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Potential for Microscale Bacterial Fe Redox Cycling at the Aerobic-Anaerobic Interface

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Recent studies of bacterial Fe(II) oxidation at circumneutral pH by a newly-isolated lithotrophic β-Proteobacterium (strain TW2) are reviewed in relation to a conceptual model that accounts for the influence of biogenic Fe(III)-binding ligands on patterns of Fe(II) oxidation and Fe(III) oxide deposition in opposing gradients of Fe(II) and O_2. The conceptual model envisions complexation of Fe(III) by biogenic ligands as mechanism which alters the locus of Fe(III) oxide deposition relative to Fe(II) oxidation so as to delay/retard cell encrustation with Fe(III) oxides. Experiments examining the potential for bacterial Fe redox cycling in microcosms containing ferrihydrite-coated sand and a coculture of a lithotrophic Fe(II)-oxidizing bacterium (strain TW2) and a dissimilatory Fe(III)-reducing bacterium (*Shewanella algae* strain BrY) are described and interpreted in relation to an extended version of the conceptual model in which Fe(III)-binding ligands promote rapid microscale Fe redox cycling. The coculture systems showed minimal Fe(III) oxide accumulation at the sand-water interface, despite intensive O_2 input from the atmosphere and measurable dissolved O_2 to a depth of 2 mm below the sand-water interface. In contrast, a distinct layer of oxide precipitates formed in systems containing Fe(III)-reducing bacteria alone. Voltammetric microelectrode measurements revealed much lower concentrations of dissolved Fe(II) in the coculture systems. Examination of materials from the cocultures by fluorescence in situ hybridization indicated close physical juxtapositioning of Fe(II)-oxidizing and Fe(III)-reducing bacteria in the upper few mm of sand. Together these results indicate that Fe(II)-oxidizing bacteria have the potential to enhance the coupling of Fe(II) oxidation and Fe(III) reduction at redox interfaces, thereby promoting rapid microscale cycling of Fe.

Keywords chemolithotrophy, Fe(II) oxidation, Fe redox cycling, metabolic energetics, microenvironment, microscale

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INTRODUCTION

Bacterial Fe Redox Cycling

The redox cycling of iron (Fe) exerts a strong influence on the behavior of various organic and inorganic compounds in aquatic systems (Stumm and Sulzberger 1992; Davison 1993) (see Figure 1). Ferric oxyhydroxides (e.g., $Fe(OH)_3$, FeOOH, Fe_2O_3) comprise the stable (and highly insoluble) form of Fe in aerobic environments at circumneutral pH (Cornell and Schwertmann 1996). Fe(III) oxides generally possess high surface areas covered by reactive –OH functional groups, and are thus important sorbents for a wide variety of organic and inorganic contaminants in both open water and soil/sedimentary environments (Stumm 1992). Fe(III) oxides are subject to nonreductive dissolution as well as reductive dissolution under anaerobic conditions (Luther et al. 1992). When these processes occur, Fe(II) and OH^-, together with sorbed and coprecipitated species, are released to solution through generalized reactions such as

$$Fe(OH)_3 \sim X + e^- \rightarrow Fe^{2+} + 3OH^- + X \qquad [1]$$

where X represents a sorbed species released to solution during Fe(III) oxide dissolution. Direct microbial (enzymatic) reduction coupled to oxidation of organic carbon and H_2 is recognized as the dominant mechanism for Fe(III) oxide reduction in nonsulfidogenic anaerobic soils and sediments (see Lovley 1991, 2000 for review). This process contributes to both natural and contaminant (hydrocarbon) organic carbon oxidation in sedimentary environments, and exerts a broad range of impacts on the behavior of trace and contaminant metals and radionuclides (Lovley and Anderson 2000). Reduced sulfur (S) oxidation processes (both biotic and abiotic) drive much of Fe(III) oxide reduction in sulfur-rich marine sediments (e.g. Thamdrup, Fossing, and Jorgensen 1994), although the potential for substantial organic carbon oxidation coupled to direct enzymatic Fe(III) oxide reduction in marine sediments is now recognized (Thamdrup 2000). Bacterial catalysis is also likely to play an

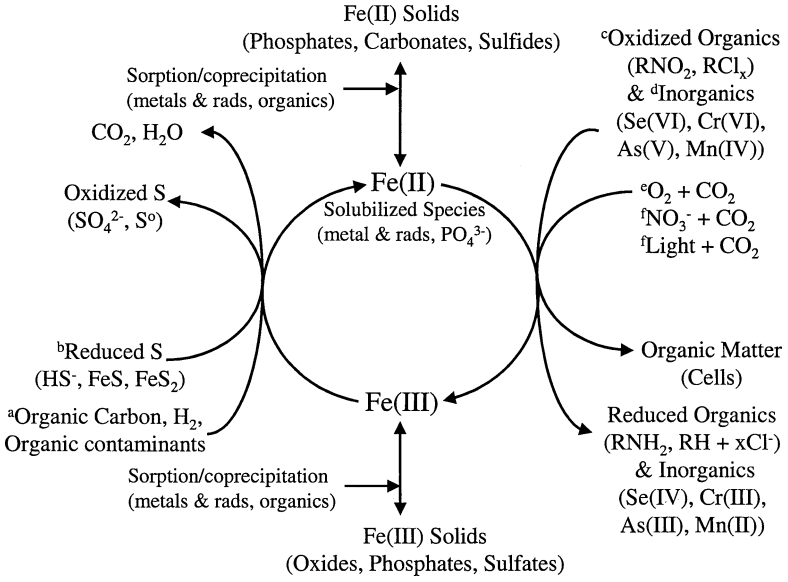

Figure 1. The role of Fe redox transformations in biogeochemical cycling. Modified with permission from Figure 1 in Tebo and He (1999), copyright 2000 American Chemical Society. Recent reviews of the role of microbial activity in the various processes are indicated by the superscripted letters: (a) circumneutral pH: Lovley (2000), Thamdrup (2000); acidic pH: Johnson (1998), Blake and Johnson (2000), see also Küsel et al. (1999) and Peine et al. (2000) (b) circumneutral pH: direct enzymatic catalysis doubtful; see Thamdrup et al. (1993), Lovley (1994), and Schippers and Jørgensen (2001); acidic pH: Blake and Johnson (2000), Pronk and Johnson (1992). (c) involvement of enzymatic catalysis not yet known. (d) circumneutral pH: Emerson (2000); acidic pH: Johnson (1998), Blake and Johnson (2000); see also Edwards et al. (2000). (e, f) Straub et al. (2001).

important role in Fe(III) reduction coupled to oxidation of organic carbon and reduced S in acidic sedimentary environments (Johnson, McGinness, and Ghauri 1993; Kusel et al. 1999; Kusel and Dorsch 2000; Peine et al. 2000).

When Fe(II) comes into contact with O_2 or other suitable oxidants, Fe(II) can be reoxidized to soluble Fe(III), which eventually precipitates as Fe(III) oxyhydroxides (Taillefert, Bono, and Luther 2000). The dominant role of microbial catalysis in Fe(II) oxidation in acidic environments (e.g., acid mine drainage and acid hot springs) is well-established (Brock and Gustafson 1972; Singer and Stumm 1972; Johnson et al. 1993). In contrast, Fe(II) is subject to rapid chemical oxidation by dissolved O_2 at circumneutral pH (Davison and Seed 1983; Millero, Sotolongo, and Izaguirre 1987), and the Fe(III) produced eventually hydrolyzes and precipitates through overall reactions such as

$$Fe^{2+} + 0.25O_2 + 2.5H_2O \rightarrow Fe(OH)_3 + 2H^+$$
$$\Delta G^{\circ\prime} = -109 \text{ kJ mol}^{-1} \quad [2]$$

In view of the speed of this spontaneous abiotic reaction (the half-life of dissolved Fe^{2+} in air-saturated water is on the order of a few minutes), bacterial Fe(II) oxidation with O_2 as an electron acceptor at circumneutral pH, though energetically more favorable than Fe(II) oxidation at low pH, has been considered doubtful from a geochemical perspective (e.g., Davison and Seed 1983). Nevertheless, bacteria have been associated with circumneutral Fe(II) oxidation and Fe(III) oxyhydroxide deposition for a over a century (Ghiorse 1984; Ehrlich 1995). A review by Emerson (2000) provides an excellent overview of the history of research on circumneutral bacterial Fe(II) oxidation, as well as the physiology and systematics of currently known FeOB. It is relevant to note that the potential for both anaerobic phototrophic (Widdel et al. 1993; Ehrenreich and Widdel 1994) and chemolithotrophic nitrate-reducing bacteria (Straub et al. 1996; Benz, Brune, and Schink 1998; Straub and Buchholz-Cleven 1998) to catalyze circumneutral Fe(II) oxidation is now recognized (Straub et al. 2001). However, the overview and experimental studies presented in this paper focus on chemolithotrophic organisms capable of oxidizing Fe(II) with O_2 as an electron acceptor.

Circumneutral Bacterial Fe(II) Oxidation

Recent enrichment and isolation studies with Fe(II)-O_2 opposing gradient systems (Kucera and Wolfe 1957; Jones 1983) have expanded the range of organisms known to be involved in aerobic circumneutral Fe(II) oxidation beyond the traditional stalk-forming *Gallionella* and sheathed bacteria of *Sphaerotilus-Leptothrix* group, to include unicellular organisms from the α-, β-, and γ-*Proteobacteria* (Emerson and Moyer 1997; Emerson 2000; Edwards et al. 2003; Sobolev and Roden 2004). The involvement of unicellular bacteria in circumneutral Fe(II) oxidation is consistent with the observation of high numbers of unicellular organisms closely associated with Fe(III) oxides in

microbial mats present at a groundwater Fe seep in Denmark (Emerson and Revsbech 1994a), and the Fe(III) oxide-rich plaque of aquatic macrophyte roots (Emerson, Weiss, and Megonigal 1999). Studies by Emerson and colleagues (Emerson and Revsbech 1994b; Emerson and Moyer 1997; Neubauer, Emerson, and Megonigal 2002) have clearly demonstrated the potential for chemolithotrophic growth of such organisms coupled to Fe(II) oxidation at circumneutral pH.

FeOB are likely to play a significant role in circumneutral Fe(II) oxidation at redox interfacial environments where diffusion-limited O_2 transport leads to low dissolved O_2 partial pressure (microaerobic conditions) within the zone of Fe(II)-O_2 overlap, i.e., environments characterized by opposing diffusion gradients of O_2 and Fe(II). Such environments include stream sediments, groundwater Fe seeps, wetland surface and rhizosphere sediments, cave walls, irrigation ditches, subsurface boreholes, municipal and industrial water distribution systems, and hydrothermal vents (Emerson 2000). Bacterial Fe(II) oxidation may also occur in association with microscale redox zonation within low-porosity matrix aggregates in subsurface sediments (Hunter, Wang, and VanCappellen 1998).

In order to understand how bacterial Fe(II) oxidation can compete effectively with chemical oxidation by O_2 under microaerobic conditions, it is useful to consider the kinetics vs. the thermodynamics of Fe(II) oxidation by O_2 as a function of Fe(II) and O_2 concentration at circumneutral pH (Figure 2). Assuming a constant dissolved Fe(II) concentration of 100 μM at pH 7.0, $\Delta G'$ calculations (using thermodynamic data from Stumm and Morgan 1996) indicate that the free energy associated with Fe(II) oxidation (Equation 2) decreases only slightly (<5%) as the dissolved O_2 concentration decreases from 300 μM to 1 μM; in contrast, the half-life of Fe(II) (estimated using the rate expression for abiotic Fe^{2+} oxidation by O_2 given in Singer and Stumm 1972) increases by a factor of 300. Thus, assuming

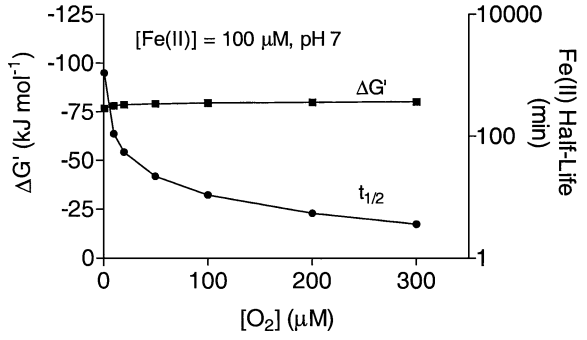

Figure 2. Change in the free energy yield ($\Delta G'$) versus the kinetics of abiotic dissolved Fe(II) oxidation as a function of dissolved O_2 concentration. A constant pH of 7.0 and dissolved Fe(II) concentration of 100 μM was assumed for the calculations, and activities were assumed to be equal to concentrations. Rates of abiotic Fe(II) oxidation used to compute half-lives ($t_{1/2}$ values) for Fe(II) were computed using the rate expression given in Singer and Stumm (1972).

FeOB can compete with abiotic Fe(II) oxidation under microaerobic conditions, considerable amounts of energy should still be available for chemolithotrophic metabolism. Flow chamber experiments have in fact demonstrated that FeOB can compete effectively with abiotic Fe(II) oxidation under microaerobic conditions (Emerson and Revsbech 1994b).

Potential for Coupling of Bacterial Fe(II) Oxidation and Fe(III) Oxide Reduction in Natural Systems

Compared to reduced Mn, S, and N oxidation processes in which the quantitative importance of enzymatic catalysis by chemolithotrophic bacteria is well-recognized (Jorgensen 1989), the significance of bacterial Fe(II) oxidation as a biogeochemical process is still not well understood. The activity of FeOB leads to the deposition of mainly amorphous Fe(III) oxide phases which are excellent substrates for anaerobic dissimilatory FeRB (Emerson and Revsbech 1994a). Since this deposition typically takes place within redox interfacial environments characterized by low dissolved O_2 concentration, the possibility exists that bacterial Fe(II) oxidation and Fe(III) reduction are tightly coupled in such environments (Emerson and Moyer 1997), at least in freshwater systems where the impact of S cycling on Fe dynamics is much lower than in marine systems. This proposed interaction is analogous to the well-known coupling between bacterial sulfate reduction and lithotrophic or phototrophic reduced S oxidation in microbial mats and marine environments (Jorgensen 1989).

Emerson and Moyer (1997) found that FeOB do not alter the rate of Fe(III) oxide accumulation in circumneutral pH diffusion-controlled opposing-gradient culture systems, a result verified by our own studies with opposing-gradient FeOB cultures (Sobolev and Roden 2004). In addition, Neubauer et al. (2002) showed that FeOB only slightly enhanced (18%) total rates of Fe(II) oxidation over cell-free controls in well-mixed batch reactors. This situation differs from radically from oxidation of Fe(II) at low pH and oxidation of dissolved sulfide at neutral pH, the kinetics of which may be accelerated by a factor of 1,000 or more in the presence of organisms such as *Thiobacillus* (Singer and Stumm 1972) and *Beggiatoa* (Jorgensen and Revsbech 1983; Nelson, Jorgensen, and Revsbech 1986), respectively. Together these results suggest that the unique role of FeOB in Fe redox cycling in nature is likely to stem from their ability to alter the spatial separation between zones of Fe(II) oxidation and Fe(III) oxide precipitation in a way that promotes Fe redox cycling. It is important to note in this context that FeOB activity generally leads to the development of a narrow band of cells and Fe(III) oxides in the vicinity of aerobic/anaerobic interface in Fe(II)-O_2 opposing gradient systems (Emerson and Moyer 1997; Benz et al. 1998); see also Figure 3), in contrast to a much more diffuse zone of Fe(III) oxide deposition in abiotic systems. These observations suggest that FeOB have the capability to focus the zone of Fe(III) oxide deposition very close to (i.e. within a few mm) the zone where FeRB are expected to be active. This capability is significant in light of the fact that

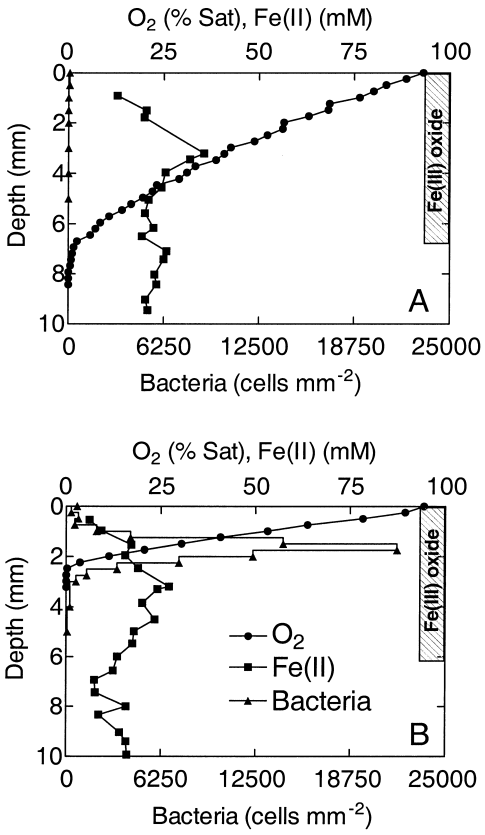

Figure 3. Distribution of Fe(II), particulate Fe(III) oxides, O_2, and bacteria in 1-week old sterile (A) and TW2-inoculated (B) opposing-gradient cultures. Note that Fe(II) profiles determined by densitometric analysis of the ferricyanide-fixed diffusion probes were confounded by the presence of Fe(III) compounds in the probe (see text). Fe(II), O_2, and bacterial profiles are averages of measurements in triplicate cultures. Reproduced with permission from Sobolev and Roden (2001), copyright 2001 American Society for Microbiology.

the presence of even traces of dissolved O_2 are likely to either inhibit strictly anaerobic FeRB metabolism (Lovley 2002), or strongly depress Fe(III) reduction activity in facultative FeRB (Arnold et al. 1990). FeOB stand to benefit greatly from altering the geometry of the Fe(II)-O_2 reaction zone such that little or no spatial separation exists between the zones Fe(III) oxide deposition and Fe(II) regeneration (discussed in detail later).

The following sections of this paper provide a review of our recent studies on FeOB metabolism and associated geochemical phenomena in Fe(II)-O_2 opposing gradient systems, and on the potential for coupled FeOB/FeRB metabolism in such systems. Although much of the reviewed research has already been published (Sobolev and Roden 2001, 2002, 2004), several previously undescribed aspects of our studies are considered here, including calculations of the energetics of FeOB metabolism in relation to production of Fe(III)-binding ligands, and microelectrode measurements of dissolved Fe(II) and O_2 distributions in FeOB/FeRB cocultures. These studies are discussed and interpreted in relation to conceptual models which account for the influence of biogenic Fe(III)-binding ligands on patterns of Fe(III) oxide deposition and microbially-catalyzed Fe redox cycling in opposing gradients of Fe(II) and O_2.

NOVEL EFFECTS OF FeOB ACTIVITY ON Fe(II) OXIDATION AND Fe(III) OXIDE DEPOSITION

We have recently conducted a series of experimental studies to evaluate quantitative aspects of bacterial Fe(II) oxidation and its potential consequence for Fe cycling in redox interfacial environments. This work was conducted with a chemolithotrophic Fe(II)-oxidizing bacterium isolated from a freshwater wetland in the Talladega National Forest in northcentral Alabama, United States (Sobolev and Roden 2001). This organism, designated strain TW2, falls within the β-subclass of the *Proteobacteria* with 91% 16S rRNA gene sequence identity with *G. ferruginea* (Sobolev and Roden 2004). The sediments in the Talladega Wetland (TW) from which strain TW2 was isolated are typical of organic-rich freshwater wetlands (Westermann 1993), characterized by steep gradients of dissolved O_2 and high concentrations of dissolved and solid-phase Fe(II) within mm of the sediment-water interface (Roden and Wetzel 1996; Sobolev and Roden 2002). It is important to acknowledge that our findings with strain TW2 may not necessarily apply to other phylogenetically and physiologically different neutrophilic FeOB, in particular the unicellular FeOB from the γ-subclass of the *Proteobacteria* that have been isolated from a variety of environments by Emerson and colleagues (Emerson and Moyer 1997; Emerson et al. 1999; Emerson and Moyer 2002; Neubauer et al. 2002; Weiss et al. 2003). For example, the ability of the latter organisms to alter geochemical conditions at the Fe(II)-O_2 interface through production of soluble/colloidal Fe(III) compounds, and/or to grow in close association with FeRB, has not yet been evaluated. In addition, the extent to which the results obtained in our laboratory pure culture systems reflect what occurs in nature is unknown. Hence, the studies discussed here should be viewed as a starting point for future evaluations of the unique physiological and biogeochemical activities of FeOB in circumneutral sedimentary environments.

Opposing-Gradient Studies

Studies of the growth of strain TW2 in Fe(II)-O_2 opposing gradient cultures have revealed several novel effects of bacterial metabolism on the dynamics of Fe(II) oxidation and Fe(III) oxide deposition at circumneutral pH (Sobolev and Roden 2001). The opposing-gradient culture systems used in these studies were analogous to those employed by Emerson and Moyer (1997), except that a relatively high concentration of soluble Fe(II) (50 mM $FeCl_2$) was employed in the bottom layer in order to stimulate FeOB growth and better observe the effects of FeOB activity on patterns of Fe(III) oxide deposition. Dissolved Fe(II) concentrations were likely on the order of several hundred

μM in the zone of Fe(II) oxidation a few cm above the bottom layer (see Figure 5). These reaction systems mimic Fe-rich surface sediments in freshwater wetlands (e.g., Roden and Wetzel (1996)), as well as certain highly reducing groundwater environments (e.g., the landfill leachate-contaminated aquifers studied by Christensen et al. 1994) in which porefluids containing 10s to 100s of micromoles of dissolved Fe(II) per L may impinge on downstream aerobic groundwaters.

Oxygen gradients were much steeper in culture systems inoculated with FeOB than in sterile controls (Figure 3), similar to observations made by Emerson and Moyer (1997) and more recently by Edwards et al. (2003). Analysis of microscope slides embedded in the agar-stabilized cultures, to which FeOB became attached during a 1-week growth period, revealed a peak in bacterial biomass at a depth where the dissolved O_2 concentration was less than 20% saturation at room temperature (<50 μM) (Sobolev and Roden 2001). An interesting observation emerged during recording of the O_2 microprofiles in inoculated vs. sterile cultures: the lower boundary of the zone of Fe(III) oxide deposition (Figure 4, hatched bars), defined by the depth at which the tip of the O_2 microelectrode became visible after exiting the oxide layer, was consistently below the depth of O_2 penetration in the inoculated cultures. In contrast, the entire zone of oxide deposition was aerobic in the abiotic controls. Time course measurements of O_2 distributions indicated that the observed suboxic deposition of Fe(III) oxides in the live cultures was not due to an upward-retreating O_2 front, because the depth of O_2 penetration increased steadily over time as a result of progressive depletion of soluble Fe(II) in the bottom layer of the cultures (Figure 4). In addition, the depth of O_2 penetration was above the bottom boundary of the oxide band at all times except for the first two time points in inoculated cultures, whereas O_2 always penetrated to depths below the lower boundary of the oxide band in sterile controls (Sobolev and Roden 2001). As discussed further below, these findings provide evidence for the ability of FeOB activity to alter patterns of Fe(III) oxide deposition in a way that would be conducive to coupling of bacterial Fe(II) oxidation and Fe(III) reduction, since Fe(III) compounds deposited below the depth of O_2 penetration would be immediately available as electron acceptors for FeRB.

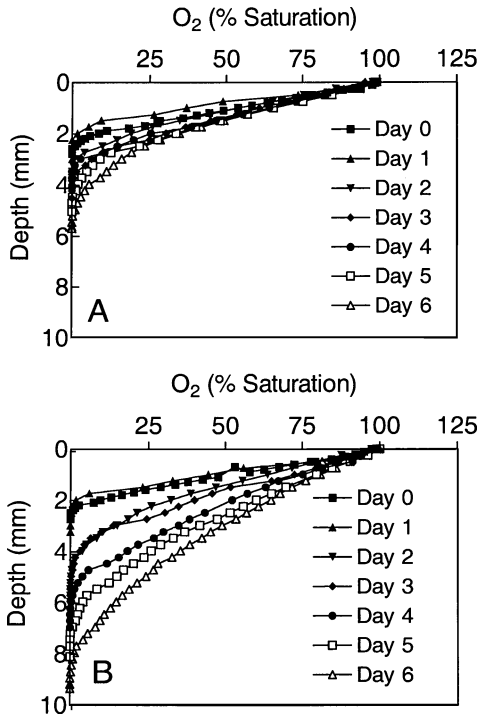

Figure 4. Time course measurements of dissolved O_2 microprofiles in Fe(II)-opposing gradient cultures in the presence (A) and absence (B) of FeOB.

Thin film diffusion probes (see Figure 5) were deployed in an attempt to assess the influence of FeOB on Fe(II) microgradients at the aerobic/anaerobic interface, in a manner analogous to how S^{2-} microelectrodes have been used to quantify the effect of chemolithotrophic S-oxidizing bacteria on dissolved sulfide microgradients (Jorgensen and Revsbech 1983; Nelson et al. 1986). Unfortunately, because of interference by Fe(III) with the densitometric measurements, the diffusion probes did not

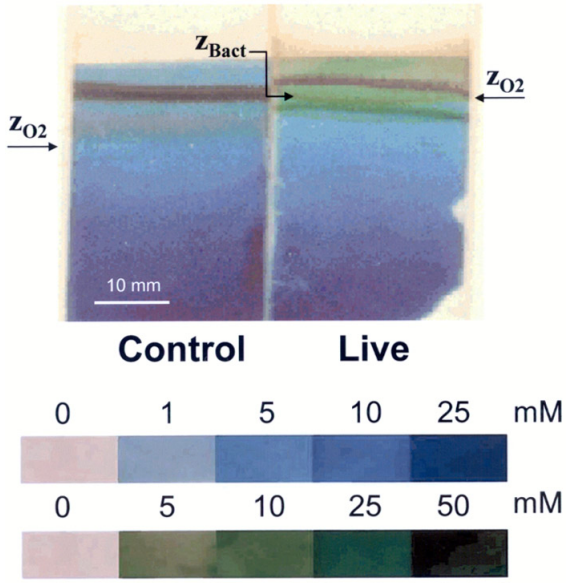

Figure 5. Photos of ferricyanide-fixed thin-film diffusion probes from control (left) and TW2-inoculated (right) opposing gradient cultures shown in Figure 4. Arrows indicate the depth of O_2 penetration (z_{O2}) and the location of the bacterial plate (z_{Bact}). Note that heavy Fe(III) deposits in the probe from the control culture (partially evident as a grayish band gray just above z_{O2}) were obscured by the presence of Fe(II) in the probe. The bottom bars are results from probes submerged overnight in standard Fe(II)-EDTA and $FeCl_3$ solutions. The standards were photographed under different light conditions and are not directly comparable with probes from the cultures. Modified with permission from Sobolev and Roden (2001), copyright 2001 American Society for Microbiology.

provide accurate measurements of dissolved Fe(II) microgradients at the aerobic/anaerobic interface. However, they revealed two previously undescribed effects of FeOB activity on circumneutral Fe(II) oxidation, which are described next.

Quantitative Contribution of FeOB to Fe(II) Oxidation

Time-course studies have shown that bulk rates of Fe(III) oxide deposition are similar in abiotic control and FeOB-inoculated opposing-gradient cultures (Emerson and Moyer 1997; Sobolev and Roden 2004). In contrast, visual analysis of diffusion probes emplaced in TW2 cultures (Figure 5) revealed the presence of much heavier Fe(III) oxide deposits in probes from control cultures compared to those from inoculated ones. In order to verify quantitatively the lower abundance of particulate oxides in probes from the live cultures, the concentration of Fe(III) in probes (determined by scraping the film off the glass slide, followed by extraction in 0.5 M HCl and Ferrozine analysis) as well as samples of whole medium were measured in replicate (n = 3) live and control cultures. When corrected for the presence of soluble/colloidal Fe(III) (see Figure 6B and discussion below), the estimated concentration of particulate Fe(III) in probes from the live cultures was ca. 4-fold lower than in probes from the abiotic controls (Figure 6A). In addition, the concentration of Fe(III) was ca. 3-fold lower in probes compared to whole medium in live cultures, whereas probe and whole medium Fe(III) concentrations were nearly identical in abiotic systems (Figure 6A). These findings confirmed the consistent visual observation of lower Fe(III) oxide abundance in probes from live vs. control cultures.

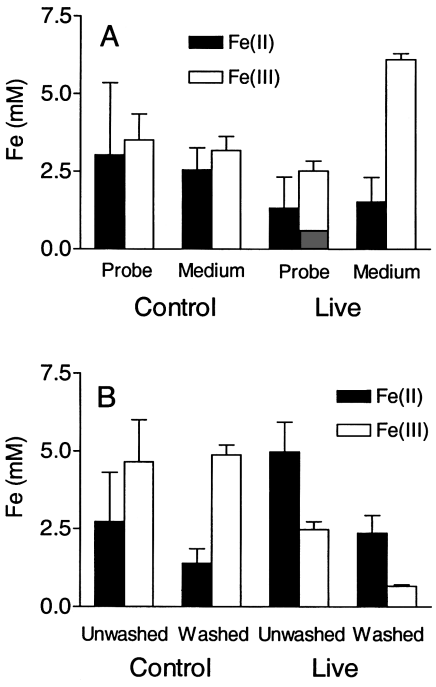

Figure 6. Fe(II) and Fe(III) in whole medium samples and diffusion probes from control and TW2-inoculated opposing-gradient cultures. (A) Comparison among probes and whole medium. The smaller shaded bar superimposed on the Fe(III) bar for live culture probes represents the estimated particulate Fe(III) concentration after correction for the presence of soluble Fe(III). (B) Comparison between washed and unwashed probes (see text). Data represent averages of triplicate cultures; error bars show 95% confidence interval. Reproduced with permission from Sobolev and Roden (2001), copyright 2001 American Society for Microbiology.

The relative scarcity of particulate oxides in diffusion probes from the live cultures suggests that Fe(II) oxidation was dominated by bacterial activity. Because the 5% agar content of the probe would be expected to effectively exclude bacteria, only chemical oxidation is expected to have occurred within the agar film. The low abundance of oxide deposits in probes from the live cultures therefore suggests that FeOB activity in the bulk medium scavenged Fe(II) rapidly enough to strongly depress Fe(II) diffusion into the probe and subsequent abiotic oxidation. Mass balance calculations on the Fe content of probes vs. bulk medium, which accounted for the presence of soluble Fe(III) in probes from live cultures (discussed next), indicated that bacterial activity could account for ca. 90% of Fe(II) oxidation occurring in the live cultures (Sobolev and Roden 2001). This fraction might be higher in steady-state systems, since some Fe(II) oxidation inevitably occurred abiotically before the FeOB plate was established in the live cultures. These results demonstrate that FeOB can compete successfully with abiotic Fe(II) oxidation in diffusion-limited opposing-gradient systems.

Production of Soluble or Colloidal Fe(III) Compounds: Experimental Evidence and Theoretical Considerations

A second important observation from the thin-film diffusion probes was the presence of a green band in ferricyanide-treated probes from the live cultures (Figure 5, top right). The green band was likely due to the presence of soluble or colloidal Fe(III) compounds which reacted with ferricyanide reagent (note that ferricyanide forms a bright blue precipitate with Fe(II); see Sobolev and Roden (2001) for further details on the use of ferricyanide with the diffusion probes). Ferricyanide was shown to form a green precipitate with Fe(III) at neutral pH (Figure 5, standards), and no other components in the media were present in quantities sufficient to produce such a colored precipitate. Since green bands were not evident in the control cultures, the formation of soluble or colloidal Fe(III) can be attributed to FeOB activity. Washing of nonferricyanide-treated probes (by repeated dipping in anaerobic distilled water inside an anaerobic chamber) from the live culture resulted in removal of approximately 70% of their Fe(III) content (Figure 6B, right); in contrast, identical treatment of probes from the abiotic control cultures resulted in no change in Fe(III) content, whereas the amount of Fe(II) was significantly decreased (Figure 6B, left). These results verified the presence of soluble/colloidal Fe(III) in probes from the live cultures, and further indicated that the presence of mobile

Fe(III) is a bacterially mediated phenomenon in these cultures. pH decrease associated with bacterial Fe(II) oxidation at O_2-Fe(II) boundary could potentially account for Fe(III) remaining in solution. However, in our experiments such a decrease was far less than would be required to stabilize any significant amount of Fe(III), as the lowest pH value observed (with a 1-mm diameter pH minielectrode; Orion Instruments) was 6.6 in the zone of oxide deposition, compared to 7.2 at the surface.

It is possible that Fe(III) was kept in the solution in the live cultures by metal-binding ligands excreted by the bacteria specifically for the purpose of retarding cell surface encrustation with oxide precipitates. Although speculative, this suggestion is consistent with the fact that encrustation of the FeOB cells with particulate oxides, leading to their eventual entombment, is likely to be one of the major environmental challenges faced by gradient-dwelling, solid-phase oxide-producing microorganisms (Emerson and Moyer 1997). Formation of soluble or colloidal Fe(III) compounds and eventual remote deposition of the oxides may reduce or delay such encrustation. This suggestion is supported by the observation that certain phototrophic FeOB have been shown to avoid cell encrustation with Fe(III) oxides while accumulating significant amounts of soluble Fe(III); in contrast, self-encrusting organisms did not form significant amounts of soluble Fe(III) (Straub et al. 2001). We hypothesize that, as soluble or colloidal Fe(III) complexes diffuse away from the locus of Fe(II)-oxidation activity, they become destabilized, resulting in precipitation of Fe(III) oxides both within and below the zone of oxygen penetration (Figure 7). This process could account for the suboxic deposition of Fe(III) oxides observed in FeOB-inoculated cultures (Figure 3B).

The validity of the above hypothesis requires that neutrophilic FeOB gain sufficient energy from Fe(II) oxidation to support the synthesis of relatively large quantities of Fe(III)-binding ligands. In order to evaluate this assumption quantitatively, a metabolic energetics analysis was conducted following the approach described in Rittman and McCarty (2001). The analysis involves comparison of the amount of energy required to convert a carbon source to cellular carbon with the amount of energy liberated from energy-generating reactions, taking into account the efficiency of cellular energy transfer. Following Rittman and McCarty (2001), we assume that the carbon source (here dissolved HCO_3^-) is converted to pyruvate as a representative intracellular intermediate. The energy required for this conversion (referred to as ΔG_p, which is equal to the $\Delta G^{\circ\prime}$ of the reaction assuming standard conditions at pH 7) was estimated from the following reaction:

$$0.3HCO_3^- + Fe^{2+} + 2.4H_2O \rightarrow 0.1CH_3COCOO^- + Fe(OH)_3 + 1.8H^+ \quad \Delta G^{\circ\prime} = +5.8\ kJ/e^- \qquad [3]$$

in which the electrons required for carbon fixation are obtained from oxidation of Fe(II) to $Fe(OH)_3$ (amorphous Fe(III) oxyhydroxide). The free energies of formation required for this and other energetics calculations were obtained from Stumm and Morgan (1996) and Thauer, Jungermann, and Decker (1977). An energy requirement of $+18.8$ kJ/e$^-$ was assumed for conversion of pyruvate to cellular carbon (ΔG_{pc}), based on a value of 3.33 kJ per gram cells with NH_4^+ as the nitrogen source, and a standard cellular composition of $C_5H_7O_2N$ (McCarty 1971). This stoichiometry corresponds to 5.65 g cells per electron equivalent assuming the following biosynthesis reaction:

$$0.05NH_4^+ + 0.25HCO_3^- + 1.2H^+ + e^- \rightarrow 0.05C_5H_7O_2N + 0.65H_2O \qquad [4]$$

The total energy required for cellular biosynthesis (ΔG_s) is then obtained from the equation

$$\Delta G_s = \Delta G_p/\varepsilon + \Delta G_{pc}/\varepsilon \qquad [5]$$

where ε is the energy transfer efficiency. A standard ε value of 0.6 (Rittmann and McCarty 2001) was used in the calculations presented here, which produces a value of $+41.0$ kJ/e$^-$ for ΔG_s. Next, the overall amount of energy available for cellular metabolism through Fe(II) oxidation (ΔG_r, equal to $\Delta G^{\circ\prime}$ assuming standard conditions at pH 7) was estimated from the reaction

$$Fe^{2+} + 0.25O_2(aq) + 2.5H_2O \rightarrow Fe(OH)_3(s) + 2H^+ \quad \Delta G^{\circ\prime} = -111.3\ kJ/e^- \qquad [6]$$

Assuming that the transfer efficiency for this reaction is the same as for biosynthesis reactions, the actual amount of energy available is $\varepsilon\Delta G_r = -66.8$ kJ/e$^-$. Finally, an energy balance is defined in which oxidation of A equivalents of electron donor are required to supply energy for cell synthesis:

$$A\varepsilon\Delta G_r + \Delta G_s = 0 \qquad [7]$$

According to equation 7, the fraction of total electron equivalents devoted to cell synthesis (f_s^0 in Rittmann and McCarty 2001) is $1/(1 + A)$, which is equal to 0.613 using the ΔG_p,

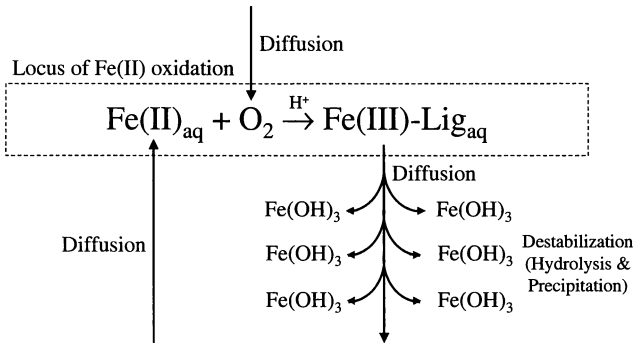

Figure 7. Conceptual model for suboxic Fe(III) oxide deposition coupled to production of soluble Fe(III)-ligand complexes by FeOB.

ΔG_{pc}, ΔG_r, and ε values given before. With this value for f_s^0, the stoichiometry in equation 4, and the fact that one electron equivalent is liberated per mol of Fe(II) oxidized, the estimated the biomass yield (not accounting for maintenance energy or endogenous decay) for FeOB growth is

$$0.613 \times 0.25 \text{ mol cell-C/e}^- \text{equiv} \times 1\text{e}^- \text{equiv/mol Fe(II)} = 0.153 \text{ mol cell-C/mol Fe(II)}.$$

This yield is several-fold in excess of the observed net growth yield for TW2 in opposing gradient cultures (Y_{Net}, equal to ca. 0.02 mol cell-C/mol Fe(II); Sobolev and Roden 2004). These results suggest that a substantial amount of energy is available for synthesis of other cellular components, e.g., Fe(III)-binding ligands, by neutrophilic FeOB. They also clearly illustrate the major difference in potential growth yield for neutrophilic vs. acidophilic FeOB: analogous calculations in which Fe(II) was assumed oxidize to soluble Fe^{3+} at pH 2 produced growth yield estimates on the order of 0.03 mol cell-C/mol Fe(II), in line with both early (e.g., Silverman and Lundgren 1959) and more recent (e.g., Harvey and Crundwell 1997) published values for *Thiobacillus ferrooxidans*.

In order to evaluate the potential significance of nonstandard conditions on the amount of energy that may be available for synthesis of Fe(III)-binding ligands, a range of dissolved O_2 and Fe(II) concentrations in the zone of Fe(II) oxidation (10–100 μM and 100–1000 μM, respectively) was estimated based on the microelectrode (Figure 3) and diffusion probe (Figure 5) measurements. Although the estimated range of concentrations is only a rough approximation, the calculations indicated (consistent with the results shown in Figure 2) that the energetics of circumneutral Fe(II) oxidation are not highly sensitive to dissolved O_2 and Fe(II) concentrations. Revised growth yields of 0.137–0.139 mol cell-C/mol Fe(II) were obtained by pairing the lowest assumed dissolved O_2 concentration (10 μM) with the highest dissolved Fe(II) concentration (1000 μM) and vice-versa. If we assume a maximum total yield (Y_{Total}) of 0.138 mol cell-C/mol Fe(II), then the potential yield for carbon allocation to ligand biosynthesis (Y_{Ligand}) can be estimated as follows:

$$Y_{Ligand} = Y_{Total} - Y_{Net} - bX_t/R_t \quad [8]$$

where Y_{Net} is the observed growth yield, b is a coefficient accounting for endogenous cellular decay, and X_t and R_t are the biomass (mol C/L) and rate of Fe(II) oxidation (mol/L/d) at time t. The last term on the right-hand side of equation 8 accounts for the influence endogenous decay on growth yield (Rittmann and McCarty 2001). A value of 0.02 mol cell-C/mol Fe(II) was assumed for Y_{Net} based on measured TW2 growth yields (Sobolev and Roden 2004). A value of 0.05 d^{-1} was assumed for b, which is appropriate for relatively slow-growing microorganisms (Rittmann and McCarty 2001). X_t was estimated at ca. 1×10^{-4} mol C/L based on maximum cell densities obtained in opposing gradient cultures (Sobolev and Roden 2004), assuming that all the cells in the cultures were contained in a ca. 2 mm thick band in the culture tubes. R_t was estimated at ca. 1×10^{-3} mol Fe(II)/L/d, using the pH-dependent second-order rate equation given in Singer and Stumm (1972), assuming a pH of 7 and the range of dissolved O_2 and Fe(II) concentrations mentioned previously. Note that use of this equation, which was originally developed to predict pH-dependent rates of abiotic Fe(II) oxidation, to estimate rates of biotic Fe(II) oxidation implicitly assumes that biotic oxidation was limited by O_2 and Fe(II) abundance, and that all Fe(II) oxidation was biologically catalyzed. Both of these assumptions are consistent with our studies of strain TW2 in opposing gradient cultures (Sobolev and Roden 2001; Sobolev and Roden 2004). Plugging the estimated Y_{Total}, Y_{net}, b, X_t, and R_t values into equation 8 yielded a Y_{Ligand} value of 0.113 mol ligand-C/mol Fe(II). Multiplying this value by the estimated rate of Fe(II) oxidation in turn yielded a value of ca. 1×10^{-4} mol ligand-C L^{-1} d^{-1} for the rate of ligand biosynthesis. At this rate of ligand biosynthesis, ligand concentrations on the order of 1 mmol C L^{-1} could be expected to accumulate in the vicinity of the growth band after 1–2 week reaction period.

Although the capacity for biogenic ligands to complex or otherwise stabilize Fe(III) (e.g., through the formation of polynuclear species with nm-scale hydrodynamic radii; vonGunten and Schneider 1991; Taillefert et al. 2000) is unknown, the energetic and biosynthesis calculations clearly suggest that accumulation of significant quantities of extracellular ligands is possible in conjunction with FeOB activity in diffusion-controlled opposing-gradient systems. The formation of such compounds has important implications for the coupling of Fe oxidation and reduction at redox interface environments. Such compounds, as well as the reactive Fe(III) oxides generated upon their destabilization, would be readily available for dissimilatory Fe(III) reduction just below the depth of O_2 penetration. This phenomenon represents unique mechanism whereby FeOB activity may alter the Fe(II)-O_2 reaction system so as to generate conditions favorable for rapid microscale Fe redox cycling (see below). This process would reduce the diffusion distance (travel time) between sites of Fe(II) consumption and regeneration, and alleviate the possible negative influence of Fe(II) sorption and/or precipitation reactions on delivery of Fe(II) to FeOB.

EXPERIMENTAL STUDIES OF COUPLED BACTERIAL Fe(II) OXIDATION AND Fe(III) REDUCTION

A preliminary investigation of the potential for coupled Fe(II) oxidation and Fe(III) reduction was conducted in experimental microcosms in the presence and absence of strain TW2 (Sobolev and Roden 2002). Based on the ability of TW2 to cause unique alterations in patterns of Fe(III) oxide deposition in opposing gradients of Fe(II) and O_2, we hypothesized that this organism would lead to enhanced coupling of Fe redox cycling at the aerobic-anaerobic interface of the microcosms. The design and results of these experiments, supported by previously unpublished voltammetric microelectrode measurements of dissolved

O_2 and Fe(II) distributions in the microcosms, are summarized next. The findings are subsequently interpreted in relation to a conceptual model of bacterially catalyzed Fe redox cycling at the aerobic-anaerobic interface.

Coculture Systems

Strain TW2 was grown in opposing-gradient cultures as described previously (Sobolev and Roden 2001), and the oxide-rich band containing bacterial cells was collected from a 7–14 d culture and used as the inoculum for the experiments described next. *Shewanella algae* strain BrY, a facultative FeRB (Caccavo, Blakemore, and Lovley 1992), was grown for 16 hr in tryptic soy broth (TSB), harvested by centrifugation, washed (3X) with Pipes buffer (10 mM, pH 7), and resuspended in anaerobic Pipes buffer supplemented with Na-formate (10 mM) and vitamins and minerals (Lovley and Phillips 1986). This cell suspension was used as the inoculum for the coculture systems. Although strain TW2 is capable of aerobic heterotrophic growth with acetate as a carbon and energy source, it is not able to utilize formate (Sobolev and Roden 2004). Hence, heterotrophic growth and O_2 consumption by strain TW2 did not occur in the coculture systems.

Amorphous Fe(III) oxide-coated sand was prepared by mixing 75 mL of a suspension of hydrous ferric oxide gel (ca. 500 mmol Fe(III) L^{-1}, prepared by neutralization of $FeCl_3$ as described in Lovley and Phillips 1986), with 300 g of quartz sand, −50 +70 mesh (Sigma Chemicals). The mixture was shell-frozen in an alcohol bath and freeze-dried. The resulting oxide-coated sand had a 0.5M HCl-extractable Fe(III) content of ca. 125 μmol g^{-1}.

Bacterial Fe cycling microcosms were prepared be adding 40 g of the oxide-coated sand to 50 mL beakers (50 mm tall, 35 mm ID), to which 15 mL of the *S. algae* cell suspension was added, resulting in ca. 1 mm of liquid over the sand surface. Rossi-Cholodny buried slides for visualization/enumeration of attached bacterial were then inserted. The cultures were incubated in an anaerobic chamber (2–3% H_2, balance N_2) for 7 days, after which they were removed from the chamber and inoculated with the TW2 by evenly distributing the cell/oxide mixture over the sand-liquid interface. A set of cultures not inoculated with FeOB served as control. In addition, a set of cultures was killed with 1 mM Na-azide (final concentration) to provide abiotic controls. The cultures were covered loosely with aluminum foil and allowed to sit open to the air on the laboratory bench.

After 2–3 d of incubation, the presence of Fe(III) oxide deposits on the surface of the microcosms was assessed visually and photographed. Oxygen microprofiles were recorded with a Clark-style oxygen microelectrode as described previously (Sobolev and Roden 2001). In addition, microprofiles of dissolved O_2 and Fe(II) were obtained simultaneously using a gold amalgam voltammetric microelectrode (Brendel and Luther 1995). A detailed description of the methodology employed in these measurements is provided in Brendel and Luther (1995) and Luther et al. (1999). Rossi-Cholodny slides were retrieved from the cultures and hybridized with a *Shewanella*-specific 16S rRNA probe under conditions described previously (DiChristina and Delong 1993). The *Shewanella* probe was labeled with the fluorochrome Oregon Green (Molecular Probes, Inc.). The slides were then counter-stained with DAPI and visualized under an epifluorescent microscope with UV/visible light illumination.

EXPERIMENTAL RESULTS

Reduction of amorphous Fe(III) oxide by *S. algae* caused a blackening of the oxide-coated sand (see Figure 8), presumably due to production of magnetite or other mixed Fe(II)-Fe(III) solid-phases (Fredrickson et al. 1998). Visual inspection of microcosms 2–3 d after inoculation with TW2 indicated a paucity of surface Fe(III) oxide deposits in the cocultures (Figure 8A, left). These results suggest that rates of Fe(II) oxidation and Fe(III) reduction were approximately in balance within the upper few mm of the coculture systems, such that very little Fe(III) oxide deposition took place. In contrast, controls lacking FeOB (Figure 8A, right) or killed with azide (not shown) accumulated an approximately 2 mm-thick oxide layer at the surface. Similar results were observed in triplicate cultures in this and several other experiments. No obvious difference in O_2 penetration or patterns of oxide deposition were detected between the cultures lacking FeOB and the azide-killed controls, which suggests that O_2 scavenging due to aerobic respiration of *S. algae* did not exert a significant influence on Fe(III) oxide deposition and inferred Fe cycling in the cocultures (Sobolev and Roden 2002). Voltammetric microelectrode measurements revealed much lower concentrations of dissolved Fe(II) in cocultures compared to those containing *S. algae* only (Figure 9). Fluorescence in situ hybridization (FISH) analysis of buried slides from the coculture microcosms indicated that FeOB and FeRB were found in close proximity to one another within the aerobic zone (Figure 8B), frequently in tight clusters with *S. algae* generally concentrated towards the center of the clusters and TW2 on the periphery. These findings suggest intimate involvement of FeOB in Fe recycling processes within the microcosms.

Conceptual Model for Microscale Bacterial Fe Redox Cycling

A conceptual model is provided in Figure 10 to explain the differences in surface Fe(III) oxide accumulation in Fe(III)-reducing microcosms with and without FeOB activity. Close juxtapositioning of FeOB and FeRB and rapid microscale Fe redox cycling within the Fe(II)-O_2 reaction zone in the cocultures is hypothesized to maintain the majority of the Fe in the reduced state at the sand-water interface, despite the presence of detectable O_2 in the bulk aqueous phase. The observed clumping of FeOB and FeRB around Fe(III) oxide aggregates suggests that bacterial Fe(II) oxidation and Fe(III) reduction activities are likely to have coexisted within the same horizon, with the FeRB taking advantage of anaerobic microzones within the aggregates.

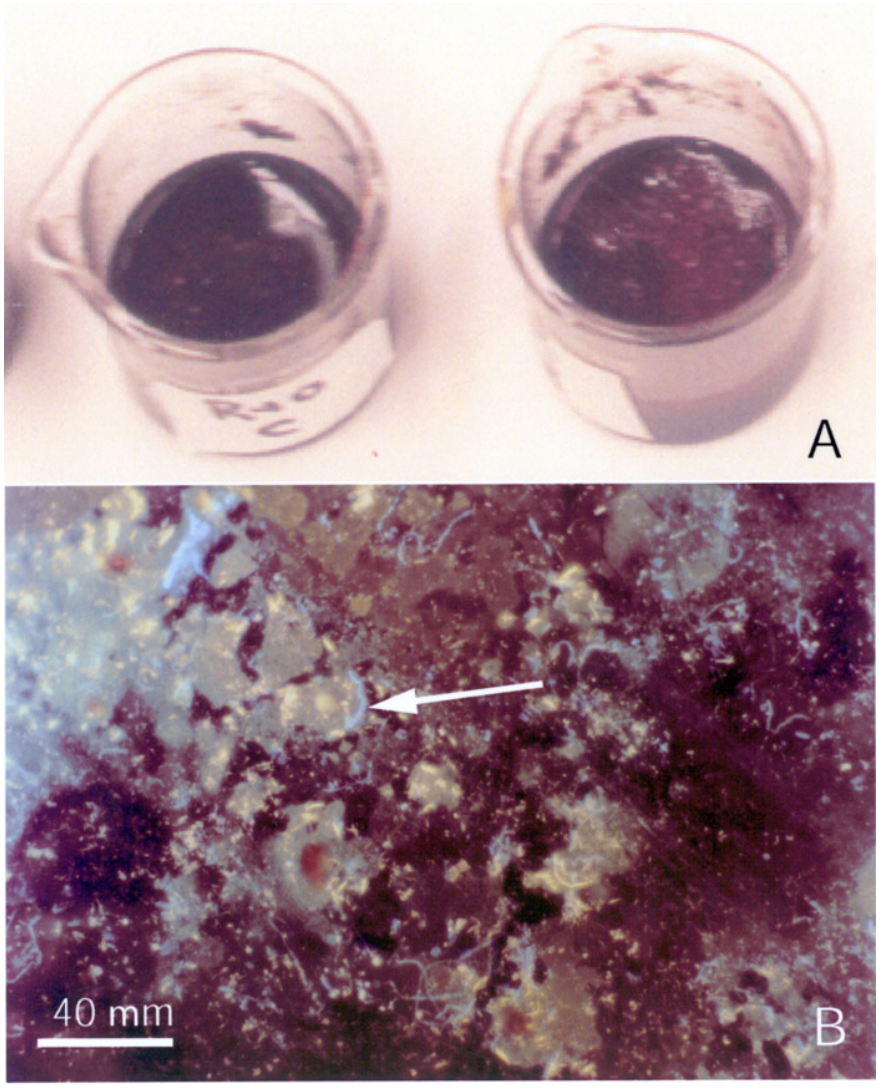

Figure 8. (A) Photo of bacterial Fe cycling microcosms containing both *S. algae* and TW2 (left) or *S. algae* only (right). Note paucity of Fe(III) oxide surface precipitates in the coculture system. (B) FISH image of buried slide (ca. 1.5 mm depth) from a coculture system hybridized with a *Shewanella*-specific 16S rRNA probe and counter-stained with DAPI. *S. algae* cells appear yellow, TW2 cells appear blue. Arrow indicates TW2 cells surrounding an aggregate of amorphous Fe(III) oxide. Reproduced with permission from Sobolev and Roden (2002), copyright 2002 Kluwer Academic Publishers.

These microzones are postulated to be generated by O_2 scavenging via Fe(II) oxidation by FeOB on the periphery of the aggregates, leading to the development of "ultramicrogradients" of O_2 at the surfaces of aggregates, with O_2 declining from the bulk aqueous phase concentration to essentially zero at some distance from the surface (Figure 10A, lower left). The lower concentration of dissolved Fe(II) in microcosms inoculated with TW2 compared to those containing *S. algae* only (Figure 9) provides evidence for enzymatically enhanced Fe(II)/O_2 scavenging. As a result of these interactions, anaerobic conditions would be maintained at the aggregate surface, allowing Fe(III) reduction to proceed within a bulk aerobic environment. Production and inward flux of biogenic ligand bound-Fe(III) would presumably facilitate rapid Fe cycling at the aggregate surface. With Fe trapped in a cycle between FeOB and FeRB, little or no net oxide deposition would occur. In the absence of FeOB activity, a lower degree of O_2 scavenging would allow O_2 to contact aggregate surfaces (Figure 10B, lower left), where Fe(III) reduction would thus be inhibited, leading to the accumulation of oxide precipitates.

The above conceptual model, though speculative, is consistent with existing models of the potential for anaerobic bacterial respiration within anoxic microniches in otherwise oxidized aquatic sediments (Jorgensen 1977; Jahnke 1985). It is also consistent with the recent demonstration of syntrophic growth of sulfate-reducing and colorless sulfide-oxidizing bacteria

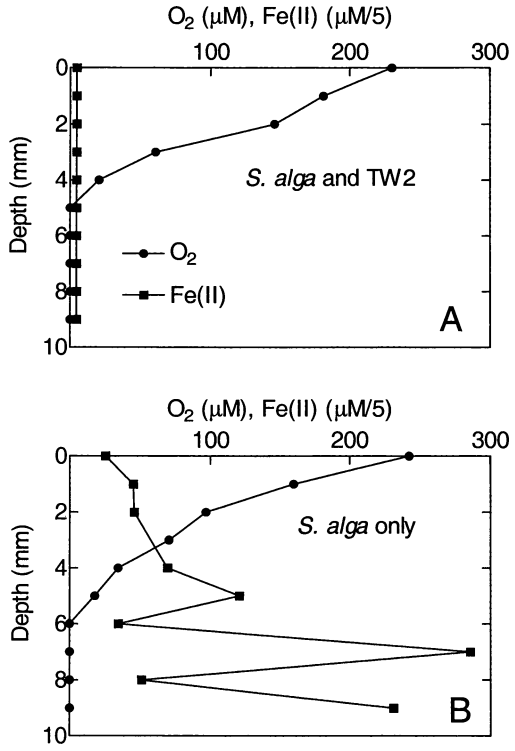

Figure 9. Voltammetric microelectrode profiles of dissolved O_2 and Fe(II) in Fe cycling microcosms containing both *S. algae* and TW2 (A) or *S. algae* only (B). Data are averages of triplicate profiles.

coupled to S redox cycling in O_2-limited reaction systems (vandenEnde, Meier, and vanGermerden 1997), in which O_2 scavenging by the sulfide-oxidizer (*Thiobacillus thioparus*) allowed the sulfate-reducer (*Desulfovibrio desulfuricans*) to remain active (mainly through reduction of highly reactive elemental S produced during sulfide oxidation) despite the continuous input of dissolved O_2. More detailed studies of the distribution of FeOB and FeRB within Fe cycling aggregates, as well as more rigorous quantification of dissolved and solid-phase Fe(II) and Fe(III) pools at the redox interface, are required to verify this model for the behavior of our coculture systems. Development and application of such techniques may also shed light on the potential for bacterially catalyzed Fe redox cycling in natural sedimentary environments. Preliminary studies in TW surface sediments indicate the presence of comparable numbers of FeOB and FeRB, as well as the presence of soluble Fe(III) at the aerobic-anaerobic interface (Sobolev and Roden 2002). The latter finding is consistent with other recent studies (employing voltammetric electrodes as well as fine scale sectioning techniques) which have demonstrated the presence of soluble Fe(III) complexes in aquatic surface sediments (Huettel et al. 1998; Ratering and Schnell 2000; Taillefert et al. 2000; Nevin and Lovley 2002). Our findings suggest that FeOB activity could be partly responsible for the generation of such compounds, whose presence could signal the occurrence of rapid microscale bacterial Fe redox cycling phenomena.

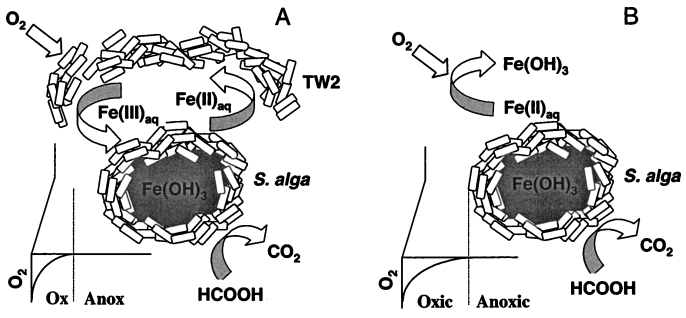

Figure 10. Conceptual model of Fe redox cycling in Fe(III)-reducing microcosms with (A) and without (B) strain TW2. $Fe(OH)_3$ refers to an aggregate of amorphous Fe(III) oxide. Reproduced with permission from Sobolev and Roden (2002), copyright 2002 Kluwer Academic Publishers.

CONCLUSIONS

Recent studies indicate that although chemolithotrophic FeOB do not accelerate the oxidation of Fe(II) at circumneutral pH in a manner analogous to how S-oxidizing bacteria such as *Beggiatoa* accelerate dissolved sulfide oxidation at the aerobic-anaerobic interface (Jorgensen and Revsbech 1983; Nelson et al. 1986), they can nevertheless dominate Fe(II) oxidation in diffusion-limited opposing gradient systems. Since opposing gradients of O_2 and Fe(II) are ubiquitous in nonsulfidogenic sedimentary environments (including the rhizosphere of aquatic plants, where a dynamic, microbially catalyzed Fe redox cycle is likely to exist; (Roden and Wetzel 1996; Emerson et al. 1999; Frenzel, Bosse, and Janssen 1999; Weiss et al. 2003), bacterial catalysis may be a widespread mechanism for Fe(II) oxidation in nature. In addition, by altering the spatial locus of Fe(III) oxide deposition in the redox transition zone (e.g. through production of Fe(III)-binding ligands), FeOB appear to have the potential to induce rapid microscale coupling of Fe oxidation and reduction at aerobic-anaerobic interfaces. Further studies of the distribution and activity of FeOB in relation to their in situ chemical microenvironment will be required in order fully understand the biogeochemical cycling of Fe in redox-stratified systems, and to predict the influence of Fe redox cycling on other biogeochemical processes in natural environments.

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