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Comparing Capabilities of Shewanella oneidensis MR-1 and the Microbial Community of Iron Caves to Reduce Fe(III)

Aaron Douglas Pham
University of Akron, ap237@uakron.edu

Honors Research Project

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Aaron Pham

The University of Akron

Department of Biology

Akron, OH 44325-3601

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Abstract:

Caves are generally formed by the erosion and/or dissolution of rock and its subsequent removal by water. Iron ore caves (IOCs) form despite being hosted by relatively insoluble and weathering-resistant rock. Due to the discovery of a microbial community behind the walls of these caves, it was hypothesized that these bacteria could be responsible for speleogenesis. Iron ore exists in an oxidized (Fe(III)) state, but reduced (Fe(II)) form is soluble. It was further reasoned that the bacteria might be able to reduce Fe(III) through direct metabolic activity, which uses iron as an electron acceptor. Here we show that cave microorganisms appear to reduce Fe(III) to a greater degree than a known iron reducer, *Shewanella oneidensis* MR-1. The results demonstrate that cave formation could be due to microbial activity which provides an additional model for cave formation.

Introduction:

Caves form through dissolution and mass transport by groundwater. There are other caves, specifically iron ore caves (IOCs), in Brazil that may form directly through the activity of microorganisms (Parker et al. 2013). The iron (Fe(III)) in the caves are localized in the banded iron formations (BIF) and canga, both of which are insoluble and hard to dissolve in its oxidized state. For this reason, the mechanism by which these caves are formed is poorly understood (Parker et al. 2017; Shuster et al. 2012; Auler et al. 2019). Additionally, these caves exist above the water table and are occasionally dry without the flowing water. This episodic water flow and the naturally weathering-resistant iron formations means that a new model for cave formation is needed.

A recent study found a community of microbes behind the cave walls (Parker et al. 2018). This layer behind the cave walls is called *sub muros*. It is soft and porous which allows for the movement of water and has less iron than the adjacent layers (Parker et al. 2017). It was then

suggested that microbes within the *sub muros* reduce the Fe(III) in the BIF-canga layer to soluble Fe(II), allowing it to be dissolved and washed away. This softens the cave walls, and eventually they collapse and increase the size of the cave (Parker et al. 2018). We hypothesize the rate and extent of iron reduction and dissolution by the *sub muros* will be higher than a model organism that is well known to reduce iron.

To do this, we compared the reduction rates of a model Fe(III) reducing microorganism to those in the *sub muros*. The bacterial species that will be used is *Shewanella oneidensis* MR-1 (Lies et al. 2005), and the concentration of dissolved and solid associated Fe(II) between *S. oneidensis* and the microbial community, was measured and compared. The results of this experiment showed that the rate and extent of reduction and dissolution of the iron found in the canga due to the activity of the model organism, *S. oneidensis* compares to those organisms found in the *sub muros*. They further demonstrated whether the iron reduction seen in the caves is most likely due to microbial activity, rather than abiotic chemical processes.

Materials and Methods:

200 g of canga were processed into a fine powder using a ball mill (SPEX Industries, Inc., Metuchen NJ) and stored in a beaker, which was covered with aluminum foil and secured with autoclave tape. The powdered canga was double autoclaved on a gravity cycle (in which pressurized steam heated to 121° C enters chamber to achieve sterilization) for 30 minutes, cooled to room temperature, autoclaved again on gravity for 30 more minutes, and dried in an oven set to 75° C overnight. Twelve serum bottles and rubber caps were rinsed then set in an overnight acid bath. The bottles and a beaker filled with rubber caps were filled with 0.5 M HCl and left overnight. After the acid bath, the bottles and rubber caps were rinsed 2-3 times with deionized water and, for sterilization, double autoclaved: once on liquid cycle for 30 minutes, they were allowed to cool

to room temperature, and autoclaved again on gravity cycle for 30 minutes to ensure that any spores were killed.

To provide the *S. oneidensis* and *sub muros* with sources of phosphate, sulfate, chloride, and lactate, and to mimic the conditions of the IFCs, the cells were suspended in 900 mL of synthetic pore water (SPW) made with 0.1 mM potassium phosphate, 0.1 mM sodium sulfate, 5 mM sodium chloride and 5 mM sodium lactate. The pH was adjusted to 6.8 using 1.0 M HCl, and the 900 mL of SPW was split equally between two 1 L bottles and autoclaved on liquid cycle for 30 minutes. The SPW was bubbled with N_2 gas to remove any oxygen in the liquid.

Shewanella oneidensis MR-1 was streaked onto TSA and grown at room temperature. From the streak plate, TSB was inoculated in a flask with a sterile loop and left on a shaker at 85 rpm for two days. The cells were then washed three times (until the supernatant was mostly clear) using sterile milliQ water and centrifuged at 35000 rpm for 15 minutes each time. The cells were suspended in 15 mL of sterile milliQ water.

The twelve serum bottles contained the following: three with canga and inoculated with *S. oneidensis*, three with sterile canga, three with canga and inoculated *sub murous*, and three with *sub muros* without canga. 20 g of sterile canga were put into nine serum bottles. In an anaerobic chamber, 60 mL of SPW were then added into six serum bottles. The six bottles were then capped with rubber stoppers. In a sterile 500 mL bottle, 30 g of *sub muros* was weighed out and mixed with 360 mL of SPW. An autopipettor was used to add 60 mL of the mix into each of the six bottles treated with *sub muros*. The bottles were also capped and removed from the glovebag. 2.5 mL of suspended *S. oneidensis* was added into three bottles using a sterile needle and syringe. Cell densities were not determined; however, this method usually yields densities of 7×10^7 cells/mL

(Parker et al. 2013). Finally, the headspace of all bottles was filled with H_2 gas to act as an electron donor.

The concentration of Fe(II) in the incubations were calculated using absorbance measured by ferrozine assay (Stookey, 1970). Every five days, 1.5 mL of samples were pulled from each bottle and transferred into centrifuge tubes. To measure total iron, using a pipettor, 100 μL of the samples were mixed and added to a separate group of test tubes into which 400 μL of 0.5 M HCl was also added; these samples were incubated overnight. For aqueous iron: the remaining samples in latter test tubes were centrifuged for 5 minutes at 3,400 rpm, and 100 μL of the supernatant were added to another set of twelve test tubes into which 400 μL of 0.5 M HCl were added. Standards for spectrophotometric quantification of Fe(II) were made by creating five solutions with the following concentrations of dissolved iron: 0.0 mM, 0.025 mM, 0.1 mM, 0.25 mM, 0.5 mM, and 1.0 mM. These standards were used to create a calibration curve against which the samples were measured. 20μL of samples were pipetted into cuvettes, and 1 mL of ferrozine reagent was added to the cuvettes and absorbance was measured at 562 nm. If samples measured an absorbance higher than the 1.0 mM standard, the samples were diluted. The dilution was performed by pipetting 100 µL of the sample into a clean centrifuge tube and adding 400 µL of 0.5 M HCl.

The hypothesis was tested using a two sample t-test. The average Fe(II) concentrations for both soluble and total iron of the final day samples for the incubations inoculated with *S. oneidensis* and *sub muros* were compared against one another.

Results:

It was previously observed that canga had the highest reducibility among the various iron formations in the caves (Parker et al. 2013). This experiment was done to compare *S. oneidensis* to *sub murous* in canga. Similar experiments tested whether *S. oneidensis* were able to reduce the

Fe(III) in BIF (Parker et al. 2017). Since it was confirmed that *S. oneidensis* could reduce Fe(III) from samples found in the IFCs, the next step was to test the bacterial community of *sub muros* against *S. oneidensis.*

After three months of weekly sample collections and measurements, it was expected that the *sub muros* bacteria would outperform *S. oneidensis* in its rate and extent of iron reduction. Figure 1 illustrates the Fe(II) average concentrations of each of the four treatment groups that were calculated and plotted over time (Figure 1). Most notably, the average concentration of total Fe(II) peaked at 176 mM for the treatments that were inoculated with *sub muros*. This is in contrast to the highest measurement for *S. oneidensis* which was 3.22 mM. Due to the low levels of reduced iron that were observed during the first 8 weeks of data collection, samples 1-3 were re-inoculated with additional *S. oneidensis* using the same method as the initial injection. As a result, there was a subtle increase in Fe(II) concentration, but it mostly followed the trend observed in the sterile control (Figure 1a) which may indicate that the extent to which *S. oneidensis* can reduce iron is much lower than *sub muros.* Overall, the measurements for aqueous iron concentrations were consistently lower than total iron which was expected since the former is only dissolved Fe(II) that is present in solution whereas total iron also has Fe(II) that is extracted from solids.

Sub muros demonstrated a cyclical pattern of iron reduction, and this means that Fe(II) was re-oxidized to Fe(III). It has been reported that there are iron-oxidizing microbes also present within the *sub muros* (Parker et al. 2013), which could explain this pattern of iron-cycling despite the incubations being kept under anaerobic conditions; however, it is possible that the incubations were not kept under perfectly anaerobic conditions. Furthermore, it appears that this pattern was occurring in the total iron measurements due to the drop in Fe(II) concentrations during the final four weeks of the experiment (Figure 1b).

With a null hypothesis that the average concentrations between the two incubations would be identical, the average concentrations of Fe(II) for *S. oneidensis* and *sub muros* from the final day of sample extraction were compared using a two sample t-test. It was calculated that there is a statistically significant difference between the two groups. The calculated p-values were 0.0124 and 0.0054 (< 0.05) for aqueous and total iron respectively. It can be concluded that *sub muros* more proficient than *S. oneidensis* at reducing Fe(III) within canga.

Discussion:

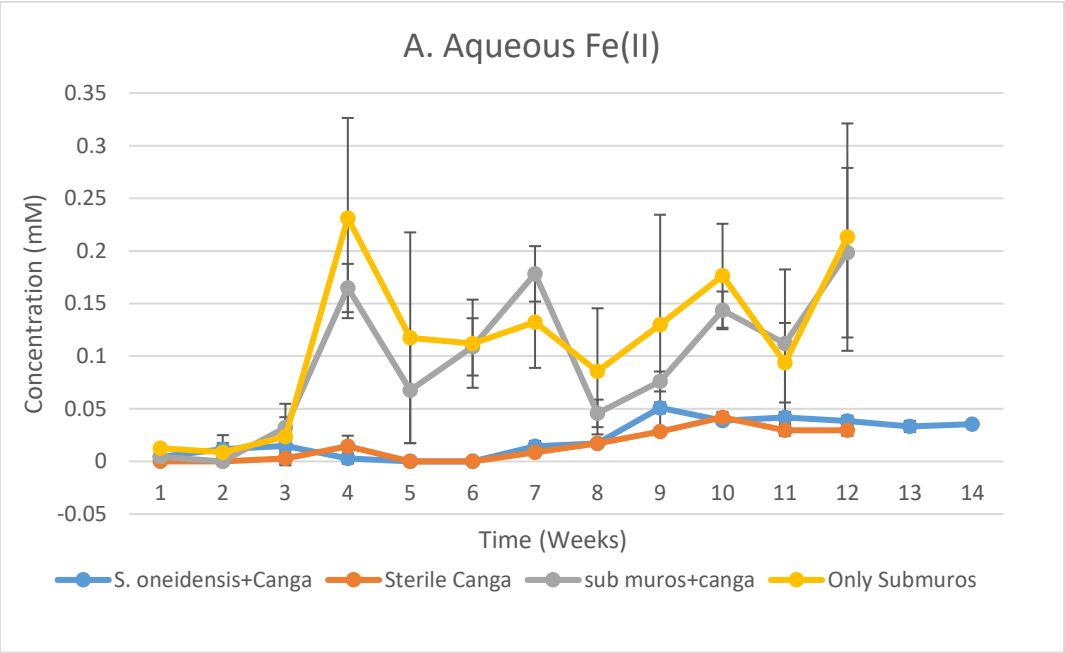
The goal of this experiment was to compare the extent of iron reduction by *S. oneidensis* and *sub muros* bacteria with canga as the iron source. It was hypothesized that the latter would outperform the former. Not only has this experiment shown that *sub muros* can reduce iron-rich canga to a greater degree than the model organism *S. oneidensis*, but it also has reinforced that IOC microbes could play a major role in cave formation as a result of their reductive capabilities.

Speleogenesis requires the breakup or dissolution of the rock that makes up the cave and its removal (Palmer 1991), but in the case of the IOCs, the iron-rich walls are weathering resistant and insoluble (Schuster et al. 2012). A new model of cave formation was proposed in which the reduction of Fe(III) to Fe(II) allowed for the breakdown and transport of the cave walls. This is supported by the existence of a microbial community in the *sub muros* and experiments, which show that *sub muros* can reduce the Fe(III) present in the cave rocks to an extent that is greater than a model organism.

Although the data show that *sub muros* could be responsible for hastening the formation of iron-rich caves, it is impossible to perfectly mimic the conditions in which the microbial community lives. Our limited understanding of the complex relationships between biotic and abiotic factors make it difficult to accurately document the activities of a microbiome *in vitro*

(Madsen, 1998). Furthermore, our methods for testing the reduction of Fe(III) are limited by having to take and store samples away from its original place for extended periods of time

Figures:



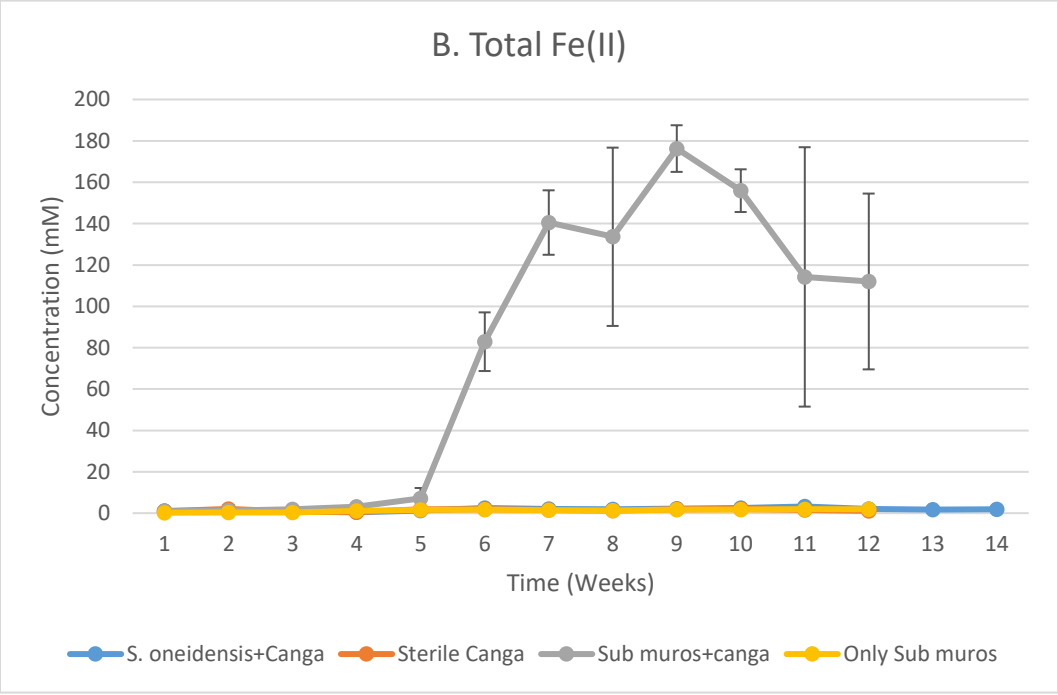


Figure 1. Aqueous (top) and total extractable (bottom) Fe(III) concentrations over time: blue represents treatment group inoculated with S. oneidensis with canga as source of Fe(II); orange was a control with SPW and canga that was left sterile to

measure possible abiotic chemistry that could affect iron reduction; grey represents incubations with sub muros and canga; yellow represents sub muros suspended in SPW to measure reduction as a result of the iron that is already present in the sub muros layer without canga. Error bars represent one standard deviation.

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