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# Fungal Iron Oxidation in Brazilian Iron Caves

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## Abstract

The iron caves in Brazil are thought to form through a cycle of Fe(III)-reduction and Fe(II)-oxidation; the Fe(II) is washed out of the cave walls by rainwater, where it is oxidized to Fe(III) and deposited in iron-oxide crusts. Bacteria are known to reduce Fe(III) in the caves, but what species may be oxidizing Fe(II) is still unknown. Our hypotheses was another bacterial species was involved and our initial assays were aimed at isolating Fe(II)-oxidizing bacteria; however all our Fe(II)-oxidizing cultures were contaminated with fungi when Fe(II)-oxidation was seen. Antifungals were used to inhibit growth of fungal species, which inhibited both fungal growth and Fe(II)-oxidation. Serially diluting cave samples also did not lead to isolation of bacterial species. Antibacterial agents added to the media had no effect and there was no inhibition of fungal growth on Fe(II)-oxidation. Together these data suggested that fungi were the dominant Fe(II)-oxidizing species in our sample. Six fungal isolates were screened by PCR amplification and restriction fragment length polymorphism analysis of the ITS sequence. These data suggested a single species was present based on the ITS sequence. Sequencing of the ITS gene and phylogenetics showed it was closely related to *Sagenomella stratispora*. This particular species of fungus has never been previously described in a cave setting, however given that this species lives in soils and the proximity of the cave to the surface and jungle, this discovery was no surprise.

## Introduction

### *Banded Iron Formation and Canga*

The Quadrilátero Ferrífero (Iron Quadrangle) of Brazil contains expansive deposits of an ancient (~2.4 – 2.6 Gyr) Banded Iron Formation (BIF) known as itabirite, which hosts one of the most significant iron-ore deposits in the world (covering 8.51 million km<sup>2</sup> the deposits contains >20% of global iron reserves) (Jacobi et al., 2015; Rosière et al., 2006; Selmi et al., 2009). The iron ore deposits post-date BIF formation by almost 2.0 Gyr, and understanding the

mechanisms of ore deposition is complicated by its association with canga (a 2-4 m thick, surficial layer of weathered BIF cemented by iron oxides) and extensive cave development (Mabesoone, 1965; Piló, 2009; Schuster et al., 2012). The much younger (~50 Myr) canga formation is poorly understood, as it does not exist as a separate bedded layer within the BIF,



**Figure 1.** Iron-oxide crusts found throughout the Brazilian iron ore caves (IOCs).

but rather overlies the BIF landscape topology like a thick blanket (Monteiro et al., 2014; Schuster et al., 2012; Simmons, 1963). The extremely weathering-resistant nature of the canga (denudation rate  $\sim 0.16 - 0.54 \text{ m Myr}^{-1}$ ) is in direct contrast to the presence of associated caves at the canga-BIF interface (some of which exceed 1,000 m in length with passage dimensions approaching 10 m) (Piló, 2009; Schuster et al., 2012). Indeed, of the ~8,000 known caves in Brazil, >3,000 are associated with canga-BIF contact, making it one of the most cave dense areas in the country (Piló, 2009).

There is increasing evidence that microorganisms play a role in the formation of the iron-oxides found in canga (Levett et al., 2016). Within the iron ore caves (IOCs) there are also a number of iron oxide structures, including thin iron-oxide crusts covering surfaces on the walls and floors (Fig 1) that might form through a similar chemistry. Given that we suspect the caves are made by Fe(III) reduction, the iron oxides may be produced from the subsequent oxidation of the liberated Fe(II). Previous studies analyzing the geochemistry of IOCs have indicated that there are high levels of reduced Fe(II) present in the cave water, and it is possible that iron oxidizing microbes play a role in the formation of the Fe(III) oxides seen in IOCs. Nonetheless, Fe(II) is highly reactive with atmospheric oxygen above pH 4, and can be auto-oxidized to Fe(III), although some bacteria can utilize Fe(II) to obtain energy for growth (Emerson and Moyer, 1997). In this study we have attempted to

determine whether Fe(II)-oxidizing microorganisms play a role in the formation of iron oxide deposits within the IOC.

## Methods

### *Culturation*

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Samples were collected from three pools within the IOCs that demonstrated rust-like Fe-oxide deposits, including one from Triangle Cave, and two pools in Poop Cave, including one heavily influenced by bat guano (Table 2; Pool B). Samples for culture were collected sterilely via hand sample and kept at 4°C upon return to lab. Culturing of iron-oxidizing microbes (FeOM) was performed on media adapted from Johnson (1995), and referred to as Johnson oxidizing media (JOM). The media contained 1.25g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g MgSO<sub>4</sub> • 7 H<sub>2</sub>O, 0.25 g Trypticase Soy Broth, 5 mL Tanner Trace Metals, 5 mL Tanner Vitamins (Hurst et al., 2007) and 15g agar in 1L of deionized water at pH 3.5. The cave conditions were close to 3.5 pH (Table 2), and the low pH would inhibit auto-oxidation of iron. In order to prevent hydrolyzation of the agar during autoclaving due to the low pH, 500 mL of deionized water and the agar were combined in one flask, while the rest of the ingredients were added to 470 mL of deionized water in a second flask. Both flasks were autoclaved. Once the autoclaved flasks cooled to ~50°C, the components of both flasks were combined along with 20 mL of filter sterilized 0.5 M FeSO<sub>4</sub> solution.

To aid in the isolation of iron-oxidizing bacteria or fungi, nystatin (NYS) and cycloheximide (CYO) were used to prevent the growth of fungi, and ampicillin (AMP) and streptomycin (S) were used to prevent the growth of bacteria. Due to solubility concerns, CYO was initially dissolved in 100% methanol and then adjusted to 50% methanol/dH<sub>2</sub>O, at a final concentration of 40 mg/mL. Stock NYS, AMP and S solutions were also prepared to 40mg/mL in 50% methanol. Several concentrations of CYO were used in JOM; 10 µg/mL, 50 µg/mL, 100 µg/mL, 1,000 µg/mL, and 5,000 µg/mL. NYS had a concentration of 10 µg/mL in JOM.

For dilution to extinction JOM was used without FeSO<sub>4</sub> and agar, and there was no separation of the ingredients before autoclaving. The solution was adjusted to pH 3.5 and autoclaved. To isolate individual species sonication was used to break up colonies of FeOM from iron oxides. To do this FeOM colonies were scraped off plates and resuspended in 10 mL JOM, then sonicated at 20% interval and 20% power for 30 seconds, 90 seconds, and 4 minutes. After each time increment 1 mL of sample was used for dilution plating, each sample was diluted from 1 x10<sup>-1</sup> to 1 x10<sup>-5</sup>. After 4 minutes of sonication the interval was increased to 50% and 50% power for 30 seconds before sampling. The dilution series were plated on JOM with NYS (10 µg/mL), with another series without sonication as a control. Each treatment is shown in Table 1.

Table 1. Treatments and conditions to deter fungal growth.

Treatments and Conditions to Isolate Fe(II)-Oxidizing bacteria (FeOB) from Fungal Growth		
Media Used	Additional Treatment	Growth Temperature (°C)
<b>1.</b> JOM with NYS	N/A	Room (~21)
<b>2.</b> JOM with NYS	100 µL CYO spread-plated on, allowed to dry	Room (~21)
<b>3.</b> JOM with NYS	20 µg, 40 µg, 60 µg NYS spread-plated on, allowed to dry	Room (~21)
<b>4.</b> JOM with 0.1 mg/mL, 1 mg/mL, and 5 mg/mL CYO	N/A	Room (~21), 33
<b>5.</b> Liquid JOM, JOM with NYS	A dilution series of eight dilutions was done, with each dilution being a power of ten lower than the previous (1x10 <sup>-1</sup> , 1x10 <sup>-2</sup> ... 1x10 <sup>-8</sup> ). These liquid dilutions were then spread-plated onto JOM with NYS (150 µL per dilution per plate)	Room (~21)

### *Molecular Techniques*

A Maxwell 16 cell DNA Purification Kit SEV configuration (Promega, Madison WI) was used to obtain FeOB DNA and fungal DNA from colonies. The DNA Blood/Cells program was run according to Promega's standard protocol. After extraction, the DNA was stored at -80°C. To test whether iron contaminants were affecting the PCR amplification of the FeOB DNA, a poisoning control was run with FeOB DNA spiked with *Escherichia coli* DNA. If PCR products

were observed this suggested that iron was interfering with the PCR reaction. For samples where iron interfered with PCR amplification, the sample was placed into 1 mL of 0.3M ammonium oxalate to remove Fe(II) (Mehra and Jackson, 1958) on an oscillating tabletop shaker (Gyrotory Shaker-Model G2, Edison, N.J.) shaking at ~300-350 rpms for 30 minutes. The sample was centrifuged at 21,000 x g with an Eppendorf Centrifuge 5424 (Mississauga, Ontario) and the pellet was resuspended in 1 mL of 0.3 M ammonium oxalate. This process was repeated three times to remove the iron. The sample was then washed in 1 mL sterile deionized water for three rinses. The pelleted sample had DNA extracted using a Maxwell DNA extraction kit as previously described.

For 16S rRNA gene sequence amplification, PCR reactions used Taq Mastermix (New England BioLabs) along with 100nM 1391R (3' - GACGGGCGGTGTGTRCA - 5', Turner et al., 1999) reverse primer, 100nM 8F (3' - AGAGTTTGATCCTGGCTCAG - 5', Turner et al., 1999) forward primer, and 2µL of DNA template to give a final volume of 20µL. PCR amplification was carried out with an Eppendorf Mastercycler Nexus Gradient PCR machine (Eppendorf Company) at a hot start at 95°C for 2 min, followed by 45 s at 95°C, 45 s at 58°C, and 90 s at 72°C for 32 cycles. This was followed by an elongation cycle at 72°C for 10 min.

The fungal ITS DNA sequence PCR amplified with fungal primers ITS-4 (Sequence: 5' TCCTCCGCTTATTGATATGC 3', Bellemain et al., 2010) and ITS-5 (Sequence: 5' GGAAGTAAAAGTCGTAACAAGG 3', Bellemain et al., 2010). PCR amplification was carried out with a hot start at 94°C for 5 min, followed by 45 s at 94°C, 60 s at 60°C, and 90 s at 72°C for 36 cycles. This was followed by an elongation cycle at 72°C for 5 min.

All PCR products were gel purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine CA) and cloned using a TOPO TA cloning kit with pCR 4-TOPO vector (Invitrogen, Carlsbad CA), following the manufacturer's protocol. Transformation was then performed with One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, Carlsbad CA). Clones were analyzed via restriction fragment length polymorphism (RFLP) using the restriction

enzymes *HinI1* and *Msp1*. Unique clones were sent to the Advanced Genetics Technology Center (Lexington, KY) for Sanger sequencing.

### *Phylogenetics*

DNA sequences were imported into Geneious 6.1.2 software, (<http://www.geneious.com>, Kearse et al., 2012), de novo assembled and trimmed to remove poor quality base calls. The sequenced fungal isolates were assembled separately and reached at least 3x depth coverage each. Initial analysis was performed using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). GenBank database was used to identify fungal isolates with similar ITS sequences (Clark et al., 2016). These selected GenBank sequences were aligned with our fungal assemblies using MUSCLE (Edgar, 2004) and then trimmed in MEGA version 6.06 (Tamura et al., 2013). The alignment was then used to produce a maximum likelihood phylogenetic tree using the RAxML-HPC Blackbox version 8.2.10 (Stamatakis, 2014) hosted within the CIPRES Science Gateway (Miller et al., 2010). The phylogenetic tree (Figure 3) was created using Figtree version 1.4.2 (Rambaut, 2006).

## **Results**

Given the role of bacteria in the precipitation of Fe(III)-oxides in canga, we began our experiment under the assumption that it would be possible to isolate bacteria involved in iron oxidation from our samples. To look for these bacteria we began by serially diluting cave samples, from three cave sites (Table 2), down to  $1 \times 10^{-5}$  and plating on Fe(II) media at pH 3.5, to mimic the environmental chemistry of the caves (Treatment 1; Table 1). Using this approach we detected Fe(II)-oxidizing species by screening for the presence of colonies that precipitated Fe(III) in the media, indicated by the production of orange Fe(III) oxides.

Table 2. Geochemistry of tested sample sites.

Sample Site	pH	Dissolved O <sub>2</sub> (mg/L)	NO <sub>3</sub> <sup>-</sup> (mg/L)	NO <sub>2</sub> <sup>-</sup> (mg/L)	Total Fe (mg/L)	Fe(II) (mg/L)
Triangle Cave Pool	4.86	nd	0.83	0.02	0.27	0.17
Poop Cave Pool A	6.40	1.8	2.50	0.05	<i>nd</i>	<i>nd</i>
Poop Cave Pool B	4.2	5.4	2.40	0.03	0.19	1.55

*nd* = not done

Our initial experiments revealed the presence of numerous Fe(II)-oxidizing colonies after 6-8 days of growth from multiple sample sites; however, almost all of the colonies appeared to be fungal on visual inspection, with one culture displaying a mix of fungi and bacterial colonies. We therefore began an exhaustive process in an attempt to differentiate any FeOB from the fungi (Table 1).

We made several attempts to select for FeOB away from the fungi in our samples. This included reducing the amount of fungal material in the samples by sonication. Media was made with several concentrations of NYS and CYO to attempt and isolate FeOB, however only fungus grew up to 100 µg/mL or nothing grew above 1,000 µg/mL. We also used discs soaked in different antifungal agents to allow the growth of FeOB within the antifungal zone. Several solutions were used with the discs: 50% methanol and 50% water with CYO, 100% methanol with CYO, 50% methanol and 50% water with NYS, 100% methanol with NYS (Table 1). The final concentrations for NYS and CYO was 40 mg/mL. The discs were placed in the center of a media plate where a fungal colony was cross-streaked to form an X-pattern presumably allowing the growth of the bacteria closer to the disc.

NYS was much less lethal as an antifungal than CYO, and had a much larger effective zone of inhibition. NYS had a small amount of fungus near the diffusion disc, and the first growth outside of the zone of inhibition was severely diminished fungal with no oxidized iron, then further out was fungal associated with oxidized iron. The iron deposits were completely surrounded by fungus suggesting the isolated fungi were NYS resistant. Each sample was

repeated in duplicate, comparing the 50% methanol and 100% methanol with 40 mg/mL CYO solutions to see if there was a difference in effectiveness between the two solutions. Despite these repeated attempts, all the plates with Fe(II) oxidation contained fungal growth. We used a sonication dilution series to break up the iron-oxidizing colonies to increase the likelihood of separating the fungal and bacterial species in pure culture, but still did not isolate FeOB in culture (Table 1).

These data suggested that we were unable to isolate FeOB from our samples and indicate that observed Fe(II)-oxidation might be due to fungal activity. To determine if this was the case we repeated the X-pattern streaking but added the anti-bacterial antibiotics of AMP and S. At the inhibitory concentration of these antibiotics AMP and S there was no zone of inhibition, but we did observe oxidized iron and fungal growth up to the diffusion disc. This suggests that it is very likely fungus that is oxidizing and depositing the iron oxides in the media. Figure 2 shows NYS, CYO, AMP, and S diffusion discs all on a single plate and demonstrates the point of fungal iron oxidation.

To confirm the lack of bacterial involvement a Kirby Bauer test was also done on a JOM plate streaked with the fungi. Six different antibiotics were used comparative analysis; Vancomycin (VA, 30 µg/mL), Tetracycline (TE, 30 µg/mL), Penicillin (P, 2 µg/mL), S (10 µg/mL), Gentamicin (GM, 10 µg/mL), and Chloramphenicol (C, 30 µg/mL). No antibiotic proved effective against Fe(II) oxidation, and therefore the results led us to the conclusion that fungal Fe(II) oxidation was taking place. This suggested that bacteria are not involved in the observed Fe(II) oxidation seen inside the IOCs. We therefore isolated six fungal species from our assays in pure culture and extracted DNA for identification via the ITS sequences. Restriction Fragment Length Polymorphism suggest that all six isolates represented the same species, so one clone, BIC 311, was sent for Sanger sequencing. BLAST analysis of the ITS sequences followed by identification suggested that this organism is closely related to *Sagenomella stratispora*. Phylogenetic placement within the genus confirmed this identification (Fig 3).

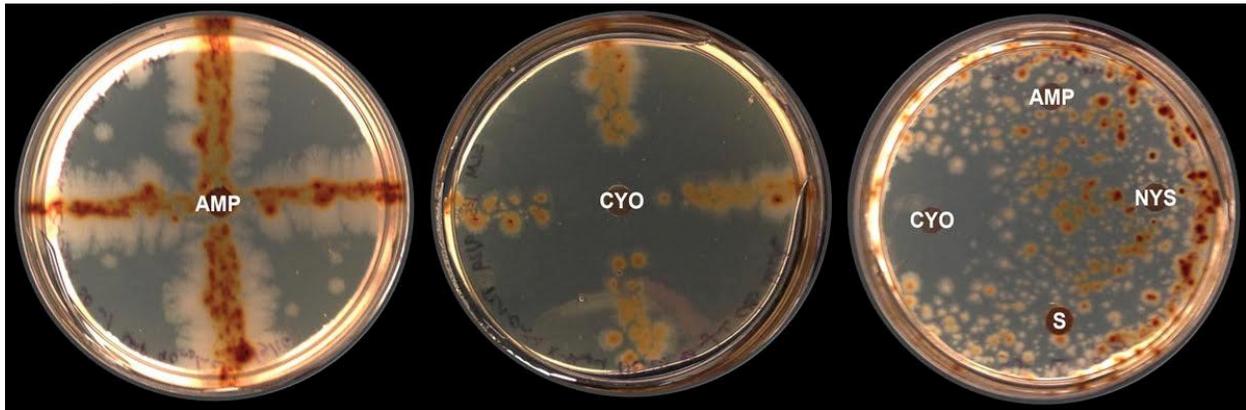


Figure 2. Production of iron oxides in the presence of antibacterial (AMP and S) and antifungal (NYS and CYO) agents.

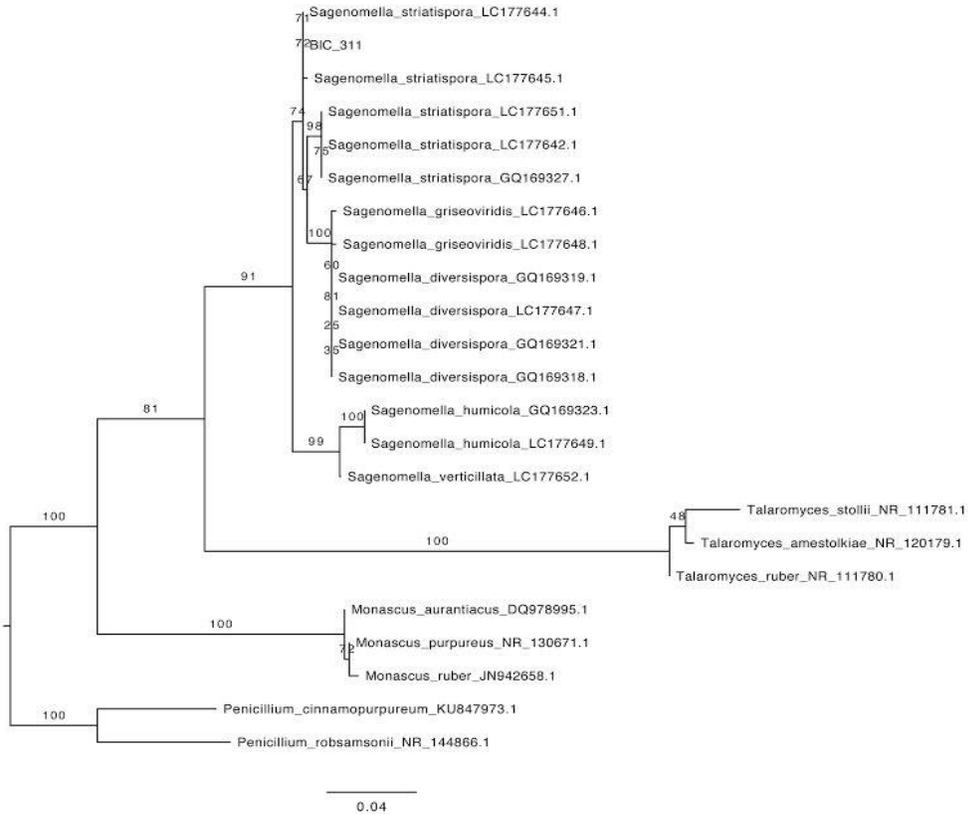


Fig 3. Phylogenetic tree including our sample (BIC 311) and relevant fungal species. BIC 311 was shown to be closely related to *Sagenomella stratispora*.

Table 3. Sample sites tested and antifungal methods attempted for FeOM isolation.  
*Sagenomella stratispora* came from Triangle Cave Pool.

Sample type	Sample Site					
	Triangle Cave Pool		Poop Cave Pool A		Poop Cave Pool B	
	Sediment	Dilutions	Sediment	Dilutions	Sediment	Dilutions
<b>Media methods used</b>						
1. No Treatment	+	nd	+	nd	+	nd
2. 10 ug NYS	+	nd	nd	+	+	nd
3. 10 ug NYS, 100 ul CYO	+	+	nd	nd	nd	nd
4. Up to 1,500 ul NYS	+	+	+	nd	nd	nd

nd = not done

## Discussion

The canga that blankets the BIF surface above the IOCs is made of hematite cemented together by iron oxides. The work of Levett et al. (2016) has demonstrated the presence of microfossils within these oxides, which are indicative of microbial biofilms approximately the size of bacterial cells. Such data suggested that FeOB might therefore be involved in the production of similar iron-oxide deposits within the cave. To test whether this was the case we attempted to purify bacterial species from rust-like iron oxide pool deposits within the cave.

Despite repeated attempts to isolate FeOB in culture, we were only able to recover fungi that we initially thought were contaminants within the cultures. While FeOB can oxidize iron using it as an electron donor in metabolism (Neubauer et al., 2002), fungi deposit iron as an extracellular detoxification process (Bumpus et al., 1985). The fungal species *Coniophora puteana* has an enzyme (cellobiose dehydrogenase) that has been shown to couple oxidation of cellobioses to conversion of Fe(III) to Fe(II) (Hyde et al., 1997). Since fungi have been shown to reduce iron, the fungal “contamination” could actually be responsible for the observed Fe(II)-oxidation.

We tested antifungals and antibacterials on growth and Fe(II)-oxidation in the cultures, and antibacterials seemed to have no effect on inhibition of fungal growth or Fe(II)-oxidation, while antifungals had varying levels of effectiveness in inhibition of fungal growth and Fe(II)-oxidation. CYO greatly inhibited the growth of the fungus while NYS had a minor impact on fungal growth, suggesting that the isolated fungi were NYS resistant. The ineffectiveness of antibacterials point to a fungal agent as the oxidizing agent, as shown in Figure 2 with antifungals having a zone of inhibition with no oxidized iron. Outside of this zone there is minimal iron oxidation associated with fungal growth, leading to the hypothesis that it is an extracellular process to detoxify the environment as the antifungal is more stressful than the Fe(II). CYO has been proven to inhibit protein synthesis in the fungal species *Saccharomyces pastorianus*, which would play a role in reducing Fe(II)-oxidation if it represents a detoxification strategy (Siegel et al., 1963). Antifungal resistance in fungi has previously been seen in high iron environments as the iron-detoxification systems often share a common mechanism with resisting antifungals (Hosogaya et al., 2013)

*Sagenomella stratispora* was originally isolated from rotting wood in a creek (Lesley, 1993) and its discovery in a cave in Brazil is no surprise, given the close proximity of the cave to the surface and the organic detritus of the surface jungle; however, this is the first time this genus has been described in a cave (Vanderwolf et al., 2013). The next step would be to test the levels of iron oxidation and determine if this fungus plays an active role in Fe(II)-oxidation.

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