Iron Metabolizers and the Banded-Iron Formations
or
How I Learned Microbiology with a Lot of Help from my Friends

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Introduction

The banded-iron formations (BIFs)—thin, interspersed layers of ferrous/ferric and silica-rich minerals—are the oldest sedimentary rocks on Earth, with ages up to 3.8-3.9Ga (Walker et al., 1983). Their formation appears to have ended fairly abruptly 1.7Ga ago, a transition generally attributed to the oceans becoming oxic. Three types of formation mechanisms for the BIFs have been proposed, all beginning with abundant soluble ferrous iron in the reduced Archean oceans. A purely abiotic mechanism involves ultra-violet photolysis of water, with the free oxygen thus produced forming the more oxidized, insoluble iron precipitates. A second mechanism, indirectly biotic, involves iron oxidation by free oxygen resulting from oxygenic photosynthesis (Walker et al., 1983). A third class of mechanisms, proposed most recently, involves biotic processes more directly. In one, anaerobic phototrophs (Ehrenreich and Widdel, 1994) or hyperthermophilic chemolithotrophs (Madigan et al., 2000 pg. 561) oxidize ferrous to ferric in the absence of free oxygen. In another, ferric iron is reduced by hyperthermophiles closely related to the last common ancestor to produce the immediate precursor of the banded iron mineralogy (Vargas et al., 1998).

The first type of mechanism is challenged by the large volume of the BIFs (basin areas are often 100,000km^2 or more), requiring more oxygen than is thought to have been available from photolysis alone. The second mechanism, if applied to the oldest BIFs, would have required a very early development of oxygenic photosynthesis. The third type of mechanism (i.e., biologic processes) is consistent with conditions thought to prevail on early Earth, and warrants more detailed investigation as a possible formation mechanism for the BIFs.

Bacterial iron oxidation and reduction under restricted environmental conditions have been known for many years (Straub et al., 2001). For example, ferrous iron is soluble only at acidic pH. The acidophile Thiobacillus ferrooxidans can oxidize ferrous iron under these conditions, and is one of the main biological agents in nature for this process (Madigan et al., 2000, pg. 463). Bacteria such as Gallionella ferruginea and Leptothrix can oxidize ferrous iron at neutral pH at the interface between ferrous-rich ground water and air (Madigan et al., 2000, pgs. 598, 689; Hanert, H. 1992). Dissimilatory iron reduction occurs in several organisms, including anaerobic sulfur reducers such as Geobacter metallireducens and Desulfuromonas acetoxidans, and facultative aerobes such as Shewanella putrefaciens (Nealson and Saffarini, 1994).

The reevaluation of possible biological mechanisms for producing the BIFs was stimulated in part by the recognition that microbes could accomplish the key step of oxidizing Fe(II) in the anoxic oceanic waters of ancient Earth. One type of these anaerobic iron oxidizers, strains of phototrophic purple bacteria, were isolated and studied by Ehrenreich and Widdel. It was my intention in this mini-project to isolate these iron oxidizers as well as anaerobic iron reducers from

the environment, and attempt to develop a co-culture in which an iron cycle occurs similar to what may have transpired in the Hadean or Archaean oceans.

Experimental Strategy and Design

The general design of this investigation was predicated on finding a site or sites in the local environment that were naturally enriched in the iron metabolizers of interest. My plans were to prepare anoxic enrichments for both phototrophic iron oxidizers and chemotrophic iron reducers that grow at near-neutral pH. I also planned to characterize the 16S rDNA diversity of the environmental samples, and correlate that characterization with the 16S rDNA of any isolates obtained. I did not expect to complete all of the work implicit in this experimental design, but wanted to go as far as possible to learn both the techniques and principles involved, and to develop a better understanding of the role this type of investigation could play in understanding the origin of the BIFs.

Environmental Sites

Several marine or brackish and fresh water samples were used in this project. One site sampled was in the School Street Swamp behind the MBL where rain water ran off a corroding iron staircase into anoxic swamp waters. This was treated as a marine sample despite the source of the iron-rich water, since the Swamp is fairly brackish. Other marine samples were taken from a piece of Sippewisset salt marsh microbial mat brought into the laboratory by D. Patterson and from the back of a trowel immersed in a bucket half-filled with School Street Swamp mud and flooded with sea water. Both of the latter samples showed abundant rusty deposits indicative of iron oxidation.

Fresh water samples were obtained from a well drilled by the US Geological Service to monitor a sewage plume on the Massachusetts Military Reservation (formerly Otis Air Force Base) and from a nearby site on the shore of Ashumet Pond where iron-containing ground water had apparently emerged and flowed into the Pond.

Enrichments

I prepared sea-water (SW) and fresh-water (FW) enrichment media in the manner of Ehrenreich and Widdel (1994). For freshwater enrichments, the following salt quantities were used for each liter of medium (prepared with 18Mohm deionized water): 0.3g NH_4Cl, 0.5g KH_2PO_4, 0.05g $MgSO_4.7H_2O$, 0.36g $MgCl_2.6H_2O$, and 0.1g $CaCl_2.2H_2O$. The sulfate concentration was deliberately kept to a minimum so that the same medium could be used in both oxidizer and reducer enrichments. Higher sulfate concentration could have led to competition between sulfate and Fe(III) as electron acceptors in the iron reducer enrichments. (Some sulfate was necessary to ensure a sulfur source for anabolic reactions.) For marine enrichments, the following salt quantities were added per liter of medium: 22g NaCl, 3.5g $MgCl_2.6H_2O$, 0.5g KCl, and 0.05 $CaCl_2.2H_2O$. The media thus prepared were then autoclaved in air in an apparatus suitable for anaerobic handling similar to that described by Widdel and Bak (1992).

Following autoclaving, the following were added under nitrogen atmosphere: 22mL of a 1.0M $NaHCO_3$ buffer solution, autoclaved separately under a CO_2 atmosphere; 1mL each of two trace

element mixtures, 1mL of a mixture of vitamins, 1mL of vitamin B_{12} solution, and 200µmol/liter of FeS as a reducing agent. (For most of the medium, the FeS was freshly prepared stoichiometrically by titrating Na_2S into $FeSO_4$, centrifuging to collect the precipitate, and washing it twice with deionized water.) For the iron reducer medium, 20mmol of freshly prepared $Fe(OH)_3$ (prepared by titrating NaOH into $FeCl_3$ and then centrifuging and washing as for FeS) was also added for each liter of medium. Finally, the pH of the medium was adjusted to 6.8-6.9 by addition of small amounts of Na_2CO_3 as necessary. The medium was immediately transferred anoxically to 50mL screw-cap (Pfennig) bottles which were filled so as to minimize head space and tightly capped. (There is evidence, described below, that air enters the head space of these bottles even when tightly capped.)

Iron reducer enrichments were prepared by adding small quantities of environmental samples (~.25g of solid, ~0.5-1mL of liquid) to the Pfennig bottles containing $Fe(OH)_3$ along with 250µL of a sterile 1M acetate solution yielding an acetate concentration of 5 mM. The initial appearance of the enrichments was a rust-colored precipitate overlain by clear liquid. Iron oxidizer enrichments were prepared by adding the same quantities of environmental sample to Pfennig bottles containing medium without $Fe(OH)_3$. A 1.0M stock solution of $FeCl_2$ was prepared in an anoxic glove-box (>90% N_2/<10%H_2 atmosphere) from a fresh bottle of $FeCl_2.4H_2O$ stored for shipment under argon. $FeCl_2$ was used rather than the more common choice of $FeSO_4$ because of the desire to minimize sulfate in the enrichments. The disadvantage of this approach is that $FeCl_2$ is readily oxidized and must be carefully protected (both in solid and solution form) from exposure to oxygen. 500µL of the 1.0M $FeCl_2$ stock solution was added to each enrichment for a final Fe(II) concentration (excluding the FeS reductant) of 10mM. The $FeCl_2$ addition lowered the pH of the enrichments by ~0.4 units, which was corrected by adding 400-500µL of 1.0M Na_2CO_3. Upon the addition of $FeCl_2$, the enrichment liquid turned cloudy owing to the formation of a white-ish $FeCO_3$ precipitate. The pH adjustment by Na_2CO_3 yielded a substantial additional amount of this precipitate, which combined with the small amount of FeS present to yield an overall gray precipitate overlain by clear-to-slightly-cloudy liquid.

I inoculated 8 iron-reducing bacteria (FeRB) and 14 iron-oxidizing bacteria (FeOB) enrichments. The FeRB enrichments were evenly divided between sea and fresh water and were inoculated in air. Samples used for the four SW enrichments were two from School Street Swamp (surface water showing a metallic sheen and bottom sediments), one from the back of the trowel, and one from the box containing the rusty Sippewisset salt marsh mat. Samples used for the four FW enrichments were from the Ashumet Pond "iron site" (surface water sheen, 2 bottom sediment samples that were extremely red, and sediment from beneath a dead vegetative mat that may have been comparatively anoxic). Uninoculated enrichment medium served as a control. The enrichments were incubated in the dark at 30C.

Of the 14 FeOB enrichments, 4 were sea water and 10 were fresh. The SW enrichments used were the same four environmental samples as the SW FeRB enrichments. Of the 10 FW FeOB enrichments, 7 used iron site samples (3 separate samples of surface water with a metallic sheen, 3 separate red sediment samples, and the possibly anoxic sediment described above. The other 3 were well-water samples, two of which were iron-rich (>10ppm) and one of which was essentially iron-free. All FeOB enrichments were inoculated and capped in the anoxic glove box. A control for the FeOB enrichments was prepared by similarly inoculating with deionized water. The

enrichments were placed on a light table in air, approximately 20-25cm from a 60watt incandescent bulb. (The three well-water enrichments were placed somewhat closer to a 25w light bulb.)

Enrichment Results

Iron Reducers

The rust-colored $Fe(OH)_3$ precipitate in the trowel and Sippewisset mat SW FeRB enrichments turned black within a few days, indicating iron reduction. The change in color generally began as dark spots in the $Fe(OH)_3$ precipitate, as expected for microbial action. One of the School Street Swamp samples turned black after about 2 weeks, and one has not turned black of this writing.

The FW FeRB enrichments began turning black within a few days of inoculation. Again, the change generally began as dark spots in the rust-colored precipitate. All 4 enrichments were black within 1-2 weeks.

Iron Oxidizers

The four SW enrichments all turned black within a few days, the trowel sample enrichment changing most rapidly and resulting in the darkest enrichment (black precipitate, dark supernatant, and black streaks on inside of the glass). However, blackening was not the expected effect for iron oxidizers. Rather, a conversion of the initially gray precipitate ($FeCO_3$ plus FeS) to rust-colored was expected. Most of the FW enrichments also turned black, but more slowly and less intensely. The only FW FeOB enrichments that did not turn black were the well-water enrichments, which appeared unaltered almost 2 weeks after inoculation.

To help determine the cause of the change in appearance of the enrichments, I inoculated a second trowel sample enrichment and a dark control. Both turned black within a few days, indicating that the darkening was not associated with phototrophic activity.

The nature of the darkening, and the manner in which it varied between enrichments (greatest in the SW samples, less in the iron site FW samples, absent in the well-water samples), strongly indicates that it was the result of sulfide production by sulfate reducing bacteria (SRBs)using environmental organic material as an electron donor and carbon source. The SW samples had the most sulfate in the inoculum and probably the greatest amount of reduced organic material as well. The iron site samples had little sulfate and probably less organics, and the well-water samples were most likely extremely low in both sulfate and organics. Very little FeS is required to cause an intense blackening, so sulfide production by SRBs leading to FeS precipitation can likely account for what occurred both qualitatively and quantitatively.

The sulfide precipitation, however, is not really a problem. The iron in the enrichment remains in the ferrous form, and is only somewhat less available as a substrate as the more insoluble FeS than the more soluble $FeCO_3$. In the experiments of Ehrenreich and Widdel (1994), rusty deposits took up to 4 weeks to begin developing. So the sulfide precipitation does not suggest that a change in the enrichment strategy is necessary. Continued incubation under light (weaker illumination than was used thus far would more closely approximate the conditions used by Ehrenreich and Widdel) may still produce phototrophic iron oxidation.

Some iron oxidation did occur in most of the enrichments, but it was not the result of microbial activity. The small gas bubble in the head space of the enrichment bottles became coated with rust-colored patches. Turning the bottles on their sides revealed these strikingly colored and textured bubbles in the midst of otherwise gray-black or clear liquid medium. The caps of the Pfennig bottles apparently allow gas exchange between the head space and the ambient atmosphere, so that even though the bottles were inoculated and capped in the anoxic glove box, incubation in air allowed some oxygen to enter the headspace resulting in chemical oxidation of a small amount of Fe(II). The quantity of Fe(II) oxidized in this manner should not be significant in relation to the amount of Fe(II) contained in the enrichments.

Molecular Analysis

The first molecular analysis step in the experimental strategy described above was polymerase chain reaction (PCR) amplification of 16S rDNA from selected environmental samples and corresponding enrichments. I chose three iron site environmental samples (sheen, red sediments, and anoxic sediments) and the corresponding FeRB enrichments, all of which showed evidence of iron reduction. These enrichments showed a low-to-moderate abundance of small motile rods, 1-2μm long and <1μm wide.

Because of the limited amount of time available for repeated PCR amplifications and the subsequent analysis and cloning that were part of the experimental strategy, I chose to do a multi-aspect PCR run that was, in retrospect, too ambitious. I used both bacterial (8F, 1492R) and archaeal (89F, 915R) 16S rDNA primers. The forward primers were used both in unlabeled and HEX-labeled forms (i.e., Bac8F and Bac8F-HEX; Arc89F and Arc89F-HEX), respectively, for subsequent cloning and 16S Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis. The labeled primers were used for all 6 samples, while the unlabeled primers were used only for the red sediment sample and its corresponding FeRB enrichment.

Because of the complexity of this set of reactions and their associated controls, and my inexperience in doing PCR, I chose to set up all reactions using a particular pair of primers in sequence, and then move on to the next pair of primers. In this process, I did the positive and negative controls in batches, the positive controls in the middle of the 6-hour reaction set-up and the negative controls at the end. Inhibition controls were set up with bacterial DNA (E. coli) and labeled forward primer, generally at the same time as the corresponding sample reaction mixtures.

The bacterial and archaeal reactions were run simultaneously on separate PCR cyclers programmed by the course staff. At the conclusion of the reactions, the cyclers held the reaction mixtures at 4C for 2-3 hours until I removed and froze them. After thawing the completed reaction mixtures several hours later, I ran them on two standard agarose gels.

The results showed inconsistencies between the samples and the controls. In general, the bacterial positive controls showed no DNA amplification, even though several of the samples showed bacterial DNA. The inhibition controls, which were all done with bacterial DNA, all showed inhibition, suggesting that the E. coli DNA used in both the positive and inhibition controls was not amplified for some reason. The labeled-primer Attila positive control showed amplification, but the unlabeled-primer positive reaction tube was found to contain only about 10% of the amount of fluid

expected, so I must have made some error in setting up that reaction. (All other tubes had the expected quantity of reaction mixture.)

The unlabeled primers (both bacterial and archaeal) used for the red sediment environmental sample and the corresponding FeRB enrichment both showed DNA amplification, meaning I could have proceeded to insert the amplified DNA into plasmid vectors and clone them using E. coli and standard procedures. The environmental samples showed only weak amplification with the labeled primers, so T-RFLP analysis would have been somewhat more problematic. However, the course was within a few days of ending at the time I obtained these PCR results, and I elected to conclude the molecular portion of the mini-project at this point.

Follow Up

Because both the red sediment environmental sample and the corresponding FeRB enrichment showed archaeal DNA amplification, I tested the enrichment for methanogens by two methods. The first was examination of a drop of the enrichment under an epifluorescence microscope to determine if the F420 fluorescence characteristic of methanogens was present. The fluorescence was observed, but only in association with grains of the precipitate. Because there is not reason to think that methanogens would be closely associated with these grains, and because the fluorescence pattern fairly closely followed the particle thickness, I concluded that the fluorescence was most likely mineralogical, not biological. I also took a sample of the enrichment liquid, transferred it to a small closed bottle, allowed any dissolved methane to enter the head space, and analyzed the head space gas for methane on a gas chromatograph (GC). There was no clear evidence of methane in the GC measurements. The archaea in the environmental sample and in the enrichment thus remains unidentified.

The enrichments will continued to be incubated in the lab of Jen Giegerich, another student in the course, at Penn State. If the oxidizer enrichments are successful and "the crick don't rise" I may yet try to develop the co-culture. Should I have the opportunity to continue this work more extensively, I would find the best iron site I could, obtain a new set of samples, and redo the project essentially as originally planned, but with better technique. The question of what this will tell us about the possible biogenic origin of the BIFs is still an open one, but continuing the project should yield more insight into this question, as well as continuing my education about the theory and practice of microbiology.

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