GENOMIC ANALYSES OF NOVEL IRON-OXIDIZING *THIOMONAS* ISOLATES FROM ACID MINE DRAINAGE

by

Michelle Hallenbeck

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Michelle Hallenbeck

Approved: _____
Clara Chan, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Fidelma Boyd, Ph.D.
Committee member from the Department of Biological Sciences

Approved: _____
Christopher Kloxin, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____
Earl Lee II, Ph.D.
Deputy Faculty Director, University Honors Program

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"A scientist lives with all of reality. There is nothing better. To know reality is to accept it and eventually to love it."

-Sir Charles Scott Sherrington

This thesis would not have been possible without the aid and assistance of so many people. First, I want to thank my advisor, Dr. Clara Chan, for supporting my growth as a scientist and providing valuable mentorship and guidance. I would like to thank our collaborators, Dr. Denise Akob and Dr. Kirsten Küsel, for providing us with the FB strains and their genomes. I would also like to thank the other members of my committee, Dr. Fidelma Boyd and Dr. Christopher Kloxin, for taking the time to read my thesis and to support my undergraduate research career.

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ABSTRACT

Certain acid mine drainage (AMD)-derived microorganisms can sequester and detoxify metals, which could be useful for bioremediation if we can determine the mechanisms and controls. Here we describe the isolation, physiological characterization, and genomic analysis of two new species, *Thiomonas ferrovorans* FB-6 and *Thiomonas metallidurans* FB-Cd, isolated from Fe-rich AMD sediment in Ronneburg, Germany. While *Thiomonas* spp. are known as mixotrophic sulfur-oxidizers and As-oxidizers, the FB strains could oxidize Fe, which would allow them to efficiently remove Fe and other metals via co-precipitation. However, *Thiomonas* Fe oxidation physiology and mechanisms are not well-established. Therefore, we conducted a genomic analysis to investigate their genetic mechanisms of Fe oxidation, other metal transformations, and additional adaptations, comparing the two FB strains with 12 other isolates. The FB strains fall within a relatively novel group of *Thiomonas* (Group II), which includes the one other strain (b6) with evidence of Fe oxidation. Most *Thiomonas* isolates, including the FB strains, have the putative iron oxidation gene *cyc2,* but only the FB strains possess the putative Fe oxidase genes *mtoAB.* The FB strain genomes contain the highest numbers of strain-specific gene clusters, greatly increasing the known *Thiomonas* genetic potential. Our results reveal that the FB strains are two distinct novel species of *Thiomonas,* with the genetic potential for bioremediation of AMD via iron oxidation.

Chapter 1

INTRODUCTION

Acid mine drainage (AMD) contaminates groundwater and surface water with arsenic and heavy metals as a result of mining practices (Klein et al., 2013). The extremely low pH of AMD allows for greater solubility of these metals, a phenomenon known as metal leaching (ML) that has devastating effects on local ecosystems (Hallberg, 2010; McCauley et al., 2009). Co-precipitation of metals with iron and other mineral oxides has previously been observed to occur in AMD-contaminated groundwater, indicating the potential for removal of heavy metal contaminants from AMD by co-precipitation and adsorption to iron oxides (Gault et al., 2005). AMD-derived microorganisms can contribute to this process by oxidizing iron, which could be useful for bioremediation if we can determine the mechanisms and controls. *Thiomonas* spp. are members of the Betaproteobacteria class and are usually grown as mixotrophic sulfur-oxidizers (Chen et al., 2004). They are widespread in AMD-impacted environments, and despite their low abundance they are known to play a role in natural attenuation of As in AMD by As oxidation (Hovasse et al., 2016). However, they may also be able to remediate AMD by oxidizing Fe, resulting in co-precipitation of As and other metals with Fe oxides. Although prior studies of *Thiomonas* have focused primarily on their As oxidation ability (Battaglia-Brunet et al., 2006; Duquesne et al., 2008; Freel et al., 2015; Hovasse et al., 2016), this work will examine their Fe oxidation mechanisms because the latter is more widely applicable to bioremediation of not only As but other heavy metals present in AMD.

Two new Fe-oxidizing *Thiomonas* spp., strains FB-6 and FB-Cd, were isolated from AMD-affected, heavy metal-rich sediment in a former uranium mining area in Ronneburg, Germany (Beulig, 2010). The Ronneburg AMD sediment has much higher Fe concentrations than the other AMD-impacted environments from which *Thiomonas* spp. have been isolated, consistent with the presence of iron-oxidizing bacteria (FeOB) (Bruneel et al., 2003; Casiot et al., 2003; Hallberg and Johnson, 2003; Johnson and Hallberg, 2005; Beulig, 2010; Hovasse et al., 2016). The Ronneburg isolates were isolated by growth on ferrous carbonate, and they have also been shown to tolerate the presence of various heavy metals commonly found in AMD (Beulig, 2010). Further study of the Ronneburg FB-6 and FB-Cd isolates may reveal their genetic mechanisms of Fe oxidation as well as highlighting their genetic distinctiveness from and commonalities with other *Thiomonas* isolates.

Thiomonas Fe oxidation physiology and mechanisms are not well-established, despite the widespread nature of *Thiomonas* spp. in AMD-impacted environments and the potential of Fe(III) precipitation as an AMD remediation mechanism. Several Fe-oxidizing *Thiomonas* spp. have previously been isolated from AMD, although their genomes have not been sequenced (Hallberg and Johnson, 2003; Johnson and Hallberg, 2005). The sequenced isolate *Thiomonas delicata* b6 was isolated from AMD-impacted sediment at the former Cheni gold mine site in Limousin, France, and it was shown to grow by oxidizing Fe(II) in shake flasks in ferrous iron/thiosulfate medium (Battaglia-Brunet et al., 2006). Other AMD-derived *Thiomonas* isolates have shown inconsistent evidence of Fe(II) oxidation. Four strains isolated from the Carnoules AMD (CB1, CB2, CB3, and CB6) could oxidize iron when grown on medium containing both Fe(II) and thiosulfate or in filter-sterilized spring water, but

they did not grow in media containing only Fe(II), nor did they oxidize Fe(II) when incubated in creek water from their AMD site (Bruneel et al., 2003; Casiot et al., 2003). Another Carnoules strain, *Thiomonas arsenitoxydans* 3As, grew on ferrous iron/thiosulfate/tryptone soya broth medium and formed colonies with ferric iron-stained centers, but ferrous iron oxidation was not observed when tested in a fixed pH (3.2) bioreactor culture and in shake flasks (Slyemi et al., 2011). The Ronneburg FeOB are unique among the *Thiomonas* isolates in that they were isolated lithoautotrophically on ferrous iron (Beulig, 2010), which makes them ideal candidates for studying putative iron oxidation mechanisms in *Thiomonas*. If the iron oxidation mechanisms of *Thiomonas* spp. can be identified and characterized, it may be possible to apply their Fe oxide formation capabilities to treatment of metal leaching, which can lead to bioremediation of AMD.

This work focuses on two putative Fe oxidation mechanisms: the outer membrane fused cytochrome-porin Cyc2 and the outer membrane cytochrome and porin MtoAB. Cyc2 has been functionally verified as an iron oxidase in *Acidithiobacillus ferrooxidans* (Yarzabal et al., 2002), and MtoA has been implicated in iron oxidation in *Sideroxydans lithotrophicus* ES-1 (Liu et al., 2012). We searched for these mechanisms in the genomes of all known *Thiomonas* isolates, focusing on two new Fe-oxidizing isolates from the Ronneburg AMD site. In this study, we show through genomic and phylogenetic analysis that the FB strains are two distinct novel species in the genus *Thiomonas* by comparing their genomes to those of the 12 other sequenced *Thiomonas* isolates. Further, we show that the FB strains represent the most genetically distinct members of the *Thiomonas* genus, in terms of genomic content, phylogenetic diversity, and their possession of multiple putative Fe(II) oxidation

mechanisms, one or both of which they may be using to oxidize iron *in situ* and to contribute to natural remediation of heavy metals in the Ronneburg AMD.

Chapter 2

MATERIALS AND METHODS

2.1 Isolation and genome sequencing of FB strains

Thiomonas strains FB-6 and FB-Cd were previously isolated from creek sediment in a former uranium mining area in Ronneburg, Germany (Beulig, 2010). Collaborators Denise Akob and Kirsten Küsel submitted their DNA for genome sequencing by the DOE Joint Genome Institute (JGI). Their genomes were annotated in the Integrated Microbial Genomes (IMG) database under the genome ID 2523533526 for FB-6 and 2574179766 for FB-Cd. The FB-6 16S rRNA sequences can be found in IMG under the locus tags D466DRAFT_0316 and D466DRAFT_1646. The FB-Cd 16S rRNA sequences can be found in IMG under the locus tags CD04DRAFT_0503 and CD04DRAFT_3334.

2.2 Genomic analyses

2.2.1 Genome collection, annotation, and analyses

A total of 14 *Thiomonas* isolate genomes (including the FB strains; Table 1) were acquired from the National Center for Biotechnology Information (NCBI) database (NCBI Resource Coordinators, 2017) and uploaded to the Rapid Annotations based on Subsystem Technology (RAST) Prokaryotic Genome Annotation Server (Aziz et al., 2008; Overbeek et al., 2013) for annotation. All 14 genomes were also submitted to BlastKOALA (Kanehisa et al., 2016) for KEGG annotation. KEGG

metabolic pathways in the IMG database (Chen et al., 2018) were used to identify key energy metabolisms and related genes in the genomes, and RAST was used to browse the genomes for genes of interest, in particular genes unique to the FB strain genomes with respect to other *Thiomonas* isolates.

The genomes were found in the NCBI database under the following GenBank assembly accession numbers: FB-6- GCA_000377645.1; FB-Cd- GCA_00733775.1; b6- GCA_900088825.1; X19- GCA_900089495.1; 3As- GCA_000253115.1; CB1- GCA_900005065.1; CB2- GCA_00947035.1; CB3- GCA_900004415.1; CB6- GCA_900004565.1; ACO3- GCA_900004955.1; ACO7- GCA_900004405.1; S10- GCA_001418255.1; K12- GCA_000092605.1; *T. intermedia*^{T}- GCA_002028405.1.

2.2.2 Phylogenetic analyses

Nucleotide and protein sequence alignments were generated in Geneious v. 10.1.3 (Geneious version 10.1 created by Biomatters. Available from https://www.geneious.com) using MUSCLE, and the alignments were used to construct maximum likelihood trees using RAxML (Stamatakis, 2014) with 100 replicates. Cyc2 sequences were found in the IMG and NCBI databases using BLAST (Stephen et al., 1997) by comparing the Cyc2 sequence from *Acidithiobacillus ferrooxidans*. An alignment of the *Thiomonas* Cyc2 protein sequences (present in 13 of the 14 genomes) was produced, which was used to construct a maximum likelihood tree. The FB strain MtoAB/MtrAB sequences as well as those of several known iron oxidizers and reducers were found in the IMG and NCBI databases. The FB strain MtoAB homologs were identified by BLAST using the MtoAB sequences from *Sideroxydans lithotrophicus* ES-1 (Liu et al., 2012; Emerson et al., 2013). Additional MtoAB/MtrAB sequences from other known iron-oxidizers and iron-reducers were

obtained using the IMG locus tags provided in Shi et al., 2012 and IMG gene IDs provided in He et al., 2017. MtoA/MtrA and MtoB/MtrB protein sequence alignments were generated and subsequently concatenated, and the concatenated alignment was used to generate an MtoAB/MtrAB maximum likelihood tree. Ribosomal proteins in the core genome were identified from the pangenome analysis using the COG annotations assigned to the gene clusters. An alignment was generated for each ribosomal protein, and the alignments were concatenated and used to generate a maximum likelihood tree. Outgroups for all trees were obtained using IMG and NCBI BLAST.

2.2.3 Pangenome analysis

Anvi'o v5.1 (Eren et al., 2015) was used to conduct a pangenome analysis of the *Thiomonas* isolates using the anvi'o pangenomic workflow as described in Delmont and Eren, 2018. RAST was used to call ORFs, and a contigs database was generated for each genome using the command anvi-gen-contigs-database. The anvi'o command anvi-run-ncbi-cogs was used to annotate the contigs databases by NCBI's Clusters of Orthologous Groups (COG) before the pangenome analysis was conducted using the command anvi-pan-genome. The anvi-pan-genome command was run using the flag '--use-ncbi-blast' and the parameters "--minbit 0.5" and "--mcl-inflation 8" which govern the determination of protein sequence similarity and identification of gene clusters, respectively. Geometric and functional homogeneity of each gene cluster in the pangenome was determined using the anvi'o v5.2 command anvi-compute-gene-cluster-homogeneity. Average nucleotide identity (ANI) was calculated using the command anvi-compute-ani, which used PyANI (Pritchard et al., 2016) to compute ANI across a given set of genomes. Average amino acid identity (AAI) of

bidirectional best hit proteins was calculated using a web-based calculator (Rodriguez-R and Konstantinidis, 2014). ANI, AAI, and 16S rRNA heatmaps were generated using the R package gplots heatmap.2 (v 3.0.1.1); the dendrograms were calculated using hierarchical clustering with complete agglomeration.

Chapter 3

RESULTS AND DISCUSSION

3.1 Genome Features of Strains FB-6 and FB-Cd

The FB-6 and FB-Cd genomes consist of 8 and 6 contigs, respectively, and are >98% complete (Table 1). They fall above the average *Thiomonas* genome size (3.85 Mb; range 3.20-4.64 Mb) and at either end of a 62.5-69.9% GC content range (average 64.6%) for *Thiomonas* (Table 1). The genome of FB-6 is 4.28 Mb with a GC content of 69.9% and 4012 protein coding genes, 82.12% with function prediction. The genome of FB-Cd is 4.39 Mb with a GC content of 62.5% and 4096 protein coding genes, 78.42% with function prediction. FB-6 has 70 RNA genes (including 46 tRNA genes), while FB-Cd has 60 RNA genes (including 44 tRNA genes). FB-6 and FB-Cd are the only *Thiomonas* isolates that have two 16S-5S-23S rRNA operons. The two FB-6 16S rRNA sequences are identical to each other, and the two FB-Cd 16S rRNA sequences are identical except one sequence has an extra guanine nucleotide.

Table 1: Genomic and Physiological Characteristics of the *Thiomonas* Isolates

Strain	Isolation site	pH range	Temperature range	Fe(II)	As(III)	Thiosulfate	Sulfur	Tetrathionate	Organics	Genome size (Mb)	Percent completeness	# contigs	GC content	References
FB-6	AMD sediment	5	isolated at room temperature	+	ND	-	+	-	+	4.28	99.3	8	0.70	12, this study
FB-Cd	AMD sediment	3-6	isolated at room temperature	+	ND	+	+	-	+	4.39	98.6	6	0.63	12, this study
X19	AMD sediment	isolated at 9.8	incubated at 25°C	ND	+	ND	ND	ND	ND	4.64	98.6	11	0.65	4, 20
b6	AMD sediment	optimum: 4.0-7.5	optimum: 20-30°C	+	+	+	+	+	+	3.84	100.0	58	0.66	8, 9, 10, 14, 38, 39
S10	hot spring sediment	6.0-8.5 (optimum 7.5-8.0)	25-45°C (optimum 30-37°C)	ND	ND	+	ND	ND	mixotrophic growth	3.20	98.6	25	0.65	47, 50
K12	corroded concrete sewer wall	grows at 5.0	grows at 30°C	ND	+	ND	+	+	ND	3.34	99.3	2	0.65	28, 33, 44, 58, 59
intermedia (T)	freshwater stream bank mud	optimum: 5.5-6.0	optimum: 30-35°C	inconclusive	+	+	+	+	+	3.34	99.3	2	0.65	9, 37, 39, 43, 45, 54
CB3	AMD water	isolated at 7.0-7.2; grows at 5.0	incubated at 30°C	inconclusive	+	+	ND	ND	ND	4.33	100.0	65	0.64	13, 15, 25, 26, 28
CB2	AMD water	isolated at 7.0-7.2; grows at 5.0	incubated at 30°C	inconclusive	+	+	ND	ND	ND	3.74	100.0	92	0.64	13, 15, 25, 26, 28
ACO3	AMD sediment	grows at 5.0	isolated at 30°C	ND	+	ND	ND	ND	ND	3.61	100.0	65	0.64	25, 28
ACO7	AMD sediment	grows at 5.0	isolated at 30°C	ND	+	ND	ND	ND	ND	3.66	100.0	68	0.64	25, 28
CB1	AMD water	isolated at 7.0-7.2; grows at 5.0	incubated at 30°C	inconclusive	+	+	ND	ND	ND	3.88	100.0	31	0.64	13, 15, 25, 26, 28
CB6	AMD water	grows at 5.0	incubated at 30°C	inconclusive	+	ND	ND	ND	ND	3.81	100.0	43	0.64	15, 25, 28
3As	AMD water	optimum: 5.0	25-37°C (optimum 30°C)	inconclusive	+	+	+	+	+	3.79	100.0	3	0.64	3, 14, 22, 23, 54

3.2 Phylogenetic Analyses

The 16S rRNA, ANI, and AAI percent identity heatmaps all clearly display two distinct phylogenetic groups within *Thiomonas* (Figures 1-3). These groups correspond to those indicated in the concatenated ribosomal protein tree (Figure 4) and have been referred to previously in the literature as Group I and Group II (Delavat et al., 2012; Hovasse et al., 2016; Arsene-Ploetze et al., 2017). FB-6 and FB-Cd fall within Group II, according to all three metrics. The FB strains are two separate species according to previously established criteria (Konstantinidis et al. 2017); they share less than 98.6% 16S rRNA identity, less than 95% ANI, and less than 95% AAI with each other and with the other *Thiomonas* isolates (supplemental tables 1-3). For these two novel species, we propose the names *Thiomonas ferrovorans* FB-6 and *Thiomonas metallidurans* FB-Cd.

The FB strains are more closely related to the other members of *Thiomonas* Group II than to the members of Group I. According to AAI and 16S rRNA, they are part of the same genus as the other Group II isolates, which in turn are members of the same genus as the Group I isolates (Konstantinidis et al., 2017). However, the FB strains themselves fall just below or just above the minimum percent identity to be considered the same genus (AAI- 65%, 16S- 95%; Konstantinidis et al., 2017) when compared to the Group I isolates. A strict interpretation of the genus/species definitions may not be entirely valid in the case of these two strains. The FB strains are clearly the most distantly related members of *Thiomonas*.

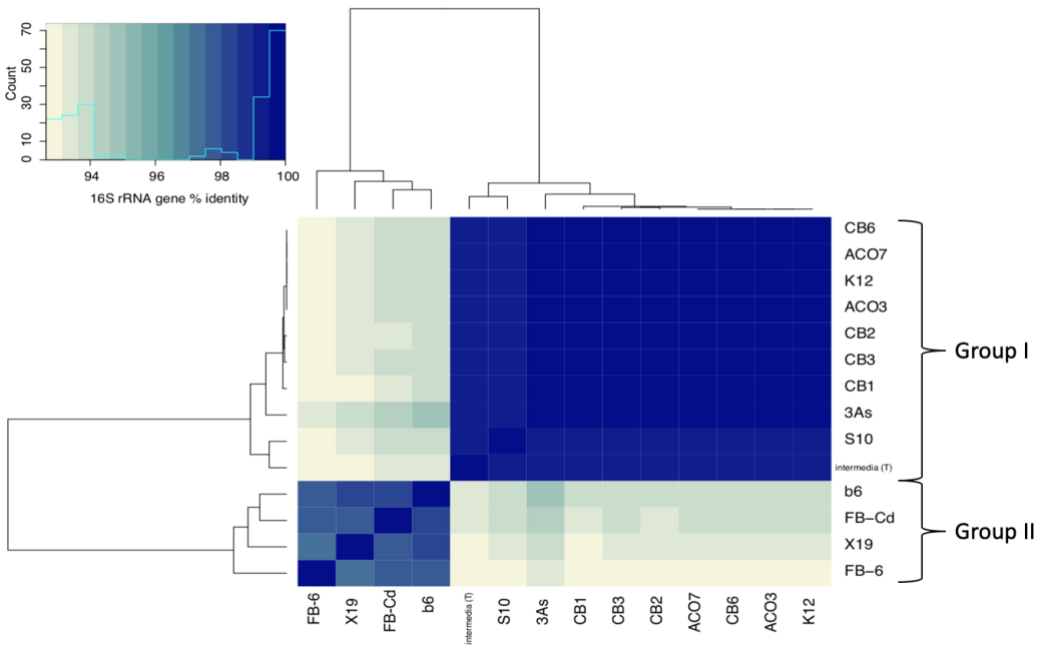

Figure 1: 16S rRNA gene percent identity heatmap of the *Thiomonas* isolates.

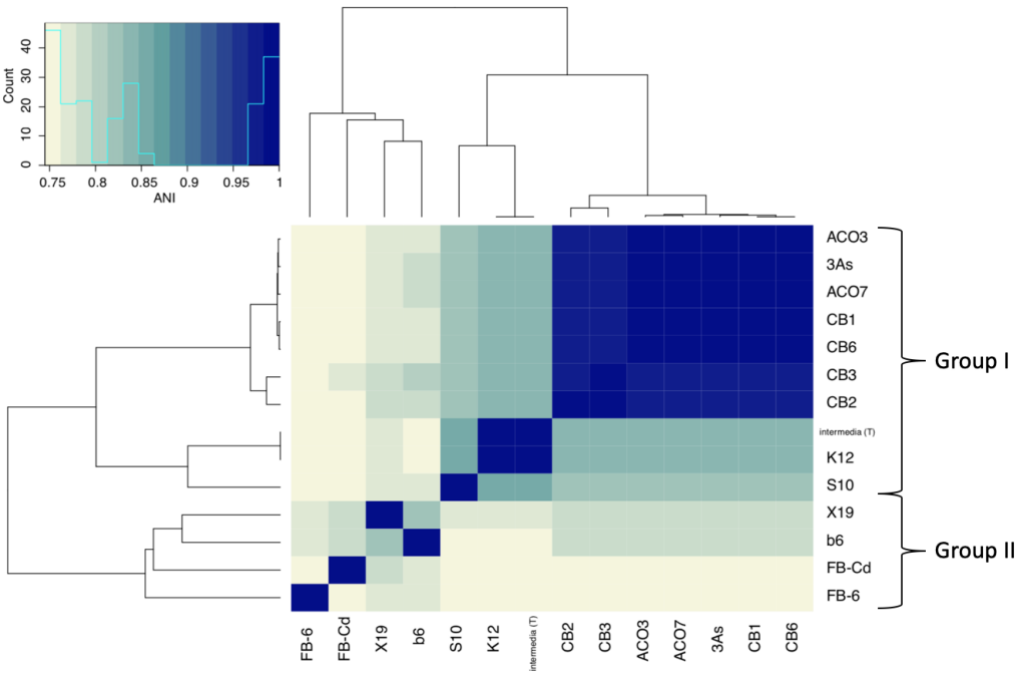

Figure 2: Heatmap displaying average nucleotide identity (ANI) of the *Thiomonas* isolates.

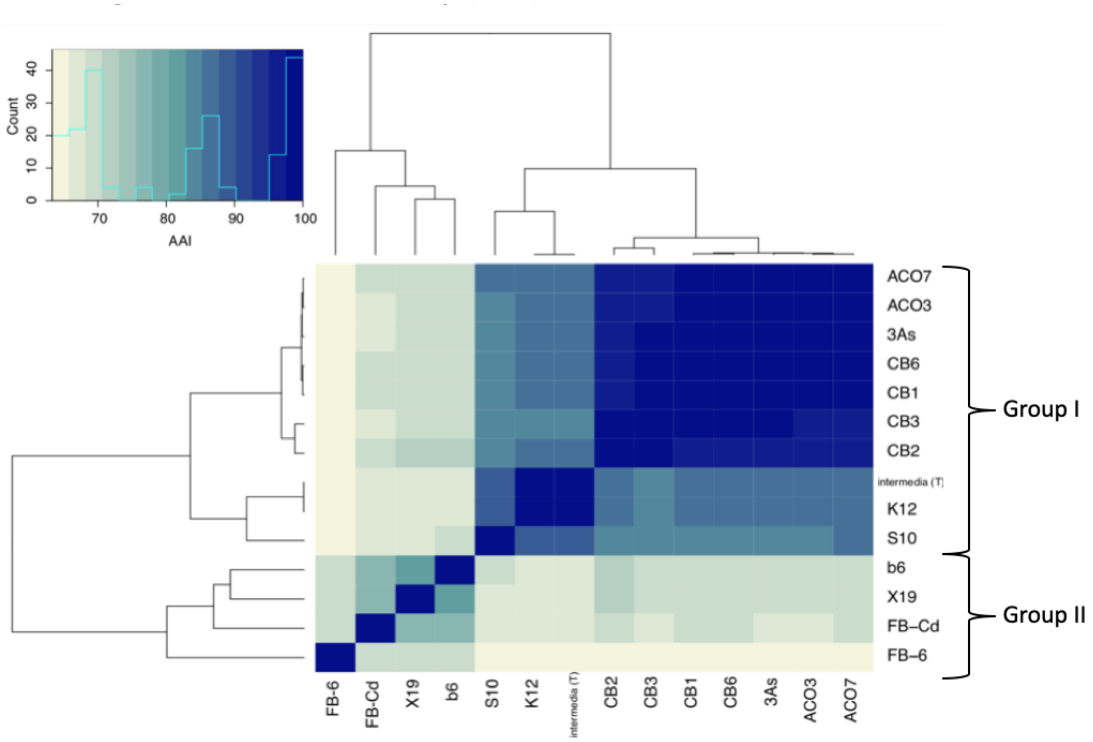

Figure 3: Average amino acid identity (AAI) of the *Thiomonas* isolates.

Figure 4: Maximum likelihood tree based on the concatenated alignments of 47 ribosomal proteins. *Burkholderia cenocepacia* AU 1054 and *Burkholderia ambifaria* MC40-6 were used as outgroups (not shown).

3.3 *Thiomonas* Isolate Pangenome

The purpose of the pangenome was to compare the genomes of all 14 *Thiomonas* isolates to identify the *Thiomonas* core genome and to determine which gene clusters were shared by subsets of *Thiomonas*. Here a gene cluster is defined as a set of homologous genes found in one or more genomes. Homology was determined by using the minbit heuristic (Benedict et al., 2014) to eliminate weak matches between amino acid sequences, and the MCL algorithm (van Dongen and Abreu-Goodger, 2012) was then used to identify gene clusters. The FB strains have the largest numbers of strain-specific gene clusters (Table 2; supplemental table 5), making them the most genetically distinct of the *Thiomonas* isolates, consistent with their phylogenetic distinctiveness. The other Group II strains, X19 and b6, have the third and fourth largest numbers of strain-specific gene clusters (Table 2).

The Group I strains have fewer strain-specific gene clusters than the Group II strains (Table 2), and there are more Group I-specific gene clusters than Group II-specific gene clusters (Figure 5). This is expected, since there are more Group I isolates than Group II isolates. There are more gene clusters differentiating Groups I and II than there are differentiating the AMD-derived isolates from the non-AMD isolates, indicating that diversification of Group I and Group II was not driven by AMD as a source environment.

Table 2: Gene Clusters of the *Thiomonas* Isolate Pangenome

	Number of gene clusters	Singleton gene clusters
Pangenome	10428	-
Core genome	1296	-
Group I-specific	416	-
Group II-specific	184	-
AMD group-specific	19	-
non-AMD group-specific	67	-
Carnoules group-specific	20	-
Group I AMD group-specific	120	-
FB strain-specific	122	-
FB-6	**3679**	**1393**
FB-Cd	**3943**	**1181**
X19	3924	937
b6	3409	532
S10	3010	337
K12	3058	8
intermedia T	3053	2
CB3	3618	230
CB2	3369	153
ACO3	3315	3
ACO7	3327	4
CB1	3471	12
CB6	3420	6
3As	3409	24

We looked for any gene clusters in the pangenome that were specific to the AMD-derived isolates and which may play a role in their ability to survive in AMD. We found only 19 AMD group-specific gene clusters, none of them appearing to have a function associated with AMD resistance. However, some metal resistance genes are present in only a subset of the AMD isolates. The arsenite oxidase genes *aioAB* were present in all AMD-derived strains but FB-6, and in two of the three non-AMD strains (K12 and *T. intermedia*^{T}). Additionally, some metal resistance genes are shared by all *Thiomonas* regardless of whether they were isolated from AMD. The core genome had several gene clusters involved in cobalt-zinc-cadmium resistance as well as metal ion efflux pumps and a tripartite multidrug resistance system. Evidently, the genetic potential for heavy metal resistance is common to all *Thiomonas*, and is not characteristic of only the AMD-derived isolates.

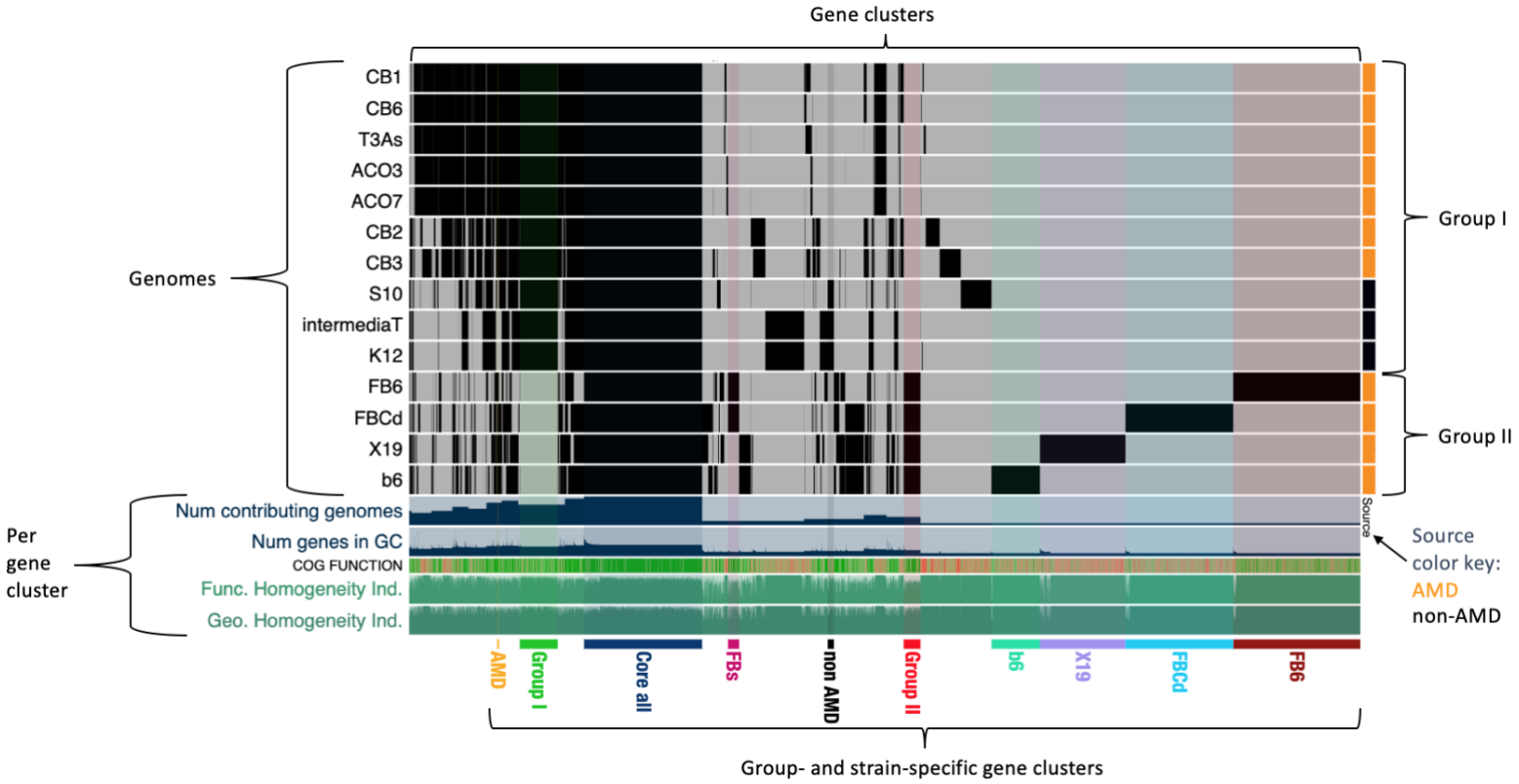

Figure 5: *Thiomonas* isolate pangenome generated with anvi'o v5.1. Functional and geometric homogeneity of gene clusters were determined with anvi'o v5.2. The FB strains clearly have the largest numbers of strain-specific gene clusters, followed by the other two members of Group II. Additionally, there are few gene clusters specific to the AMD-derived isolates.

3.4 Fe(II) Oxidation Pathways in *Thiomonas*

We identified two possible Fe(II) oxidation mechanisms within *Thiomonas* (Figure 6): the putative iron oxidase gene *cyc2*, shared by all strains except for *T. intermedia*^{T}, and the putative iron oxidation genes *mtoAB*, which were among the 122 gene clusters shared by and unique to both FB strains (Table 3; supplemental table 5). Mto is homologous to the iron reductase Mtr, which was initially characterized in iron-reducing bacteria and identified prior to Mto (Shi et al., 2012). To determine whether the FB strain *mtoAB* homologs were Mto or Mtr, we constructed a concatenated MtoAB/MtrAB phylogenetic tree (Figure 8) with known iron oxidizers and reducers. The FB strain *mtoAB* homologs are more closely related to those of known iron-oxidizers than known iron-reducers, providing further support for their putative iron oxidation function (Shi et al., 2012; He et al., 2017). The conserved cytochrome regions of the *Thiomonas* Cyc2 sequences all have 61.1% identity with that of the *Acidithiobacillus ferrooxidans* Cyc2 cytochrome region, while the less conserved porin regions have 29-33% identity with that of *A. ferrooxidans*. The Cyc2 phylogenetic tree is generally congruent with the phylogeny of Groups I and II in the concatenated ribosomal protein tree, which suggests that *cyc2* was inherited vertically from the *Thiomonas* common ancestor rather than via horizontal gene transfer (Figures 4 & 7). The FB strains could have acquired *mtoAB* horizontally, since only the FB strains have *mtoAB* in addition to *cyc2*. Having two putative iron oxidation mechanisms gives them the option of using one or the other depending on various environmental conditions. Additionally, the higher number of hemes in MtoA compared to Cyc2 might indicate greater efficiency of MtoA (Liu et al., 2012).

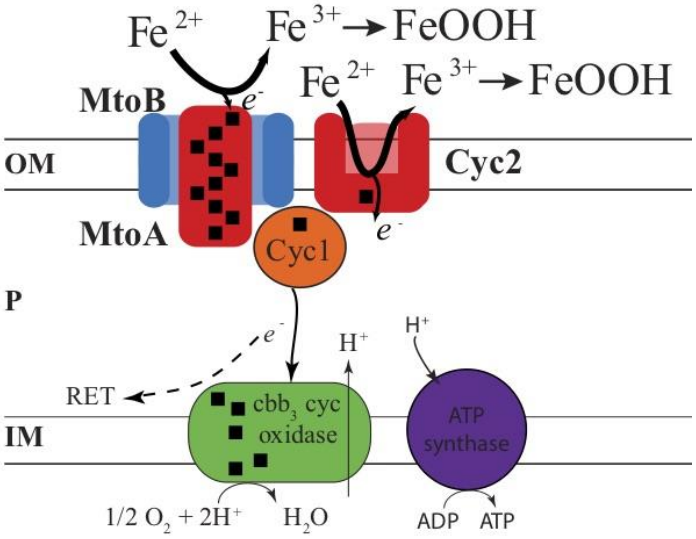

Figure 6: Proposed Fe(II) oxidation mechanisms in the FB strains based on genomic analysis. Black squares indicate hemes. See text for further description.

The FB strains are also distinct in having two cytochrome c biogenesis systems, the purpose of which is to transport heme from the cell interior to the periplasm and covalently link it to cytochromes (such as Cyc2 and MtoA) (Kranz et al., 2009). Most bacteria have only one of two cytochrome c biogenesis systems, system I or system II, and whether they have system I or system II is generally determined by phylogeny (Kranz et al., 2009). System I consists of eight genes, and studies have shown one of its components can serve as a heme reservoir which allows the system to function even at low heme levels (Feissner et al., 2006). However, its function requires energy in the form of ATP hydrolysis (Kranz et al., 2009). On the other hand, system II is simpler, consisting of only two genes at minimum, and requires less energy to synthesize but does not have any advantages under heme-limited conditions (Kranz et al., 2009). Bacterial species with both systems may have

horizontally acquired one system, and depending on selection pressures may subsequently lose the less advantageous system, or keep both systems for use under different growth conditions (Kranz et al., 2009).

Since the FB strains have both system I and system II while the other *Thiomonas* only have system II, the FB strains could have acquired system I by horizontal gene transfer. The FB strain system I genes are encoded in a complete *ccm* operon (Allen et al., 2002) located almost directly adjacent to *mtoAB*, which is also unique to the FB strains, and likely also acquired horizontally. Since MtoA has 10 hemes, it may require system I's higher heme capacity in order to function. Conversely, Cyc2 has only one heme, and so it could use system II for heme transport. The FB strains may be inclined to keep both system I and system II due to this differing utility; when they are using Cyc2, they could save energy by using the less energy expensive system II, and when they are using MtoAB, they could use system I to provide all the necessary heme.

Table 3: Locus Tags of FB Strain *cyc2* and *mtoAB* Homologs

	FB-6	E-value	FB-Cd	E-value
Cyc2 (ref: *A. ferrooxidans*)	D466DRAFT_0260	4.00E-47	CD04DRAFT_3625	9.00E-38
MtoA (ref: *S. lithotrophicus* ES-1)	D466DRAFT_2462	3.30E-133	CD04DRAFT_2152	1.30E-133
MtoB (ref: S. lithotrophicus ES-1)	D466DRAFT_2461	1.70E-149	CD04DRAFT_2153	0

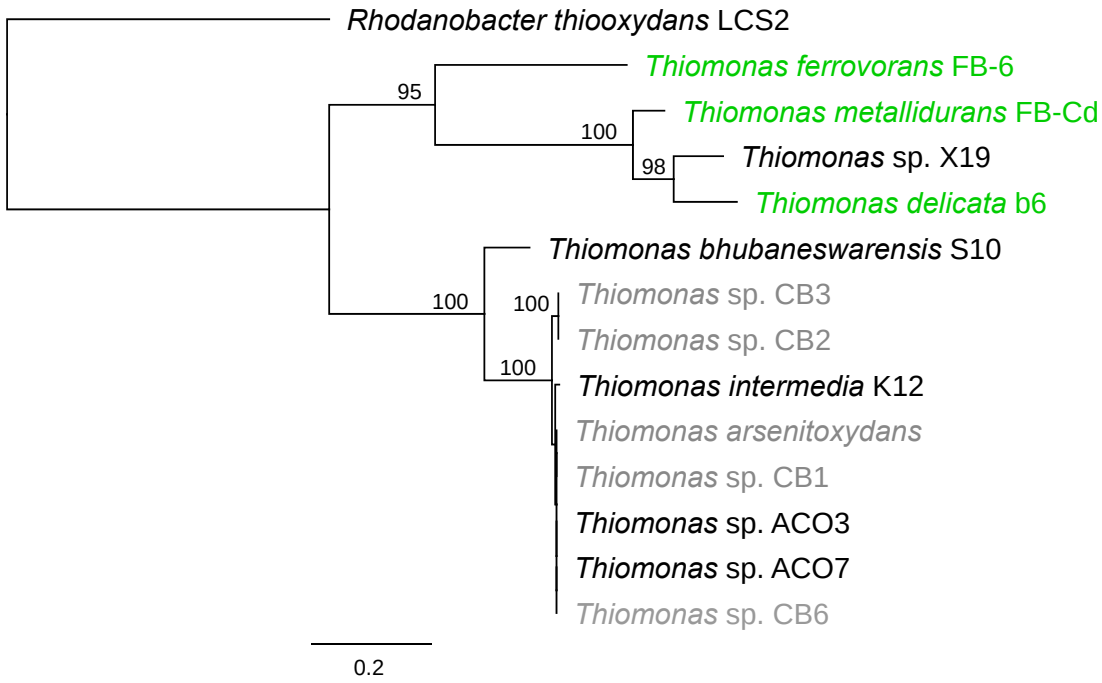

Figure 7: Maximum likelihood tree based on Cyc2 amino acid sequences. Bootstrap values are shown next to branch nodes. *Rhodanobacter thiooxydans* LCS2 was used as outgroup. Green= known Fe(II) oxidizers. Grey= inconsistent evidence of Fe(II) oxidation.

A hypothetical iron oxidation pathway has been previously proposed for the iron oxidizers *Mariprofundus aestuarium* CP-5 and *Mariprofundus ferrinatatus* CP-8 (Chiu et al., 2017). The pathway consists of genes encoding an iron oxidase (*cyc2* or *mtoAB*) and a complete oxidative phosphorylation pathway, including genes encoding an NADH dehydrogenase, succinate dehydrogenase, cytochrome bc1 complex, cytochrome c oxidases (cbb_3- or aa_3-type), ATP synthase, and at least one homolog of *cyc1* (Figure 6; Chiu et al., 2017). The *cyc1* gene encodes the periplasmic electron carrier Cyc1 that transfers electrons between the outer membrane protein Cyc2 and the cbb_3-type cytochrome c oxidase (Yarzabal et al., 2002; Barco et al., 2015). All 14

isolates have all these components, as well as a cytochrome c biogenesis system, except for *T. intermedia*^{T}, which is missing both *cyc2* and *mtoAB*. Although only the FB strains and strain b6 have so far been conclusively shown to oxidize iron (Battaglia-Brunet et al., 2006; Beulig, 2010), our data indicates that all *Thiomonas* isolates except for *T. intermedia*^{T} have the genetic potential for iron oxidation using the putative iron oxidase Cyc2.

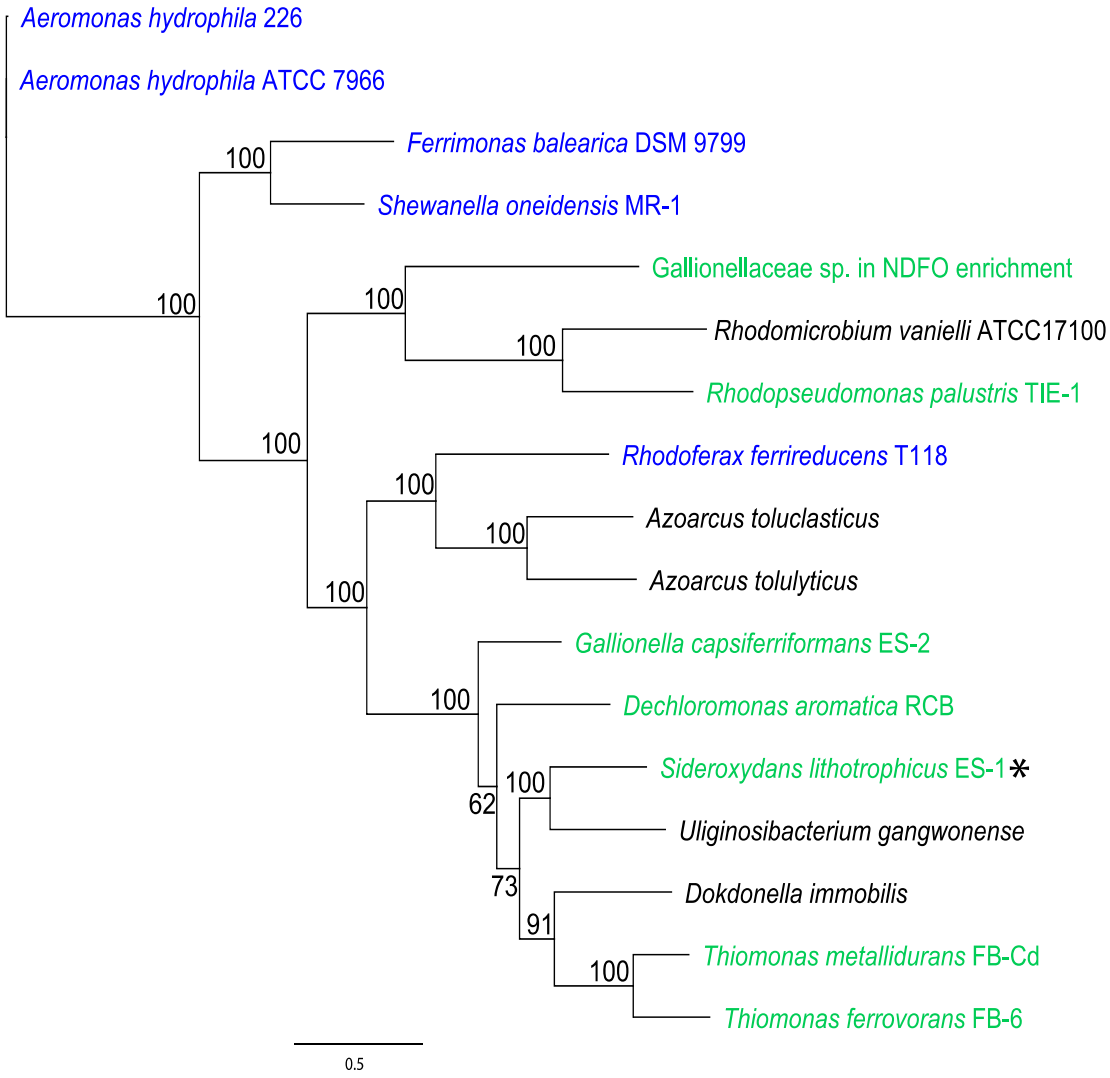

Figure 8: Maximum likelihood tree based on the concatenated alignments of MtoAB/MtrAB amino acid sequences. Bootstrap values are shown next to branch nodes. Names of known iron-oxidizers are in green, and names of known iron-reducers are in blue. *= MtoA has been implicated as an Fe(II) oxidase (Liu et al., 2012).

3.5 Carbon Metabolism

The FB strains have the genetic potential for autotrophy, as indicated by complete Calvin-Benson-Bassham cycle (including form I RuBisCo) and TCA cycle

pathways. All Group I isolates have both form I and form II RuBisCo, while the Group II strains only have form I. Form I is better suited for higher O_2 conditions than form II (Badger and Bek, 2008), which could indicate the Group I strains are adapted to a wider range of O_2 concentrations. This is also supported by the presence of both cbb_3- and aa_3-type terminal oxidases. The cbb_3-type is adapted to low O_2 while the aa_3-type is adapted to high O_2 (Arai et al., 2014), suggesting that most *Thiomonas* can tolerate a wide range of O_2 concentrations. The Group II strains FB-6, FB-Cd, and b6 have both types of terminal oxidases, even though they only have form I RuBisCo, indicating they could still tolerate a wide range of O_2 concentrations. The Group II strain X19 has the high O_2 adapted varieties of both RuBisCo and terminal oxidase, indicating a preference for higher O_2 conditions.

The FB strains both have multiple glycolytic pathways: they both have complete Entner-Doudoroff (ED) and glycolysis (EMP) pathways, and FB-Cd also has a complete oxidative pentose phosphate (OPP) pathway. Most isolates have the complete ED pathway, including the key enzyme 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase (Chen et al., 2016). The exception is strain b6, which is missing two components, including KDPG aldolase. Most isolates have the complete glycolysis pathway, including the key enzyme phosphofructokinase (Chen et al., 2016). Strains b6 and X19 are missing phosphofructokinase, and b6 is also missing another component. Since the genome of strain b6 is complete (Table 1), it likely does have incomplete ED and glycolysis pathways. However, b6 does have a complete OPP pathway. Similar to b6, the cyanobacterium *Prochlorococcus* lacks phosphofructokinase but has a complete OPP pathway, which it could be using to catabolize glucose (Gomez-Baena et al., 2008). Strain b6 may also use the OPP

pathway for glucose catabolism. The only other *Thiomonas* isolates with complete OPP pathways are FB-Cd and X19; the other strains are all missing one component. Since all the isolates have at least one complete glycolytic pathway, they should all have the genetic potential for mixotrophic or heterotrophic metabolism.

Both FB strains can grow heterotrophically on organic carbon sources (Beulig, 2010), although FB-6 has a wider variety of organic carbon transporters than the other isolates (supplemental table 4). FB-6 is unusual among the *Thiomonas* isolates in that it has a fructose ABC transporter system, while FB-Cd, along with 3As, K12, *T. intermedia*^{T}, the CB strains, and the ACO strains, have a PTS system for fructose transport. While FB-6 has several other organic carbon transporters which are rare or absent in the other *Thiomonas* isolates, all isolates have the ABC-type branched-chain amino acid transport system components *livFGMHK* (supplemental table 4). Strains FB-6, FB-Cd, b6, 3As, and *T. intermedia*^{T} have been shown to grow heterotrophically on several forms of organic carbon (Beulig, 2010; Battaglia-Brunet et al., 2006; Duquesne et al., 2007; London, 1963; Slyemi et al., 2011). S10 is an obligate mixotroph and can grow mixotrophically on thiosulfate with several forms of organic carbon (Panda et al., 2009). The CB and ACO strains were isolated on R2A agar medium (Bruneel et al., 2003; Freel et al., 2015), so they may have been able to use the agarose as a carbon source. The presence of organic carbon transporters in the *Thiomonas* isolates, together with the presence of at least one glycolytic pathway, indicates that all of them have the genetic potential for heterotrophy or mixotrophy.

3.6 Heavy Metal Resistance Mechanisms

All 14 *Thiomonas* isolates have the genetic potential for heavy metal resistance; they possess multiple copies of various heavy metal efflux pumps used in the export of cobalt, zinc, cadmium, and chromate, as well as the cobalt-zinc-cadmium resistance genes *czcA* and *czcD*. The presence of these genes in the FB strain genomes is consistent with the high levels of cobalt, zinc, and cadmium detected in the Ronneburg AMD sediment from which they were isolated. All the isolates also have a three-part multidrug resistance system, consisting of an inner membrane component, an outer membrane component, and a membrane fusion protein, which can confer resistance to a variety of antimicrobial substances (Moreira et al., 2004). Most isolates also have the genetic potential for arsenite oxidation; they all have the arsenite oxidase genes *aioAB* except for FB-6 and S10. Most isolates have the genetic potential for mercury resistance. Every isolate except for S10 and *T. intermedia*^{T} has the mercuric ion reductase gene *merA*. Most isolates have the mercuric transport genes *merC* and *merT*, except for the FB strains, S10, and *T. intermedia*^{T}. Strain K12 also lacks *merC*, but it has *merT*. The FB strains, S10, and *T. intermedia*^{T} are also the only strains which do not have the gene for the periplasmic mercury(+2) binding protein MerP.

The FB strains and most of the other isolates have the genetic potential for urea transport and degradation. Urea degradation has previously been associated with acid tolerance and ferric iron precipitation (Freel et al., 2015). Media acidification was prevented when CB2 was grown in the presence of urea, and when CB2 was incubated in AMD-impacted water supplemented with urea, an orange precipitate accumulated, which was not observed when there was no urea degradation activity (Freel et al., 2015). All isolates except for strains CB3 and K12 have the urea transport system genes *urtABCDE*, the urease gene *ureC*, and the urease subunits *ureAB*. Every isolate

except for K12 has the genes for the urease accessory proteins UreF and UreG. All 14 isolates have the gene for the urease accessory protein UreE. Strains CB2, 3As^{T}, CB1, and CB6 have been shown to degrade urea, although the urea degradation activity of CB2 was much greater than that of the other three strains, and urea degradation and transport genes have previously been identified in these four genomes (Farasin et al., 2015).

Chapter 4

CONCLUSIONS

The FB strains represent two distinct novel species of *Thiomonas* according to phylogenetic and pangenomic analyses, for which we propose the names *Thiomonas ferrovorans* FB-6^T sp. nov. and *Thiomonas metallidurans* FB-Cd^T sp. nov. The FB strains are distinct among the *Thiomonas* isolates regarding their possession of two putative iron oxidation genes and two cytochrome c biogenesis systems. They may preferentially use either Cyc2 or MtoAB depending on various environmental conditions, which remain to be fully understood. Additionally, the FB strains may be inclined to keep both mechanisms due to a difference in efficiency, which may result from the higher number of hemes in MtoA compared to Cyc2.

The presence of multiple putative iron oxidation mechanisms in the FB strains presents an opportunity to study alternative mechanisms of iron oxidation in a group of organisms who are not typically known as iron oxidizers. The fact that strain b6 can oxidize iron yet only has one putative mechanism provides an opportunity for comparison to the FB strains, who can also oxidize iron yet have multiple putative mechanisms for doing so. The higher Fe(II) concentrations in the Ronneburg AMD sediment compared to other AMD-impacted *Thiomonas* environments is also an exciting indicator of the presence and activity of iron oxidizers (Bruneel et al., 2003; Casiot et al., 2003; Hallberg and Johnson, 2003; Johnson and Hallberg, 2005; Beulig, 2010; Hovasse et al., 2016). Overall, the FB strains are good candidates for the study of iron oxidation mechanisms in AMD-derived bacterial isolates.

Thiomonas ferrovorans FB-6 and *Thiomonas metallidurans* FB-Cd are the newest and most genetically distinct members of *Thiomonas*, contributing genetic and metabolic diversity to the genus. Our analyses demonstrate that the FB strains have the genetic potential for iron oxidation by two putative mechanisms, one of which they do not share with the other *Thiomonas* isolates. Now that their putative iron oxidation mechanisms have been identified, the iron oxidation activity of the FB strains can be tracked via differential expression experiments, and it could also be determined which of these mechanisms they are using *in situ*. The FB strains may be able to use one or both mechanisms to remove heavy metals from AMD by iron oxidation, and therefore contribute to bioremediation of AMD.

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Appendix A

List of Supplementary Tables

ST1: 16S rRNA gene percent identity matrix

ST2: Average nucleotide identity (ANI) matrix

ST3: Average amino acid identity (AAI) matrix

ST4: Presence/absence of key genes in *Thiomonas*

ST5: Gene clusters specific to both FB strains, FB-6 only, and FB-Cd only

Appendix B

Appendix B

Culturing *Thiomonas* on Ferrous Iron

Thiomonas strains FB-6, FB-Cd, and b6 have previously been reported to oxidize ferrous iron (Beulig, 2010; Battaglia-Brunet et al., 2006). We cultured these three strains in ferrous carbonate gradient tubes and ferrous chloride serum bottles to observe their growth. Gradient tubes have a ferrous iron source at the bottom of the tube and an oxygenated headspace at the top, and the ferrous iron and oxygen gradients proceed in opposite directions, as depicted in Figure B1.

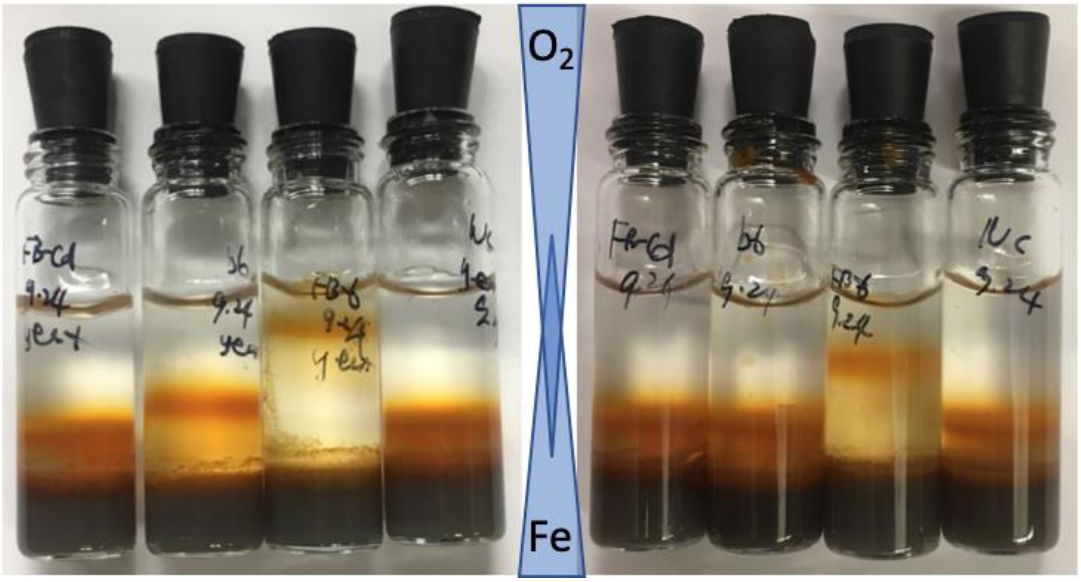

Figure B1: FB-Cd, b6, and FB-6 in gradient tubes containing an $FeCO_3$ plug and 10 mM MWMM-HEPES media at pH 7.0. The four tubes on the left have 0.1 g/L yeast extract added, and the four tubes on the right are without yeast extract. The fourth tube in each set of four is a negative (uninoculated) control.

When we grew these strains in gradient tubes with and without yeast extract added (Figure B1), we observed that FB-6 grows at a higher position in the tube than the other two strains, whether or not yeast extract is present. Strain b6 appears to prefer the presence of yeast extract, and the FB-Cd growth band is not easily distinguishable from the negative control.

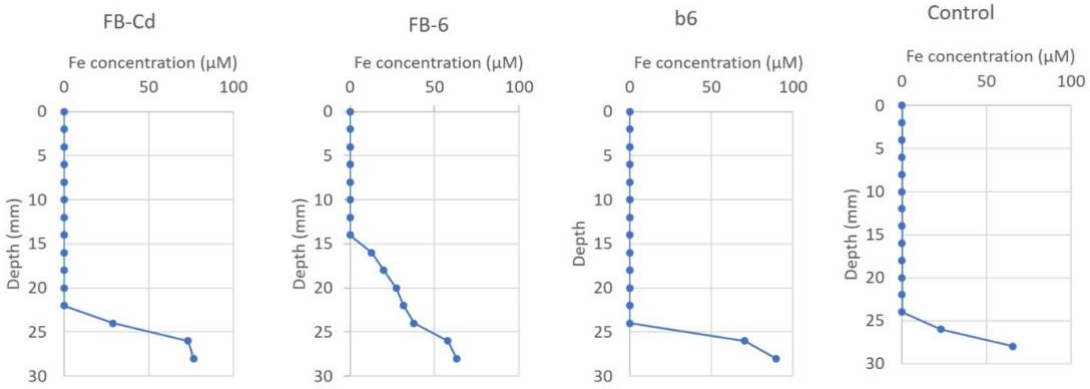

Figure B2: Ferrous iron profiles of the gradient tubes in figure B1 without yeast extract.

We then measured the ferrous iron gradient in the gradient tubes without yeast extract (Figure B2); we could not measure the ferrous iron gradient in the tubes with yeast extract because the yeast extract would interfere with the measurement of iron. We observed that the iron gradient in the FB-6 tube is steeper than for the other two strains, and the point at which ferrous iron appears to vanish corresponds with the level at which FB-6 grows in the tube. This indicates that FB-6 is consuming ferrous iron. It also indicates that FB-6 is consuming more oxygen than the other two strains

and needs to grow at a point along the oxygen gradient where the oxygen concentration is high enough for its needs.

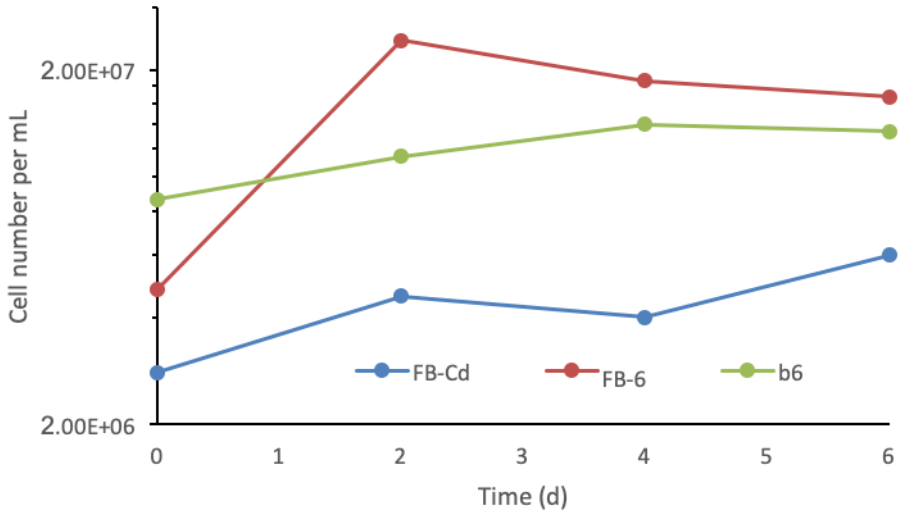

Figure B3: Growth curve of FB-6, FB-Cd, and b6 in 10 mM MWMM-HEPES liquid culture (pH 7) amended daily with 100 uM $FeCl_2$. The headspace was 5% O_2, 20% CO_2, and 75% N_2.

Since the agarose in the gradient tubes may provide a carbon source to support cell growth, we also cultured FB-6, FB-Cd, and b6 in liquid media containing ferrous chloride and constructed a growth curve (Figure B3). While none of the strains increased in cell number by more than an order of magnitude, all three strains did increase in cell number, and FB-6 showed higher growth than either FB-Cd or b6.