL, yielding an estimated concentration of bacterial surface acid functional groups of  $\sim 4\text{--}5\ \mu\text{M}$  (14). If it is assumed that each available acid functional group on the bacterial surface can complex one  $\text{Fe}^{3+}$  cation, optimal Fe(III) reduction rates could be sustained if only 0.1% of initially available Fe(III) ( $5\ \mu\text{M}$  capacity/5000  $\mu\text{M}$  initial Fe(III)) sorbs onto the cell surface. Thus, competitive speciation between the aqueous phase and the bacterial surface requires only that a small fraction of available Fe(III) be sorbed for optimal Fe reduction rates to be sustained. The results of mixed-ligand Fe(III) reduction experiments support this interpretation and indicate a low per cell concentration of active sites involved in dissimilatory Fe(III) reduction. Because the activity of competing chelating ligands will govern the equilibrium fraction of uncomplexed or weakly complexed Fe(III), Fe(III) reduction rates are likely to depend on the ability of the bacterial surface to compete for adsorbing Fe(III) species. Our experimental results point to the potential importance of organic ligands in solubilizing bioavailable Fe(III) from sedimentary or biogenic iron oxide minerals. Recent work suggests that biofilms augment the capacity of FRB to access insoluble Fe(III) by chelation-promoted solubilization (28). Chelating agents such as oxalate or other low molecular weight organic acids would tend to strongly complex Fe(III) and promote its solubility, yet would not be sufficiently stable as aqueous complexes to sequester Fe(III) from FRB.

**Fe(III) Sorption Experiments.** The results of Fe(III) reduction experiments demonstrate that Fe(III) bioavailability is dependent on Fe(III) chemical speciation. Although supportive of this interpretation, the results of reduction rate experiments do not provide information separating surface complexation effects from the results of Fe(III) reduction rate measurements. Equilibrium Fe(III) sorption experiments provide this information by measuring the potential of the cell surface to reversibly bind Fe(III) under conditions that are not favorable for dissimilatory Fe(III) reduction.

Partitioning experiments were conducted in which chloramphenicol-amended cell suspensions were equilibrated with solutions containing soluble Fe(III). Partitioning experiments were conducted as a function of bacteria/Fe(III) concentration, time, and chelator concentration. The effect of pH was not examined due to the very low solubility of  $\text{Fe}(\text{OH})_3$  under basic conditions and fast (<1 h) heterogeneous abiotic Fe(III) reduction at acidic pH conditions. Heterogeneous Fe(III) reduction was observed in all trials containing bacteria in which pH was adjusted below  $\sim 5$ . During all sorption experiments, pH was between 6.5 and 7.0.

Uptake of Fe(III) by 200R was initially rapid, achieving 80% of equilibrium sorption in 20 min and 90% within 1 h (Figure 4). After 2 h incubation, approximately 98% of Fe(III) was sorbed by the bacteria, and this value did not change significantly after 3 h (99% sorption). No measurable Fe(III) reduction took place during sorption experiments at neutral pH. Dissimilatory Fe(III) reduction was effectively precluded during sorption experiments because experiments were conducted in air and because a readily available carbon source was not provided.

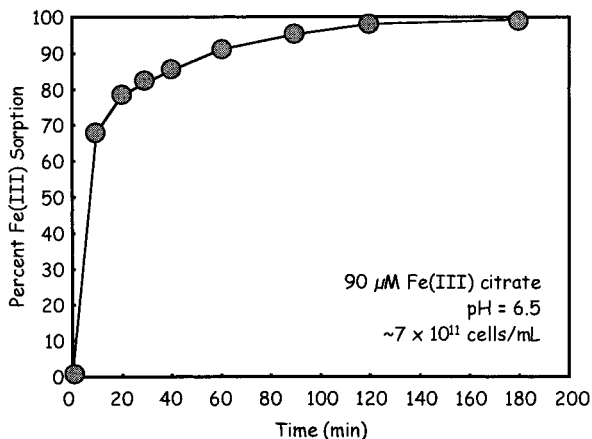


FIGURE 4. Fe(III) sorption by 200R vs time.

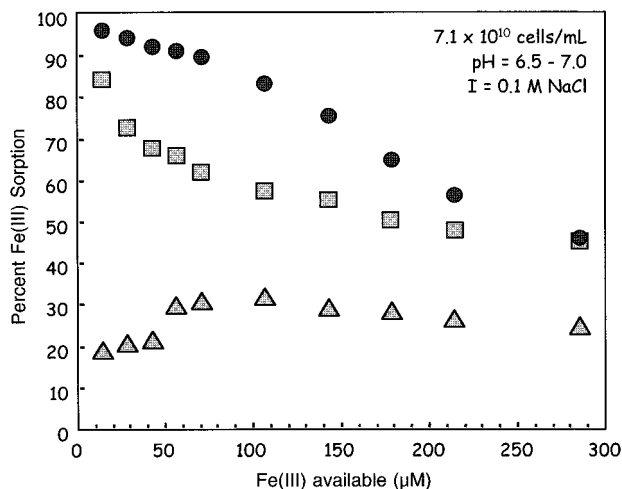


FIGURE 5. Fe(III) sorption by 200R vs initial concentration of aqueous Fe(III). Circles represent 50 mM citrate only trials, triangles stand for 50 mM salicylate trials, and squares depict trials in 50 mM citrate using cells treated with 0.5 M NaCl.

The rate of Fe(III) sorption by 200R is similar to that of U(VI) sorption (14) by this strain. Sorption of Cd(II), Pb(II), Cu(II), and Al(III) onto *Bacillus subtilis* and *Bacillus licheniformis* similarly achieve equilibrium within 1–3 h (29, 30), as does U(VI) sorption onto *B. subtilis* (31). In all of these examples, cation uptake by the cell membrane is rapid and reversible, supporting a model of equilibrium adsorption of cations onto active functional groups on the cellular surface. Acid elution experiments with 200R further indicated (data not shown) that Fe(III) sorption is fully reversible after up to 4 h exposure; in this study recovery of Fe(III) by acid elution from cells centrifuged and resuspended in 0.1 M NaCl was  $\sim 95\text{--}100\%$  efficient.

Sorption of Fe(III) by 200R was also investigated as a function of the concentration ratio of Fe(III) and bacteria. Trials were conducted in which bacterial cell density was held constant, but the concentration of Fe(III) ions in solution was varied. Bacteria were equilibrated with solutions containing Fe(III)–citrate and sodium citrate (50 mM total citrate) for 4 h. Experiments were also conducted in the presence of sodium salicylate (50 mM) in place of sodium citrate.

In experiments with citrate and Fe(III) plus cells, the extent of Fe(III) sorption by 200R correlated strongly with the concentration of Fe(III) relative to the bacteria (Figure 5). Sorption is nearly 100% at  $14\ \mu\text{M}$  Fe and  $7.1 \times 10^{10}$  cells/mL ( $\sim 1.2 \times 10^5$  Fe(III) ions per cell), decreasing as the concentration of Fe(III) increases. Sorption drops to  $\sim 30\%$  at  $286\ \mu\text{M}$  Fe(III) ( $\sim 2.4 \times 10^6$  Fe(III) ions per cell). Fe(III) concen-

tration is reported here in micromoles and also in terms of Fe(III) cation abundance per cell to facilitate a direct comparison with reported concentrations of active ionizable functional groups per 200R cell (14) and also to allow comparison among experiments at different cell densities and Fe(III) concentrations. Titration studies (14) show that 200R cells exhibit approximately  $1.9 \times 10^7$  putative carboxyl sites,  $\sim 6 \times 10^6$  phosphoryl sites, and  $\sim 2.3 \times 10^7$  amine sites per cell. Thus, optimal Fe(III) sorption by 200R occurs at bulk Fe(III) concentrations that are far below the potential saturation concentration. For example, if it is assumed that one  $\text{Fe}^{3+}$  cation can sorb onto every  $\text{H}^+$ -exchange-active carboxyl site at the cell surface, then the potential saturation concentration of Fe(III) on 200R would be  $\sim 1.9 \times 10^7$   $\text{Fe}^{3+}$  cations/cell. Optimal Fe(III) sorption occurs on 200R at a relative concentration that is approximately 2 orders of magnitude lower than this value, indicating that the majority of ionizable acid sites on the 200R surface are weak metal-binding sites. In contrast, a small minority of these sites appear to act as strong metal-binding sites, as shown by Fe(III) uptake at relatively low cation/cell concentrations. The occurrence of weak and strong metal-binding sites on the 200R surface appears analogous to the occurrence of abundant weak metal-binding type II sites and less common strong metal-binding type I sites on hydrated ferric oxide (HFO) (32) and on humic acids (33). These results are consistent with those of Fe(III) reduction rate experiments in this study, which imply that Fe(III) complexation at the cell surface probably involves a small number of sites having a strong metal-binding potential.

The results of Fe(III) reduction rate experiments show that chelation in solution reduces Fe(III) bioavailability. To test this hypothesis, another set of Fe(III) sorption experiments was performed in which 50 mM salicylate was added as a strong Fe(III) chelator. Results (Figure 5) show that 200R sorbs much less Fe(III) in the presence of salicylate than in the presence of citrate. This result is consistent with expectations based on the complex-forming ability of salicylate ( $\log K_{\text{Fe-salicylate}^+} = 16.3$  at  $I = 0.1$ ) relative to citrate ( $\log K_{\text{Fe-citrate(aq)}} = 11.5$ ). Maximum uptake of Fe(III) by 200R cells in the presence of 50 mM salicylate was  $\sim 30\%$  and was not strongly sensitive to Fe(III) concentration. Under similar conditions (but a lower cell density), rates of Fe(III) reduction measured in this study in the presence of 50 mM salicylate were  $\sim 10\%$  those recorded in the presence of citrate alone.

Another set of Fe(III) adsorption experiments was performed, under conditions identical to citrate-only and salicylate-only experiments, except that for these studies 200R cells were pretreated with 0.5 M NaCl to promote elution of proteins from the outside aspect of the outer cell membrane. For these experiments, a 50-mL suspension of 200R cells in 0.1 M NaCl was pelleted by centrifugation (30 m at 5000g) and resuspended in 50 mL of 0.5 M NaCl. Cells equilibrated with 0.5 M NaCl for 1 h and were then reconcentrated by centrifugation, the supernatant was decanted, and then the cells were suspended in 0.5 M NaCl for a second time. The cells were concentrated by centrifugation a second time and resuspended in 0.1 M NaCl, followed by a final centrifugation-resuspension cycle into a final solution of 50 mL 0.1 M NaCl. Intact cell density was measured following the NaCl treatment by AODC; no measurable loss of cells was recorded.

Results of Fe(III) sorption experiments (Figure 5) show that treatment with 0.5 M NaCl results in a significant loss in Fe(III)-sorbing capacity, especially at low relative concentrations of Fe(III). At high Fe(III) concentrations, the extent of sorption observed was essentially identical to that of untreated cells. These results are consistent with loss of metal adsorption potential as a result of protein solubilization. Protein solubility increases dramatically with increasing ionic strength (34), and this property is used routinely to separate

proteins from cell wall fractions in the laboratory. Retention of metal-binding capacity at higher Fe(III) concentrations supports a model in which adsorption occurs onto strong (type I) and weak (type II) sites and that a significant portion of type I sites are removed from the cell surface during 0.5 M NaCl treatment. One interpretation of these data locates a majority of type I Fe(III)-sorbing sites onto active sites of Fe(III) reductase enzymes secreted to the outside aspect of the outer cell membrane. However, further work describing the sorption of other metals onto *S. putrefaciens* at a wide range of metal/cell concentrations are needed to assess the importance of type I versus type II sites to metal uptake by this and similar bacteria. To constrain the mechanism of dissimilatory Fe(III) reduction, additional work is also needed to quantify the proton-exchange properties of bacteria before and after treatment with high ionic strength solutions and the Fe(III)-reducing and -complexing ability of treated bacteria, untreated bacteria, and eluted protein fractions.

Fe(III) adsorption data such as those presented in this study can also be used to evaluate quantitatively the stoichiometry and thermodynamics governing Fe(III) coordination onto FRB. As adsorption appears to govern dissimilatory metabolism, a quantitative and predictive depiction of the bioavailability of Fe(III) to FRB is needed to assess the role of FRB in Fe cycling and the reactive transport of environmentally sensitive siderophilic trace metal species in natural systems. To provide this level of quantification requires assessment of all reactions controlling Fe(III) coordination at the cell-aqueous interface, including ligand sorption onto cells, the formation of ternary cell-Fe(III)-ligand and cell-ligand-Fe(III) complexes, and the attachment of cells to Fe(III)-bearing colloids or mineral particles. Further investigations are needed to address these questions.

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