

Assessing Mutualism Between Dissimilatory Ferric Iron- and Sulfate-Reducing Bacteria

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ABSTRACT

In this project, I tested the hypothesis growing iron reducing and sulfate-reducing bacteria in co-culture would demonstrate a mutualistic relationship between the two groups. The precipitation of iron sulfide minerals would remove product from solution and maintain the thermodynamic driving force behind the terminal electron accepting reaction, allow both organisms to consume more substrate than either could when growing in isolation. I examined this process using the model organisms *Geobacter sulfurreducens* and *Desulfovibrio vulgaris*. After 90 hours of incubation, the data clearly indicate that rather than being mutually beneficial, the growth of both organisms is inhibited when grown in co-culture. In *D. vulgaris* this is likely due to substrate uptake inhibition due to the presence of ferric citrate, and *G. sulfurreducens* is likely inhibited as well due to the buildup of sulfide minerals which may interfere with extracellular electron transport. This inhibition of iron reduction by sulfide may indicate a possible mechanism by which sulfate reducers can compete with iron reducers in the environment.

INTRODUCTION

Microbially mediated oxidation-reduction (redox) reactions are primary agents of geochemical change in the subsurface. As microorganisms exploit redox disequilibria to generate energy in the form of ATP, they serve as catalysts for global nutrient cycles (Whitman et al., 1998) and control many aspects of water quality (Chapelle and Lovley, 1992; Park et al., 2006). These processes also serve to break down anthropogenic pollutants such as petroleum hydrocarbons (Bolliger et al., 1999; Lovley, 1997), chlorinated solvents (Azizian et al., 2005; Mohn and Tiedje, 1992), and toxic radionuclides (Barkay and Schaefer, 2001; Chang et al., 2001; Sanford et al., 2007). While the geochemical implications of these reactions have been known for many years, determining which redox processes are active in a given area of the subsurface is not a straightforward task (Banfield et al., 2005; Tiedje et al., 1999).

Generally, aquifers are zoned according to a single, dominant terminal electron accepting process (TEAP). Aerobic metabolism is the most energetically favorable, providing the greatest amount of free energy (ΔG) per mole oxidized. When molecular oxygen is depleted along a flow path, metabolic processes transition to other, less energetically favorable TEAPs such as ferric iron and sulfate reduction, depending on substrate availability (Chapelle and Lovley, 1992). Generally speaking, Fe(III)-reduction is more energetically favorable than sulfate reduction (Cozzarelli et al., 2007) although the availability of for biological reduction is strongly dependent on the crystal structure and surface chemistry of ferric minerals (Roden, 2006). Unlike other TEAPs, however, iron and sulfate reduction often appear to be occurring simultaneously in the subsurface (Jakobsen and Postma, 1999; Park et al., 2006). Recently, reactive transport modeling studies indicate that this may be a result of a mutually beneficial relationship whereby the products of both TEAPs (ferrous iron and sulfide) react to form iron sulfide minerals (Bethke

et al., 2008). This process of product removal allows the thermodynamic driving force (Jin and Bethke, 2005) of the TEAP to remain high and may indicate a mutualistic relationship between iron and sulfate reducers.

In this project, I will test the hypothesis that the co-metabolism of iron and sulfate reducing bacteria will be beneficial to each microorganism by maintaining thermodynamic driving force through product removal. I will test this in noncompetitive co-culture experiments using two model organisms, an iron reducer (*Geobacter sulfurreducens*) and a sulfate reducer (*Desulfovibrio vulgaris*).

MATERIALS AND METHODS

Microbial Growth Media

Pure strains were obtained from culture collections at the University of Illinois (*D. vulgaris*) and the University of Massachusetts (*G. sulfurreducens*). They were both grown up to high cell density in media containing a standard freshwater base (17.1 mM NaCl, 1.97 mM MgCl₂*6H₂O, 0.15 mM CaCl₂*2H₂O, and 6.71 mM KCl) with 10 mM NH₄Cl, various trace elements (20 μM HCl, 7.5 μM FeSO₄*7H₂O, 0.48 μM H₃BO₃, 0.5 μM MnCl₂*4H₂O, 6.8 μM CoCl₂*6H₂O, 1.0 μM NiCl₂*6H₂O, 12 nM CuCl₂*2H₂O, 0.5 μM ZnSO₄*7H₂O, 0.15 μM Na₂MoO₄*2H₂O, 2.0 μM NaVO₃, 75 nm Na₂WO₄*2H₂O, and 23 nm Na₂SeO₃*5H₂O) and vitamins (1.0 μg/L riboflavin, 0.3 μg/mL biotin, 1.0 μg/mL thiamine HCl, 1.0 μg/mL L-ascorbic acid, 1.0 μg/mL d-Ca-pantothenate, 1.0 μg/mL folic acid, 1.0 μg/mL nicotinic acid, 1.0 μg/mL 4-aminobenzoic acid, 1.0 μg/mL pyridoxine HCl, 1.0 μg/mL lipoic acid, 1.0 μg/mL NAD and 1.0 μg/mL thiamine pyrophosphate. We buffered the media with both NaHCO₃ (20 mM) and K₂HPO₄/KHPO₄ (1 mM each), which also served as a source of phosphorus. A matrix showing additional amendments for each test microcosm can be seen in Table 1. All experiments were

performed in 160 mL serum bottles (Bellco Glass, Vineland, NJ) sealed with blue butyl rubber stoppers and an anaerobic headspace of 80/20 N₂/CO₂. Media was bubbled for 30 minutes with pure N₂ gas that had been passed through a heated copper column in order to remove all oxygen. All amendments were added aseptically from sterile, anaerobic stock solutions. Cultures were incubated in the dark at 30°C.

In the first control, *D. vulgaris* was grown alone in media amended with 21 mM sodium lactate and 10 mM sodium sulfate. This experiment was designed to test the rate of substrate consumption by *D. vulgaris* in the absence of ferric oxide. A second experiment was conducted under identical conditions to the first with the addition of 10 mM sodium acetate in order to control for the effects of elevated acetate concentrations on the consumption rate *D. vulgaris*, as would be experienced in the co-culture experiments. A third experiment was set up under identical conditions to the first with the addition of 30 mM ferric citrate in order to monitor the effect ferric iron alone would have on *D. vulgaris*. A control for *G. sulfurreducens* was grown in media containing 30 mM ferric citrate and 15 mM sodium acetate. The co-culture experiments were conducted in a similar manner to the controls by the addition of substrate (21 mM lactate, 10 mM sulfate, 10 mM acetate, and 30 mM ferric citrate) and were inoculated with equivalent cell concentrations measured using optical density (OD₆₀₀). Initial cell inoculations were identical across all experiments

Analytical Methods

Conditions within the cultures were monitored using a combination of colorimetry, high-pressure liquid chromatography (HPLC) and epifluorescent microscopy. The ferrozine assay (Lovley and Phillips, 1986) was used to monitor consumption of ferric citrate and production of ferrous iron in the cultures. A 1 g/L solution of ferrozine 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-

triazine-4',4''-disulfonic acid) was buffered with 50 mM HEPES and brought to pH = 7 with 10N NaOH. Samples were first diluted in 0.5N HCl in order to stabilize ferrous iron, which is rapidly oxidized at circumneutral pH under atmospheric levels of oxygen. Following dilution, 20 μ L of sample was added to 980 μ L of ferrozine solution and allowed to react for 5 minutes, after which the absorbance at 562 nm was measured using a UV-vis spectrophotometer (Cary 50 Scan, Varian). Standards were run periodically to ensure that instrumental drift did not affect the readings.

Sulfate reduction was monitored using the barium sulfate turbidity method for sulfate (Kelly and Wood, 1998) and the methylene blue method for sulfide. A 200 μ L sample was added to 500 μ L of 20% (v/v) glycerol and mixed thoroughly, after which 100 μ L of a 10% (w/v in 0.5N HCl) BaCl₂ solution was added. This causes the precipitation of BaSO₄ micro-crystals, and the turbidity of the resulting solution is then read on the spectrophotometer at 460 nm. Sulfide was preserved by first adding 100 μ L of sample to 400 μ L of 20% (w/v) Zn-acetate. The sulfide concentration was then determined colorimetrically using the Hach Hydrogen Sulfide kit, following the manufacturer's instructions.

Consumption and production of organic acids were monitored using a Shimadzu LC-2010C-HT HPLC system with an Aminex[®] HPX-87H ion exclusion/reversed phase column. Samples were first acidified by adding 100 μ L H₂SO₄ to 900 μ L of undiluted sample. Each was then centrifuged at 13100 rpm in order to remove cell debris and particulate matter. If particles were unable to be pelleted by centrifugation, the sample was filtered using a 0.45 μ m filter. Standards were measured periodically to ensure proper calibration of the instrument.

Microscopic Enumeration

Total cells in each microcosm were enumerated using a combination of DAPI (4',6'-diamidino-2-phenylindole) staining and fluorescent in-situ hybridization (FISH). Cells were first fixed for 24 hours at 4°C in a PBS solution containing 1% formaldehyde, then filtered through a 0.22 µm filter. Hybridization and DAPI staining were performed following the instructions in the Microbial Diversity Manual 2008. Cells were hybridized with the probes EUB338 (Amann et al., 1990) which targets all Eubacteria, GEO-A,B and C (Richter et al., 2007) which targets the *Geobacteraceae* including *G. sulfurreducens* and a non-specific probe which does not bind to any known 16S sequences and is used as a control.

RESULTS

Pure Culture Controls

Incubating *D. vulgaris* in media containing ferric citrate significantly reduced its rate of lactate consumption (Fig. 1). After a lag phase of approximately 24 hours, substrate consumption increased rapidly to nearly 60 µmol per hour in the *D. vulgaris* culture grown without ferric citrate, and nearly all of the lactate was consumed after 50 hours of growth. In the culture grown with ferric citrate, lactate was continually consumed throughout the experiment, albeit at a rate six times lower than in the absence of ferric citrate (10 µmol/ hr). After 90 hours of incubation, there was no detectable lactate in those cultures grown without ferric citrate, and more than 50% of the initial substrate remained in those where ferric citrate was added. The addition of 10 mM acetate had no effect on the substrate consumption rate of *D. vulgaris*.

Co-culture Experiments

The growth of *D. vulgaris* and *G. sulfurreducens* in co-culture appeared to inhibit the growth of both organisms. As is seen in Figure 2, when *G. sulfurreducens* was grown alone, all of the initial ferric citrate was consumed after 41 hours, while nearly one-third of the initial

amount remained when it was grown with *D. vulgaris*. Only after more than twice that amount of time (90 hours) had passed was all of the ferric citrate consumed in the co-culture experiments.

Although the substrate consumption rate of *D. vulgaris* was inhibited when grown in co-culture with *G. sulfurreducens* in comparison to when it is grown in the absence of ferric citrate, more substrate was consumed in co-culture than when grown in pure culture with ferric citrate (Figure 3). In the co-culture experiments, very little lactate was consumed until nearly all of the ferric citrate had been reduced, after which the rate of lactate consumption increased dramatically. Qualitatively, the cultures where *D. vulgaris* was grown alone with ferric citrate remained black after 90 hours, while the co-culture experiments were clear with large clumps of black, presumably sulfidic precipitate on the bottom of the serum bottle.

Microscopic Enumeration

The results of the direct cell counts using DAPI and FISH are summarized in Table 2. The data appear to indicate that the growth yield of *D. vulgaris* was approximately an order of magnitude less when grown with ferric citrate than when grown without. Total cell yields were similarly lower in the co-culture experiments than when grown separately. The data also indicates that after 90 hours of growth, 70-75% of the total cells in the co-culture experiments appear to be *D. vulgaris*. It should be emphasized, however, that the cell numbers counted per field were very low, and therefore these experiments would have to be repeated and a larger volume of media filtered in order to get a more statistically significant result.

Analytical Problems

The results of the barium sulfate turbidity assay were suspect and are not reported here due to interference in the absorbance spectrum from the ferric citrate, which shows strong absorbance at the wavelength (460 nm) at which turbidity is measured. These data indicated that

sulfate was being consumed at a rate commensurate with the consumption of lactate, although this was only a rough approximation. Sulfide data is also not reported due to a spectrophotometer cuvette contamination issue in which the cuvettes contained residual acid from an acid wash. The methylene blue assay is strongly pH dependent and even the trace amounts of acid left on the cuvettes was enough to render all data unreadable.

“Missing” Carbon

As is seen in Figure 1, only 61% the initial lactate in the cultures containing *D. vulgaris* was converted to acetate, which is significantly lower than other reported values, which range from 84% to 97%. This “missing” carbon was accompanied by the appearance of an unknown peak in the HPLC traces between the peaks representing lactate and acetate in these same samples. This was initially thought to be formate being produced after pyruvate was converted to acetyl-CoA by pyruvate formate lyase, although subsequent analyses using formate-spiked samples disproved this hypothesis (data not shown). The peak was also shown to not be fumarate, which has a similar elution time, as it was not detected in the refractive index detector of the HPLC. The identity of this peak remains unknown pending further analysis.

DISCUSSION

The presence of ferric citrate clearly inhibits the rate of substrate consumption in *Desulfovibrio vulgaris*. Citrate has been shown to have an inhibitory effect on other sulfate reducing bacteria (Roden and Lovley, 1993) and may be the cause of the lower rates of consumption. As is seen in Figure 3, however, once the ferric citrate is reduced to ferrous citrate by *G. sulfurreducens*, this inhibitory effect is lost and sulfate reduction can proceed at a rate equivalent to that seen in the absence of any ferric citrate. This may be because ferrous citrate is less inhibitory than ferric citrate, or because it is more readily precipitated with the other sulfide

minerals. A more quantitative analysis of the sulfidic precipitates would be required to confirm this, however.

The inhibitory effect of *D. vulgaris* on the substrate consumption rate of *G. sulfurreducens* is likely a result of the production of sulfide, which is toxic to most cells, even sulfate reducers (Rabus et al., 2006). Sulfide reacts readily with ferrous and ferric iron (Morse et al., 1987) and sulfide minerals are often found encrusted around cells grown in the presence of both (Li et al., 2006). Because *Geobacter* species often respire insoluble electron acceptors such as ferric iron (Lovley, 2006), they must be able transfer electrons to a suitable acceptor extracellular, such as with the use of conductive pili (Reguera et al., 2005). The precipitation of copious ferrous sulfide minerals may interfere with this cellular machinery and inhibit the consumption of substrate. This might also explain why the yields of *G. sulfurreducens* cells were comparatively lower in co-culture. The inhibition of iron reduction by *D. vulgaris* may represent an important mechanism by which sulfate reducers can compete with iron reducing bacteria in the environment. When ferric iron is available in an aquifer, it is usually more energetically favorable to reduce iron than sulfate (Chapelle and Lovley, 1992; Cozzarelli et al., 2007), and inhibition by sulfide may allow sulfate reducers to compete on a more level playing field. Further research on the relative toxicity of sulfide on various sulfate reducers and iron reducers would be needed to confirm this.

Clearly, the interactions between iron and sulfate reducing bacteria are complex. Further experiments using ferric compounds that do not contain citrate would enable a more realistic evaluation of the effect of the presence of Fe(III) on dissimilatory sulfate reduction. In-depth tracking of cell populations over the course of the experiment using FISH would also help to elucidate the effects of co-metabolism on the growth rates of *D. vulgaris* and *G. sulfurreducens*.

Further experiments could also examine the role of substrate competition by using a sulfate reducing bacterium such as *Desulfobacter*, which can grow on acetate. A flow-through column experimental design would provide a more realistic growth environment that would serve to prevent the excessive build-up of sulfide minerals. More research is needed, however, in order to understand the dynamics of even this simplified model system.

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FIGURES

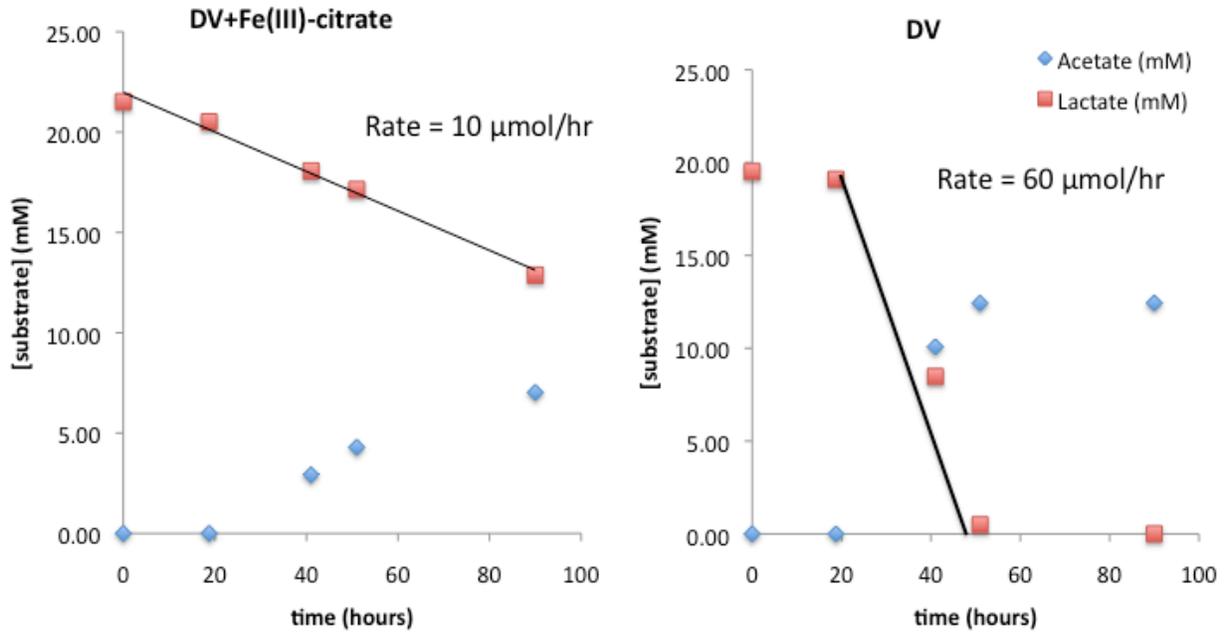


Figure 1. A comparison of lactate consumption and acetate production rates for *Desulfovibrio vulgaris* when grown in standard freshwater media and media amended with 30 mM ferric citrate. This experiment was repeated in duplicate, and these plots are representative of both replicates.

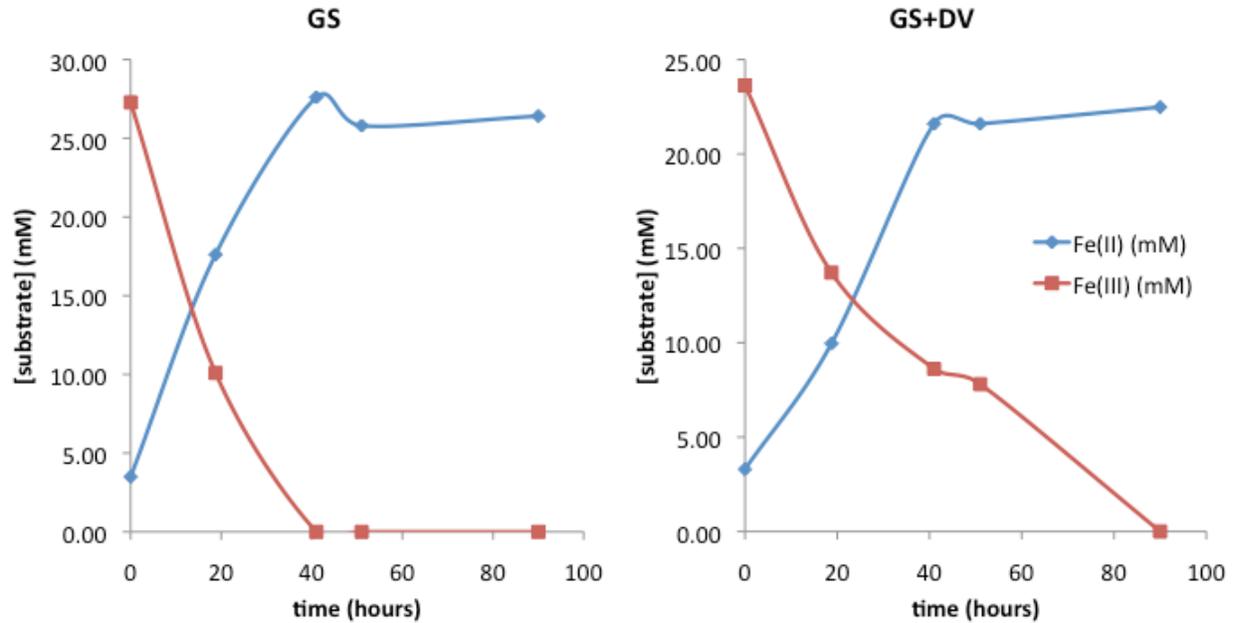


Figure 2. Rates of ferric citrate consumption and the production of Fe(II) in *Geobacter sulfurreducens* when grown alone in 30 mM ferric citrate and in co-culture with *Desulfovibrio vulgaris*. These results were done in duplicate and are representative of both.

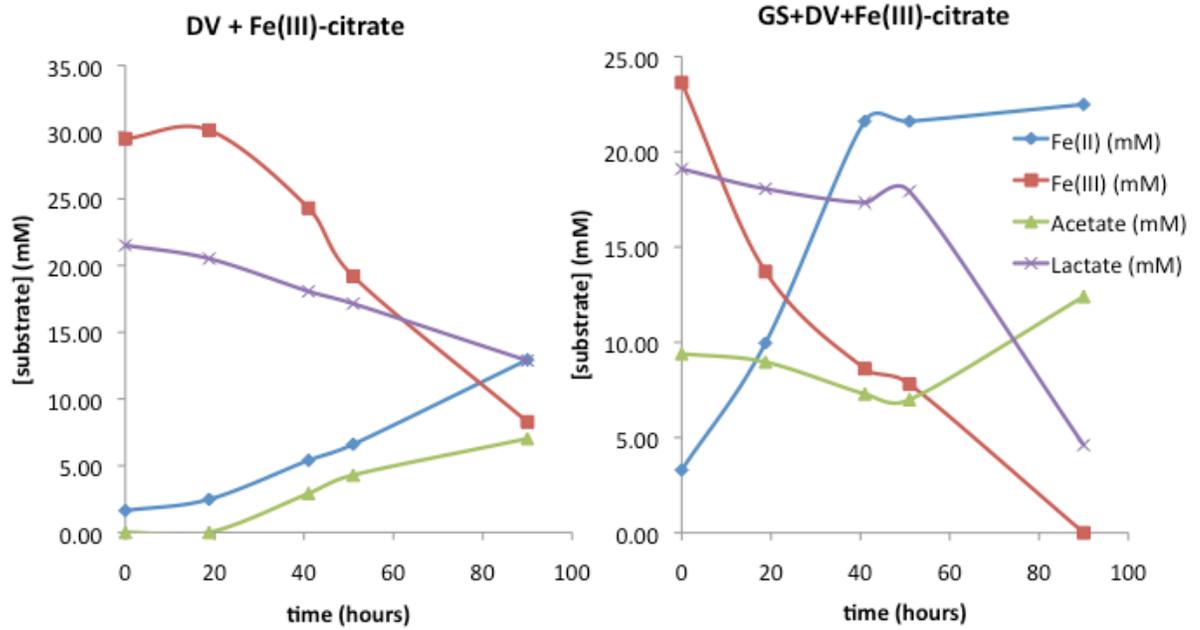


Figure 3. A comparison of substrate consumption rates for *D. vulgaris* when grown in pure culture with 30 mM ferric citrate and in co-culture with the same inoculum plus *Geobacter sulfurreducens*.

TABLES

Treatment	Total cells	Geobacter sulfurreducens	Desulfovibrio vulgaris
DV – Fe(III)-cit	2.0e7	0	2.0e7
DV + Fe(III)-cit	2.3e6	0	2.3e6
GS + Fe(III)-cit	7.5e6	7.5e6	0
GS+DV+Fe(III)-cit (1)	5.9e6	1.8e6	4.1e6
GS+DV+Fe(III)-cit (2)	2.7e6	6.9e5	2.1e6

Table 1 Results of direct cell count enumeration using epifluorescent microscopy. Total cell numbers were obtained using DAPI and FISH with the Eubacterial probe EUB338 (Amann et al., 1990). Geobacter cells were enumerated using the *Geobacteraceae*-specific probes GEO-A, GEO-B, and GEO-C (Richter et al., 2007).