THE EFFECTS OF ACID-TOLERANT SULFATE REDUCING BACTERIAL ACTIVITIES ON IRON SPECIATION[1]

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Abstract Fe^{2+} may be removed from acid mine drainage (AMD) by the activities of Fe(II) oxidizing bacteria that mediate the oxidative precipitation of Fe(III) (hydr)oxide phases, but the stability of these phases under anoxic conditions is unclear. Activities of Fe(III) and/or sulfate-reducing bacteria may lead to 1) reductive re-solubilization of iron and 2) accumulation of sulfide phases. We examined how the activities of a sulfate- and Fe(III) reducing bacterium, *Desulfosporosinus* sp. GBSRB4.2 would affect the speciation of iron under anoxic conditions at Fe(II):Fe(III) concentrations (mM) of 40:0, 30:10, 10:30, and 0:40. In incubations containing Fe(III), it was provided as Fe(III) (hydr)oxide. The presence of Fe(III) had little impact on sulfide production, suggesting that GBSRB4.2 did not preferentially reduce Fe(III) over sulfate. Acid-extractable (0.5 M HCl) Fe(II) decreased in incubations containing 40 mM Fe(II):0 mM Fe(III). Similarly, in incubations containing 0 mM Fe(II):40 mM Fe(III), after an initial increase in Fe(II) concentration due to Fe(III) reduction, acid-extractable Fe(II) concentrations decreased in the incubations. These data suggest the maturation of biogenic iron sulfide mineral phases to more stable forms as the incubations proceeded.

Additional Key Words: sulfate reducing bacteria; acid mine drainage; iron reduction

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Introduction

The microbiologically mediated oxidation of ferrous iron (Fe^{2+}) may be exploited for low-cost, sustainable removal of dissolved Fe from acid mine drainage (AMD). Under such a scenario, Fe(II) oxidizing bacteria (FeOB) catalyze the oxidation of Fe^{2+} to Fe^{3+} coupled with O_2 reduction (Equation 1) (Senko *et al*., 2008). In the pH range often observed in Appalachian coalmine-derived AMD (3.0-4.5) (Cravotta *et al.,* 1999), the resulting Fe^{3+} will rapidly hydrolyze and precipitate from solution (Equation 2) (Stumm and Morgan, 1996).

$$4\ Fe^{2+} + O_2 + 4\ H^+ \rightarrow 4\ Fe^{3+} + 2\ H_2O \quad (1)$$

$$Fe^{3+} + 3\ H_2O \leftrightarrow Fe(OH)_3 + 3\ H^+ \quad (2)$$

We have designated this process "oxidative precipitation of iron," and we have observed such activities in a variety of AMD-impacted systems in Pennsylvania and Ohio where AMD flows as a sheet over the terrestrial surface. In these systems, the sustained oxidative precipitation of iron has lead to the accumulation of Fe(III) (hydr)oxide crusts that may be tens to a hundred cm in depth (Senko *et al.,* 2008). We have estimated that some crusts may accumulate at rates of 0.5 – 0.7 cm yr^{-1}, and are composed almost exclusively of Fe(III) (hydr)oxides (Senko *et al.,* 2008). Oxygen gradients may develop within the crusts with depth (Tarutis *et al.,* 1992). Low O_2 availability may limit the efficiency of FeOB and stimulate the activities of anaerobic microorganisms. Indeed, much of our knowledge of microbial processes associated with these sheet-flow systems is based on investigations focusing on the upper 1-2 cm of the Fe(III) (hydr)oxide crusts (Senko et al., 2008), but our knowledge of the distribution and activities of anaerobic microorganisms in such systems is limited.

The most abundant terminal electron acceptors for anaerobic respiration in AMD-impacted systems are Fe^{3+} and SO_4^{2-}. Ferric iron- and sulfate-reducing bacteria (FeRB and SRB, respectively) may couple the oxidation of organic carbon (or H_2) to the reduction of Fe(III) (hydr)oxides (Equation 3) or sulfate (Equation 4) (Senko *et al.,* 2009).

$$CH_2O + 4\ Fe(OH)_3 + 8H^+ \rightarrow CO_2 + 4\ Fe^{2+} + 11\ H_2O \quad (3)$$

$$2\ CH_2O + SO_4^{2-} + 2\ H^+ \rightarrow 2\ CO_2 + H_2S_{(aq)} + 2\ H_2O \quad (4)$$

Under sulfate-reducing conditions, Fe(III) (hydr)oxides may also be reduced by biogenic sulfide (Equation 5) and Fe^{2+} ions may react with sulfide to form insoluble iron-sulfide phases that we depict broadly (Equation 6) (Neal *et al*., 2001).

$$2\ Fe(OH)_3 + H_2S_{(aq)} + 4\ H^+ \rightarrow 2\ Fe^{2+} + S^0 + 6\ H_2O \quad (5)$$

$$Fe^{2+} + H_2S_{(aq)} \rightarrow FeS + 2\ H^+ \quad (6)$$

While SO_4^{2-} and Fe^{3+} iron reduction may lead to an increase in the pH of acidic fluids (Equations 3-5), these processes are undesirable in the context of systems designed to exploit the oxidative precipitation of iron for two reasons. First, it may lead to the reductive resolubilization of Fe^{2+}. Second, it may lead to the concentration of iron sulfide phases near the terrestrial surface that, should O_2 be introduced into the sediments, could be reoxidized and lead to the formation of fluids that are more acidic than the originally treated AMD (Johnson and Hallberg, 2002, Johnson and Hallberg, 2005a, Johnson and Hallberg, 2005b).

While evidence of SO_4^{2-} reduction under acidic conditions has been reported (e.g. Herlihy and Mills, 1985), such activity is unpredictable (Gould and Kapoor, 2003, Walton-Day, 2003), and few acidophilic or acid tolerant SRB have been recovered in pure culture (Küsel *et al.*, 2001, Church *et al.*, 2007). We have isolated an acid tolerant SRB from an AMD-impacted system in McKean County, PA. This organism is affiliated with the genus *Desulfosporosinus* and has the strain designation GBSRB4.2. It is capable of growth in media with an initial pH as low as 4.0, and is able to enzymatically reduce Fe(III) and Mn(IV) phases (Senko *et al.*, 2009). To better understand anaerobic microbial processes associated with AMD-derived Fe(III)-rich crusts, we examined the dynamics and geochemical consequences of Fe and S redox cycling under anaerobic conditions by *Desulfosporosinus* sp. GBSRB4.2.

Materials and Methods

Growth Conditions and Media

Desuolfosporosinus sp. GBSRB4.2 was routinely grown anaerobically at room temperature (approximately 25°C) in a medium described by Senko et al. (2009) (Table 1) in serum tubes or serum bottles that were sealed with butyl rubber stoppers. Additional sulfate was provided as $FeSO_4$ (40 mM). For experiments to assess the effect of iron oxidation state on coupled iron and sulfur redox reactions, additional sulfate was provided as either $FeSO_4$ and/or $Fe_2(SO_4)_3$, and was provided at Fe(II):Fe(III) ratios of 1:0, 3:1, 1:3, and 0:1 at concentrations shown in Table 2. Upon addition of $Fe_2(SO_4)_3$, an orange-brown Fe(III) (hydr)oxide precipitate formed. Fe(II)- and/or Fe(III)-containing media were inoculated with *Desuolfosporosinus* sp. GBSRB4.2 that was in late log/early stationary phase of growth. Uninoculated media containing various ratios

of Fe(II):Fe(III) served as controls. Three inoculated cultures and one uninoculated control were used for these experiments, except for the inoculated incubation containing 20 mM $Fe_2(SO_4)_3$, in which no replicates were included.

Table 1. Components of *Desulfosporosinus* sp. GBSRB4.2 media.

Solute	Concentration
$(NH_4)_2SO_4$	10 mM
$MgSO_4$	2 mM
Glucose	5 mM
Trypticase soy broth (TSB)	0.5 g l^{-1}
Fe(II) or Fe(III) sulfate	shown in Table 2
pH of complete media	4.2
Vitamins and trace metals were provided to the media as described by Tanner (1997)	

Table 2. Iron sulfate additions to *Desulfosporosinus* sp. GBSRB4.2 media

Sample Number	$FeSO_4$ (mM)	$Fe_2(SO_4)_3$ (mM)
1 Fe(II):0 Fe(III)	40	0
3 Fe(II):1 Fe(III)	30	5
1 Fe(II):3 Fe(III)	10	15
0 Fe(II):1 Fe(III)	0	20

Sampling and Analytical Techniques

Daily sampling of *Desuolfosporosinus* sp. GBSRB4.2 cultures was conducted by using a needle and syringe to remove portions of the media from serum bottles in an anoxic glovebag containing approximately 98% N_2 and 2% H_2. Fe(II) was extracted using 0.5 M HCl, and solids were removed by centrifugation in the anoxic glovebag. Fe(II) was subsequently quantified by ferrozine assay (Lovley and Phillips, 1987). Sulfide was preserved in the anoxic glovebag by placing samples in 10% zinc acetate (anoxic), and was subsequently quantified by methylene blue assay (Cline, 1969). pH was measured in samples using a meter outside of the glovebag immediately after removal from the anoxic environment. At the conclusion of the experiments, Fe phases, including 0.5 M HCl-extractable Fe(II) ("total" Fe(II)) and 0.5 M hydroxylamine-

HCl-extractable Fe(III) (Fe(III) (hydr)oxide phases) were quantified as described by Lovley and Phillips (1987).

Results and Discussion

To determine the effects of sulfate-reducing bacterial activities on the oxidation state and mineralogy of different iron-containing phases, we cultured *Desulfosporosinus* sp. GBSRB4.2 in media with Fe^{2+} and Fe(III) (hydr)oxide phases at a variety of ratios. After an initial lag, sulfide was produced in cultures regardless of the Fe(II):Fe(III) ratio of the amendments, and sulfide concentrations reached similar levels in all incubations (Fig. 1 I-L). No sulfide production was observed in uninoculated controls (Fig. 1 I-L). These results suggest that the presence of Fe(III) has little effect on sulfate reduction by GBSRB4.2, despite the ability of GBSRB4.2 to reduce Fe(III) (hydr)oxides enzymatically, and the slightly greater energetic favorability of Fe(III)

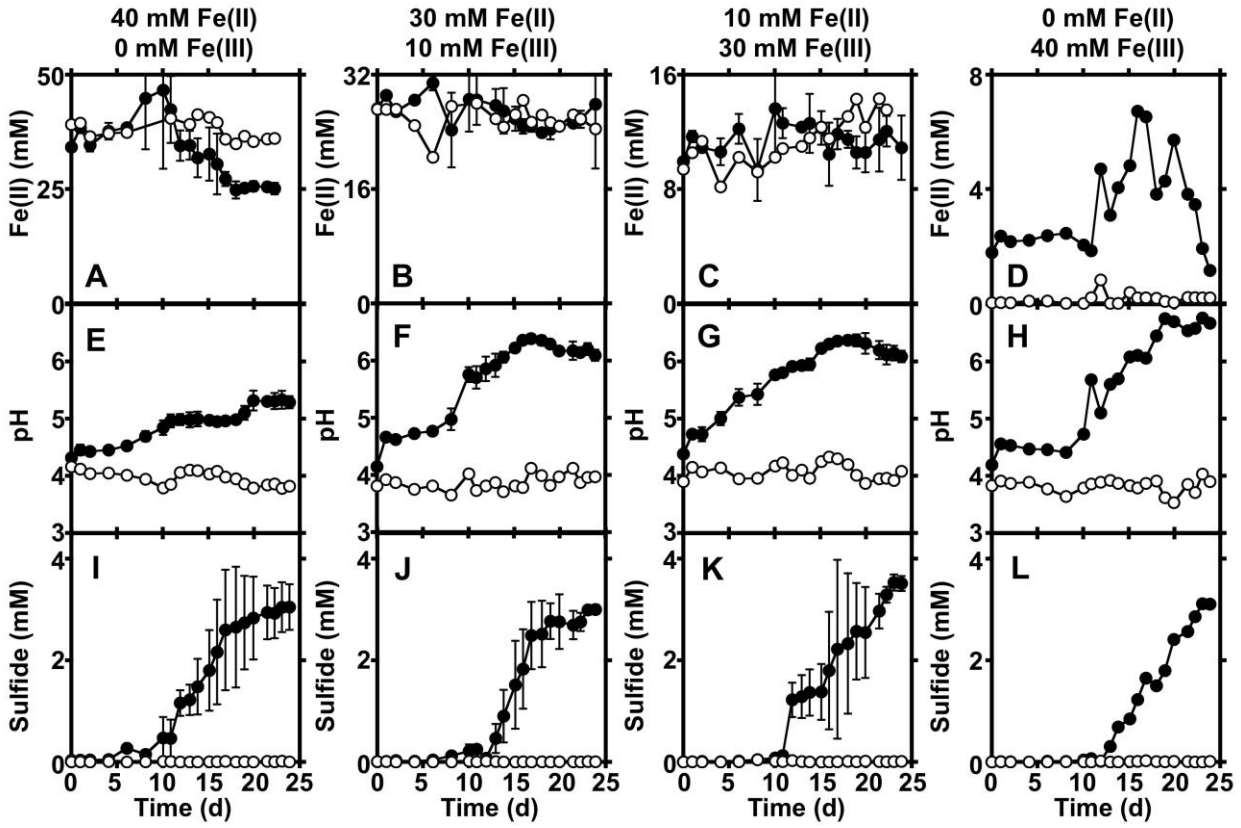

Figure 1. Fe(II) concentrations (0.5 M HCl-extractable Fe(II); panels A-D), pH (panels E-H), and sulfide concentrations (I-L) in *Desulfosporosinus* sp. GBSRB4.2 cultures (●) and uninoculated control incubations (○) containing Fe(II) and Fe(III) at various ratios. Error bars represent one standard deviation of triplicate inoculated cultures. Values shown in panels D, H, and L represent Fe(II) concentration, pH, and sulfide concentration of a single bacterial culture.

reduction in comparison to sulfate reduction (ΔG^{o}_{R} = -75 kJ.mol^{-1} and -47 kJ mol^{-1}, respectively; values calculated from Dean, 1985). These results are similar to those reported by Senko et al. (2009), in which the presence of U(VI) and Mn(III/IV) (hydr)oxides inhibited sulfate reduction by GBSRB4.2, but Fe^{3+} iron did not.

In cultures amended with 40 mM ferrous iron, pH increased to approximately 5.5, which is consistent with previous work (Senko *et al.*, 2009). The increase in pH of these cultures is attributable to the conversion of strongly acidic sulfate ($H_2SO_4 \leftrightarrow HSO_4^-$ pK_a = -3.0, $HSO_4^- \leftrightarrow SO_4^{2-}$ pK_a = 1.99) to weakly acidic sulfide ($H_2S \leftrightarrow HS^-$ pK_a = 6.9, $HS^- \leftrightarrow S^{2-}$ pK_a = 14) (Equation 4), the increased alkalinity associated with oxidation of organic carbon to CO_2 (Equation 4) (Senko et al., 2009). In cultures amended with Fe(III) (10 mM, 30 mM, and 40 mM), pH increased to approximately 6.5, suggesting that pH modulation in the cultures was attributable to both sulfate and ferric iron reduction (Equations 3, 4, and 5).

Despite evidence of Fe^{3+} iron reduction based on pH measurements, we did not observe increase in Fe(II) concentrations in incubations amended with 30 mM Fe(II):10 mM Fe(III) or 10 mM Fe(II):30 mM Fe(III), relative to uninoculated controls (Fig. 1 B and C). Indeed, an increase in Fe(II) concentration was only observed in cultures amended with 40 mM Fe(III) (and no additional Fe(II)) (Fig. 1 D). With continued sulfidogenesis in cultures amended with 40 mM Fe(III), Fe(II) concentrations decreased (Fig. 1 D), as did Fe(II) concentrations in cultures containing 40 mM Fe(II) (and no Fe(III)) (Fig. 1 A). Extraction of Fe^{3+} iron using hydroxylamine-HCl at the conclusion of the incubations suggested that Fe(III) (hydr)oxides in cultures amended with Fe^{3+} iron were reduced, compared to uninoculated controls (Fig. 2). However, the Fe^{2+} iron that we would expect to result from Fe^{3+} iron reduction could not be extracted from the solid phases with 0.5 M HCl, leaving a pool of iron unaccounted for (Fig. 2). These results and the observation that all GBSRB4.2-containing incubations turned black suggest that iron-sulfide phases were formed in the cultures, but that phase transformations of the iron-sulfides may have occurred rendering them resistant to dissolution by 0.5 M HCl.

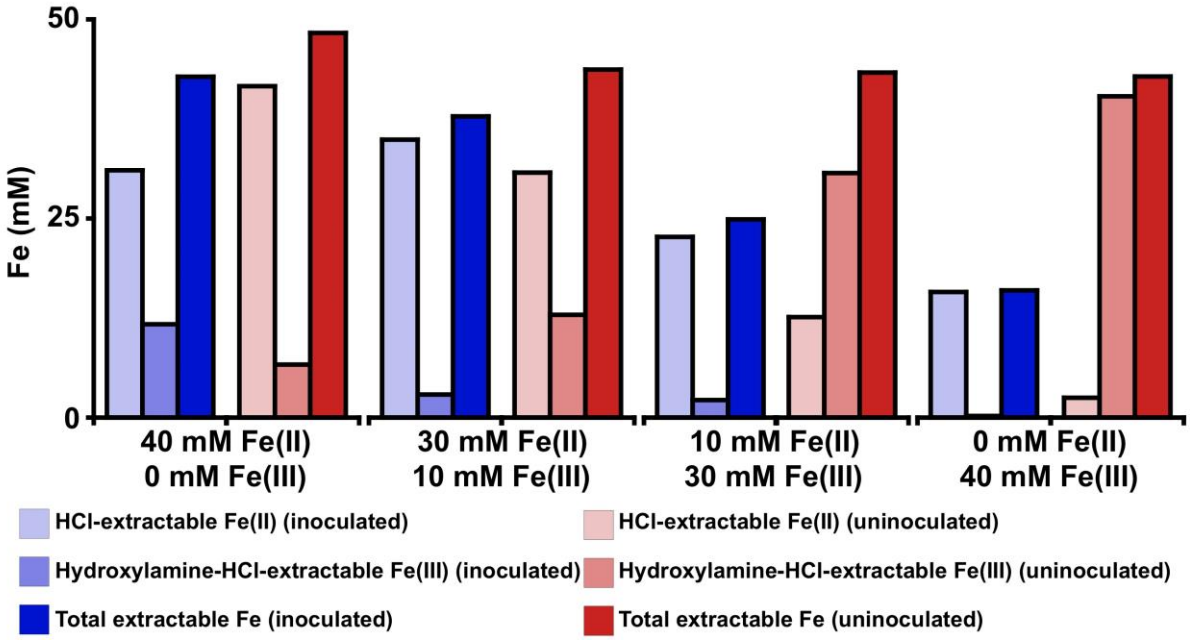

Figure 2. Extractability of Fe(II) and Fe(III) phases in cultures of *Desulfosporosinus* sp. GBSRB4.2 after 24 d of growth (inoculated) and in uninoculated culture medium amended with various proportions of Fe(II) and Fe(III).

Mackinawite (FeS) is generally the predominant iron-sulfide phase produced in Fe(II)-containing sulfate reducing bacterial cultures and is susceptible to dissolution by 0.5 M HCl (Rickard, 1969; Herbert *et al.*, 1998; Snowball and Torii, 1999; Rickard and Luther, 2007). However, other, more thermodynamically stable iron-sulfide phases, including pyrrhotite ($Fe_{1-x}S$; where $0 < x < 0.2$), greigite, Fe_3S_4, pyrite, and markasite (FeS_2) may be more resistant to dissolution by 0.5 M HCl (Snowball and Torii, 1999). These iron-sulfide phases have been observed in sulfate reducing bacterial cultures, particularly in cultures containing Fe(III) (hydr)oxides (Neal *et al.*, 2001; Watson *et al.*, 1999; Watson *et al.*, 2000; Watson *et al.*, 1995). Our results suggest that phase transformations of iron-sulfide phases occur under sulfate reducing conditions in the presence of Fe(III) (hydr)oxides. These transformations may influence the mobility of iron in AMD systems as well as the susceptibility of iron-sulfide phases to reoxidation in the presence of O_2. Further characterizations of iron-sulfide phases will be done using magnetometry and X-ray diffraction.

Acknowledgements

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