INVESTIGATING THE RELATIONSHIP BETWEEN ZETAPROTEOBACTERIA AND CYANOBACTERIA AND ITS IMPLICATIONS FOR THE GEOLOGICAL RECORD

By

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Abstract

Banded Iron Formations (BIFs) are geological relics of the ancient oceans that were partially formed by microorganisms. This includes oxygenic phototrophs and iron-oxidizing bacteria (FeOB) whose relative contributions are likely influenced by their interactions with each other. Based on co-occurrence in modern environments, I propose that oxygenic phototrophs and microaerophilic FeOB may have a syntrophic relationship that aided their ability to contribute to BIF genesis. Ancient Cyanobacteria could have produced oxygen that oxidized iron while simultaneously stimulating the growth of marine microaerophilic iron-oxidizers, such as Zetaproteobacteria, by providing them oxygen in the largely anoxic ancient oceans. I first aim to identify evidence of this syntrophy within Zetaproteobacteria genomes. I constructed profile Hidden Markov Models for oxygen tolerance proteins to determine their presence in Zetaproteobacteria genomes and compared Zetaproteobacteria genomes from hydrothermal vent ecosystems to those from environments more likely to harbor Cyanobacteria. From these comparisons, we found little genomic evidence of a long term syntrophy, suggesting that their

relationship may be opportunistic rather than tightly coupled. Currently a Cyanobacteria is being isolated from the same environment that the Zetaproteobacterium *Mariprofundus erugo* was cultivated. Microscopy suggests that a *Synechococcus sp*. and other coccoid Cyanobacteria are present in those phototrophic enrichment cultures. This isolate can be used in future co-culture experiments to further investigate this relationship and its implications for BIFs.

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Introduction

Marine, iron oxidizing Zetaproteobacteria have been found growing alongside Cyanobacteria in environments with low levels of oxygen, sufficient light, and an availability of reduced iron (Field et al. 2016). These environmental conditions, capable of supporting both metabolic strategies, were abundant throughout the late Archaean and early Proterozoic oceans (Poulton and Canfield 2011) and the geological record supports the existence of both organisms during that time period (Dodd et al. 2017; Planavsky et al. 2014; Lin et al. 2019). This suggests that Zetaproteobacteria and Cyanobacteria have a relationship that began in the late Archaean Eon and that their interactions are relevant to ancient phenomena persevered in the geological record such as banded iron formation (BIF) precursors and the Great Oxidation Event (GOE) (Chan, Emerson, and Luther 2016; Farquhar, Zerkle, and Bekker 2011; Posth, Konhauser, and Kappler 2013). In fact, it has been hypothesized that Cyanobacteria may benefit and enrich the growth of Zetaproteobacteria (Field et al. 2016). Through this interaction it is possible that ancient oxygenic phototrophs and microaerophilic iron oxidizers may have contributed to BIFs mutually. There is evidence that oxygen accumulated in localized marine environments preceding the GOE by up to 500 million years, potentially enabling microaerophilic metabolisms (Anbar et al. 2007; Planavsky et al. 2014), which means that a mutualism may have begun upwards of 3 billion years ago impacting both the GOE and BIF genesis.

Prior to the emergence and surge of oxygenic photosynthesis, the Archaean Ocean was largely devoid of oxygen and was ferruginous (Poulton and Canfield 2011). This changed between 2.3 and 2.4 billion years ago during the GOE when oxygen accumulated in the atmosphere due to oxygenic photosynthesis by ancient Cyanobacteria (Kump 2008; Lyons, Reinhard, and Planavsky 2014; Schirrmeister, Gugger, and Donoghue 2015). Sedimentary rocks

dating between 2.3 and 2.4 Gyr ago show the disappearance of mass-independent fractionation (MIF) of sulfur isotopes, which can only form when there is little oxygen in the atmosphere therefore indicating a rapid rise in atmospheric oxygen during that time (Lyons, Reinhard, and Planavsky 2014; Farquhar, Zerkle, and Bekker 2011; Farquhar, Bao, and Thiemens 2000). However, it has been proposed that oxygen existed in localized environments preceding the Great Oxidation Event by up to 500 million years (Anbar et al. 2007; Planavsky et al. 2014). This was suggested due to the appearance of metals such as molybdenum and rhenium in oceanic shale (Anbar et al. 2007) as well as evidence of manganese oxidation within the ancient geological record (Planavsky et al. 2014), which indicates oxidative weathering occurred along the ocean margins prior to the GOE. *Figure 1* illustrates this emerging model of the GOE compared to the classic model. These localized "whiffs" of oxygen may have enabled microaerophilic metabolisms along the continental and coastal margins preceding the GOE. Despite evidence of oxidative weathering prior to the GOE, however, there remains debate about when oxygenic photosynthesis first appeared, with the maximum estimate being around 3.6 Gyr ago (Frei et al. 2016) and the minimum estimates coinciding with the GOE (Raymond and Segrè 2006).

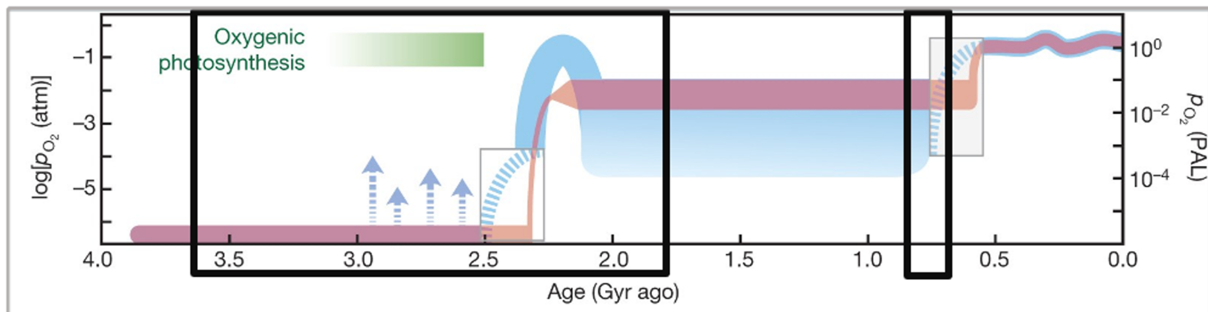


Figure 1: Atmospheric oxygen over time
The blue line represents the emerging model of the GOE, with the arrows indicating evidence of localized oxygen production. The pink line represents the classical model. The green bar indicates the origin/presence of oxygenic photosynthesis. Black boxes (my addition) outline time periods corresponding to BIF genesis. Adapted from: (Lyons, Reinhard, and Planavsky 2014).

The geological record also contains BIFs, which are sedimentary deposits made of iron-rich and silica-rich layers (Posth, Konhauser, and Kappler 2013; Kappler et al. 2005). These iron formations date from about 3.8 Gyr to 0.6 Gyr, with a gap between 1.6 Gyr and 0.8 Gyr (*Figure 1*) (C. Klein 2005). Iron-rich minerals within BIFs contain both ferrous and ferric iron with an average oxidation state of $Fe^{2.4+}$ and include magnetite, siderite, greenalite, and hematite (Posth, Konhauser, and Kappler 2013; C. 2005; C. Klein 1992). The minerals observed today are a result of metamorphosis and diagenesis and therefore differ from the Fe(III) oxide and Fe(III) oxyhydroxide primary BIF minerals that were formed in the ancient oceans (Posth, Konhauser, and Kappler 2013; C. Klein 2005). The main source of iron in the ancient oceans was Fe(II), so formation of Fe(III) oxides and oxyhydroxides, which are BIF precursors, required a source of iron oxidation (Poulton and Canfield 2011; C. Klein 2005). Possible sources include 1) photochemical oxidation, 2) abiotic oxidation from oxygen produced by ancient Cyanobacteria, 3) anoxygenic photoferrotrophy, and 4) microaerophilic iron oxidation (Chan, Emerson, and Luther 2016; Braterman, Cairns-Smith, and Sloper 1983; Posth, Konhauser, and Kappler 2013). Because the ancient Earth lacked an ozone layer, UV light has been speculated as one source of iron oxidation, but simulated experiments indicate that such photochemical oxidation would have been trivial compared to other oxidants (Konhauser et al. 2007). This means that microorganisms likely played a significant role in the formation of BIF precursors.

It is generally accepted that BIFs dating before the GOE were a result of ancient photoferrotrophy (Kappler et al. 2005; Chan, Emerson, and Luther 2016). This is because iron oxidation by O_2 occurs at a relatively slow rate at low oxygen concentrations, which would have been kinetically unfavorable compared to photoferrotrophy at pre-GOE oxygen concentrations (Kappler et al. 2005; Sung and Morgan 1980). Furthermore, photochemical oxidation has been

shown to be negligible in ancient seawater conditions (Konhauser et al. 2007). Because the origin of oxygenic photosynthesis may have preceded the GOE, however, it is possible that microaerophilic iron oxidation also played a role in generating BIF precursors prior to the GOE.

As atmospheric and oceanic oxygen levels rose, abiotic iron oxidation by O_2 and biotic iron oxidation by microaerophilic bacteria, such as the Zetaproteobacteria, could have become increasingly important players in BIF genesis. Fe(III) oxyhydroxides, which are BIF precursors (Sun et al. 2015; C. Klein 2005), are produced by Zetaproteobacteria as mineral stalks, sheaths, or dreads during iron oxidation (Laufer et al. 2017; Fleming et al. 2013; Chiu et al. 2017). Cyanobacteria can form Fe(III) oxyhydroxides indirectly by producing oxygen which chemically oxidizes Fe(II) (Bekker et al. 2010). In this proposed model of mutual BIF formation, Cyanobacteria produced oxygen leading to the formation of BIF precursors while also stimulating the growth of microaerophilic Zetaproteobacteria that oxidized iron biotically (Field et al. 2016). The oldest BIF was formed approximately 3.8 Gyr (Mloszewska et al. 2012) and BIFs are largely absent from the geological record at 1.8 Gyr until a brief reappearance around 0.8 Gyr (Bekker et al. 2010). Because marine oxygen concentrations and Cyanobacteria abundance fluctuated within those time frames, understanding when Zetaproteobacteria played a role in BIF genesis requires an understanding of their oxygen tolerance and relationship to Cyanobacteria.

Microbially facilitated iron oxidation at circumneutral pH, the metabolic strategy used by Zetaproteobacteria, is generally restricted to low oxygen environments due to near instantaneous abiotic oxidation rates of Fe(II) at high concentrations of O_2 (Sung and Morgan 1980; Hedrich, Schlömann, and Johnson 2011). Zetaproteobacteria isolates from the Chesapeake Bay, an ancient ocean analog where Cyanobacteria were also recovered, grew in bands within the gradient tubes

that contained less than 2 μM O_2 (Chiu et al. 2017). *Mariprofundus ferrooxydans* PV-1, a strain of Zetaproteobacteria isolated from the Lo'ihi Seamount hydrothermal vent environment, was shown to outcompete abiotic iron oxidation with Fe(II) concentrations starting at 154 μM and oxygen concentrations below 49 μM (McAllister et al. 2019). These may be general proxies for environmental limitations among Zetaproteobacteria, but genomic evidence suggests that OTUs within the class differ in their oxygen-related adaptations (Field et al. 2015), influencing which clades may form a relationship with Cyanobacteria.

In the present day, Cyanobacteria and Zetaproteobacteria have been found in environments alongside one another. This includes the open ocean where Zetaproteobacteria and Cyanobacteria were both detected in 87 out of 139 locations sampled by the Tara Oceans Microbiome Project, although environmental oxygen data is available, reduced iron data is not (Sunagawa et al. 2015). Another environment is the sub-oxic zone of the Chesapeake Bay which yielded phototrophic enrichment cultures that cultivated both Zetaproteobacteria and Cyanobacteria with Fe(II) concentrations as high as 100 μM Fe(II) (Field et al. 2016). Conditions in the Chesapeake Bay become ideal for this interaction seasonally, with around 3μM of both Fe(II) and O_2 in depths ranging between about 6 to 12 m during the summer months (MacDonald et al. 2014). Cyanobacteria and Zetaproteobacteria have also been found coexisting in a hot spring environment with Fe(II) concentrations as high as 150 μM and O_2 concentrations as low as 5μM (Ward et al. 2019). In this environment, the interaction was spatially constrained by opposing gradients of dissolved oxygen and Fe(II).

Coexistence in ferruginous environments is surprising because Cyanobacteria can be intolerant to high concentrations of Fe(II) (Swanner, Mloszewska, et al. 2015; Swanner, Wu, et al. 2015). They have been shown to undergo iron toxicity by accumulating intracellular reactive

oxygen species (ROS) in ferruginous environments and experience oxidative stress at 10 μM of Fe(II) (Swanner, Mloszewaska, et al. 2015; Shcolnick et al. 2009). Additionally, the Cyanobacterium *Synechococcus* PCC 7002 has decreased growth rates at 100 μM Fe(II) and significantly reduced pigment levels at 70 μM Fe(II) (Swanner, Mloszewska, et al. 2015; Swanner, Wu, et al. 2015). In experiments with *Synechococcus* PCC 7002, cultures starting with 4800 μM Fe(II) reached the same cell counts as those with 7.5 μM Fe(II) once the majority of iron was oxidized abiotically, indicating that iron oxidation alleviated iron toxicity (Swanner, Wu, et al. 2015). At lower concentrations of oxygen, iron oxidation by Zetaproteobacteria happens at a faster rate than abiotic oxidation by oxygen (McAllister et al. 2019) which means that the presence of microaerophilic iron oxidizers, such as Zetaproteobacteria, may benefit Cyanobacteria by the removal of Fe(II).

Because they are found together in modern environments, it is possible that Zetaproteobacteria and Cyanobacteria share an active relationship. Here, I propose that they share a syntrophic relationship that has aided their ability to survive in unlikely environments. Microbial syntrophy has been defined as an “obligately mutualistic metabolism” where the metabolism of each individual is dependent on the metabolic activity of the other in a mutually beneficial way (Morris et al. 2013). However, it has been noted that all syntrophic organisms may be capable of surviving if provided with the byproducts typically produced by their partner organisms, so syntrophic relationships may be facultative (Morris et al. 2013). Syntrophy is a type of mutualism because both organisms in the relationship experience a positive outcome or benefit (Carrara et al. 2015). Syntrophy has been chosen to describe this proposed relationship because the metabolic activity of each organism is central to this model (Zetaproteobacteria

benefit from the O_2 produced by Cyanobacteria and the Cyanobacteria benefit from iron oxidation by the Zetaproteobacteria).

While we are knowledgeable about iron metabolism and tolerance in Cyanobacteria, there remains limited data on oxygen tolerance in Zetaproteobacteria. Consequently, my first research question is: *Is there a genomic signature for specific Zetaproteobacteria that suggests it can form a syntrophic relationship with a Cyanobacteria?* I hypothesize that Zetaproteobacteria from environments where Cyanobacteria can be found will have more ROS scavenging genes and oxygen-tolerance genes, indicating their potential to form a syntrophic relationship with Cyanobacteria. To investigate the relationship *in vitro,* co-culture experiments will be necessary to investigate my second question: *Is the growth of Zetaproteobacteria and Cyanobacteria enhanced when grown together in BIF/GOE relevant Fe(II) concentrations compared to grown individually?*

Chapter 1: Genomic evidence suggests a passive relationship between Zetaproteobacteria and Cyanobacteria

1.1 Introduction

While there is considerable research about Fe(II) metabolism and tolerance in Cyanobacteria, there few experiments relating to oxygen tolerance in Zetaproteobacteria. Although O_2 is necessary for microaerophilic FeOB due to its function as a terminal electron acceptor, it can cause difficulties by generating reactive oxygen species (ROS) when it is partially reduced. These ROS may be even more important in ferruginous conditions because iron reacts with hydrogen peroxide, which naturally occurs in marine ecosystems (Miller and Kester 1994), to form damaging hydroxyl radicals through a process known as Fenton chemistry (Wardman and Candeias 1996; Winterbourn 1995). Furthermore, ROS are a byproduct of aerobic metabolisms and intracellular H_2O_2 concentration increases with O_2 concentration, indicating that ROS scavenging is necessary for oxygen tolerance and therefore a relationship with oxygen producing Cyanobacteria (J. A. Imlay 2008; Seaver and Imlay 2004). To survive in marine environments that give access to both oxic and ferruginous conditions, Zetaproteobacteria need mechanisms to inactivate hydroxyl radicals, hydrogen peroxide, and other ROS. These mechanisms can include utilizing ROS scavenging proteins such as catalases and peroxidases (Mishra and Imlay 2012) which are summarized in *Box 1*. Genes encoding ROS scavenging proteins have been studied in the class's model genome (Singer et al. 2011) and have been found in other Zetaproteobacteria genomes (Field et al. 2015; Garrison, Price, and Field 2019; Mori et al. 2017), indicating the potential for their involvement in a mutualism with Cyanobacteria.

Box 1: Summary of ROS scavenging proteins used for HMM analysis

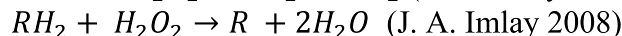
Alkyl Hydroperoxide Reductase: $NADH + H^+ + H_2O_2 \rightarrow 2H_2O + NAD^+$ (J. A. Imlay 2013)

Alkyl Hydroperoxide Reductase (AHP) is an NADH peroxidase capable of scavenging both organic hydroperoxides and H_2O_2 (Mishra and Imlay 2012). AHP has been shown to be necessary for oxygen tolerance in microaerophilic bacteria (Baillon et al. 1999) and is the primary scavenger of H_2O_2 in *E. coli* when H_2O_2 concentrations are low (Seaver and Imlay 2001).

Catalase: $2H_2O_2 \rightarrow 2H_2O + O_2$ (J. A. Imlay 2013)

Catalase scavenges H_2O_2 and produces water and O_2 as a byproduct. It is the primary scavenger in *E. coli* when H_2O_2 levels are elevated beyond what is typically found in nature (Baillon et al. 1999; Mishra and Imlay 2012; Seaver and Imlay 2001).

Catalase Peroxidase: $2H_2O_2 \rightarrow 2H_2O + O_2$ (J. A. Imlay 2013)



$RH_2 + H_2O_2 \rightarrow R + 2H_2O$ (J. A. Imlay 2008)

Catalase-peroxidase (CP) enzymes are bifunctional and can carry out both catalase and peroxidase reactions as listed above (Mishra and Imlay 2012). Although they are both H_2O_2 scavengers, CPs are less abundant than catalases (Mishra and Imlay 2012; Passardi et al. 2007).

Superoxide Dismutase: $2O_2^- \rightarrow H_2O_2 + O_2$ (J. A. Imlay 2013)

Superoxide dismutases catalyze the conversion of superoxide into O_2 and H_2O_2 and have been shown to be necessary for oxygen tolerance in strains of *E. coli* (Carlioz and Touati 1986). SODs are thought to have evolved independently three times and can be grouped based on their metal cofactors: Cu/Zn, Ni, and Fe/Mn (A.F. Miller 2012).

Cytochrome c Peroxidase: $RH_2 + H_2O_2 \rightarrow R + 2H_2O$ (Atack and Kelly 2006; Mishra and Imlay 2012)

Bacterial cytochrome c peroxidases are located within the periplasm and contain two c-type haems (Atack and Kelly 2006). Although it plays a role in mitigating oxidative stress, it has also been shown to use H_2O_2 as a terminal electron acceptor in oxygen limiting environments.

Glutathione Peroxidase: $2GSH + H_2O_2 \rightarrow GS - SG + 2H_2O$ (Margis et al. 2008)

Glutathione peroxidase (GPX) uses reduced glutathione (GSH) as an electron donor to convert H_2O_2 into glutathione disulfide (GS-SG) and water (Margis et al. 2008). Although bacterial GPX has been shown to function as an ROS scavenger, its physiological function is unclear (Mishra and Imlay 2012).

Beyond increasing oxygen tolerance, ROS scavenging proteins in Zetaproteobacteria may also benefit Cyanobacteria. Iron toxicity in Cyanobacteria can be mediated through ROS scavenging by other microorganisms, as was shown with co-culture experiments using *Synechococcus* and *Shewanella* species (Szeinbaum et al. 2021). Furthermore, catalase in heterotrophic bacteria has been shown to successfully mitigated harmful effects of H_2O_2 on *Prochlorococcus* (Morris et al. 2011). Alkyl hydroperoxide reductase (AHP) and catalase in *E. coli* are each capable of scavenging extracellular H_2O_2 before it enters the cell, further supporting the idea that ROS scavenging can be involved in microbial mutualisms (Mishra and Imlay 2012). Zetaproteobacteria with higher numbers of ROS scavenging-related genes may be more likely to share a syntrophic relationship with Cyanobacteria than other Zetaproteobacteria.

If Cyanobacteria and Zetaproteobacteria developed a syntrophy in the ancient oceans that persists today, we can expect to see evidence of coevolution in Zetaproteobacteria genomes. Specifically, those Zetaproteobacteria would likely have an increased number and diversity of genes relating to oxygen tolerance compared to Zetaproteobacteria that did not remain alongside Cyanobacteria. Since some Zetaproteobacteria have been found in environments conducive Cyanobacterial growth and others have been found in deep hydrothermal vent systems, which are inhospitable to Cyanobacteria, we can compare the genomes of Zetaproteobacteria from each group to look for evidence of a syntrophy.

1.2 Research Question

Is there a genomic signature for specific Zetaproteobacteria that suggests it can form a syntrophic relationship with a Cyanobacteria?

1.3 Hypothesis

I hypothesize that Zetaproteobacteria from environments where Cyanobacteria can be found will have more ROS scavenging genes and oxygen-tolerance genes, indicating their potential to form a syntrophic relationship with Cyanobacteria, than those Zetaproteobacteria that live in the absence of Cyanobacteria.

1.4 Methods

Profile Hidden Markov Models

Fifty Zetaproteobacteria genomes representing at least 14 OTUs from 13 different environments were downloaded from the IMG database of the DOE's Joint Genome Institute for analysis (Chen et al. 2020; Mukherjee et al. 2020). These consisted of 16 isolate genomes, 15 metagenome-assembled genomes, and 19 single amplified genomes (*Table 1*). Profile Hidden Markov Models (HMMs) were used to search the chosen Zetaproteobacteria genomes for homologs of the following ROS scavenging-related proteins: catalase, catalase peroxidase, cytochrome c peroxidase, alkyl hydroperoxide reductase, Cu/Zn superoxide dismutase, Fe/Mn superoxide dismutase, and glutathione peroxidase (*Box 1*). For each protein, reviewed bacterial protein sequences were downloaded from UniProt (Boutet et al. 2007) and aligned using Clustal Omega (Sievers et al. 2011). To avoid bias in the HMMs, sequences were collapsed based on 90% identity as demonstrated in the literature (Garber et al. 2020). Next, the HMMs were built using HMMER 3.3 along with the collapsed and aligned sequences (S. R. Eddy 1995; S. R. Eddy 2011). To verify that the HMMs accurately identified the correct proteins, each HMM was

searched through a bacterial genome with known genes encoding the corresponding protein. Because the cytochrome c peroxidase HMM was non-discriminately pulling out sequences with the cytochrome c domain (with low E-values), the CCP (PF03150) HMM from Pfam was used instead of the built HMM for further analyses (Mistry et al. 2020). Once all HMMs were verified for accuracy, Zetaproteobacteria genomes (*Table 1*) were searched with each HMM using HMMER 3.3 (S. R. Eddy 1995; S. R. Eddy 2011). Cut-off E values were tailored for each protein by searching returned sequences in Pfam and NCBI BLAST to verify the results (Mistry et al. 2020; Altschul et al. 1990).

Table 1: 50 Zetaproteobacteria genomes used for analysis
"Type" refers to the representative that was sequenced. SAG = single cell amplified genome, MAG = metagenome assembled genome.
* Indicates OTU designation differs depending on method used.

IMG ID	Name	OTU	Type	Environment	Study
2775506949	*Mariprofundus ferrinatatus CP8*	37	Isolate	Chesapeake Bay, water column	(Chiu et al. 2017)
2775506950	*Mariprofundus aestuarium CP-5*	18	Isolate	Chesapeake Bay, water column	(Chiu et al. 2017)
2724679736	*Mariprofundus micogutta ET2*	18	Isolate	Hydrothermal field sediment	(Makita et al. 2018)
2571042359	*Mariprofundus sp. DIS-1*	18	Isolate	Steel Coupon - West Boothbay Harbor	(Mumford, Adaktylou, and Emerson 2016)
2571042360	*Mariprofundus sp. EKF-M39*	36	Isolate	Loihi Iron Mat	(Field et al. 2015)
2513237158	*Mariprofundus ferrooxydans M34*	11	Isolate	Loihi Iron Mat	(McAllister et al. 2011)
639857004	*Mariprofundus ferrooxydans PV-1*	11	Isolate	Loihi Iron Mat	(Singer et al. 2011)
2648501925	*Mariprofundus ferrooxydans JV-1*	11	Isolate	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2582580733	*Ghiorsea bivora TAG-1*	9	Isolate	MAR Hydrothermal Vent Iron Mat	(Mori et al. 2017)
2524614796	*zeta proteobacterium SCGC AB-137_C09B*	6	SAG	Loihi Iron Mat	(Field et al. 2015)
2264867015	*Zeta proteobacterium SCGC AB-602-E04*	6	SAG	Loihi Iron Mat	(Field et al. 2015)
2264867009	*Zeta proteobacterium SCGC AB-137-C09*	6	SAG	Loihi Iron Mat	(Field et al. 2015)
2264867013	*Zeta proteobacterium SCGC AB-133-G06*	1/6*	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614781	*zeta proteobacterium SCGC AB-602_F03*	2	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614794	*zeta proteobacterium SCGC AB-137_G16:*	2	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614793	*zeta proteobacterium SCGC AB -133_M17*	2	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614792	*zeta proteobacterium SCGC AB-604_P22*	2	SAG	Loihi Iron Mat	(Field et al. 2015)
2265123003	*Zeta proteobacterium SCGC AB-137-I08*	2	SAG	Loihi Iron Mat	(Field et al. 2015)
2264867014	*Zeta proteobacterium SCGC AB-133-C04*	2	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614795	*zeta proteobacterium SCGC AB-604_O11*	4	SAG	Loihi Iron Mat	(Field et al. 2015)
2264867012	*Zeta proteobacterium SCGC AB-604-O16*	4	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614790	*zeta proteobacterium SCGC AB-137_M18*	1	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614791	*zeta proteobacterium SCGC AB-602_L11*	1	SAG	Loihi Iron Mat	(Field et al. 2015)
2524614788	*zeta proteobacterium SCGC AB-133_D10*	14/1	SAG	Loihi Iron Mat	(Field et al. 2015)
2264867010	*Zeta proteobacterium SCGC AB-137-J06*	14/1	SAG	Loihi Iron Mat	(Field et al. 2015)

2264867008	*Zeta proteobacterium SCGC AB-602-C20*	14/1	SAG	Loihi Iron Mat	(Field et al. 2015)
2786546605	*Zetaproteobacteria bacterium CG03_land_8_20_14_0_80_59_51*	10	SAG	Loihi Iron Mat	(Field et al. 2015)
2264867011	*Zeta proteobacterium SCGC AB-604-B04*	10	SAG	Loihi Iron Mat	(Field et al. 2015)
2757320575	*Unclassified zetaproteobacterium sp. KV*	3	Isolate	Coastal sediment	(Blackwell et al. 2020)
2757320574	*Unclassified zetaproteobacterium sp. NF*	3	Isolate	Coastal sediment	(Blackwell et al. 2020)
2895121536	*Mariprofundus erugo P3*	N/A	Isolate	Steel Coupon - Mallard Creek, Pamlico River, NC	(Garrison, Price, and Field 2019)
2895150109	*Mariprofundus erugo P7*	N/A	Isolate	Steel Coupon - North Creek, Pamlico River, NC	(Garrison, Price, and Field 2019)
2898187551	*Mariprofundus ferrooxydans O-1*	N/A	Isolate	Shipwreck Pamlico Sound, NC	(Price et al. 2020)
2786546601	*Zetaproteobacteria bacterium CG06_land_8_20_14_3_00_59_53*	N/A	MAG	Subsurface Groundwater	(Probst et al. 2018)
2836439418	*Unclassified Zetaproteobacteria bacterium 664-BS3_FULL_No.7_0.4_17*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836504328	*Unclassified Zetaproteobacteria bacterium 476-BS1_FULL_No.2_1.0_85*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836506071	*Unclassified Zetaproteobacteria bacterium 479-BS3_FULL_No.1_66.1_97*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836510713	*Unclassified Zetaproteobacteria bacterium 479-BS3_FULL_No.3_3.0_26*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836511590	*Unclassified Zetaproteobacteria bacterium 479-BS3_FULL_No.4_1.8_52*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836508227	*Unclassified Zetaproteobacteria bacterium 479-BS3_FULL_No.2_3.9_94*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836513220	*Unclassified Zetaproteobacteria bacterium 479-BS4_FULL_No.1_63.1_19*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836514127	*Unclassified Zetaproteobacteria bacterium 479-BS4_FULL_No.2_6.8_91*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836516342	*Unclassified Zetaproteobacteria bacterium 479-BS4_FULL_No.3_3.6_69*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836518008	*Unclassified Zetaproteobacteria bacterium 479-BS4_FULL_No.4.1_2.6_47*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836519699	*Unclassified Zetaproteobacteria bacterium 479-BS4_FULL_No.4.2_2.6_27*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836520566	*Unclassified Zetaproteobacteria bacterium 479-BS4_FULL_No.5_2.1_15*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)
2836521333	*Unclassified Zetaproteobacteria bacterium 479-BS4_FULL_No.6_1.8_70*	N/A	MAG	Loihi Iron Mat	(Fullerton, Hager, and Moyer 2015)

2781125668	*Zetaproteobacteria bacterium EBB1*	14	Isolate	pyrrhotite biofilm	(Lopez et al. 2019)
2617270712	*Zetaproteobacteria bacterium SV108*	9	Isolate	Mariana black arc hydrothermal vent	(Mori et al. 2017)
2786546582	*Zetaproteobacteria bacterium CG2_30_46_52*	N/A	MAG	Crystal Geyser, Utah,	(Emerson et al. 2016)

Genome Comparisons

For genomes without completeness estimates reported in the literature, percent completeness was determined using Checkm (Parks et al. 2015). 13 complete or near complete (>97%) Zetaproteobacteria isolate genomes were uploaded into the Rapid Annotation using Subsystem Technology (RAST) server for comparison (Aziz et al. 2008; Overbeek et al. 2014; Brettin et al. 2015). Genomes were grouped into one of two categories based on the type of environment from which the Zetaproteobacteria were isolated: "Cyano-friendly" and "hydrothermal vent." Cyano-friendly Zetaproteobacteria were isolated from environments that include: the water column in the Chesapeake Bay, metal coupons incubated in estuarine systems, coastal sediment surface, and a shallow-water shipwreck. Three of these environments have confirmed Cyanobacteria present either through amplicon sequencing data (Garrison, Price, and Field 2019; Price et al. 2020) or cultivation (Field et al. 2016). Other environments were placed into this category because they are similar to environments where Cyanobacteria are found such as shallow coastal and benthic ecosystems (Caires et al. 2018; Brito et al. 2012), estuarine biofouling communities (Abed, Al Fahdi, and Muthukrishnan 2019), and incubated metal coupons (Messano et al. 2014). Most of the Zetaproteobacteria genomes in the hydrothermal vent category came from the Loihi Seamount hydrothermal vent systems which are >1000m below the sea surface, leaving no light for phototropic growth.

RAST categorizes each gene into one of 27 subsystems or leaves them uncategorized. For each genome, the number of genes in each subsystem was divided by the total number of genes

categorized into a subsystem to get the proportion in each subsystem (*Table 6*). The number of genes that RAST was able to categorize into a subsystem varies for each genome, which may possibly skew the percentages shown in *Table 6*. Genomes were compared in RAST based on functional and sequence similarity. Common genes found within the oxidative stress subsystems of Zetaproteobacteria genomes were used to construct *Table 8*. In addition, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo), type was compared since different RuBisCo types are known to function best in differing O_2 and CO_2 partial pressures. To verify that *Mariprofundus ferinatatus CP8* does not have ferric uptake regulator (FUR) genes, the FUR gene sequence from *Mariprofundus aestuarium CP5* was ran through the *M. ferinatatus CP8* genome via BLAST on IMG (Chen et al. 2020; Mukherjee et al. 2020). Sequences categorized as phytochromes in RAST were ran through each corresponding genome in IMG using BLAST to compare annotations. The number of PAS domains in each genome was determined by searching IMG and RAST COGs for proteins annotated as PAS domain containing proteins.

Phylogenetic Trees

16S SSU rRNA gene sequences were gathered for Zetaproteobacteria genomes from IMG. 16S rRNA gene sequences for other members of Proteobacteria were downloaded from NCBI, with equal representation from each class within the Proteobacteria phylum. Protein sequences for Fe/Mn superoxide dismutase for Zetaproteobacteria were collected based on the HMM results. Rubrerythrin sequences were identified and downloaded based on the RAST annotation. Additional sequences used to build each tree were downloaded from NCBI. For all trees, sequences were uploaded into Unipro UGENE (Okonechnikov et al. 2012; Rose et al. 2018) and aligned using MAFFT (Katoh et al. 2002) with a gap opening penalty of 1.53, gap extension penalty of 0.12, and maximum iterative refinement of 1,000. Sequences were trimmed

to equal length and positions that had gaps for over 50% of the genomes were removed. In some cases, short sequences were omitted to optimize subsequent bootstrap support. Aligned sequences were used to build maximum likelihood trees using W-IQ-TREE (Trifinopoulos et al. 2016). For each tree, the model parameters were set to find and apply the best model and both ultrafast, with 1000 bootstrap iterations, and standard, with 100 bootstrap iterations, were used. The trees with the best bootstrap support were used for final analyses.

Statistical Analysis

Percent genome completeness (determined using Checkm) was plotted with the number of ROS scavenging genes found per genome to determine if there is a strong correlation between genome completeness and number of ROS scavenging genes found in the HMM analysis (*Figures 3a and 3b*). A Poisson regression model was used to model the trends. The shaded regions on the graphs represent a 95% confidence interval. All t-tests were two-tailed assuming equal variances and were conducted in Microsoft Excel.

1.5 Results

HMM Analysis

All analyzed genomes from isolated representatives coded at least three of the reactive oxygen scavenging (ROS) proteins and at least five OTUs contained sequences for four ROS proteins. Results from SAGs and MAGs were more variable. As shown in *Table 2*, the majority of the Zetaproteobacteria genomes analyzed, and all genomes over 90% complete, had genes for both cytochrome c peroxidase and alkyl hydroperoxide reductase. 11 of the 50 genomes (22%), recovered genes for catalase peroxidase whereas only 3 genomes recovered genes for catalase. No sequences were recovered for Cu/Zn superoxide dismutase, but 18 genomes (36%) recovered

genes for Fe/Mn type superoxide dismutase. Five out of the 50 genomes recovered sequences for glutathione peroxidase.

Table 2: Results from HMM analysis
# of Genomes out of 50 indicates how many genomes contained genes for the corresponding protein. Percentage indicates the percent of genomes analyzed that recovered genes for the given protein.

Protein	Cytochrome c peroxidase	Alkyl hydroperoxide reductase	Catalase peroxidase	Catalase	Fe/Mn Superoxide dismutase	Glutathione peroxidase
# Genomes out of 50	**39**	**32**	11	3	18	5
Percentage	**78%**	**64%**	22%	6%	36%	10%

Results from Zetaproteobacteria that are from environments with confirmed Cyanobacteria presence showed that there was a core set of ROS genes recovered: cytochrome c peroxidase, alkyl hydroperoxide reductase, and superoxide dismutase. However, upon comparison with isolate genomes from hydrothermal vent systems, where Cyanobacteria are not found, it is evident that this core repertoire is not unique to Zetaproteobacteria that are more likely to encounter Cyanobacteria (*Table 3*).

Table 3: The core set of ROS genes is not unique to "cyano-friendly" Zetaproteobacteria. Comparison of ROS genes recovered in Zetaproteobacteria isolates known to be found with Cyanobacteria (green) and those from hydrothermal vents (orange).

	Cytochrome c peroxidase	Alkyl hydroperoxide reductase	Catalase peroxidase	Catalase	Superoxide dismutase	Glutathione peroxidase
Mariprofundus aestuarium CP-5	X	X	X		X	
Mariprofundus ferrinatatus CP-8	X	X			X	
Mariprofundus ferrooxydans O-1	X	X			X	
Mariprofundus erugo P3	X	X			X	X
Mariprofundus micogutta ET2	X	X	X		X	
Mariprofundus sp. EKF-M39	X	X			X	
Mariprofundus ferrooxydans M34	X	X			X	
Mariprofundus ferrooxydans PV-1	X	X			X	
Ghiorsea bivora TAG-1	X	X	X			

16S Phylogenetic Tree

The 16S rRNA maximum likelihood phylogenetic tree (*Figure 2*) shows evolutionary relationships among a subset of the 50 Zetaproteobacteria genomes used for HMM analysis. All Zetaproteobacteria genomes with sequences recovered for Fe/Mn superoxide dismutase fall into one clade (marked with a star in *Figure 2*). This clade consists entirely of genomes from cultivated isolates of Zetaproteobacteria with at least three of the seven ROS scavenging genes recovered, suggesting that we may be selectively isolating Zetaproteobacteria with greater oxygen tolerance. This also suggests that there may be vertical inheritance of oxygen tolerance through that clade of Zetaproteobacteria.

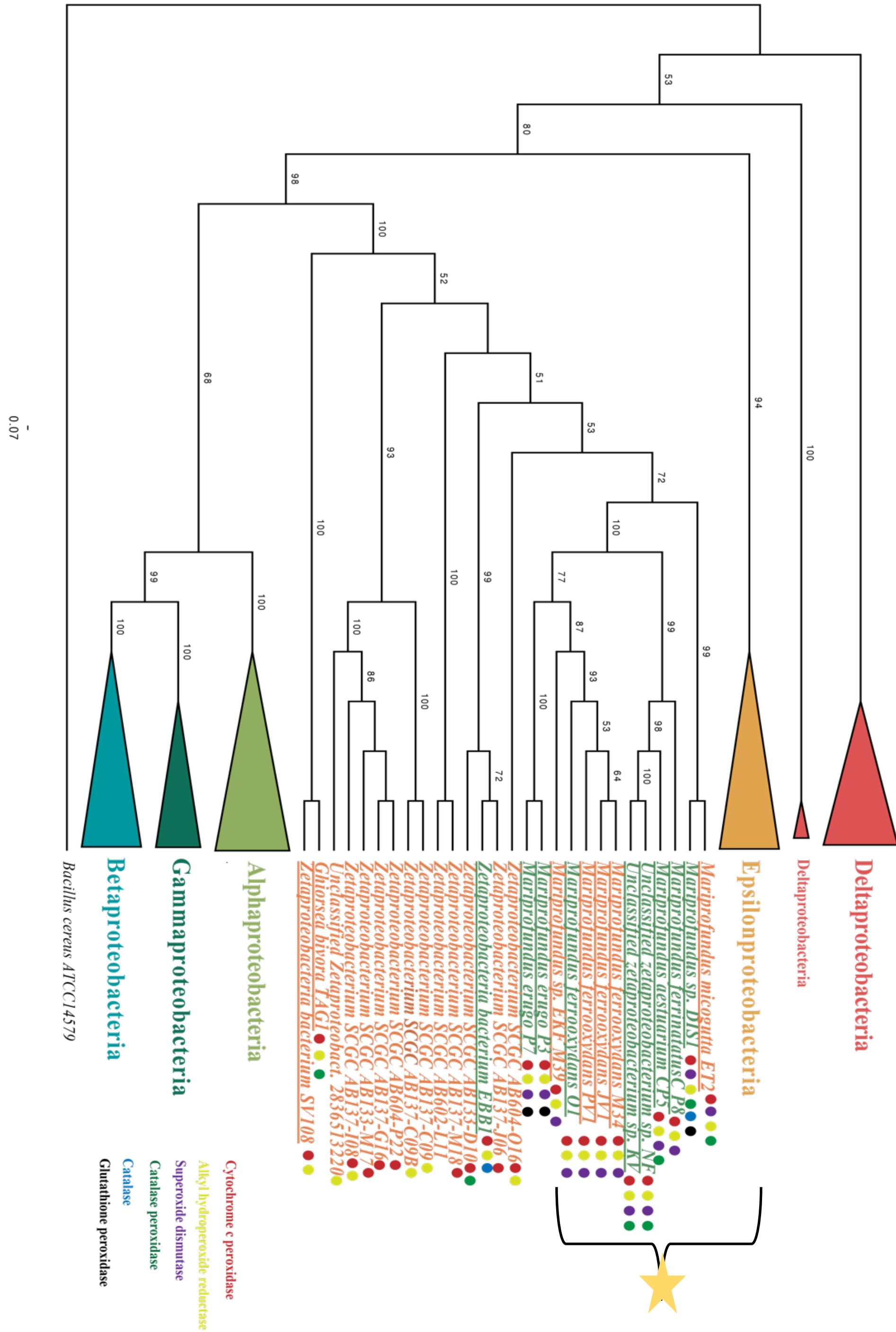


Figure 2: 16S rRNA Maximum Likelihood Phylogenetic Tree

Zetaproteobacteria in green correspond to those found in Cyanobacteria friendly environments and those in orange correspond to those found in hydrothermal vent systems. Symbols represent the presence of genes for the corresponding protein within the genome. Underline indicates isolate genome. All members within the starred clade have genes for cytochrome c peroxidase, alkyl hydroperoxide reductase, and Fe/Mn superoxide dismutase.

Comparison of Isolate and Environmental Genomes

A higher proportion of genomes from isolate representatives recovered each ROS gene compared to MAGs and SAGs (*Table 4*). Notably, all isolate genomes contained genes for cytochrome c peroxidase and alkyl hydroperoxide reductase whereas approximately 68% and 47% of environmental genomes recovered them respectively. Over 81% of isolate genomes had genes for Fe/Mn superoxide dismutase compared to less than 15% of environmental genomes. To assess whether these differences are due to discrepancies in genome completeness, the percent completeness and number of genes were plotted and analyzed with a Poisson regression model (*Figure 3*). *Figure 3a* shows a trend between percent completeness and number of genes recovered, but these results may be biased since all isolate genomes are near complete (>97%) and have relatively high numbers of ROS genes recovered. To account for this, the same analysis was done using MAGs/SAGs only (*Figure 3b*). This shows that while ROS genes may be missed due to incomplete genomes, it likely does not account for the entirety of differences observed (*Table 5*).

Table 4: Isolate genomes tend to have more ROS scavenging genes than MAGs/SAGs. The numbers represent the number of genomes analyzed that have genes for the corresponding protein. The percentage corresponds to the percent of genomes within the category (isolate or MAG/SAG) with genes for each protein.

Protein	Cytochrome c peroxidase	Alkyl hydroperoxide reductase	Catalase peroxidase	Catalase	Superoxide dismutase	Glutathione peroxidase
Isolate Genomes	16 **100%**	16 **100%**	6 37.5%	2 12.5%	13 **81.3%**	4 25%
MAG/SAG Genomes	23 ~68%	16 ~47%	5 ~14.7%	1 ~3%	5 ~14.7	1 ~3%

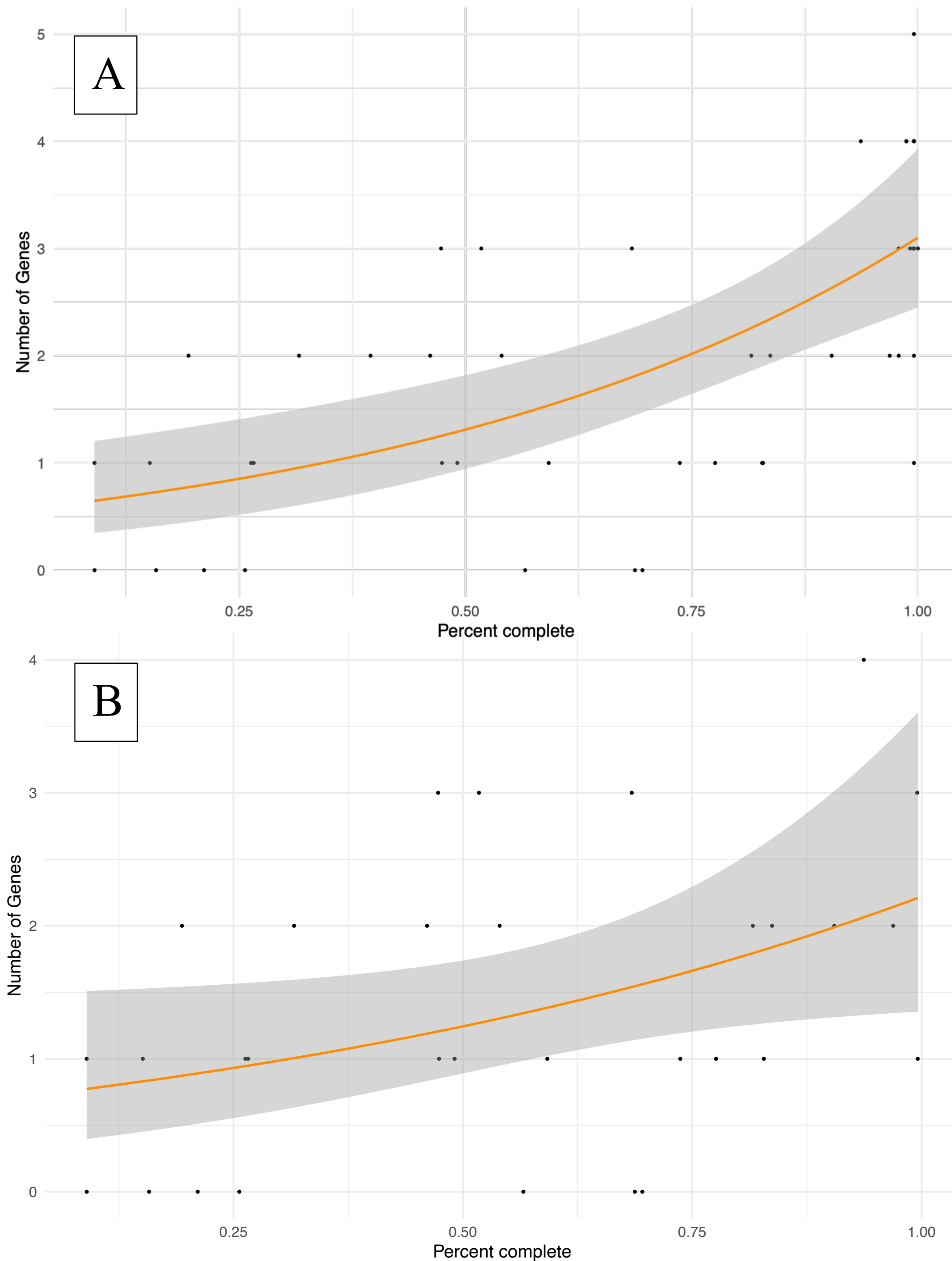


Figure 3: Correlation between the number of ROS genes found in genomes and percent completeness

The relationship was analyzed using a Poisson regression model and the shaded region corresponds to a 95% confidence interval. Graph A was constructed with all 50 Zetaproteobacteria genomes and graph B was constructed with only MAGs/SAGs.

Table 5: Statistics from Poisson regression analysis depicted in figure 3
Table A corresponds to regression using all 50 genomes. Table B corresponds to regression using only MAG/SAGs.

A

Coefficient	**Estimate**	**Standard Error**	**Z value**	**Pr(>|z|)**
Intercept	-0.5899	0.3515	-1.678	0.0933
% Complete	1.7216	0.4060	4.240	2.24×10^{-5}

B

Coefficient	**Estimate**	**Standard Error**	**Z value**	**Pr(>|z|)**
Intercept	-0.3500	0.3867	-0.905	0.3655
% Complete	1.1128	0.5532	2.011	0.0443

Fe/Mn Superoxide Dismutase Phylogenetic Tree

Since all Zetaproteobacteria genomes in the starred clade in *Figure 2* possess genes for Fe/Mn superoxide dismutase (SOD), a maximum likelihood phylogenetic tree was constructed with SOD protein sequences to see how the protein evolved throughout the Zetaproteobacteria class (*Figure 4*). Most of the Zetaproteobacteria SOD proteins fall within a single clade except for *Mariprofundus micogutta ET2* which is more closely related to SOD proteins of members of the Gammaproteobacterial class. *Mariprofundus ferrooxydans O1* has two distinct copies of SOD genes, each with a different evolutionary distance from SOD sequences from other *M. ferrooxydans* strains. The SOD cladogram was compared to the 16S rRNA cladogram in *Figure 5* revealing similarities in placement for most Zetaproteobacteria except for *M. micogutta ET2* and *Mariprofundus sp. DIS-1*.

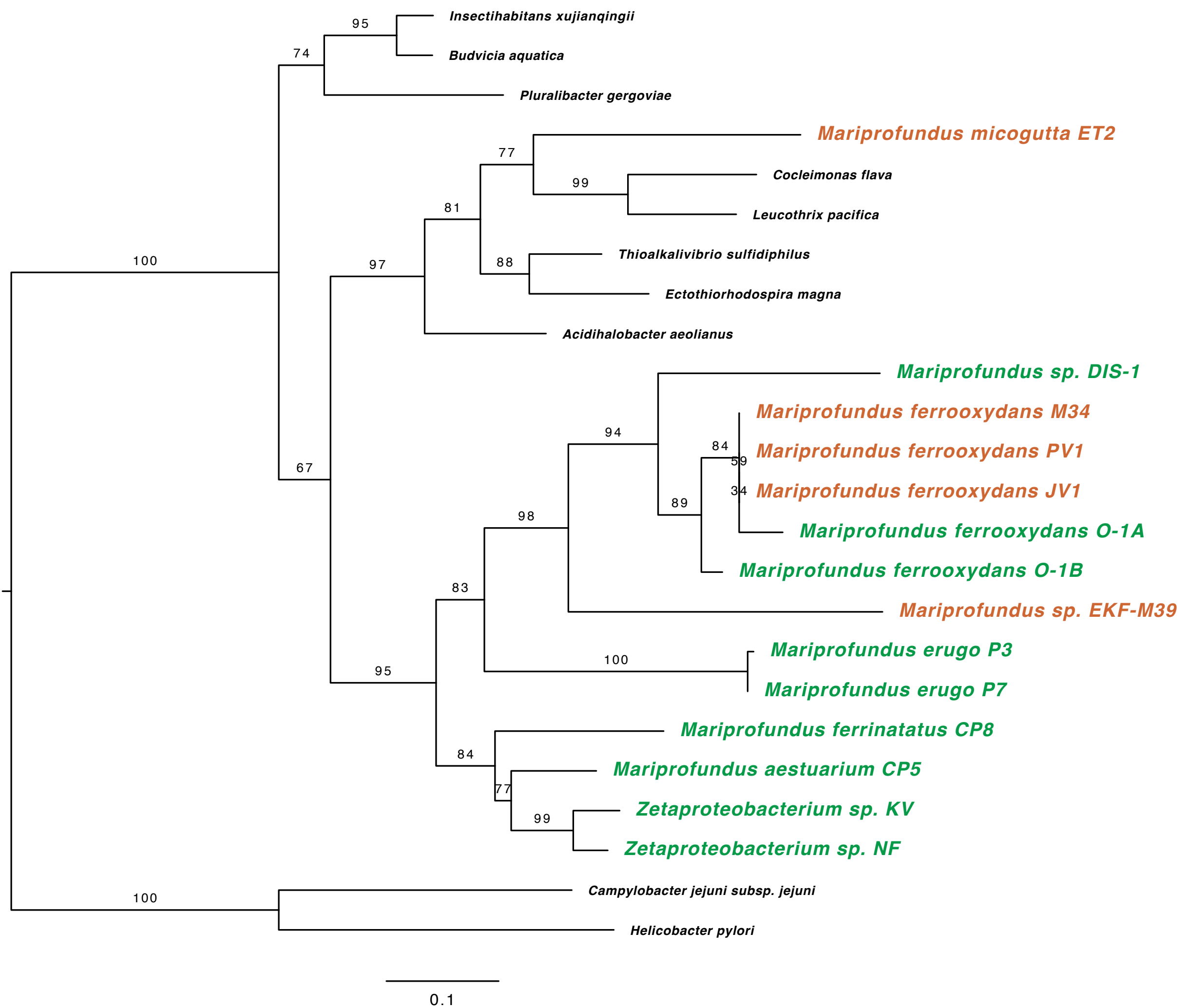


Figure 4: Superoxide Dismutase (SOD) maximum likelihood tree.
Zetaproteobacteria in green correspond to those found in Cyanobacteria friendly environments and those in orange correspond to those found in hydrothermal vent systems.

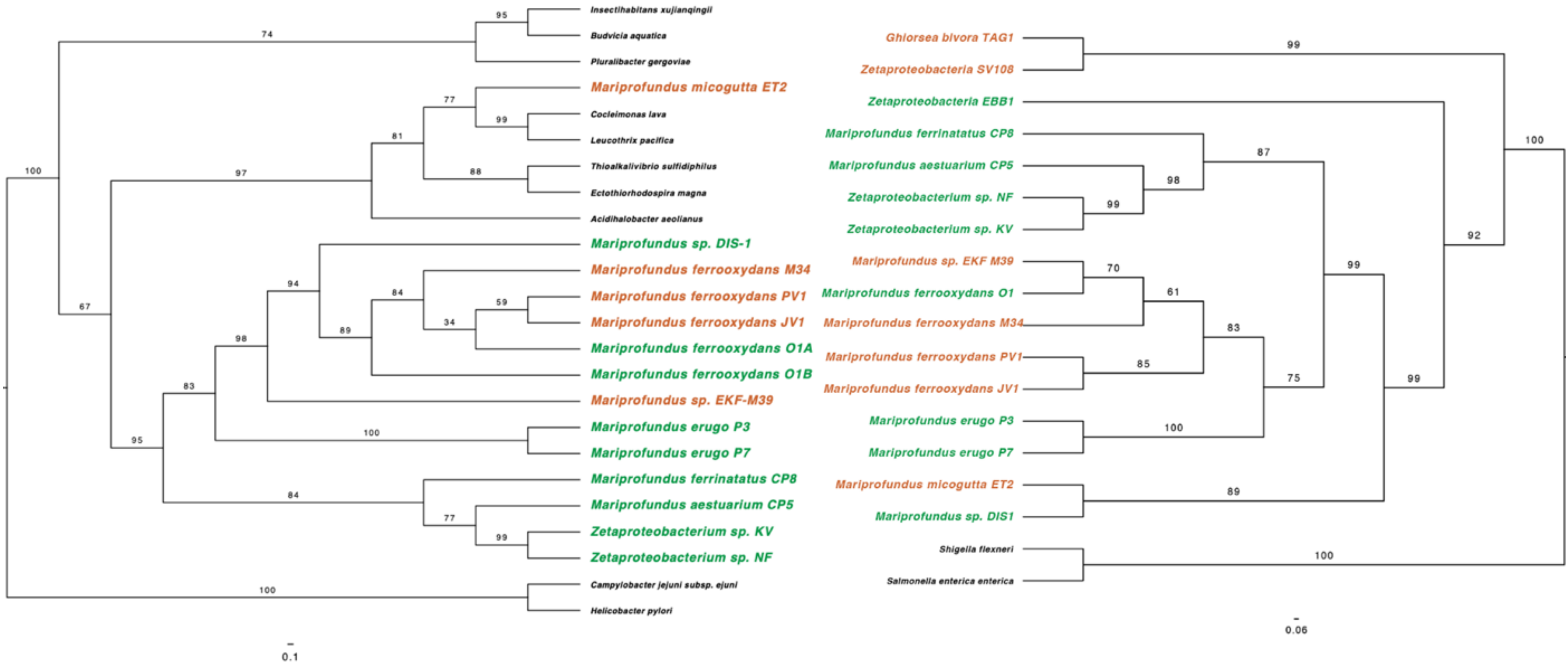


Figure 5: Maximum likelihood SOD tree (left) compared to 16S rRNA tree (right) Zetaproteobacteria in green correspond to those found in Cyanobacteria friendly environments and those in orange correspond to those found in hydrothermal vent systems.

Genome Comparisons

To determine if there is a genomic signature of a syntrophy beyond the six ROS proteins, whole genomes of a subset of Zetaproteobacteria isolates were compared using RAST. COGs were grouped into 27 different subsystems (*Figure 6*). Comparisons between the percentage of COGs falling into each subsystem reveals that there are no noticeable differences between organisms that may encounter Cyanobacteria and those that do not. Rather, the differences seem to be on an individual basis independent from environment. Because no subsystems were markedly different between the two groups, the oxidative stress subsystem was analyzed due to its relevance to oxygen tolerance and therefore a possible relationship with Cyanobacteria. A t-test was performed to determine if there are overall differences in the proportion of genes related to oxidative stress in genomes from each environmental group (*Table 6*). No significant difference was found ($p = 0.339$, $t=2.228$, $df= 10$).

Figure 6**:** Comparison between subsystems of "Cyanobacteria-friendly" Zetaproteobacteria (green box) and hydrothermal vent Zetaproteobacteria (orange box) Percent indicates how many genes out of all categorized into a subsystem fall within a given subsystem for that genome.

Table 6**:** Comparison of the percentage of subsystem genes categorized as relating to oxidative stress in RAST. Orange indicated Zetaproteobacteria from hydrothermal vents and green indicates Zetaproteobacteria from Cyanobacteria friendly environments. There was no significant difference between the two groups (**t = 2.228, p=0.339, df =10).**

Hydrothermal vent Genome	Percent
Mariprofundus ferrooxydans JV1	2.2%
Mariprofundus micogutta ET2	2.24%
Mariprofundus sp. EKF M39	2.79%
Zetaproteobacteria sp. SV108	1.72%
Mariprofundus ferrooxydans M34	2.61%
Ghiorsea bivora Tag1	1.9%

Cyano- friendly Genome	Percent
Mariprofundus erugo p7	2.81%
Mariprofundus aestuarium CP5	2.61%
Mariprofundus ferrooxydans O1	2.65%
Zetaproteobacteria sp. EBB1	2.19%
Mariprofundus ferrinatatus CP8	2%
Mariprofundus sp. DIS-1	2.92

Next, the 10 most abundant cluster of orthologous groups (COGs) categorized in IMG for each genome were compared (*Table 7*). All genomes analyzed had signal transduction histidine kinases and all but *Zetaproteobacteria SV108* had GGDEF domain diguanylate cyclase within their most abundant COGs, which are related to signaling and biofilm formation, respectively. The “cyano-friendly” genomes all had DNA-binding transcriptional response regulators in the NtrC family, which is related to nitrogen regulation and metabolism, within their top 10 COGs. Similarly, the hydrothermal vent genomes all had nitrogen specific signal histidine kinases in their most abundant COGs.

Table 7**:** COGs within the top 10 most abundant for all genomes in each group. 13 total genomes were used: 6 were categorized as "Cyano-friendly" and 7 as "hydrothermal vent."

Top 10 Cogs of All Genomes	Top 10 Cogs of All Cyano-friendly Genomes	Top 10 Cogs of All Hydrothermal Vent Genomes
Signal transduction histidine kinase	DNA-binding transcriptional response regulator, NtrC family	Signal transduction histidine kinase, nitrogen specific
GGDEF domain, diguanylate cyclase **(all but SV108)**		

The presence of specific oxygen and iron related genes were compared among Zetaproteobacteria isolate genomes within each environmental group (*Table7*). Most genomes analyzed had genes for rubrerythin, which has been shown to have hydroperoxidase function in anaerobic bacteria (Mishra and Imlay 2013) and archaea (Weinberg et al. 2004). However, *Ghiorsea bivora Tag-1* and *Zetaproteobacteria SV108*, which are closely related, only had genes for rubredoxin. *Zetaproteobacterium bacteria EBB1* lacked genes for both rubrerythrin and rubredoxin. Out of the 13 genomes, all but *Mariprofundus ferrinatatus CP8* contained genes for a ferric uptake regulator (FUR). All genomes have genes for a nitric oxide transcriptional regulator (NsrR) and/or a nitric oxide response protein (NnrS). All genomes had genes for Form II RuBisCo, but some contained genes for both form I and form II. *Mariprofundus sp. DIS -1* and all four strains of *Mariprofundus ferrooxydans* had genes for both forms of RuBisCo, suggesting that those organisms may be able to tolerate environments with fluctuating CO_2 and O_2 concentrations. Interestingly, many Zetaproteobacteria genomes contained genes classified as

phytochromes (*Table 8*), which function as light and oxygen sensors (Rockwell, Su, and Lagarias 2006), in the RAST annotation. However, the same sequences were classified differently in IMG. Most of the sequences were annotated in IMG as histidine kinases or PAS domain containing proteins, both of which are domains within the phytochrome system (*Figure 7*), but no genes encoding proteins corresponding to the photosensory region of phytochromes were found.

Table 8**:** Comparison of oxygen-related genes between genomes. Green boxes correspond to Cyanobacteria friendly genomes and orange boxes correspond to hydrothermal vent genomes. Numbers indicate the number of copies of the gene whereas Yes/No indicates presence. Parentheses correspond to the presence of that gene within that genome.

Organism	**RuBisCo**	**Rubrerythrin**	**Phytochrome**	**FUR**	**NSRR**	**Environment**
Mariprofundus ferrinatatus CP8	Form II	1	1	No	No (NnrS)	Chesapeake Bay, water column
Mariprofundus aestuarium CP-5	Form II	1	4	Yes	Yes	Chesapeake Bay, water column
Mariprofundus micogutta ET2	Form II	1	0	Yes	Yes (NnrS)	Hydrothermal field sediment
Mariprofundus sp. DIS-1	Form I and II	2	1	Yes	Yes (NnrS)	Steel Coupon - West Boothbay Harbor
Mariprofundus sp. EKF-M39	Form II	1	1	Yes	Yes	Loihi Iron Mat
Mariprofundus ferrooxydans M34	Form I and II	1	1	Yes	Yes	Loihi Iron Mat
Mariprofundus ferrooxydans JV-1	Form I and II	1	1	Yes	Yes	Loihi Iron Mat
Mariprofundus ferrooxydans O-1	Form I and II	1	0	Yes	Yes	Shipwreck Pamlico Sound, NC
Ghiorsea bivora TAG-1	Form II	0 (1 rubredoxin)	0	Yes	Yes (NnrS)	MAR Hydrothermal Vent Iron Mat
Mariprofundus erugo P7	Form II	1	3	Yes	Yes	Steel Coupon North Creek, NC
Zetaproteobacteria bacterium EBB1	Form II	0	0	Yes	Yes (NnrS)	Coastal Sediment
Mariprofundus ferrooxydans PV-1	Form I and II	1	1	Yes	Yes	Loihi Iron Mat
Zetaproteobacteria SV108	Form II	0 (1 rubredoxin)	0	Yes	No (NnrS)	MAR Hydrothermal Vent Iron Mat

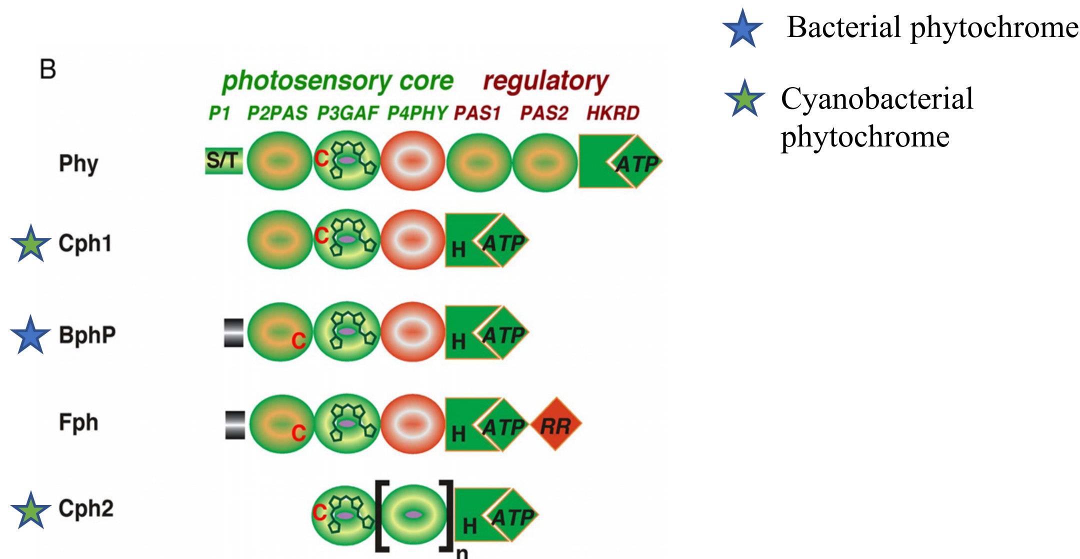


Figure 7**:** Phytochrome schematic adapted from: (Rockwell, Su, and Lagarias 2006). Proteins identified as phytochromes in RAST were annotated as PAS domain-containing proteins or histidine kinases in IMG, which fall in the regulatory portion of phytochromes, rather than the photosensory core.

All 13 of the Zetaproteobacteria genomes analyzed in RAST contained genes for multiple proteins annotated as PAS domain containing proteins, which can function as oxygen or redox sensors (Taylor and Zhulin 1999), and signal transduction (Henry and Crosson 2011). This demonstrates that all Zetaproteobacteria likely have a high need to respond to environmental stimuli, which is consistent with their metabolic physiology requiring a redox gradient. Although oxygen and redox sensing is important to microaerophilic iron oxidation in general, Zetaproteobacteria that associate with oxygen-producing Cyanobacteria may need more sensing capabilities to situate themselves along a potentially diurnal redox boundary. Because of this, the number of proteins annotated as PAS domain containing proteins were compared across

Zetaproteobacteria genomes in each group (*Table 9*). T-tests for annotations in both RAST and IMG revealed that there is no significant difference in the number of PAS domains between Zetaproteobacteria from Cyano-friendly environments and those from hydrothermal vent systems.

Table 9**:** Comparison of PAS domains containing proteins across genomes according to RAST and IMG annotations
Green boxes correspond to Cyanobacteria friendly genomes and orange boxes correspond to hydrothermal vent genomes. Two-tailed t-tests were performed to evaluate differences between the environmental groups in each annotations system. *Mariprofundus ferrooxydans PV1* was omitted due to the large discrepancy between RAST and IMG.

Organism	**# PAS domains in RAST**	**# PAS domains in IMG**
Mariprofundus ferrinatatus CP8	8	19
Mariprofundus aestuarium CP-5	21	24
Mariprofundus micogutta ET2	13	18
Mariprofundus sp. DIS-1	18	24
Mariprofundus sp. EKF-M39	29	22
Mariprofundus ferrooxydans M34	15	25
Mariprofundus ferrooxydans JV-1	14	17
Mariprofundus ferrooxydans PV1	*47*	*7*
Mariprofundus ferrooxydans O-1	13	17
Ghiorsea bivora TAG-1	5	11
Mariprofundus erugo P7	13	29
Zetaproteobacteria bacterium EBB1	10	18
Zetaproteobacteria SV108	3	11
Two tailed t-test (PV1 omitted)	t = 0.1566, p = 0.8787, df = 10	t= 1.5051, p = 0.1632, df = 10

Since many Zetaproteobacteria genomes recovered genes for rubrerythrin, a protein involved in oxygen tolerance among anaerobic bacteria (Whitham et al. 2015; Morvan et al. 2021), a maximum likelihood phylogenetic tree was constructed to see how rubrerythrin evolved within the class. Although the tree was constructed with similar protein sequences identified through NCBI BLAST, the Zetaproteobacteria rubreyrythrin sequences seem distinct from other Proteobacteria (*Figure 8*). This suggests that there may be unique pressures on the Zetaproteobacteria rubrerythrins. Since rubrerythrins fall within the ferritin super-family (Andrews 2010), it is possible that these proteins function as ferredoxins. *Mariprofundus sp. DIS1* has two distinctive copies of rubrerythrin, indicating that horizontal transfer or a gene duplication occurred in the past. Upon comparison with the 16S rRNA maximum likelihood tree, only *Mariprofundus sp. DIS1* and *Mariprofundus sp. EKF-M39* had different placement on the rubrerythrin tree (*Figure 9*). This may be due to either uncertainty in the tree or biological events such as differing selective pressures, horizontal gene transfer, or gene duplication. Gene clusters around rubrerythrin were compared across the 13 Zetaproteobacteria genomes, revealing that many of the rubrerythrin genes are adjacent to various ribosomal and metabolic genes. This suggests that rubrerythrin genes may be frequently accessed and expressed. Furthermore, gene clusters were more similar among closely related Zetaproteobacteria, providing support that rubrerythin was largely vertically transferred through the class.

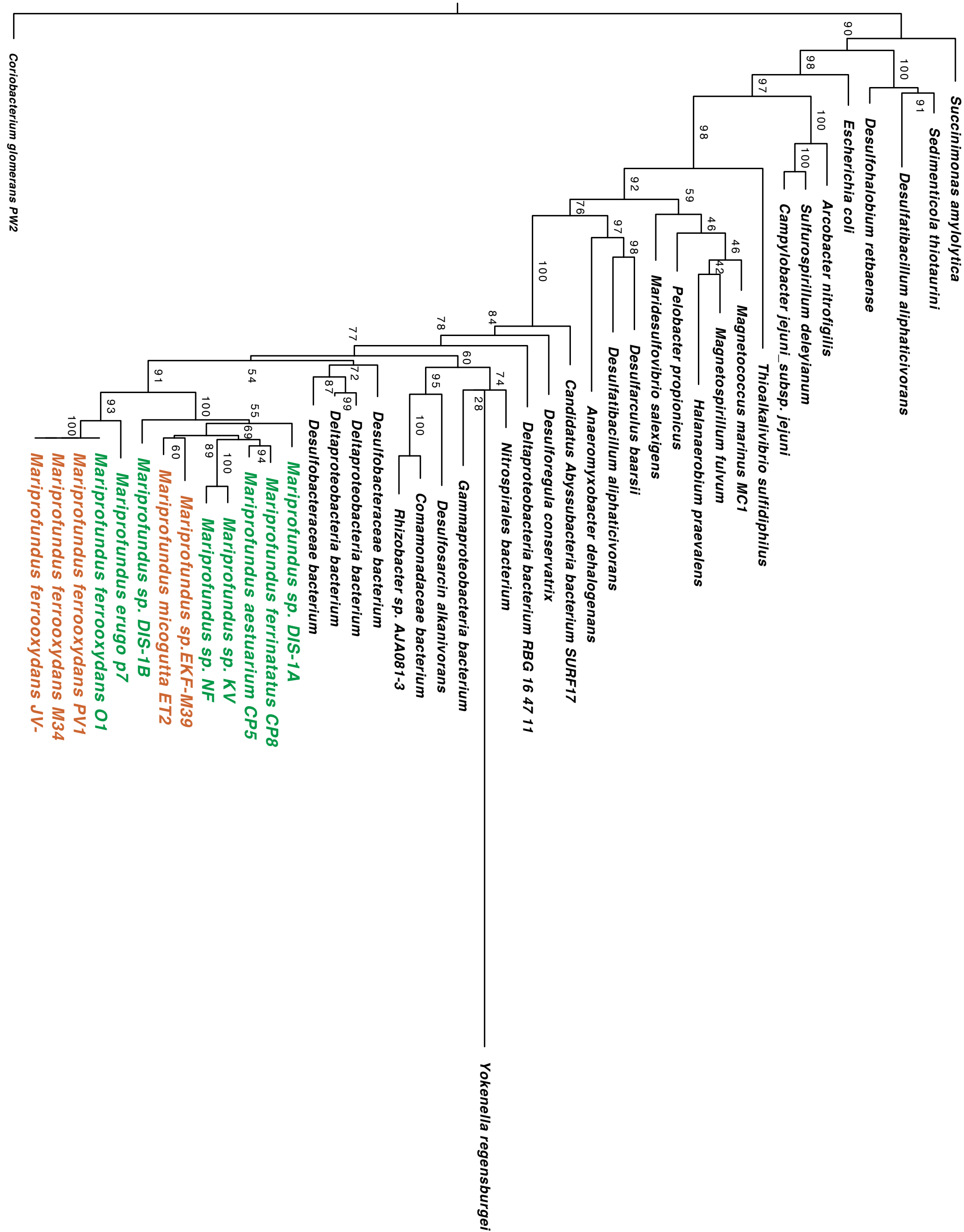


Figure 8**:** Rubrerythrin maximum likelihood tree
Zetaproteobacteria in green correspond to those found in Cyanobacteria friendly environments and those in orange correspond to those found in hydrothermal vent systems.

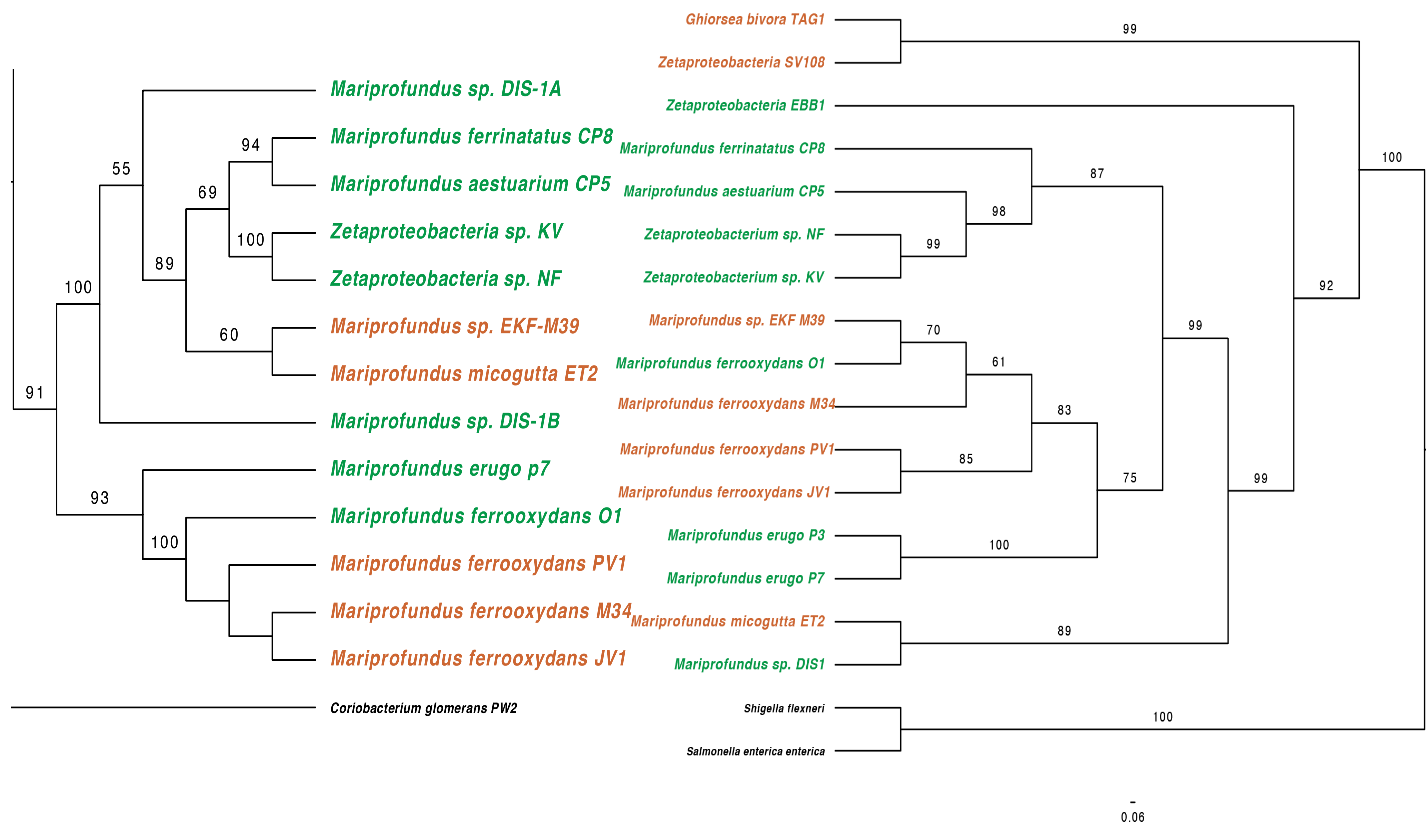


Figure 9**:** Comparison between rubrerythrin maximum likelihood tree (left) and 16S rRNA tree (right). Zetaproteobacteria in green correspond to those found in Cyanobacteria friendly environments and those in orange correspond to those found in hydrothermal vent systems.

1.6 Discussion and Conclusion

The genomes of Zetaproteobacteria from environments where Cyanobacteria are found were compared to the genomes of Zetaproteobacteria from environments that are inhospitable to Cyanobacterial growth. Overall, the results have demonstrated that many similarities and differences relating to oxygen tolerance genes among Zetaproteobacteria may be due to phylogeny or other factors rather than differences in environment. All Zetaproteobacteria genomes analyzed have a high number of genes relating to environmental sensing and signaling, indicating their need to respond to fluctuating environmental conditions. Furthermore, Zetaproteobacteria from both environmental groups have a high number of nitrogen metabolism and regulating genes, suggesting that they may have an increased demand for nitrogen and/or are equipped to cope with fluctuating nitrogen availability. It was also discovered that Zetaproteobacteria isolates tend to have higher numbers of oxygen tolerance genes than MAGs and SAGs, and that this may not be entirely explained by differences in genome completeness. This suggests that we may be overestimating oxygen concentration and fluctuations during attempted isolation of new Zetaproteobacteria species. Taken as a whole, however, these results do not reveal genomic evidence for a tightly coupled relationship with Cyanobacteria. Instead, the relationship may be akin to an opportunistic mutualism that occurs when the two are in proximity to one another.

Reactive oxygen species (ROS) scavenging genes are known to be important for oxygen tolerance among microaerophilic and facultatively anaerobic microorganisms. Analysis of these genes in 50 Zetaproteobacteria genomes revealed that alkyl hydroperoxide reductase and cytochrome c peroxidase are common throughout the entire class and superoxide dismutase is common throughout genomes of Zetaproteobacteria cultivated isolates *(Table 4)*. Cytochrome c

peroxidase and alkyl hydroperoxide reductase do not produce oxygen as a byproduct, which may make them more advantageous scavengers than oxygen-producing catalases and superoxide dismutases for the microaerophilic Zetaproteobacteria. Since alkyl hydroperoxide reductase can scavenge low concentrations of H_2O_2 more efficiently than catalase (Seaver and Imlay 2001), Zetaproteobacteria may be adapted to lower levels of H_2O_2. Marine environments tend to have H_2O_2 concentrations around $10^{-9} – 10^{-7}$ M, with the highest concentrations occurring at the surface and decreasing with depth (Zinser 2018; Price, Mantoura, and Worsfold 1998). This is below the saturation threshold for alkyl hydroperoxide reductase in *E. coli* (Seaver and Imlay 2001), suggesting that alkyl hydroperoxide reductase may provide sufficient scavenging for Zetaproteobacteria to thrive in typical marine environments. However, no studies to date have investigated whether these ROS scavenging proteins are functional in Zetaproteobacteria.

Overall, there were no significant differences in the number and type of ROS scavenging genes between Zetaproteobacteria from Cyanobacteria-friendly environments and those from deep-sea hydrothermal vent ecosystems. The largest differences were between isolate genomes and MAGs/SAGs (*Table 4*), which cannot be entirely explained by differences in genome completeness (*Figure 3*). While this suggests that we may be overestimating oxygen concentration during isolation attempts of novel species, it does not provide evidence for a sytntrophy between Zetaproteobacteria and Cyanobacteria. In a classical syntrophy, organisms rely on one another for their metabolic function, typically requiring them to be in close proximity (Morris et al. 2013). It is reasonable to expect that if some Zetaproteobacteria have a syntrophic mutualism with Cyanobacteria, they would have an increased ability to cope with O_2 and ROS byproducts. Our results indicated no differences between Zetaproteobacteria that may be in proximity to Cyanobacteria and those that aren't, supporting our conclusion that a syntrophy is

not likely present. However, it cannot be ruled out that there may be differences in unidentified ROS scavenging genes, protein function, and/or gene expression.

All 13 isolate genomes used for whole-genome comparisons encoded many genes relating to sensing, signaling, and nitrogen metabolism. This suggests that Zetaproteobacteria have the capability to respond to changing environmental conditions, which likely corresponds to their metabolic need to be along a redox gradient for access to O_2 and Fe(II). The abundance of nitrogen-related genes and the recovery of nitrogen fixation genes in some genomes indicates that Zetaproteobacteria either have an increased need for nitrogen or are adapted to nitrogen-limited environments. Some Cyanobacteria are capable of nitrogen fixation, which may benefit Zetaproteobacteria in environments with little available nitrogen. However, the timing and evolution of nitrogen fixation in Cyanobacteria is contested (Sanchez-Baracaldo, Hayes, and Blank 2005; Latysheva et al. 2012; Boyd et al. 2011), making it unclear to what extent this could have contributed to an ancient mutualism. Furthermore, no notable differences were found between Zetaproteobacteria that may encounter Cyanobacteria and those that do not, indicating that these genes are not related to a tightly coupled syntrophy or mutualism. Rather, they may be involved in a more passive beneficial relationship if the two organisms happen to be together in the same environment.

Subsystem and whole-genome comparisons in RAST revealed no significant differences between Zetaproteobacteria of the two environmental groups. Overall, oxygen-related genes seem to be most similar between closely related Zetaproteobacteria and this is supported by my phylogenetic analyses. Although there are a few exceptions, this indicates that there may have been vertical transfer of ROS scavenging genes, suggesting that these genes are important to Zetaproteobacteria function within those clades. However, if these genes are part of a long-term

syntrophy, we would expect to see a signature of this relationship among Zetaproteobacteria found alongside Cyanobacteria compared to those that are not. Since this was not observed, it may be that the two groups form an opportunistic mutualism when in proximity, but that it is not a tightly coupled or obligate syntrophy resulting from coevolution.

In the largely anoxic ancient oceans, microaerophilic iron-oxidizers would have relied on ancient Cyanobacteria for a source of electron acceptors. Although there are fewer extant environments suitable for both metabolisms, co-occurrence of Cyanobacteria and Zetaproteobacteria in modern environments indicates that a mutualism may persist today. If some Zetaproteobacteria and Cyanobacteria species share an obligate or tightly coupled mutualism, which occurs in syntrophic metabolisms, we expected to see a different in oxygen-related genes between Zetaproteobacteria groups, but our analyses revealed no significant differences. However, it is possible that the two shared a syntrophy in the ancient oceans that has diminished overtime. Future co-culture experiments with representatives of both groups will be necessary to further characterize the interactions between Zetaproteobacteria and Cyanobacteria and how it may have impacted BIF genesis.

Chapter 2: Cultivation of Cyanobacteria from the same location as *Mariprofundus erugo*: a step toward co-culture

2.1 Introduction

Due to the limitations when trying to use genomic data alone to draw conclusions about microbial interactions, culture experiments are necessary to assess any relationship between Zetaproteobacteria and Cyanobacteria. Because the ancient oceans were largely anoxic and ferruginous (Poulton and Canfield 2011), culturing in these conditions is necessary to understanding how these interactions may have impacted the formation of BIF precursors. Such ferruginous conditions may have negatively impacted Cyanobacteria since they can have reduced growth rates and accumulate intracellular ROS at elevated concentrations of Fe(II) (Swanner, Mloszewska, et al. 2015; Swanner, Wu, et al. 2015). Previous co-culture experiments with *Synechococcus* PCC 7002, a Cyanobacteria, and various *Shewanella* species have demonstrated that iron toxicity in Cyanobacteria may be mitigated by "helper" Proteobacteria (Szeinbaum et al. 2021). Similarly, heterotrophic *Alteromonas sp*. EZ55 mitigated oxidative stress in the Cyanobacterium *Prochlorococcus* by removing H_2O_2 in co-culture (Morris et al. 2011). These experiments suggest that Cyanobacteria may benefit from other microorganisms during iron and oxidative stress.

Similarly, Zetaproteobacteria may benefit from the presence of Cyanobacteria in culture. Although *Shewanella* growth declined when cultured with Cyanobacteria (Szeinbaum et al. 2021), Zetaproteobacteria from the Chesapeake Bay appeared enriched by the presence of Cyanobacteria (Field et al. 2016). Because Zetaproteobacteria require a source of oxygen, their relationship with Cyanobacteria may be an obligate mutualism or syntrophy in anoxic,

ancient ocean conditions. Because Zetaproteobacteria are microaerophilic and thrive under low oxygen conditions, so there may be a balance between oxygen production by Cyanobacteria and use by Zetaproteobacteria. In culture experiments, the Zetaproteobacterium *Mariprofundus ferrooxydans PV-1* accounted for over 90% of Fe(II) oxidation at 10.4 μM O_2 and 10.5% of Fe(II) oxidation at around 80 μM O_2 (McAllister et al. 2019). Culture experiments are one to determine how these factors impact Cyanobacteria and Zetaproteobacteria interactions.

Co-culture experiments with Zetaproteobacteria and Cyanobacteria representatives in varying iron concentrations are a direct way to investigate my second question: *Is the growth of Zetaproteobacteria and Cyanobacteria enhanced when grown together in BIF/GOE relevant Fe(II) concentrations compared to grown individually?* Specifically, *Mariprofundus erugo*, a Zetaproteobacteria species isolated from steel coupons in a North Carolina estuary (Garrison, Price, and Field 2019) and a Cyanobacteria isolate from the same coupon are ideal candidates for co-culture. Amplicon sequence data suggests that *M. erugo* and Cyanobacteria were co-existing in the steel coupon environment and thus may have a relationship *in situ*. Furthermore, *M. erugo* has a high number of ROS scavenging genes (Garrison, Price, and Field 2019). Due to a lack of early Proterozoic and late Archaean analogs, present-day conditions will be used to infer past relationships and how they may have contributed to BIFs and the GOE. I hypothesize that if there is a syntrophic relationship between Zetaproteobacteria and Cyanobacteria, the growth in co-culture experiments will be higher than individual cultures.

2.2 Research Question

Is the growth of Zetaproteobacteria and Cyanobacteria enhanced when grown together in BIF/GOE relevant Fe(II) concentrations compared to grown individually?

2.3 Hypotheses

1. I hypothesize that if there is a syntrophic relationship between Zetaproteobacteria and Cyanobacteria, the growth in co-culture experiments will be higher than when grown separately in individual cultures.
2. I hypothesize that if there is a syntrophic relationship between Zetaproteobacteria and Cyanobacteria, the difference in growth between co-cultures and individual cultures will increase as Fe(II) concentration increases.
3. I hypothesize that if there is a syntrophic relationship between Zetaproteobacteria and Cyanobacteria, the oxygen concentration in the co-culture experiments will reach an overall steady state overtime with shifts corresponding to day/night cycles.

2.4 Methods

Coupon Samples

In order to identify the best samples to use for Cyanobacteria isolation, amplicon sequencing data from previous experiments in the lab were utilized (Garrison, Price, and Field 2019). In those experiments, DNA was extracted from biofilms on 316 stainless steel coupons that were incubated in various North Carolina estuaries and material from those same biofilms were available as frozen glycerol stocks. Because I am interested in isolating a Cyanobacteria that may have an *in-situ* relationship with a Zetaproteobacteria, I chose coupon samples based on presence of both representatives in amplicon sequencing data or presence of Cyanobacteria in amplicon sequencing data and isolation of *Mariprofundus erugo* from that coupon biofilm. From this, we chose three total samples collected from 316SS coupons in May and February 2016 that were incubated in North Creek and Mallard Creek (*Table 10*).

Table 10**:** Amplicon Sequencing Data from 316SS Coupons
Each coupon material used for isolation showed evidence of Cyanobacteria presence. Although no Zetaproteobacteria were detected via amplicon sequencing for the May P3 site, *M. erugo P3* was isolated from that coupon confirming the presence of Zetaproteobacteria.

taxon	total	Feb P3 316SS	May P3 316SS	May P7 316SS
Cyanobacteria/Chloroplast	1624	54	81	63
Cyanobacteria	938	21	34	26
Cyanobacteria unclassified	530	9	22	11
Cyanobacteria unclassified	530	9	22	11
Cyanobacteria unclassified	530	9	22	11
Cyanobacteria/Chloroplast unclassified	69	1	5	3
Cyanobacteria/Chloroplast unclassified	69	1	5	3
Cyanobacteria/Chloroplast unclassified	69	1	5	3
Cyanobacteria/Chloroplast unclassified	69	1	5	3
Zetaproteobacteria	14	2	0	3
Mariprofundales	14	2	0	3
Mariprofundaceae	14	2	0	3
Mariprofundus	14	2	0	3

Cultivation methods

ASN-III, a medium frequently used for cultivation of marine Cyanobacteria (Rippka, Waterbury, and Stanier 1981), and estuary media, a medium used for the isolation of *Mariprofundus erugo* and other Zetaproteobacteria (E. K. Field et al. 2016; Garrison, Price, and Field 2019), were used to culture Cyanobacteria. For both media types, 1mL/L of ATCC MD-VS and 1mL/L of ATCC MD-TMS were added. Because Cyanobacteria media often contains a source of nitrate (Rippka, Waterbury, and Stanier 1981; Kotai 1972; Waterbury 2006), the estuary media was modified to have 0.467 g/L of $NaNO_3$ while maintaining salinity around 16ppt. 96% cyanocobalamin from Acros Organics was added in addition to the ATCC vitamin supplement for a total of 1.2×10^{-3} mg/L of cyanocobalamin in both media types, which falls within the concentration rage used for Cyanobacteria cultivation (Waterbury and Willey 1988). The estuary media pH was around 6.1 and the ASN-III was around 7.8. Cultures were grown at 20°C ± 1°C, which corresponds to the temperature ranges of the estuary environment *in situ* (Garrison, Price, and Field 2019) in addition to standard growth conditions used for cultivation of Cyanobacteria and Zetaproteobacteria (Chiu et al. 2017; Dvořák et al. 2017). Cultures were incubated under LED grow lights at 60-100 μmol photons m^{-2} s^{-1} on a 14hr/10hr day night cycle.

Initially, three cultures per coupon sample were inoculated for each media type as outlined in *Table 11*. 50mL of media were used for liquid cultures and each was inoculated with 50μL of thawed coupon sample except for negative controls which were inoculated with 50μL of sterile water. Agar plates were made with 1.5% bacteriological agar and 50μL of coupon sample were added to each plate by spread plating. The negative control for agar plates was inoculated by spread plating 50μL of sterile water. Liquid cultures were manually swirled daily, and all cultures were visually inspected each day. At least once a week, agar plates were observed under

a dissecting microscope and liquid cultures were observed microscopically at 400x. No growth was observed on the negative control cultures throughout the duration of the experiment. Subculturing was conducted as needed. For agar plates, as soon as green pigmented colonies were observed, they were aseptically transferred using a sterile loop to a new plate. For liquid cultures, between 100µL and 1mL was added to a new 50mL culture depending on the amount of growth present in the original culture as observed in microscopy. Since the majority of the Cyanobacterial growth was observed on the estuary media cultures, only estuary media was used for subculturing and future experiments.

Table 11**:** Outline of initial phototrophic enrichment cultures
Rows correspond to each coupon sample and the columns correspond to the media type. For every media/coupon combination 3 liquid broth cultures and 3 solid agar plates were inoculated. A negative control of each media type was inoculated with sterile water.

	ASN-III Media	Modified Estuary Media	Total coupon material
Feb P3 316SS	3 agar plates -1.5% (25ml) 3 broth (50ml)	3 agar plates -1.5% (25ml) 3 broth (50ml)	50ulx12 = 600ul
May P3 316 SS	3 agar plates -1.5% (25ml) 3 broth (50ml)	3 agar plates -1.5% (25ml) 3 broth (50ml)	50ulx12 = 600ul
May P7 316SS	3 agar plates -1.5% (25ml) 3 broth (50ml)	3 agar plates -1.5% (25ml) 3 broth (50ml)	50ulx12 = 600ul
Negative control Sterile H2O	1 agar plate -1.5% (25ml) 1 broth (50ml)	1 agar plate -1.5% (25mL) 1 broth (50ml)	50uL x4 = 200uL

Isolation Methods

Multiple methods were employed to attempt isolation of a Cyanobacteria from the heterotrophic bacteria and eukaryotic phototrophs that were growing concurrently in the enrichment cultures. The first method involved adding sulfide to the liquid cultures. Although high concentrations of hydrogen sulfide can be toxic to Cyanobacteria, some are tolerant (Cohen et al. 1986). These sulfide "shocks" were achieved by dissolving sodium sulfide in water to 0.25M and adding the solution to each culture to a final concentration of 50µM. Each time a sulfide shock was done, a culture without any treatment was maintained.

A 96 well plate was used as a high-throughput method of isolation (*Table 12*). Serial dilutions of cultures from the May 2016 Mallard Creek and North Creek sites were used along with various treatments for each row. Cycloheximide is an antifungal chemical that has been used to isolate Cyanobacteria from eukaryotic phototrophs in concentrations ranging from 5 – 20 µg/mL (Jaiswal et al. 2018). To see which concentration may be optimal for isolating the Cyanobacteria present in these cultures, 5, 10, 15, and 20 µg/mL were used for every dilution. To attempt to increase growth rate, a higher concentration of cyanocobalamin, 2.4×10^{-3} mg/L in the fifth row of the plate. The sixth row had no treatment and only consisted of serial dilutions of the respective cultures. The last row was inoculated with sterile culture media as a negative control to identify any possible contamination. For each treatment, the lowest dilution with growth was used to inoculate a 25mL culture which was examined under microscopy once sufficient growth was observed.

Table 12: Schematic of 96-well plate
All wells were inoculated with estuary media. The columns correspond to the dilution of the respective culture. "C" stands for Cycloheximide; "No treatment" indicates that wells were inoculated with only serial dilutions of the cultures. The negative control wells were inoculated with modified estuary media and sterile water.

	MP72 10^{-1}	MP72 10^{-2}	MP72 10^{-3}	MP72 10^{-4}	MP72 10^{-5}	MP72 10^{-6}	MP31 10^{-1}	MP31 10^{-2}	MP31 10^{-3}	MP31 10^{-4}	MP31 10^{-5}	MP31 10^{-6}
5µg/mL C												
10µg/mL C												
15µg/mL C												
20µg/mL C												
1.2 x 10^{-2} mg/L B12												
No Treatment												
Negative Control												

0.3% agar plates along with pour plating were used for isolation of individual colonies as demonstrated in the literature (Brahamsha 1996). Green pigmented colonies were picked with a 10µL pipette and transferred to liquid cultures and the process was repeated. Original cultures, subcultures, and cultures from the 96 well plate were used for this process. Because the heterotrophic bacteria seemed to be growing in close proximity to the Cyanobacteria, the most successful method involved diluting cultures and vigorously vortexing them before pour plating. Since this is a very slow process, some cultures were "bubbled" by using a sterile filter connected to a bubbler, rather than manually swirled, to attempt to increase their growth rates.

DNA Extractions

DNA was extracted from two liquid cultures with heavy growth using the DNEasy PowerSoil Pro Kit (Qiagen, Inc.) and 250 µl of pelleted culture material. Two extractions were done for each culture, resulting in four samples. DNA was quantified using a Quibit™ dsDNA BR Assay Kit (Thermo Fischer Scientific). Samples have been sent to CGEB-IMR© (Dalhousie University, Halifax, NC, Canada) for 16S rRNA gene sequencing using Illumina MiSeq.

2.5 Results

Overall, all phototrophic enrichment cultures grew a mixture of presumptive Cyanobacteria, protists, and heterotrophic bacteria. The culture from May 2016 North Creek site yielded the most Cyanobacteria growth over time (*Figure 10*) with possible growth of *Synechococcus sp.* but was a heavily mixed culture (*Figure 11a*). Shocking to 50µM sodium sulfide led to the overgrowth of eukaryotic algae with morphology consistent with *Chlorella sp*. (*Figure 11b*). Likewise, additions of sodium sulfide to cultures from Mallard Creek led to the overgrowth of eukaryotic algae with morphology close to *Klebsormidium sp*. (*Figure 11c*). The most successful treatment at promoting/maintaining Cyanobacteria growth while inhibiting the growth of eukaryotic microorganisms was the 20 µg/mL cycloheximide and the best growth was seen in the MP72 10^{-4} and MP72 10^{-5} dilutions. However, most Cyanobacteria growth observed was similar to larger coccoid Cyanobacteria without mucilage rather than *Synechococcus* and both cultures still had growth of heterotrophic bacteria (*Figure 11d*). When these cultures were subsequently aerated with "bubbling," the heterotrophs overgrew the Cyanobacteria (*Figure 11e*). Because of this, transfers to and from soft agar to liquid media were used for the remainder of the experiment. Although this technique is successful at reducing the number of undesired microorganisms, it is a slow process and is limited by the growth rate of the Cyanobacteria.

To identify the Cyanobacteria growing in these phototrophic enrichment cultures, DNA was extracted from two liquid cultures and sent for 16S rRNA gene sequencing. The first culture chosen was an original culture inoculated with May 2016 North Creek 316SS as shown in *Figure 9*. This culture was chosen because it contained heavy growth was the original culture from which the most successful subcultures came. Although this culture was mixed with heterotrophic bacteria and eukaryotic protists, it contained possible *Synechococcus sp.* (*Figure 11a*) and dark growth on the sides of the flask had growth similar to other coccoid Cyanobacteria (similar to *Figure 11d*. The second culture, which was a subculture of *Figure 9*, was chosen due to its heavy phototrophic growth. It also contained mixed microbial species, but multiple Cyanobacteria morphologies detected via microscopy. Overall, these cultures were chosen because each contained heavy growth with multiple morphologies of possible Cyanobacteria. Sequencing will give us an idea of what types of Cyanobacteria are present in these enrichment cultures and thus help guide future isolation efforts.

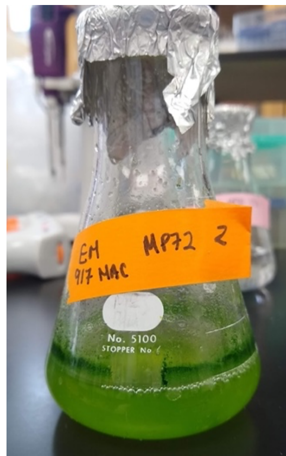


Figure 10**:** Phototrophic enrichment culture ("MP72") inoculated with May 2016 North Creek 316SS coupon material

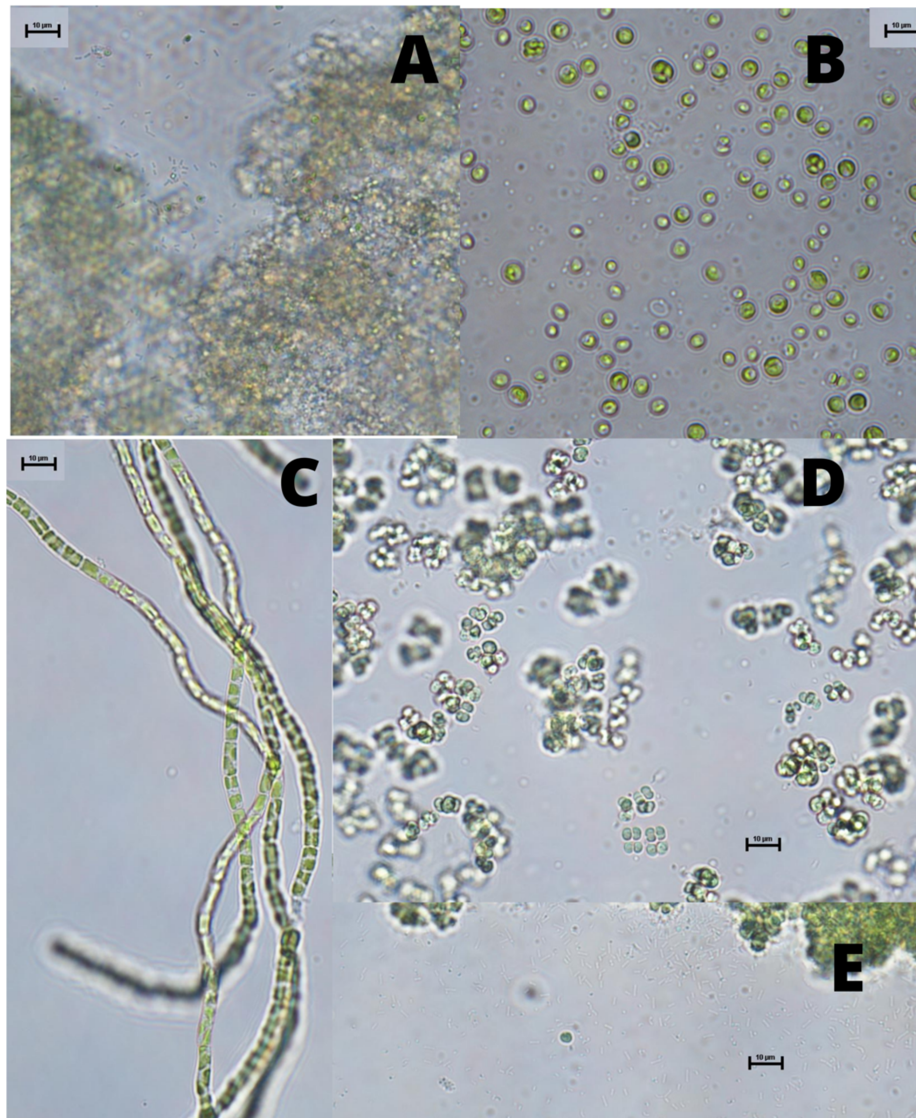


Figure 11**:** Microscopy images from phototrophic enrichment cultures
A) 2016 North Creek 316SS coupon material ("MP72") culture with the most Cyanobacteria growth. Peanut-shaped cells are possible *Synechococcus sp.* B) Possible eukaryotic *Chlorella sp.* C) Possible eukaryotic *Klebsormidium sp.* D) Coccoid Cyanobacteria growing after treatment with cycloheximide E) Increased growth of heterotrophic bacteria after bubbling.

2.6 Discussion

Axenic Cyanobacteria cultures were not obtained within the timeframe for this experiment. This is in part due to the slow growth times of the Cyanobacteria, but it may also be due to a tight coupling between the members of the microbial consortia. Heterotrophic bacteria are often found in tight associations with Cyanobacteria such as *Synechococcus sp*., making it difficult to obtain pure cultures with standard dilutions and plate transfers (Zheng et al. 2018). Similarly, low abundances of *Prochlorococcus* are unable to grow efficiently without additions of "helper" heterotrophs (Morris et al. 2008) and *Prochlorococcus* can depend on heterotrophs for help scavenging ROS (Morris et al. 2011). It is therefore possible that interactions between the heterotrophs and the Cyanobacteria in our phototrophic enrichment cultures may be making isolation difficult.

Since the liquid media contained no additions of organic carbon, it is likely that heterotrophic bacteria were utilizing byproducts from phototrophic microorganisms within the cultures. Co-cultures of heterotrophic bacteria and *Synechococcus* strains have demonstrated that certain heterotrophs secrete hydrolytic enzymes in the presence of *Synechococcus* (Christie-Oleza, Scanlan, and Armengaud 2015). Although agar within the sold and semi-solid plates may have been a source of nutrients, it is still likely that the Cyanobacteria themselves were supporting heterotrophic growth since the colonies were concentric to one another. Moving forward, the use of broad spectrum β - lactam antibiotics may assist in obtaining axenic cultures as has been demonstrated in the literature (Ferris and Hirsch 1991).

To conduct co-culture experiments, and therefore address our research questions, it is necessary to obtain an axenic culture of a Cyanobacteria. Without a pure culture, we cannot effectively determine the nature of interactions between *M. erugo* and a co-occurring

Cyanobacteria. Currently, the most successful method has been to use 20µg/ml of cycloheximide to eliminate eukaryotic growth in combination with the soft agar and pour plate method. It takes multiple transfers from 0.3% agar to liquid culture to see significant reductions in heterotrophic growth and it can take 1-2 months for Cyanobacteria colonies to appear on soft agar, making this a slow process. Since isolation has been limited by slow growth rates of the Cyanobacteria, future methods should involve adjusting media components, micronutrients, and physical parameters to increase growth while continuing isolation. Additionally, efforts should go into cultivating and isolating Cyanobacteria from other "Cyano-friendly" environments where Zetaproteobacteria are present.

Conclusion and Future Directions

Overall, these results indicate that Zetaproteobacteria and Cyanobacteria species may have a passive relationship as opposed to a tightly coupled syntrophy. This means that when the two organisms are in proximity, they may be able to benefit each other similarly to what was observed by Field et. al 2016, but that they are not obligately coupled. Our genomic data has shown that there are no differences in relevant gene categories between Zetaproteobacteria that encounter Cyanobacteria compared to those that do. However, to fully characterize the relationship between the two and determine its relevance to ancient ocean systems, it is necessary to conduct culture experiments.

Our phototrophic enrichment cultures have yielded Cyanobacterial growth from the same samples where *Mariprofundus erugo p7* was isolated. Those Cyanobacteria are ideal candidates for co-culture experiments since they may already have a relationship with *M. erugo in situ*. To utilize them, however, it is necessary to obtain axenic cultures so that results accurately reflect the relationship of interest. Microscopy suggests that *Synechococcus sp.* as well as other coccoid Cyanobacteria may be growing in the cultures, but these morphological observations do not provide insight into physiological characteristics that can be used for isolation. Because of that, sequencing of the enrichment cultures is currently being conducted so that identifications can be made. This will guide isolation methods as well as determine what types of Cyanobacteria may associate with Zetaproteobacteria.

Although we do not see evidence of a tightly coupled syntrophy today, the nature of the relationship may have been different in the ancient oceans when BIF precursors were formed. This is why co-culture experiments in simulated ancient-ocean environments are crucial to

understanding how Zetaproteobacteria and Cyanobacteria may have interacted in BIF-relevant time periods. Although marine environments where Zetaproteobacteria and Cyanobacteria are found today are conducive to the growth of both organisms, they have different geochemical properties from the ancient oceans. Because of this, it may be appropriate to study microaerophilic iron oxidizing bacteria and Cyanobacteria by using freshwater representatives from ferruginous lakes. Due to the high Fe(II) content, these lakes are often used as ancient ocean analogues. While we did not uncover evidence for a syntrophy between extant Zetaproteobacteria and Cyanobacteria, a relationship between the two may have still impacted the formation of BIFs. To fully understand this potential, future studies should focus on investigating environments that are analogous to ancient oceans in addition to conducting co-culture experiments.

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