Proteogenomic Studies on Extracellular Electron Transport in the Iron-Respiring Thermophilic Bacterium, *Thermus* sp. AO3C

by

Anders Carl Johnson

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To the Graduate Faculty:

The members of the committee appointed to examine the thesis of ANDERS C. JOHNSON find it satisfactory and recommend that it be accepted.

_____
Dr. Timothy S. Magnuson
Advisor

_____
Dr. Michael A. Thomas
Committee Member

_____
Dr. Chikashi Sato
Graduate Faculty Representative

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Proteogenomic studies on extracellular electron transport in the iron-respiring thermophilic bacterium, *Thermus* sp. AO3C

Thesis abstract- Idaho State University 2016

Iron is the fourth most abundant element on Earth. Dissimilatory Metal Respiring Bacteria (DMRB) play a role in the redox cycling of this element. In addition to iron, DMRB could be useful for use in toxic-metal bioremediation or electrical energy generation. Mesophilic DMRB genera were the first to be isolated and studied for remediation purposes. Further exploration has discovered these bacteria residing in all energetically favorable habitats throughout geographically distinct regions of the world. While mesophilic DMRB are more favorable for laboratory experimentation, extremophile DMRB tend to be over-looked due to further constraints necessary for cultivation. Their study presents an opportunity to examine inhabitants of environments that may be early Earth analogs and metabolic processes potentially related to the Great Oxygenation Event. *Thermus* sp. AO3C, a gram-negative thermophilic bacterium, was isolated from Alvord hot springs (Oregon, USA) in 2006 for its Arsenic – oxidizing ability and was later determined to have iron respiration capabilities. Our proteogenomic analysis of *Thermus* sp. AO3C produced a draft genome composed of 78 contigs that compose one circular chromosome and indicated presence of two plasmids. MS/MS Tandem Spectroscopy indicates presence of *c*-type cytochrome and pilin proteins, as well as melanin and Flavin redox active molecules potentially implicated in extracellular electron transport. We identified redox proteins and other redox molecules that appear to be bound to the outer membrane and excreted from cells and may be involved in extracellular electron transport for Fe- respiration.

Chapter 1

Literature Review: Microbial Respiration of Metals

Introduction

This review is presented to provide an understanding of Dissimilatory Metal Respiring Bacteria (DMRB) and potential mechanisms behind Extracellular Electron Transport (EET). Specifically, interest is directed toward a thermophilic (DMRB) genus, *Thermus* sp. AO3C. The function of iron (Fe)-respiration proteins may hold potential applications for biotechnological industry. By developing a broader understanding of how Fe-respiration couples to EET, those processes can be applied to investigations of bacterial reduction of Fe-species for energy generation or environmental remediation. This literature review is an effort to synthesize current understanding of the following areas: 1) bacterial respiration 2) iron, 3) biotic and abiotic iron conversion, 4) Iron Reducing Bacteria, 5) *Thermus*, 6) potential mechanisms of extracellular electron transport, 7) facilitating direct contact with ferruginous minerals 8) indirect contact: redox proteins and other redox-active molecules, 9) bacterial *c*-type cytochromes, 10) biofilms and metal metabolism, 11) current extracellular electron transport analytical tools and methodologies, 12) applications for *Thermus* in industry and biotechnology, 13) EET and its role in exobiology. Thermophilic DMRB may utilize one or a combination of proteins or cellular structures, potentially homologous with other DRMB genera, where respiratory processes transport electrons from an electron donor to a metal within the environment.

Bacterial Respiration

The mechanisms by which energy flow is encompassed within the unified themes of biology and reduction-oxidation (redox) chemistry (Nealson, Belz, & McKee, 2002). In electron-transport chained biological redox reactions, free energy is harnessed by transporting electrons from an electron donor (e.g. oxidation / loss of electron) to an electron acceptor (reduction / gain of electron) (Gralnick & Newman, 2007; Magnuson, Hodges-Myerson, & Lovley, 2000; Nealson et al., 2002; Weber, Achenbach, & Coates, 2006). Electron transport via redox chemistry is ultimately coupled to the generation of adenosine triphosphate (ATP) (Gray & Winkler, 2003). Eukaryotic aerobic organisms have evolved use of a limited pool of respiratory molecules where glucose and molecular oxygen serve as the primary electron donor and acceptor, respectively (Pirbadian et al., 2014). Prokaryotes are capable of harvesting electrons from almost any compound with a redox potential sufficient enough to yield energy; almost any solid phase or soluble mineral can serve as terminal electron acceptors (Gralnick & Newman, 2007; Kowalchuk, Jones, & Blackall, 2008; Nealson et al., 2002). This would suggest that there is a potential pool of prokaryotic candidates possessing desirable forms of respiration that humans might manipulate for environmental remediation or energy generation.

Adenosine Triphosphate (ATP) is the energy currency for life. Prokaryotes and eukaryotes both depend on a continuous flow of electrons to form requisite electrochemical gradients to enable the synthesis of ATP via electron transport systems and proton motive force formation (Harel, Bromberg, Falkowski, & Bhattacharya, 2014). The electron transport chain is housed in the mitochondria of eukaryotes and within the plasma membrane of prokaryotes. Energy is derived from electron transport chains by

passing electrons stepwise down electron carriers of descending redox potential to terminal electron acceptors.

During anaerobic respiration, the prokaryote oxidizes either organic carbon or hydrogen thereby freeing an electron (or several, 8 electrons in the case of acetate and more for glucose). The electron shuttles from one redox protein to the next following descending electronegativity (Eh). At each of these steps a small portion of energy is harvested from the electron through each protein (Richter, Schicklberger, & Gescher, 2012) and coupled to electron transport. That released energy powers the various peptides along the electron transport chain like dehydrogenases, cytochrome complexes, and quinones, that shuttle protons across the plasma membrane to cell-wall or cell-surface associated cytochromes (Gralnick & Newman, 2007). The electron is finally passed on to the terminal respiratory electron acceptor. Energy harvested from the transfer of electrons down those stepwise redox carriers powers transmembrane proteins that pump protons across the plasma membrane generating a proton motive force that powers ATP synthesis from Adenosine Diphosphate (ADP) with H^+-transporting ATP synthases (Fuller et al., 2014).

Iron

Iron (Fe) is the fourth most abundant element of the Earth's crust (Weber et al., 2006). At or above circumneutral pH, iron exists primarily as an insoluble, solid-phase, mineral in one of two oxidation states: divalent ferrous iron (Fe(II)) or trivalent ferric iron (Fe(III)) (Weber et al., 2006). Iron redox reactions occur through abiotic and biotic processes (Ionescu, Heim, Polerecky, Thiel, & de Beer, 2015). Reduction potential (Eh) measures the tendency for electrons to be acquired by a chemical species and thereby

reduced. Eh is measured in volts (V) and each chemical species has its own Eh range. For example, Fe^{3+}/ Fe^{2+} at pH 2 has an Eh of +0.77V, Fe(III)/Fe(II)-citrate has an Eh of +0.385V, and ferrihydrite has an Eh of +0.1 to -0.1 (Bird, Bonnefoy, & Newman, 2011). The more positive the chemical species Eh the greater the tendency to be reduced due to its greater affinity for electrons.

Biotic and Abiotic Iron Conversion

Abiotic iron oxidation is a function of pH and O_2 concentration and abiotic iron reduction has been shown to be catalyzed by iron-sulfur minerals or organic compounds (Ionescu et al., 2015). An abiotic Fe cycle is no longer a new idea and was first suggested by Luther et al. (1992). The great abundance of iron and iron redox reactions, taken together, have the ability to sustain substantial, diverse, microbial populations and is conceivably one of the most ancient form of metabolism on Earth (Weber et al., 2006).

In aerobic environments, bacteria may facilitate biotic iron oxidization using O_2, while anaerobic Fe(II) oxidation has been observed with NO_3 as terminal electron acceptors (Ionescu et al., 2015). Conversion rates for biotic and abiotic oxidation occur at similar rates and compete with each other at circumneutral pH. (Fe(III)) will bind to charged groups of organic substances that are in a reduced state (Ionescu et al., 2015). A nucleation matrix would be formed by this iron and further aid abiotic precipitation. Upon oxidization, this iron would then be amenable to iron reduction through abiotic chemistry or biotic metabolism. This suggests that in the presence of biologic organic matter (humic substances), a common constituent of natural systems, abiotic and biotic process of iron reduction and oxidation reactions are mathematically inseparable (Ionescu et al., 2015). Through live and dead experiments, a complete abiotic Fe cycle was shown

to be possible with presence of biological matter, even in sub-optimal conditions (Ionescu et al., 2015). Metabolic and non-metabolic components were determined to regulate Fe-redox rates and cannot be experimentally distinguished due to the complexities of natural systems. Regardless of the quantity of Fe-reducing or oxidizing bacteria, presence of any biological matter that contributes to those reactions yield similar rates for different environments. This represents limitations with separating abiotic reduction from biotic reduction of an extremophile that may reside in a hot spring or acid-mine seepage.

Iron Respiring Bacteria

DMRB collectively represent genera capable of respiring on metals, with some capable of reductions and others oxidization. With regard to iron-reducing organisms, Fe (III) serves as a terminal electron acceptor under anaerobic conditions and Fe (II) is an electron donor for Fe-oxidizing microbes under both aerobic and anaerobic conditions (Weber et al., 2006). Iron reducing bacteria (FeRB) belong to a sub-class of DMRB because of their ability to couple Fe reduction with their metabolism and iron respiration. This form of respiration has been proposed to be one of the first microbial metabolisms to have evolved (Weber et al., 2006). Their mechanisms of respiring metals, which may precede sulphate, nitrate and oxygen respiration, is of particular interest and may yield insights for biotechnological remediation, energy generation, and exobiological exploration.

The generalized Fe-reduction cycle starts when the electron donor becomes oxidized by FeRB and subsequently donates electrons that travel step-wise down through a series of redox carriers with decreasing potential finally to reduce Fe (III), the solid-phase electron acceptor. Abiotic reduction of the terminal electron acceptor reinitiates the

cycle (Lovley, Coates, Blunt-Harris, Phillips, & Woodward, 1996). As previously stated, oxygen, nitrate, or humic substances may provide a means to abiotic oxidization of the terminal electron acceptor.

Oxygenic respiration is more favorable and 'preferential' do the higher affinity oxygen has for electrons. More energy is generated from this form of respiration, but oxygen is not ubiquitous through all environments. Anaerobic Fe(III)-reduction will not be as energetically favorable as oxygenic respiration, there is still a net energy gain from this form of respiration. As a result of increased energy limitations, microorganisms that utilize Fe(III) as terminal electron acceptor will multiply more slowly than if the organism used oxygen. A hypothetical model of *Shewanella oneidensis* Fe-respiration involving extracellular electron transport (Figure 1.1) was previously proposed by Bird, Bonnefoy, & Newman, (2011). They outlined a potential electron transport chain arrangement in tandem with a tree of oxygenic and anoxic redox potential Eh ranges of each transport protein related to this electron transport pathway.

It may be that anaerobic DMRB grow more slowly, but the lack of oxygen will not allow the abiotic reduction of the terminal electron acceptor and oxidized insoluble iron should precipitate. In an aqueous environment like a hot spring, the constant flow-through of water may wash the iron terminal electron acceptor back and forth between the aerobic and anaerobic zones, thus allowing for abiotic reduction and biotic oxidization cycling. Those regeneration cycling rates may be increased in areas with lower hydraulic turn over, like pore spaces of biofilms.

Thermus

Thermus is a genus of thermophilic bacteria that is characterized as a gram-negative, non-motile, non-flagellated, pleomorphic filamentous rod. Most strains predominantly form luxuriant orange streamers. Members of the genus utilize diverse energy generation mechanisms including strict aerobic, fermentative, and facultative anaerobic metabolisms in addition to dissimilatory metal respiration (Whitman et al., 2015). Isolates have been cultured from geographically, naturally and anthropogenically formed, distinct thermal regions all over the world (Dwivedi, Sangwan, & Nigam, 2012; Murugapiran et al., 2013; Saiki et al., 1988). This broad geographical distribution of *Thermus* suggests that these bacteria are likely inhabiting any suitable niche world-wide. The most notable and widely known member of this genera is *Thermus aquaticus*, from which the biotechnologically important enzyme *Taq* polymerase was first isolated (Saiki et al., 1988) allowing for thermocyclic automation of Polymerase Chain Reaction (PCR) DNA amplification. Thermophiles represent a core group of DMRB where model organism incorporation can expand phylogenetic, eco-physiologic, and evolutionary boundaries across DMRB genera. Elucidating DMRB core metal respiratory processes could reveal a 'global' model of metal respiration that holds true across all environments.

Thermus sp. AO3C (16S rRNA gene sequence EF204914) was first isolated from Alvord Hot Springs in 2006 (N374069 –E4711380, UTM, Zone 11, NAD 83, Meters) c. 50 km north of Fields in southeastern Oregon, USA. Alvord hot spring channels are circumneutral pH. Temperatures begin at 75 ˚C and decrease along water channels. Waters have an average As concentration of 4.5mg L^{-1}(60 μM) (Connon, Koski, Neal, Wood, & Magnuson, 2008). *Thermus sp. AO3C* follows reported *Thermus* genera morphology: gram negative, facultative anaerobic metabolism, and pleomorphic

filament-forming rods. Connon (2008) reported *Thermus sp. AO3C* can derive energy from harbored respiratory arsenite (As(III)) oxidase activity that rapidly converts As(III), the primary or sole As species in that environment, to Arsenate (As (V)) via microbial oxidation. FT-IR and X-ray spectroscopy revealed the presence of amorphous Fe-oxides in mineral phases of both colorless upstream biofilms and thick orange mats of downstream biofilms. Dissolved As concentrations did not decrease significantly over the stream distance, suggesting that As incorporation into the biofilm is limited (Connon et al., 2008). Direct isolation and sequencing of 16S rRNA gene sequences indicate that *T.* AO3C is similar to *Thermus oshimai* JL-2 (BLASTn, NR102473, >99% similarity), isolated from the Great Boiling Spring geothermal system near Gerlach, Nevada (Murugapiran et al., 2013).

Potential Mechanisms of Extracellular Electron Transport

Mediating electron transfer to insoluble Fe (III) oxides via genetically-orchestrated microbial redox reactions is important due to the vast diversity of potential electron donors and acceptors available to microorganisms (Gralnick & Newman, 2007; Kowalchuk et al., 2008; Nealson et al., 2002). Multiple strategies have been proposed to facilitate the electron transfer between microorganisms and solid-phase Fe (III) surfaces where the microorganisms are in direct contact, indirect contact, or great distances from the respiratory surface (Hernandez & Newman, 2001; Lovley et al., 1996; Pirbadian et al., 2014; Reguera et al., 2005). Strategies suggested for extracellular electron transport (EET) include direct contact by nanowires (Gorby et al., 2006; Pirbadian et al., 2014; Reguera et al., 2005; Wegener, Krukenberg, Riedel, Tegetmeyer, & Boetius, 2015), redox protein shuttles (Newman & Kolter, 2000a), humic substances (Lovley et al.,

1996), substrate augmentation(Luther et al., 1992), and biofilm formation (Branda, Vik, Friedman, & Kolter, 2005). Orchestration of those potential avenues for cell-to-cell or cell-to-substrate electron transport are discussed below.

Due to their historical significance as the first strains studied for environmental remediation and relative ease of cultivation, *Shewanella* (Petrovskis, Vogel, & Adriaens, 1994; Venkateswaran et al., 1999), *Geobacter* (Lovley, Stolz, Nord, & Phillips, 1987), and *Desulfovibrio* (Payne et al., 2004) remain the primary model genera for study of dissimilatory metal or mineral reduction (Shi, Squier, Zachara, & Fredrickson, 2007). Members of those model genera have also been shown capable of living and thriving in environments laden with contaminant metals (e.g. Cr, U, Se, Tc, As, Fe) and are amenable to mesophilic and neutrophilic culture conditions. Incorporation of *Thermus* pursues a holistic understanding of DMRB respiration by exploring modifications to redox molecules that may have been selected for heat-tolerance.

Facilitating Direct Contact with Ferruginous Minerals

Extracellular electron transport proposes strategies to facilitate the transfer of electrons between the surfaces of solid-phase oxides and microorganisms (Gralnick & Newman, 2007). *Geobacter* and *Shewanella* spp. require a mechanism of direct contact with respiratory surfaces and have been shown to express extracellular, conductive 'conduit-like' appendages, that facilitate electron transfer, even over distances, to Fe(III)-oxide surfaces (Childers, Ciufo, & Lovley, 2002; Gorby et al., 2006; Reguera et al., 2005). The formation of pilin- or flagella-like structures would allow the microorganism to directly attach to the respiratory surface, or if not strictly acting as an anchoring mechanism, may function as a conduit to transfer electrons (Gorby et al., 2006; Reguera et

al., 2005). A cellular bridge may be created through cells networking and may also function as a mechanism to penetrate small soil pores, thereby connecting sediments thought to be previously unavailable to the cell. The 'networks' connect nearby cells together and may also provide a means of cell-to-cell communication (Reguera et al., 2005).

Nanowires are thought to be pilin-based structures that are required by cells to be in direct contact with respiratory terminal electron acceptors. Nanowire production favors insoluble electron acceptors. This is true in the case of reduction by *Geobacter* spp. of insoluble Fe(III) oxides (Nevin & Lovley, 2000). *G. metallireducens* can utilize chemotaxis to locate and attach to Fe(III) oxides using pilin structures (Childers et al., 2002; Mehta, Coppi, Childers, & Lovley, 2005) as one avenue to make direct contact with target electron acceptors. Nanowire production is known to increase with electron acceptor limitation (Childers et al., 2002; Gorby et al., 2006) and can be facilitated through transition from aerobic to anoxic culture conditions.

Childers et al. (2002) were able to amplify a pilin-encoding gene, *pilA* (400bp), from *G. metallireducens* and *G. sulfurreducens*. They detected mRNA signal in cultures grown with Fe (III) and Mn (IV) insoluble oxides and not soluble counterparts. Atomic force microscopy was used by Malvankar et al. (2014) to test the electrical propagation of a charge conducted along untreated *G. sulfurreducens* KN400 pili and flagella compared with knockout mutants lacking key aromatic amino acid arrangements in both of those structures. There was no propagation of charge along flagella, which were not hypothesized to be conductive. However for pili, there was a nearly homogenous distribution of charge propagated rapidly over micrometer distances following charge injection that suggests charges are mobile along the whole structure and supports that pili are molecular wires.

Conductive ‘nanowires’ were once thought to be exclusive to *Geobacter* spp. In *Shewanella oneidensis*, nanowires were shown to not be ‘wire-like’ electron conduits, but rather are composed of highly-specific arrangements of aromatic amino acids along structures that function to tunnel electrons to the respiratory surface (Pirbadian et al., 2014). Pirbadian (2014) demonstrated through qPCR that pilin gene expression remained constant or decreased when subject to electron acceptor limitation and nanowire and pilin-knockout mutants showed nanowire production. They determined *S. oneidensis* nanowires to be extensions of the outer membrane containing soluble periplasmic peptides, rather than pilin based structures as were found in G*eobacter* spp.

Indirect Contact: Redox Proteins and Other Redox–Active Molecules

Along with nanowires, redox active molecules represent a second mechanism DMRB may use to solve the problem of electron transfer over distance. Endogenous (Lovley et al., 1996) or exogenous (Nevin & Lovley, 2002; Turick, Tisa, & Caccavo, 2002) electron shuttles like quinones (Newman & Kolter, 2000b), melanins, flavins, or ligands mediate indirect electron transfer to the Fe (III)-oxide surface (Hernandez & Newman, 2001). These proposed EET shuttles potentially mitigate the need for complex respiration structures and may be more applicable to commensal biofilms; due to the decreased turnover of shuttles trapped within pore spaces and pockets inherent to biofilms structure.

Humic substances are redox-active, high-molecular weight, organic chelating substances resistant to microbial degradation and are ubiquitous throughout the biosphere. Suspensions of *G. metallireducens* were capable of slowly reducing insoluble Fe (III) oxide. Addition of purified soil humic acids caused Fe (III) to be reduced faster,

but in the absence of *G. metallireducens* negligible amounts of Fe (III) were reduced (Lovley et al., 1996). *Shewanella alga* BrY was also shown to utilize humic acids as electron acceptor intermediates in a similar manner (Hernandez & Newman, 2001). Growth of those genera required the presence of both electron donor (acetate, lactate; *Geobacter*, S*hewanella*; respectively) and electron acceptor intermediate (humic substances). *G. metallireducens* quinone-mediated electron transport pathway reduces 2,6-anthraquinone disulphonate (AQDS) to 2,6-anthrahydroquinone disulphonate (AHDS), then AHDS instantly reduces Fe (III) thus regenerating AQDS (Lovley et al., 1996). Quinones are the electron accepting moieties of humic substances, the electron shuttle. Passing electrons to electron shuttle intermediates provides an avenue for microorganisms to pass electrons to insoluble Fe (III) oxides and bridge the space between cell and electron acceptor too large to be readily incorporated within the cell.

Melanins are also well suited to serve as intermediate electron acceptors. These are quinone-containing polymers and have chemical and functional similarities with other humic compounds. Interestingly, they have the ability to act as an electrical threshold switch, amorphous semiconductor, electron donor, and electron acceptor (Turick et al., 2002). Melanins derived from homogentisic acid (HGA) or dihydroxyphenylalanine (DOPA) of *S. colwelliana*, are produced though conversion of tyrosine to HGA that is excreted, auto-oxidized and self-assembled (Coon et al., 1994). Melanin is a reported endogenous shuttle of *Shewanella alga* used for enhancing Fe (III) reduction in response to environmental stressors. Turick et al., (2002) showed that reduced-melanin reduced hydrous ferric oxide (HFO) purified from *S. algae* reduced-melanin reduced HFO in the absence of cells, but that oxidized melanin did not regenerate. However those

experiments were performed under aerobic conditions and supplemented with tyrosine. The role of melanin in the reduction of Fe (III) under anaerobic conditions, microaerophilic, and thermophilic conditions remains poorly understood.

Flavins are another potential extracellular redox shuttle. Cell membranes are relatively impermeable to external flavins, while internal flavins (e.g. riboflavin) have been noted to be efficiently secreted (Gralnick & Newman, 2007; Von Canstein, Ogawa, Shimizu, & Lloyd, 2008). In *Shewanella* sp, extracellular concentrations of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) suggest that those flavins are actively released by cells and not a product of cell lysis (Von Canstein et al., 2008). Actively secreted flavoproteins (FMN and FAD) and free riboflavin were not previously implicated in extracellular respiration, but secretion of flavins have the ability to undergo redox reactions and function as extracellular electron shuttles that promote growth coupled to insoluble Fe (III) oxides. Flavins are more electronegative than Fe (III) oxides which should also make them effective redox shuttles (Von Canstein et al., 2008).

Ligands are chemical chelators, such as citrate or nitro-triacetic acid (NTA), and are able to solubilize and deliver iron to *c*-type cytochromes within the cell (Gralnick & Newman, 2007). They pose another mechanism for solubilizing otherwise insoluble electron acceptors. Coming in direct contact with the respiration surface may prove challenging and could also be accomplished by solubilizing the insoluble surface through extracellular excretion of ligands. Organic ligand production may occur thought anaerobic decomposition of organic matter when the sedimentary environment becomes reductive (Li, Yu, Strong, & Wang, 2012). Ligand production, as seen in *Geothrix* sp., solubilizes solid-phase Fe(III)-oxides and providing a soluble Fe(III) form more available

to microbial reduction (Weber et al., 2006). The FeRB redox cycle of *Geothrix* starts by coupling the reduction of a soluble electron shuttle to the oxidation of an electron donor. The reduced shuttle diffuses from the cell and then donates the electrons to the insoluble Fe (III) oxide abiotically. This cycle automatically restarts when the electron acceptor is abiotically re-oxidized (Weber et al., 2006). There are relevant energetic risks associated with this method of energy generation. The metabolic cost associated with producing and releasing redox shuttle compounds into low-populated areas would not be favorable. Whereas releasing redox shuttles into a dense biofilm community's intracellular spaces, theoretically, would favor conservation of redox shuttles; so long as the shuttles are indiscriminate with biofilm community members and promiscuous with their counterpart electron donor and acceptors.

The redox processes presented above (nanowires, melanins or flavins, and ligands) represent three classes of redox proteins that have been demonstrated to aid in indirect metal respiration by DMRB under mesophilic and neutrophilic conditions. They have not yet been explored in *Thermus*. Those structures and proteins, if expressed in the *Thermus* genera, may be structurally similar or have been modified for resilience to high temperatures or acidic pH.

Bacterial c*-type Cytochromes*

C-type cytochromes are a large family of proteins ubiquitous through the biosphere and essential to many respiratory processes (Cusanovich, Meyer, & Tollin, 1988). *C*-type cytochromes (Cusanovich et al., 1988), can mediate electron transfer to Fe(III)-oxide surfaces (Cusanovich et al., 1988; Mehta et al., 2005). There are many examples of multi-heme *c*-type cytochromes in the bacterial world and are often

associated with the nitrogen and sulfur cycle electron transport processes (Barker & Ferguson, 1999). Soluble *c*-type cytochromes of gram-negative bacteria are thought to be predominantly periplasmic (Allen, Daltrop, Stevens, & Ferguson, 2003; Wood, 1983, 1991). Multi-heme bacterial c_3-type cytochromes have anywhere from 2-12 heme groups are arranged following CXXCH or CXXXXCH motifs with the Fe atoms coordinated axially by two histidine residues. A 25 residue polypeptide wraps the heme groups and covalent attachment decreases the need for more polypeptides in order to create four hydrophobic heme-binding pockets (Barker & Ferguson, 1999). This suggests that multi-heme *c*-type cytochromes are economical peptides of densely packaged hemes facilitated by covalent attachment (Allen et al., 2003; Barker & Ferguson, 1999).

Multiple *c*-type cytochromes have been implicated in Fe (III) reduction by *G. sulfurreducens* which associate with the inner (MacA, PpcA) or outer (OmcB, OmcS, OmcE) periplasmic membranes (Mehta et al., 2005) and may play overlapping roles in Fe (III) reduction. OmcS, and OmcT were purified from *G. sulfurreducens* in the presence of insoluble Fe(III)-oxide as the terminal electron acceptor, but not with Fe(III)-citrate. OmcS and OmcT were expressed when fumarate was the sole electron acceptor and OmcT was expressed with all three electron acceptors. Northern analysis of gene transcripts indicated that OmcS was present on two transcripts (1.5 and 2.5 kb, while OmcT was only present on one (2.5kb) suggesting that OmcS is transcribed individually and in an operon while OmcT is only transcribed with OmcS (Mehta et al., 2005). Deletion of OmcE did not impact grown on fumarate or Fe(III)-citrate, but did inhibit Fe(III)-oxide growth for 30 days (Mehta et al., 2005) then Fe(II)-oxide was also reduced at a lower rate than the wild-type. This presents an important consideration because

previous studies of Fe(III) EET mechanisms have relied heavily on soluble Fe forms and presents the possibility that important components of Fe EET may be missing in cells that are grown on soluble Fe sources. The cytochromes expression may be environmentally regulated based upon the availability and solubility of the Fe electron acceptor.

Biofilms and Metal Metabolism

Biofilms provide a structural matrix for cells, whose redox active enzymes are ideally spaced between each other and minerals and metals. By forming a biofilm consisting of a conductive matrix, the problem of long-distance electron transfer is solved by providing an electrically conductive conduit between cells and reducing metals. The intimate cell-to-cell and cell-to-surface contact facilitated by biofilm formation freely allow exchange of soluble organic nutrients, soluble inorganic nutrients, electron donors, and genetic material (Branda et al., 2005; Thomas et al., 2008) among member physiotypes. This free exchange of materials promotes better physiological conditions for mineral respiration among community members. Biofilms may provide an architecture of opportunity, helping contain redox shuttles released into the inter-cell spaces, a shuttle released by one cell need not necessarily return to the cell it was released from to facilitate electron transfer for another cell. There may not be a single method of EET that characterizes DMRB energy generation through metal respiration. The energetic costs of expressing redox proteins necessary for biofilm formation as well as respiration of soluble and insoluble Fe electron acceptors inherently must be different. Life may favor redundancy where expression of multiple redox systems may be more advantageous that any single system alone, especially in environments with low energetics where for life to exist it must persist at the organism's metabolic thresholds.

Current Extracellular Electron Transport Analytical Tools and Methodologies

Redox Tower

The iron redox tree, as presented in Figure 1.1 and Figure 1.2, is a theoretical aid refined through years of thorough geochemical analysis and assembled into a handy compendium that allows researchers to diagram redox potentials of various electron transport proteins, donors, and acceptors significant to iron redox cycling. As researchers have identified and elucidated physical and chemical properties of redox proteins, those proteins have been placed onto the redox tree. The ability to visualize where an electron may originate and the potential redox pathway it can travel from electron donor to terminal electron acceptor is an invaluable tool for mapping potential novel metabolic pathways. The redox tree illustrates potential avenues of travel electrons may take upon being released from their donor until they are ultimately captured by a terminal electron acceptor. This is a means of effectively tracing the metabolism of an organism where electron-transfer describes the capture, storage, and release of energy (Bird et al., 2011). Often the biological systems bacteria use to transfer energy to and from solid substrates *in vivo* are the same systems that those bacteria utilize when transferring electrons to electrodes in bioreactor energy generation (Bird et al., 2011) or targeted bioaccumulation in environmental remediation (Mauricio Gutiérrez, Peña Cabriales, & Maldonado Vega, 2010).

Proteogenomics

Proteogenomics utilizes a ‘multi-omics’ approach combining protein sequencing with genome analysis (Ansong, Purvine, Adkins, Lipton, & Smith, 2008). This method allows for a protein isolated through targeted-purification, from either an environmental sample or isolated bacterial strains, to be sequenced and compared with the organisms

complete genome sequence. Sequenced proteins can be searched against the six-frame translation of the gene sequence, rather than just a protein database, and facilitates improved gene annotations (Ansong et al., 2008). Genome and protein sequencing technologies are improving at rapid rates, with sequencing time and cost falling within grasp of even small laboratories. It has reached the point that a bacterial culture can have its total genetic information prepared for sequencing in under one week, sequenced by a core facility in two weeks, the sequence prepared and assembled in a few months (depending on computer processing power) for *de novo* assembly or faster with reference sequence-based assembly. Further, algorithms are being developed to align novel protein sequences with reference protein sequences and map their location on the *de novo*-assembled chromosome. The ability to compare genomes of thermophilic genera with mesophilic genera presents a valuable resource for predicting homologies of gene sequences.

Microscopy

Through the use of microscopy, there is an ever-increasing understanding of how cellular constituents interact to regulate cellular maintenance, structure, and function. Much of this knowledge was obtained through observing diverse approaches of genetic expression, *in vitro* reconstruction, and genomic sequencing experiments. Fluorescent microscopy utilizes laser light of specific wavelengths, and chemical and biological fluorophores. Highly defined wavelengths of light illuminate a sample and excite target electrons within cellular structures. As those excited electrons released energy, photons of light are emitted at a different wavelength. Some cellular constituents, like actin-filaments, are auto-fluorescent and require no additional stains for observation.

Fluorescence microscopy advanced with the discovery of green fluorescent protein (GFP), a protein that can be bound to molecular tags that have high binding affinity to target proteins. By using these tags, a specified non-autofluorescent structure can be visualized. Early fluorescent microscopy images were plagued by ambient signal noise. This was eventually mitigated through development of the confocal microscope that placed a pin hole in the optical path of the photodetector to filter all out-of-focus light except light at the focal plane. Since the specimen is only in focus along the focal plane, the specimen can be imaged along the Z-plane allowing for focal planes to be stacked together to assemble a 3-D rendition of the sample (Stephens, 2003).

Early fluorescent images provided a static view of dynamic events due to the available techniques of the time relying heavily on fixing cells to an observation plane through use of cytotoxic mordents. Live imaging facilitated observation of cells in a more natural state, often relying heavily on techniques that took the metabolic capability of the observed cells into consideration while limiting light-induced damage (Stephens, 2003). Temperature, humidity, and gasses must be addressed for the stability and vitality of the organism, where the importance of each parameter is sample specific. With live imaging, the trade-off of acquiring high quality images and damaging the sample through extended observation are express challenges, but are shadowed by the possibility of observing targeted cellular processes as they unfold.

Multiple research groups have utilized fluorescent imaging techniques to compare wild-type and knockout DMRB variants. These groups have explored potential EET avenues that these organisms may use for metal respiration. For example, live imaging the electron charge propagation along microbial nanowires (Pirbadian et al., 2014), or

nanowire attachment (Pirbadian et al., 2014). We hope to design a flow-through reactor capable of supporting *Thermus* sp AO3C biofilm formation and facilitate live-imaging through fluorescent microscopy. This research group is unaware of experiments have attempting to visualize nanowire production in *Thermus* sp. AO3C or biofilm formation on insoluble Fe surfaces.

Applications for Thermus *in Industry and Bioremediation*

Understanding unconventional electron transport chains is as important as knowledge of the range of substrates available for biological reduction and oxidation reactions. The range of bioavailable substrates that are currently known to science are expanding (Nealson et al., 2002). We should be conceptualizing contaminant metals as nutrients. This advocates for the idea that by manipulating the bacterial load of an environment to favor a specific bacterial respiration pathway to utilize the target metals, DMRB may be encouraged, or 'driven,' into cleaning up contaminated environments or generate electricity.

Fe and Mn oxides are the dominant electron acceptors of sedimentary environments (Nealson et al., 2002). Electron acceptors of sedimentary environments flow by means of gravity-driven redox cycles due to solid oxides are continuously pulled down into the increasingly anoxic soil column by water permeation and burrowing organisms (Nealson et al., 2002). Bacteria-excreted redox proteins acting as mediators of metal reduction need to be considered when those proteins can destabilize pH and Eh of Fe and MN oxides; which tend to make their reduction more favorable and quicker (Nealson et al., 2002). Unfortunately for environments contaminated with unwanted heavy metals (e.g. U(VI) and Cr(VI)) (Bender, Duff, Phillips, & Hill, 2000; Nyman,

Caccavo, Cunningham, & Gerlach, 2002), during Mn and Fe reduction there is a potential for massive releases of U(IV) or Cr(VI) contaminants (Nyman et al., 2002). Currently, U(VI) and Cr(VI) are two pollutants on the US Department of Energy's 'wanted list.' The challenge for bioremediation is determining how soluble pollutants can be precipitated via microbial redox reactions and subsequently physically removed from that environment all depending on how the system is 'driven'. Knowledge of what conditions drive a particular metabolic pathway at the cellular scale is necessary to predict impacts when applied to biotechnological industries, whether exploitation or remediation (Bird et al., 2011). In-depth understanding of chemoauxotrophic bioremediation should be motivated by the need to address intended outcomes and potential unintended outcomes prior to large-scale application of any methods or bacteria consortia.

Large scale industrial level purification of desired redox proteins could be applied to areas with known metal contamination concentrations without microbial inoculation or recombinant bacterial systems could be developed that over-express desired redox systems for a specific contaminated environment. Once the contaminant is immobilized, it may be considered inert or can be physically removed entirely. It may even be possible to couple remediation to energy generation. For example, diverting an acid mine seep laden with Fe through a bioreactor may allow the precipitate contaminant to concentrate on an electrode. This might also provide electricity while allowing the precipitate to be removed during electrode replacement (Wang & Ren, 2014). .

EET and Its Role in Exomicrobiology

Understanding respiratory alternatives is imperative to understanding Earth's developmental history and the potential for extraterrestrial life. Through billions of years of evolution, life has been found to inhabit every niche energetically favorable on Earth. As more peculiar and extreme environments are examined, the list of favorable niches continues to grow (Francesco Canganella & Juergen Wiegel, 2011; Nealson et al., 2002). Fe respiration is described as one of the most ancients types of microbial metabolism on Earth, providing a method of transferring electrons to extracellular Fe (III) with an energetic advantage over early fermenters(Vargas, Kashefi, Blunt-Harris, & Lovley, 1998). It could be a conceivable extraterrestrial metabolism on other iron rich planets (Weber et al., 2006).

As an example, spectral evidence for liquid water was recently discovered along slope lineae on Mars. It was found bound in hydrated salts like magnesium perchlorate, magnesium chlorate, and sodium perchlorate (Ojha et al., 2015). Those salts significantly lower the freezing point, reduce the evaporation rate of liquid water, and are hygroscopic. Those factors together can help form and stabilize liquid water on the surface of Mars as a brine. All known life on Earth is water-based, and that may hold true elsewhere in the solar system and beyond. If we follow the liquid water, we may find constituents of life.

In a pre-Cambrian anoxic Earth, oxidized forms of arsenic, sulfur, manganese, and ferric iron could have served the role of final electron acceptor. Prior to discovery of extent microbial Fe metabolisms, abiotic mechanisms were thought to control environmental redox chemistry (Vargas et al., 1998), but this was determined to be invalid. Redox proteins serve fundamental roles in modern environmental

biogeochemistry and having such fundamental roles has led to the idea that redox proteins may have facilitated important processes on early Earth (Weber et al., 2006). This evolutionary modification suggests that their role in, and capacity for, electron transport must be important (Allen et al., 2003; Cusanovich et al., 1988; Wood, 1991; Wozniak & Parsek, 2014).

Summary

Currently understood mechanisms of EET have yet to explore how further incorporation of thermophilic Fe reducing bacteria as model organisms can yield understanding of chemoauxotrophy, metals conversion, environmental nutrient cycling, early Earth evolution. More importantly this could apply to bioremediation as a means to clean up EPA Superfund sites or contaminated water (Bender et al., 2000; Nyman et al., 2002). There will be increased demands for clean water, and is one of three key elements required for sustainable development along with energy supply and environmental protections (Macedonio et al., 2012). The world use of fresh water resources continues to outpace replenishment. As water travels through contaminated areas, it picks up and carries those contaminants into crucial water stores (Bender et al., 2000).

High-throughput next generation sequencing has reached a point where microbial genomes and proteomes can be assembled with relative ease. Using these tool combinations we can explore the *Thermus* sp. AO3C genome for genes of interest e.g. pilin structures as seen in *Geobacter*, outer membrane extensions of *Shewanella*, and cytochromes and other redox proteins found in both. Through application of proteogenomic and fluorescent microscopy techniques, we seek to reveal protein involved in EET and Fe respiration of *Thermus* sp. AO3C and whether or not those redox structures share similarity with other DMRB genera. We would like to isolate redox proteins from other extracellular polymeric substance and the outer membrane. We also would like to visualize pilin extension to an insoluble Fe substrate in real time.

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Figures

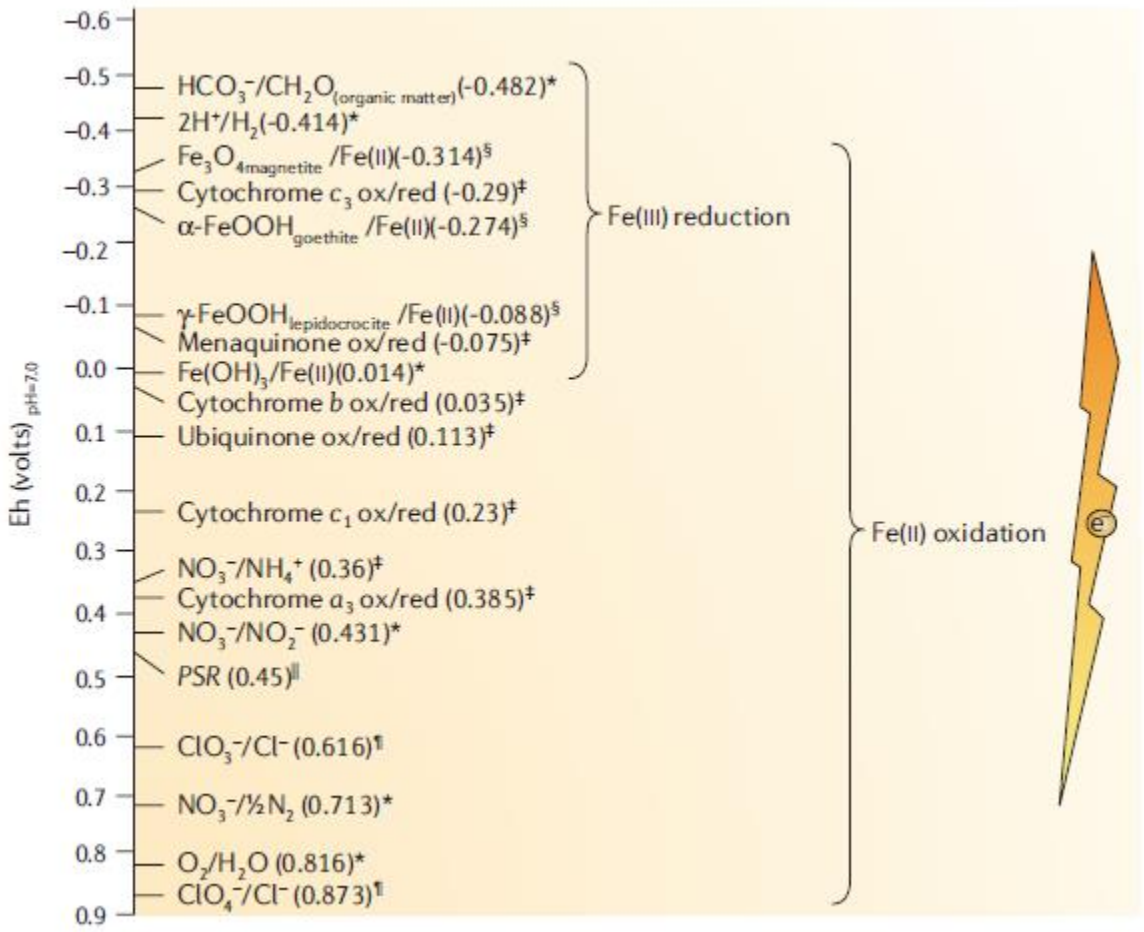

Figure 1.1. Weber et al. (2006) Redox Tower. Eh (volts) values for reduction-oxidation couples that are significant to microbially-mediated iron redox cycling, calculated at circumneutral pH, are shown. The redox tower is an effective way to visualize the potential electron donors and acceptors utilizable by microorganisms measuring the tendency of a compound to interact as an electron donor or acceptor. An electron donor will have a greater negative potential than the electron acceptor will. (Reprinted with premission: Weber et al., 2006).

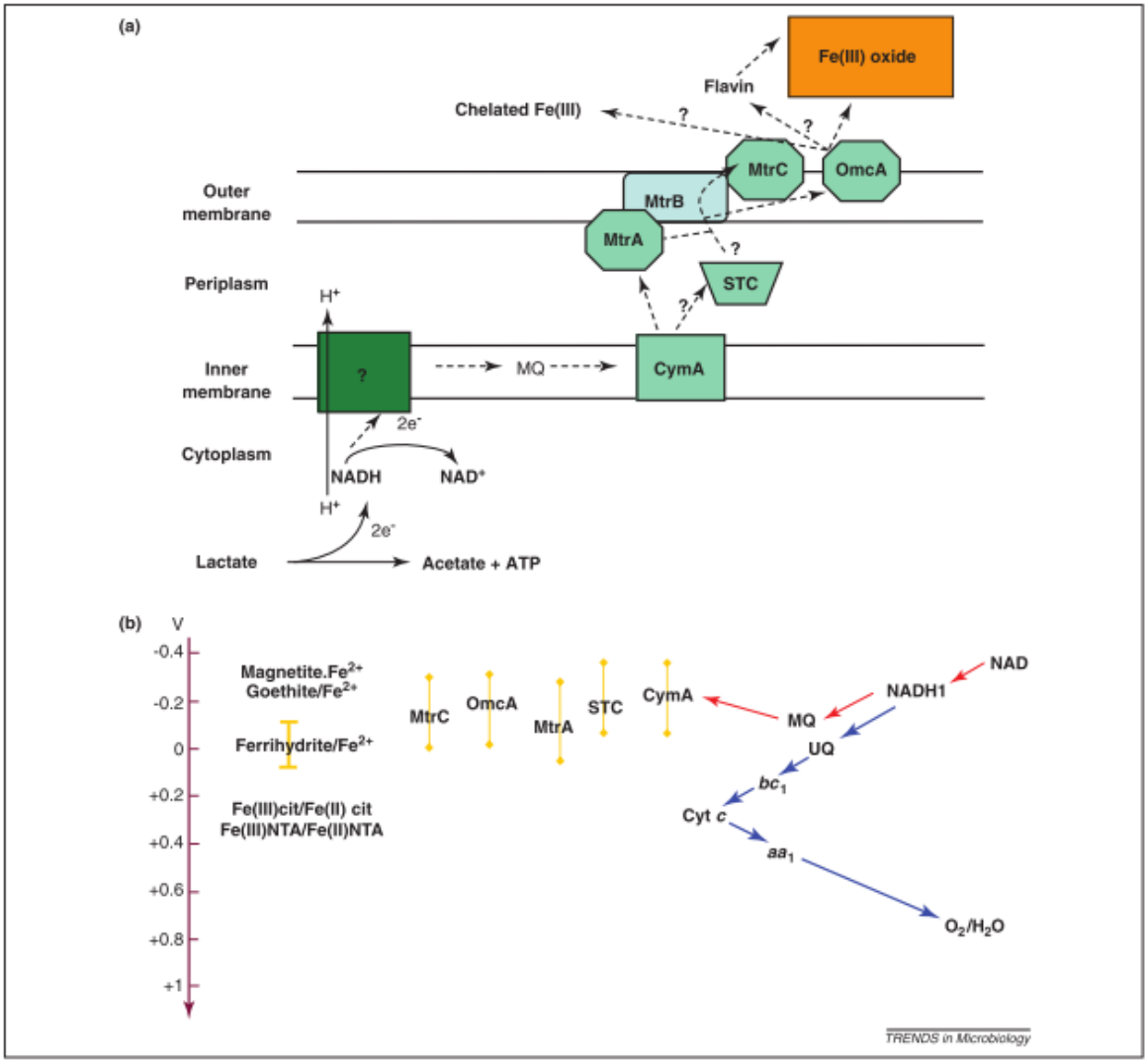

Figure 1.2. Bird et al., (2011) *Shewanella oneidensis* proposed Fe-respiration (Panel A).Protons are pumped across a membrane to generate a proton motive force while electrons are shuttled from a yet unknown electron carrier through a series of quinones and cytochromes of descending redox potential until they finally reach their terminal electron acceptor, ferric iron. Schematic of (Panel B) shows the reduction potentials of the Shewanella electron transfer pathway. The arrow on scale bar denotes the energetically favorable direction. Blue arrows denote the aerobic path electrons take to O_2, red arrows denote the path to ferric Fe(III), and yellow bars denote potential ranges of the transport proteins. (Reprinted with permission: Bird et al., 2011)

Chapter 2

Draft Genome Sequence of *Thermus* sp. AO3C: Evidence for an Iron Respiration Pathway

Anders C. Johnson[1], James Wilson, and Timothy S. Magnuson[1]

[1]Department of Biological Sciences, Idaho State University, Pocatello, ID 83209, USA

Corresponding author: Timothy S. Magnuson

Keywords

Genome assembly, *Thermus*, proteogenomics, iron respiration, extracellular electron transport, *c*-type cytochromes, redox proteins

Abbreviations

EET: Extracellular Electron Transport

DMRB: Dissimilatory Metal Respiring Bacteria

FeRB: Dissimilatory Iron Respiring Bacteria

OMV: Outer Membrane

EPS: Extracellular Polymeric Substances

PEGs: Protein Encoding Genes

Abstract

Thermus sp. AO3C, a Gram-negative pleomorphic rod, was isolated from Alvord hot springs in southeastern Oregon, USA. The draft genome of *Thermus sp. AO3C*, was determined to be 2,261,687 base pairs, and has been assembled to 78 contigs. Sequence data suggest presence of 2 plasmids. This genome contains 2,269 coding sequences, 56 RNAs, and 350 metabolic subsystems. Further analysis revealed 3 Fe acquisition and

metabolism systems, and 98 systems linked to respiration. Metabolic pathways, tested *in vitro*, were compared with genomic metabolic predictions generated through RAST and BASys. Proteomic analysis of proteins isolated from outer membrane and whole-cell lysates were compared with the draft genome assembly to elucidate proteins involved in Fe respiration and extracellular electron transport. Annotations and draft genome comparisons were directed towards *c*-type cytochromes, extracellular electron transport (EET) redox proteins, and nanowire structure discovery.

Introduction

Shewanella, Geobacter, and *Desulfovibrio* have historically been the model genera of dissimilatory metal reducing bacteria (DMRB) used to examine interfacial phenomena between biofilms and contaminant metals (e.g., As, U, Cr, Tc, Fe) (Cornell & Schwertmann, 2008; Lovley, Stolz, Nord, & Phillips, 1987; Payne et al., 2004; Petrovskis, Vogel, & Adriaens, 1994; Shi, Squier, Zachara, & Fredrickson, 2007; Venkateswaran et al., 1999). Those genera were the first to be discovered and applied to environmental remediation. Ongoing study has provided valuable insights into the world of bacterial metal cycling; however, researchers have been unable to develop a concise 'global' model of extracellular electron transport for DMRB because of limited interest in extremophile DMRB respiration. Incorporation of extremophiles as DMRB models may further that pursuit by expanding eco-physiologic, phylogenetic, and evolutionary boundaries that are currently under-explored in *extremis*.

Isolates of *Thermus* genera have been isolated from geographically distinct thermal regions all over the world including both natural and anthropogenic formed habitats. Examples of *Thermus* habitats include hot springs of the US Great Basin

(Murugapiran et al., 2013), Malaysian (Teh, Abdul Rahman, Saito, Hou, & Alam, 2012), and Himalayan (Dwivedi, Sangwan, & Nigam, 2012) thermal areas. The biotechnologically important protein *Taq* polymerase was purified from *Thermus aquaticus* isolated from a hot spring in Yellowstone National Park (Saiki et al., 1988). Energy generation mechanisms of the *Thermus* genera are diverse; with some restricted to aerobic conditions, while others are capable of anaerobic metal respiration (Whitman et al., 2015).

Mesophilic DMRB typically transfer electrons from an electron donor to cell-wall, or cell-surface, redox proteins and then on to terminal-electron acceptors using core respiration complexes (Bird, Bonnefoy, & Newman, 2011; Hernandez & Newman, 2001). Energy is harvested by the cell through the transfer of electrons along redox protein chains with sequentially descending redox potentials. In anoxic environments, the size of the terminal electron-acceptor may be insoluble or too large for transport into the cell (Gralnick & Newman, 2007). The cell is otherwise unable achieve direct contact with target respiratory surfaces. Extracellular electron transport (EET) provides pathways to overcome those issues and provides a mechanism to facilitate electron transfer across 'long' distances to metal-mineral substrates.

Microbiological Fe cycling and respiration is of particular interest with Fe being the fourth most abundant element of the Earth's crust (Weber, Achenbach, & Coates, 2006). This should include understanding what role extremophile DMRB respiration play and how these bacteria contribute to biogeochemical cycling of elements in terrestrial and subsurface environs. This unusual bacterial metabolism has been previously employed as a remediation tool in metal-contaminated environments (Cornell & Schwertmann, 2008).

Fe-respiration and EET, at the microbe-environment interface, must be orchestrated along genomic and functional levels, and common themes of function and structure should be expected across phylotypes and physiotypes. At the cellular and molecular level, flavins (Fuller et al., 2014; Von Canstein, Ogawa, Shimizu, & Lloyd, 2008), quinones (Newman & Kolter, 2000; Shi et al., 2007), pilin structures (Holmes et al., 2006; Pirbadian et al., 2014), and outer membrane cytochromes (Mehta, Coppi, Childers, & Lovley, 2005; C. R. Myers & Myers, 2004; Shi et al., 2009) have all been implicated in EET mechanisms of mesophilic DMRB genera, but little is known if *Thermus* shares similar EET architecture and composition.

The goal of this study was to utilize proteogenomic analyses of *Thermus sp. AO3C* revealed gene sequences and proteins potentially implicated in Fe-respiration and EET. We hypothesized that *Thermus* sp. AO3C biofilms can anaerobically respire using ferrihydrite as a terminal electron acceptor using multiple carbon sources and the extracellular electron transport redox molecules utilized can be isolated and identified. To test those hypothesis we used *de novo* assembly methods to generate a *Thermus* sp. AO3C draft genome assembly, used selective purification techniques that favor isolation and identification of extracellular electron transport redox molecules; fluorescent microscopy aided observation of redox molecules.

Organism Information

Thermus sp. AO3C (EF204914) was isolated in 2006 from Alvord hot springs (N374069 –E4711380, UTM, Zone 11, NAD 83, Meters) in southeastern Oregon, USA, c. 50 km north of Fields, OR (Connon, Koski, Neal, Wood, & Magnuson, 2008). Alvord hot spring waters exhibit circumneutral pH, temperatures ranging from 50˚C to about

75˚C along the flow path, and an average As concentration of 4.5mg L^{-1}(60 μM) (Connon et al., 2008). Further details regarding Alvord Hot Spring are available at http://webpages.uidaho.edu/BioGeoChemistry/alvord.html.

Thermus sp. AO3C is a gram-negative, non-motile, non-flagellated, filamentous rod. Our isolate was previously determined to harbor respiratory arsenite oxidase activity (Connon et al., 2008) and has been observed anaerobically respiring on ferrihydrite while utilizing multiple electron donors (Fig.3). The high similarity of 16S rRNA gene sequences from PCR amplifications and genome assemblies indicate that *Thermus* sp. AO3C is closely related to *Thermus oshimai* JL-2 (NR102473, >99% Identity), isolated from the Great Boiling Spring geothermal system near Gerlach, Nevada (Murugapiran et al., 2013).

Proteogenomic sequencing information

Genome project history

Thermus sp. AO3C, isolated from 'orange streamer' biofilms, was previously studied for its arsenite-oxidizing ability (Connon et al., 2008). Data and observations indicate that *Thermus* sp. A03C reduces both soluble (Fe(III)-citrate) and insoluble ferruginous minerals (ferrihydrite). Preliminary protein analysis indicated that *Thermus* sp. AO3C produces a host of redox proteins that facilitate insoluble iron reduction with multiple carbon sources (Figure 2.1). *Thermus* sp. AO3C ability to respire insoluble ferruingous minerals and underrepresentation of this extremophile genera in genomic databases would suggest that this organism a good candidate for genome sequencing and assembly. At least one reference genome is currently available among eleven *Thermus* species, including *Thermus oshimai* JL-2 (Table 2.1), to aid *de novo* and reference-guided genome assembly and annotation.

Growth conditions

Thermus sp. AO3C cultures were grown with Modified Hot Spring Medium (MHSM), formulated based on the aqueous geochemistry of Alvord thermal waters. The basal MHSM (Wolin, Wolin, & Wolfe, 1963) solution was made by combining three solutions (A, B, and C). Solution A (pH 7.0 +/- 0.2) (per L) contained 80.1 mg H_3BO_3, 341.0 mg NaCl, 4.0 mg $NaNO_3$, 54.7 mg KCl, 100 mg K_2HPO_4, 3.0 mg $MgCl_2 \cdot 7H_2O$, 10 mL Wolfe's vitamin solution, and 10 mL Wolfe's modified mineral solution (100X) (Wolin et al., 1963). Solution B contained 55g L^{-1} $NaHCO_3$ (100X) and solution C contained 5.3g L^{-1} $CaCl_2$ (100X). Solutions were autoclaved prior to combining 10 mL of solution B and C, aseptically, to 980mL of solution A.

Planktonic aerobic cultures were maintained on MHSM supplemented with 0.5% (w/v) yeast extract and grown in 150ml Erlenmeyer flasks. Anaerobic cultures were grown in Balch tubes, capped with butyl stoppers, supplemented primarily with 0.5 % (w/v) electron donor (glucose, yeast extract, cellulose), and 50 mM (excess) ferrihydrite (Cornell & Schwertmann, 2008). All cultures were incubated at 65° C.

Biofilm cultures were grown in a custom-made flow-through reactor fitted with 26mm by 75mm by 1mm Fe coupons (Figure 2.2). The bioreactor was fed MHSM supplemented with 0.5 % glucose. Following reactor inoculation, cells were allowed to adhere to the Fe coupon experimental surface for 1 hr prior to commencing media flow-through pumped at a rate of ~1 L/ 24 hr for 10 days.

Protein isolation

Planktonic and biofilm cultures were grown for 7 to 10 days. Harvesting cell pellets and extracellular polymeric substances (EPS) was accomplished through centrifugation. All centrifugation steps were performed at 8000g for 30 min. EPS

fractions were stored at -20˚C for further analysis. Cell pellets were extracted with an outer membrane (OM) cell-surface-protein extraction buffer (0.2 % Zwittergent 3-14, 50mM EDTA (disodium salt), 20 mM Tris·HCl pH 8). Cell pellets were suspended in extraction buffer (10mL extraction buffer per g of cell pellet) and continuously stirred overnight at 4˚C, centrifuged, then extracted overnight a second time. The resulting OM fraction supernatants were pooled. Remaining cell pellets were suspended in 50 mM Tris·HCl pH 8, 1 mg/mL lysozyme, incubated at 37˚C for 60 min, and then mechanically lysed with a Mini Beadbeater-8 (Biospecs products; 3X 5 min at max speed). Lysed cells were centrifuged and supernatants were stored at -20˚C for further analysis.

Supernatants were concentrated with 3000 kDa MWCO centrifugal filters (Amicon, EMD Millipore Sigma, Darmstadt, Germany). Protein concentrations were determined using bicinchoninic acid (BCA) assay (Sigma Chemical, St. Louis MO). Proteins were resolved using native and SDS-PAGE. A colorimetric, stain (3,3' ,5,5'-Tetramethylbenzidine) (Schwalb, Chapman, & Reid, 2003) was employed to identify hemoproteins and total protein banding was visualized with coomassie brilliant blue. Heme-stained bands were cut from the gels and bands were stored at -80˚C for later MS/MS tandem spectroscopy (Figure 2.4) at the University of Idaho (Moscow, Idaho).

Genomic DNA isolation

Genomic DNA was isolated from *Thermus* sp. AO3C using the ZR Fungal/Bacterial DNA mini prep kit (ZymoResearch, D6005, Irvine, California) and purified using Genomic DNA Clean & Concentrator kit (ZymoResearch, D4064, Irvine, California). Samples were quantified with a Qubit fluorometer (Life Technologies,

Carlsbad, California) prior to submission for Next-gen sequencing on the MiSeq platform at Idaho State University (Pocatello, Idaho).

Genome sequencing and library construction

Sequencing was performed by the Idaho State University Molecular Research Core Facility (MRCF) by creating two paired-end Illumina MiSEQ libraries with sonication fragmentation times of 10 min (BioA fragment sizes 540, 544, and 526) and 12 min (BioA fragment sizes 454, 470, and 460). Sonication cycles were 30 sec, followed by 90 sec rest. The first sonication time generated a paired-end library with 25,163,774 reads totaling 16.3 Gb (forward: 12,644,525 reads, 8.2 Gb; reverse: 12,519,249 reads, 8.1 Gb) The second sonication time generated a paired-end library with 20,531,071 reads totaling 13.4 Gb (forward: 10,295,143 reads, 6.8 Gb; reverse: 10,235,928 reads, 6.6 Gb). Sequence reads were 35-301bp long with >150X coverage. Sequences were randomly chosen to provide files 20% of their original read size using SeqTK. The first sub-set library contained 5,036,652 total reads (3.4 Gb) and the second sub-set library contained 4,108,232 total reads (2.8 Gb).

De Novo genome assembly

Genome sequence quality visualizations were performed with FastQC v0.11.2 (Andrews, 2010). Quality trimming (Q20 cut-off) of heads, tails, and low-quality reads were performed with Trimmomatic (Bolger, Lohse, & Usadel, 2014). Sequence assemblies were performed with SPAdes (v.3.6.0) (Nurk et al., 2013) and both sonication time sub-set libraries were assembled independently (e.g., S1 and S2), and then combined (e.g., S1S2). Assembly qualities were assessed with QUAST (v.3.1, build 29.08.2015)

(Gurevich, Saveliev, Vyahhi, & Tesler, 2013). Combining sub-set libraries provided the highest quality draft assembly (Table 2.2).

Reference-guided genome assembly

RNAmmer (v1.2) (Lagesen et al., 2007) extracted RNA sequences from draft-assembly contigs and provided a 1450bp 16S RNA gene sequence suitable for nBLAST. The 16S rRNA gene sequence for *Thermus* sp. AO3C was determined to share 99.9% similarity with *Thermus oshimai* JL-2 (RefSeq: NC_019386.1), which has had its complete genome sequenced and includes two plasmids (pTHEOS01, NC_019387.1; pTHEOS02, NC_019388.1). The available sequence data for *T. oshimai* JL-2 were compiled into a single reference FASTA file consisting of 3 contigs. QUAST assembly statistics were re-computed with *T. oshimai* JL-2 serving as a completed reference genome (Table 2.3). Mauve (v.20150226 build 10c) (Rissman et al., 2009) was used to sort and reorder the draft assembly contigs against the reference genome. Sequences that did not align with Mauve were searched against *T. oshimai* JL-2 plasmids. The sorted genome assembly was uploaded to BASys (Van Domselaar et al., 2005) for COG calling and to NMPDR RAST (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014) for annotation, gene calling, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map generation.

Genome properties

The draft genome is composed of 78 contigs, which includes one circular chromosome and indicates presence of two plasmids, for a total current size of 2,261,687bp (68.76% GC content). Those contigs contain protein-encoding genes for 350 subsystems predicted by RAST, with 2,269 coding sequences and 56 RNAs. Of 2,269

protein-coding genes, 2,235 were assigned to a putative function with 489 annotated as hypothetical proteins. 98 subsystem features were identified as respiration-associated. Of those, 9 were associated with ATP synthases, 9 were associated with terminal c type cytochrome oxidases, 3 were attributed to anaerobic respiratory reductases, and 4 were associated with ubiquinone menaquinone-cytochrome c reductase complexes. Table 2.4 presents the project information with MIGS version 2.0 compliance.

Proteogenomics of redox proteins present in biofilm and planktonic cultures

PAGE analysis compared *Thermus* sp. AO3C phenotypic variation between a biofilm population grown on Fe slides and planktonic populations grown in the absence of Fe. Analysis revealed banding pattern differences between heme-stained and coomassie brilliant blue- stained gels (Figure 2.1). Upon comparison of the heme-stained PAGE gels, the extracellular polymeric substances isolated from biofilm grown populations displayed five heme-stained bands and >11 coomassie brilliant blue stained bands, while no bands were resolved from either the heme-stained or coomassie- stained lanes of the planktonic populations. Differences were also observed between outer membrane (OM) fractions of the two populations. Five heme-stained bands were observed in the biofilm OM fraction and seven observed in the planktonic fraction. Coomassie staining intensity for the planktonic OM fraction was visibly greater than that of the biofilm OM fraction however intensity not quantitated. Heme-stained bands of interest were submitted to the University of Idaho (Moscow, Idaho) for MS/MS tandem spectroscopy.

MS/MS tandem spectroscopy identified protein sequences were binned together into biofilm-associated or planktonic-associated protein assemblages (Figure 2.6 and

Figure 2.7). pBLAST searches for potential protein reference sequences were performed using those incomplete sequences provided by MS/MS tandem analysis. Sequenced and identified proteins isolated from differing gel bands were compiled together and compared with their published reference sequences (Figures 2.6-2.16). Reference sequences were uploaded to SWISS-MODEL to create overall hypothetical protein models (Figures 2.6-2.16) and further aid phenotypic comparison.

Redox molecules

Previous studies of A*cidiphilium cryptum*, an acidophilic iron-respiring *Alphaproteobacterium* (Cort, Swenson, & Magnuson, 2011), and *Geobacter sulfurreducens* (Magnuson, Hodges-Myerson, & Lovley, 2000) indicate extracellular redox proteins pose a possible explanation for medophilic Fe(III) reduction. Endogenous (Lovley, Coates, Blunt-Harris, Phillips, & Woodward, 1996) or exogenous (Nevin & Lovley, 2002; Turick, Tisa, & Caccavo, 2002) redox active molecules such as c-type cytochromes, melanins, flavins, and humic substances may function as electron shuttles and are constituents of the extracellular matrix of biofilm communities. Availability of those redox-shuttles would mitigate the need for individual production of complex respiration structures and may be more favorable for commensal biofilms due to the increased electron cycling by shuttles trapped in pore spaces and pockets within the biofilms. Conversely, this mechanism of electron shuttling might be disadvantageous to bacterial populations residing in areas with high rates of EPS flushing through turnover of their external aqueous environment.

Significant quantities of periplasmic *c*-type cytochromes have been observed in the related DMRB species, *Acidiophilium cryptum* (ApcA and ApcB) (Magnuson et al.,

2010) and *G. sulfurreducens* (PpcA) (Leang et al., 2010); which support the hypothesis that *c*- type cytochromes and other redox proteins may be exported into the biofilm matrix as a biologically controlled response to changes in environmental conditions and availability of electron donors and acceptors. Outer membrane *c*-type cytochromes OmcB, OmcE, OmcS, OmcT, and OmcZ have previously been implicated in *Geobacter sulfurreducens* iron reduction (Richter, Schicklberger, & Gescher, 2012). *C*- type cytochromes are essential to many cell functions and are not solely being exported into the biofilm matrix.

The outer membrane (C. R. Myers & Myers, 2004; J. M. Myers & Myers, 1998; Payne et al., 2004), cell surface, and extracellular polymeric substance (EPS, biofilm matrix) (Küsel & Dorsch, 1999) of biofilm communities contain variations of c-type cytochromes. They are constituents of the newly revealed family of biofilm-associated polyheme *c*- type cytochromes. Those cytochromes are also present in both families of Shewanellaceae and Geobacteraceae (Afkar & Fukumori, 1999; Beliaev et al., 2001; Magnuson et al., 2001, 2010; Spormann, 2008) and typically contain 10 to 12 heme-*c* moieties per molecule or in one case 37 (*Anaeromyxobacter* sp. (Wozniak & Parsek, 2014)). Those heme-*c* moieties were the targets of our native and SDS PAGE staining efforts and are hypothesized to play a role in *Thermus* sp. AO3C EET.

Quinones and melanins and other redox molecules are reported to be an endogenous shuttle expressed by *Shewanella algae* for enhancing Fe (III) reduction, functioning as intermediate electron acceptors where production is thought to be a response to environmental stressors. Quinones are electron accepting moieties of humic substances (Newman & Kolter, 2000). Humic substances are redox-active, high-

molecular weight, organic chelating substances that are resistant to microbial degradation and are ubiquitous through the biosphere. For example, *G. metallireducens* is capable of slowly reducing insoluble Fe (III) oxide and the addition of purified soil humic acids caused increased Fe (III) reduction (Newman & Kolter, 2000). In the absence of *G. metallireducens,* negligible amounts of Fe (III) were reduced (Lovley et al., 1996). Passing electrons to electron shuttle intermediates provides an avenue for microorganisms to pass electrons to insoluble Fe (III) oxides that are too large to be readily incorporated within the cell.

Heme stained PAGE bands from OM and EPS fractions were sent to University of Idaho (Moscow, Idaho) for MS/MS tandem analysis for protein sequencing. Following sequencing, potential redox proteins were extracted from total protein sequencing results (Table 8, Table 9). Not surprisingly, multiple redox proteins of interest were identified. Redox proteins were not found in the EPS of planktonic cultures. C-type cytochrome (gi|410697034) was purified from both the EPS of biofilm cultures and the OM of planktonic cultures of *Thermus* sp. AO3C (figure 2.8, 2.15, respectively). C-type cytochrome (gi|140696121) was purified from the OM of planktonic cultures (Fig. 2.16), but not biofilm cultures. Electron transfer flavor proteins were found in both planktonic OM (gi|517272632, fig 2.17, gi|517272632, fig 2.21; gi|410697470, fig 2.21) and biofilm EPS fractions (gi|517272633, fig 2.13). Biofilm EPS contained NADH-quinone oxidioreductase (gi|410696703, fig 2.9) and NADH: Flavin oxidioreductase (gi|410697806, fig 2.10) not seen in planktonic cultures.

Microbial nanowires

Nanowires as well as outer membrane vesicles (OMVs) are other mechanisms hypothesized to facilitate electron transfer over long distances and deliver redox-active protein throughout the biofilm matrix (Branda, Vik, Friedman, & Kolter, 2005; Ledbetter, Connon, Neal, Dohnalkova, & Magnuson, 2007; Pirbadian et al., 2014; Renelli, Matias, Lo, & Beveridge, 2004). Gorby et al. (2006) hypothesized that the *Shewanella oneidensis* OMVs containing redox active *c*- type cytochromes were affixed to cells by nanowires functioning as tethers and suggested that fossilized versions of these nanowires may be symbolic of life on early earth, or other planets. *S. oneidensis* MR-1 expressed nanowires determined to be extensions of the periplasm and OM extracellular electron transport components (Pirbadian et al., 2014). Substantial evidence has been garnered for 'nanowires' having redox potentials as these structures have been visualized by fluorescent dyes and confocal microscopy (Pirbadian et al., 2014). In contrast, *Geobacter sulfurreducens* nanowires have been described as being pilin-based structures and having 'metallic-like' conductance properties (Malvankar, Yalcin, Tuominen, & Lovley, 2014). 'Metallic-like' conductance observed by Malvankar et al. was hypothesized to be a result of electron transfer through the aromatic amino acid sequence structures of those nanowires rather than *c*-type cytochrome binding.

We had hypothesized previously that pilin structures played a role in biofilm formation and could serve as a network to facilitate redox molecule retention within the biofilm EPS. We were able to identify pilus retraction protein PilT (WP_016328540.1, Figures 2.12 and 2.20) in both biofilm and planktonic cultures, but only type IV pilus assembly protein PilM (WP_016328709, Figure 2.19) which occurred within the planktonic culture. This is at odds with our hypothesis that pilin structures would be

found within the biofilm and not the planktonic populations, due to physical anchoring requirements of biofilm growth. Fluorescent staining and confocal microscopy eluded to potential pilin production (Figure 2.22), in the form of faint fluorescence patterning occurring between two separated bacteria. Those predictions were not supported with PAGE analysis.

Electron donors

Thermus sp. AO3C was found to be capable of anaerobically reducing Fe(III) in the presence of several electron donors: glucose, cellulose, and yeast extract. Abiotic anaerobic reduction of Fe (III) was also observed in the presence of Glucose and Yeast extract, but not cellulose (Figure 2.3). Reduction rates of biotic and abiotic Fe(III) were similar for glucose and yeast extract electron donors, but cellulose had no observable abiotic effect on Fe (III) reduction.

Conclusion

Several strongly supported hypotheses have been forwarded through experiments of other authors (Gralnick & Newman, 2007; Holmes et al., 2006; Ionescu, Heim, Polerecky, Thiel, & de Beer, 2015; Lovley et al., 1996; Newman & Kolter, 2000; Pirbadian et al., 2014; Shi et al., 2007; Vargas, Kashefi, Blunt-Harris, & Lovley, 1998) concerning the nature of interactions of FeRB EET and Fe-respiration at the cell-environment interface, and with regard to the function of *c*-type cytochromes, conductive biofilm matrices, redox protein consortia, outer membrane vesicles, and nanowires. Here, we showed that *Thermus* sp. AO3C genome assembly does not have >97% identity similarity with G*eobacter* or *Desulfovibrio* genera *c*-type cytochrome sequences searched

thus far. That does not imply an absence of ability, rather the genome assembly is likely incomplete and associated genes may have been more highly modified for thermostability than previously assumed. Further sequencing of previously unamplified regions is required in order to advance this draft genome assembly to a complete or reference genome assembly stage.

C-type cytochromes, similar to those found within the EPS of Fe-biofilm and OM of plantonic cultures, contain covalently bound heme *c* groups that are retained under denaturing conditions and vigorous treatment. Their resilience thorough isolation and purification may make them suitable targets for large-scale extraction. *S. oneidensis* Mr-1 bacterial nanowire production have been correlated to decaheme *c*-type cytochromes, MtrC (SO1778), and been implicated in Fe(III) and Mn(IV) reduction by this genus. MtrC and OmcA (SO1779) are partially exposed to the cell surface (J. M. Myers & Myers, 2003), which could favorably expose those cytochromes to external iron sources. *C*-type cytochrome ubiquity through the biosphere (Allen, Daltrop, Stevens, & Ferguson, 2003; Cusanovich, Meyer, & Tollin, 1988; Wood, 1991; Wozniak & Parsek, 2014), persistence through time, and resistance to evolutionary modification indicate that their roles in, and versatility for, electron transport are essential.

Other redox active enzymes, like flavins, melanins, quinones, appear spaced between metals and respiring bacteria within structural matrices for the cells provided by the biofilm. Biofilms enclosing a conductive matrix may solve the problem of long-distance electron transfer by providing an electrically conductive conduit between cells and metals. Intimate cell-to-cell and cell-to-surface contact facilitated by biofilm formation freely allow exchange of soluble organic nutrients, soluble inorganic nutrients,

electron donors, and genetic material among member physiotypes, promoting better physiological conditions for mineral respiration (Branda et al., 2005; Thomas et al., 2008).

Nanowires present another explanation to overcome distances between cells and respiratory surfaces. While the presence of nanowires, structural protrusions emanating from cell surfaces, have been extensively observed, there is ongoing debate regarding the action of their electron transporting roles. Further investigations must be conducted to concretely define whether a nanowire is a structure anchored within the cells membrane or an extension of the cell's outer membrane and periplasmic space. This definition becomes increasingly difficult to characterize across DMRB genera when there are supported claims for both arguments that claim that nanowires are either, anchored pilin having with electoral charge propagation occurred along pili associated globular structures that are known to localize with *c*-type cytochromes (Malvankar et al., 2014) or extensions of the outer membrane and periplasmic space with electron propagation occurring by means of tunneling as a result of the arrangement of aromatic amino acids along the outer membrane extension(Gorby et al., 2006). Further research, requiring gene knockout experiments are needed to make this particular distinction in *Thermus* sp. AO3C.

Investigations of *Thermus* sp. AO3C did not reveal an obvious keystone for development of a global model for EET that encompasses all DMRB genera. Our proteogenomic results however do suggest that *Thermus* sp. AO3C does not utilize a single EET mechanism, rather multiple reduction pathways as indicated through presence of pilin, *c*-type cytochromes, quinones, flavins, Fe chelators, and flavoproteins. This

leads us to hypothesize that a multifaceted approach to electron transport may be able to facilitate the most energy harvested for the organism across multiple environmental conditions serving as protein expression regulators.

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Tables

Table 2.1.

Comparison of basic genome features of *Thermus*, *Shewanella*, *Geobacter*, and *Desulfovibrio* stains. All available *Thermus* genomes are present in the IMG database with select genomes of related DMRB with known Fe reducing traits.

F= Finished, P= Permanent Draft, D= Draft, *=Assembled

IMG Genome ID	Status	Genome Name	Genome Size* bp	Gene Count*	Scaffold Count*	GC Count*bp	GC %*	RNA Count*	COG Count*	COG %*	KEGG Count*	KEGG %*
-	D	*T.* sp. AO3C										
2508501045	F	*T. oshimai* JL-2	2401329	2548	3	1646250	0.69	60	1735	68.09	868	34.07
2515154080	P	*T. oshimai* DSM 12092	2260954	2409	24	1553835	0.69	60	1700	70.57	858	35.62
2579778517	P	*T. amyloliquefaciens* YIM 77409	2160855	2313	6	1457281	0.67	56	1558	67.36	768	33.2
2522572193	P	*T. antranikianii* DSM 12462	2165150	2321	33	1402261	0.65	59	1654	71.26	823	35.46
645058872	P	*T. aquaticus* Y51MC23	2338193	2595	22	1591030	0.68	56	1530	58.96	816	31.45
2648501156	P	*T. aquaticus* YT-1	2205130	2419	14	1479132	0.68	62	1555	64.28	797	32.95

2617270932	D	*T. arciformis* CGMCC 1.6992	2442297	2672	88	1678713	0.69	58	1704	63.77	839	31.4
2502171156	P	*T. brockianus*	2483116	2789	130	1657729	0.67	49	1709	61.28	886	31.77
2582581225	P	*T. caliditerrae* YIM 77777	2218114	2327	4	1491036	0.67	61	1646	70.73	804	34.55
IMG Genome ID	**Status**	**Genome Name / Sample Name**	**Genome Size* bp**	**Gene Count***	**Scaffold Count***	**GC Count*bp**	**GC %***	**RNA Count***	**COG Count ***	**COG %***	**KEGG Count ***	**KEGG %***
2515154172	P	*T. igniterrae* ATCC 700962	2227749	2379	75	1532421	0.69	54	1661	69.82	850	35.73
2518645614	P	*T. scotoductus* DSM 8553	2072611	2305	85	1342185	0.65	59	1484	64.38	765	33.19
2648501702	P	*T. scotoductus* K1	2379636	2728	55	1551972	0.65	60	1566	57.4	908	33.28
2574179778	P	*T. scotoductus* KI2	2475676	2643	2	1620826	0.65	58	1808	68.41	868	32.84
649633105	F	*T. scotoductus* SA-01, ATCC 700910	2355186	2514	2	1528252	0.65	53	1704	67.78	843	33.53
2630968794	P	*T.* sp. 2.9	2404183	2702	62	1618053	0.67	54	1632	60.4	881	32.61
2511231187	F	*T.* sp. CCB_US3_UF1	2263488	2333	2	1552285	0.69	54	1655	70.94	855	36.65
2616644986	D	*T.* sp. L198	2160271	2308	4	1473497	0.68	57	1618	70.1	830	35.96
2514885041	P	*T.* sp. NMX2.A1	2293277	2522	133	1496357	0.65	54	1666	66.06	863	34.22
2513237279	P	*T.* sp. RLM	2036600	2043	17	1390857	0.69	57	1326	64.9	684	33.48

2574179781	P	*T. tengchongensis* YIM 77401	2562314	2750	5	1701428	0.66	57	1818	66.11	869	31.6
2554235155	P	*T. thermophilus* ATCC 33923	2146664	2366	112	1490122	0.69	52	1603	67.75	847	35.8
637000322	F	*T. thermophilus* HB27	2127482	2273	2	1476627	0.69	63	1562	68.72	796	35.02
637000323	F	*T. thermophilus* HB8	2116056	2302	3	1470574	0.69	64	1550	67.33	805	34.97
IMG Genome ID	**Status**	**Genome Name / Sample Name**	**Genome Size* bp**	**Gene Count***	**Scaffold Count***	**GC Count*bp**	**GC %***	**RNA Count***	**COG Count ***	**COG %***	**KEGG Count ***	**KEGG %***
2508501108	F	*T. thermophilus* JL-18	2311212	2508	3	1594227	0.69	56	1717	68.46	839	33.45
2505679077	F	*T. thermophilus* SG0.5JP17-16	2303227	2488	2	1580434	0.69	55	1700	68.33	838	33.68
643348538	F	*D. desulfuricans desulfuricans* ATCC 27774	2873437	2443	1	1668667	0.58	61	1578	64.59	840	34.38
2524614548	P	*D. desulfuricans desulfuricans* DSM 642	3391683	2958	20	1945591	0.57	65	1930	65.25	978	33.06

637000119	F	*G. metallireducens* GS-15	4011182	3663	2	2386067	0.59	87	2329	63.58	1099	30
2619619097	P	*G. metallireducens* RCH3	3778796	3502	89	2260817	0.6	75	2206	62.99	1060	30.27
2639762790	F	*G. sulfurreducens* AM-1	4566144	4198	1	2748001	0.6	89	2520	60.03	1045	24.89
648231707	F	*G. sulfurreducens* KN400	3714272	3384	1	2266892	0.61	56	2157	63.74	1009	29.82
2546826727	P	*S. algae* BRY	4930587	4560	170	2285442	0.46	149	2843	62.35	1160	25.44
2518645527	F	*S. amazonensis* SB2B	4306142	3803	1	2307506	0.54	130	2680	70.47	1113	29.27
637000258	F	*S. oneidensis* MR-1	5131416	4657	2	2354767	0.46	155	2848	61.16	1147	24.63

Table 2.2.

QUAST complied results of *Thermus* sp. AO3C *De Novo* assembly. Original file sizes were randomly reduced to 20% using SeqTK (-s11, 0.20). Sequences were first trimmed to a fixed length (f-15, l-289), then trimmed for base quality (min 100bp seq, Q20 cutoff). De novo assembly was performed with SPAdes (v.3.6)(Nurk et al., 2013) **and QUAST (v.3.1)**(Gurevich et al., 2013) **generated the assembly report. All statistics are based on contig size >=500bp, unless otherwise noted (e.g. "# contigs (>=0bp)" and "Total length (>=0bp)" include all contigs).**

	contigs	scaffolds
# contigs (> = 0bp)	78	78
# contigs (> = 1000bp)	16	16
Total Length (> = 0bp)	2261687	2261687
Total Length (> = 1000bp)	2235216	2235216
# contigs	24	24
Largest contig	690999	690999
Total Length	2239371	2239371
GC (%)	68.72	68.72
N50	313907	313907
N75	235953	235953
L50	3	3
L75	5	5
# N's per 100kbp	0	0

Table 2.3.

***De novo* sequence result for *T.* AOC compared against *Thermus oshimai* JL-2 reference genome (Panel A, Report; Panel B Misassembles Report; Panel C, Unaligned Report). Individual *T. oshimai* JL-2 reference contigs (Representative Genome, CP003249.1; pTHEOS01, CP003250.1; and pTHEOS02,CP003251.) were compiled into a three contig file. *T.* AOC file sizes were randomly reduced to 20% using SeqTK (-s11, 0.20). Sequences were first trimmed to a fixed length (f-15, l-289), then trimmed for base quality (min 100bp seq, Q20 cutoff). De novo assembly was performed with SPAdes (v.3.6) and QUAST (v.3.1) generated the assembly report. All statistics are based on contig size >=500bp, unless otherwise noted (e.g. "# contigs (>=0bp)" and "Total length (>=0bp)" include all contigs). All statistics are based on contig size >=500bp, unless otherwise noted (e.g. "# contigs (>=0bp)" and "Total length (>=0bp)" include all contigs).**

Report (Panel A)	contigs	scaffolds
# contigs (> = 0bp)	78	78
# contigs (> = 1000bp)	16	16
Total Length (> = 0bp)	2261687	2261687
Total Length (> = 1000bp)	2235216	2235216
# contigs	24	24
Largest contig	690999	690999
Total Length	2239371	2239371
Reference Length	2401329	2401329
GC (%)	68.72	68.72
Reference GC (%)	68.56	68.56
N50	313907	313907
NG50	313907	313907
N75	235953	235953
NG75	235953	235953
L50	3	3
LG50	3	3
L75	5	5
LG75	5	5
# of misassemblies	67	67
# of misassembled contigs	7	7
Misassembled contigs length	2071578	2071578
# of local misassemblies	44	44
# of unaligned contigs	12 + 2 part	13 + 2 part
unaligned length	49110	49110

Genome fraction	83.171	83.171
Duplication ratio	1.097	1.097
# of N's per 100 kbp	0	0
# of indels per 100 kbp	29.89	29.89
Largest alignment	163307	163307
NA50	45102	45102
NGA50	45075	45075
NA75	19551	19551
NGA75	12818	12818
LA50	15	15
LGA50	16	16
LA75	33	33
LGA75	41	41

Panel B, Misassemblies Report	contigs	scaffolds
# misassemblies	67	67
# relocations	67	67
# translocations	0	0
# inversions	0	0
# misassemblied contigs	7	7
Misassembled contigs length	2071578	2071578
# local misassemblies	44	44
# mismatches	21011	21011
# indels	597	597
# short indels	476	476
# long indels	121	121
Indels length	2203	2203

Panel C, Unaligned report	contigs	scaffolds
# fully unaligned contigs	12	12
Fully unaligned length	28114	28114
# partially unaligned contigs	2	2
# with misassembly	2	2
# both parts are significant	1	1
Partially unaligned length	20996	20996
# N's	0	0

Table **2.**4. **Project information**

MIGS ID	**Property**	**Term**
MIGS-31	Finishing quality	High-quality Draft
MIGS-28	Libraries used	(540, 544, 526) (454, 470, 460)
MIGS-29	Sequencing platforms	Illumina MiSeq
MIGS-31.2	Fold coverage	>150X
MIGS-30	Assemblers	SPAdes
MIGS-32	Gene calling method	NMPDR RAST, BASys
	Genome Database release	
	Genbank ID	
	Genbank Date of Release	
	GOLD ID	
	Project relevance	Fe respiration, Extracellular Electron Transport, extremophile genome

Table **2.**5. **Summary of genome: one chromosome and two plasmids**

Label	**Size (Mb)**	**Topology**	**INSDC identifier**	**RefSeq ID**
Chromosome 1	2,009,836	Linear		CP0003249.1
Plasmid 1	228,337	Linear		CP0003250.1
Plasmid 2	23,514	Linear		CP0003251.1

Table **2.6. Nucleotide content and gene count levels of the genome**

Attribute	**Genome (total)**	
	Value	% of total[a]
Size (bp)	2,239371	93.25
G+C content (bp)	68.72	100.02
Coding region (bp)		
Total genes[b]	2269	
RNA genes	56	
Protein-coding genes	78	
Genes in paralog clusters		
Genes assigned to COGs	2541	
1 or more conserved domains		
2 or more conserved domains		
3 or more conserved domains		
4 or more conserved domains		
Genes with signal peptides		
Genes with transmembrane helices		
Paralogous groups		

A)The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome. B) Also includes 54 pseudogenes and 5 other genes.

Table **2.**7. **Number of genes associated with the 25 general COG functional categories**

Code	Value	% of total[a]	Description
J	62.04	.02	Translation
A	0	0	RNA processing and modification
K	38.750	0.015	Transcription
L	50.079	0.020	Replication, recombination and repair
B	0	0	Chromatin structure and dynamics
D	13.446	0.005	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	0	0	Defense mechanisms
T	21.387	0.008	Signal transduction mechanisms
M	31.445	0.012	Cell wall/membrane biogenesis
N	8.576	0.003	Cell motility
Z	0	0	Cytoskeleton
W	0	0	Extracellular structures
U	0	0	Intracellular trafficking and secretion
O	31.339	0.012	Posttranslational modification, protein turnover,chaperones
C	50.741	0.020	Energy production and conversion
G	54.737	0.022	Carbohydrate transport and metabolism
E	101.322	0.040	Amino acid transport and metabolism
F	26.681	0.011	Nucleotide transport and metabolism

H	43.515	0.017	Coenzyme transport and metabolism
I	37.564	0.015	Lipid transport and metabolism
P	27.845	0.011	Inorganic ion transport and metabolism
Q	13.870	0.005	Secondary metabolites biosynthesis, transport and catabolism
R	85.971	0.034	General function prediction only
S	47.114	0.019	Function unknown
-	1770.865	0.697	Not in COGs

a) The total is based on the total number of protein coding genes in the annotated genome.

Table 8. Consolidated list of potentially EET related proteins sequenced from Fe grown biofilm cultures. Proteins were isolated from extra polymeric substance (EPS) and outer membrane (OM) fractionations

Fig	MS/MS call (bp)	MS/MS call accession	Description	Cellular Location
2.6	61188	gi|410697034	cytochrome c, mono- and diheme variants fam.	EPS
2.7	87396	gi|410696703	NADH-quinone oxidoreductase, chain G	EPS
2.8	37553	gi|410697806	NADH:flavin oxidoreductase	EPS
2.9	41084	gi|410696272	pilus retraction protein PilT	OM

Table 9. Consolidated list of potentially EET related proteins sequenced from planktonic cultures. Proteins were isolated outer membrane (OM) fractionations; no proteins were resolved from extra polymeric substances (EPS) extractions

Fig.	MS/MS call (bp)	MS/MS call accession	Description	Cellular Location
2.11	61188	gi|410697034	cytochrome c, mono- and diheme variants fam.	OM
2.12	25793	gi|410696121	cytochrome c, mono- and diheme variants fam.	OM
2.13	33050	gi|517272632	electron transfer flavoprotein subunit alpha	OM
2.14	41730	gi|410696445	type IV pilus assembly protein PilM	OM
2.15	41084	gi|410696272	pilus retraction protein PilT	OM
2.16	26597	gi|410697470	electron transfer flavoprotein, beta subunit	OM

Figures

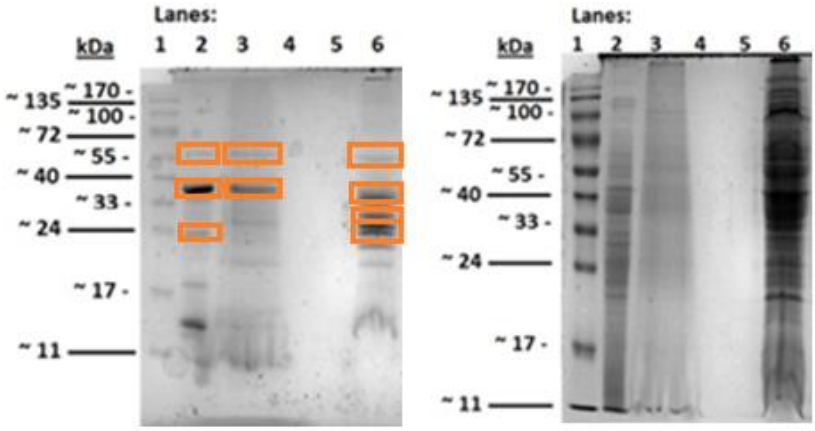


Figure 2.1. 10% SDS PAGE heme-stained (left) gel and Coomassie-stained (right) gel. Supernatants concentrated with 3000 MWCO ultrafiltration units. Lanes are designated as: 1) FisherSci EZ-rec Protein Ladder, 2) *T.* AO3C Fe-bioreactor Extracellular Polymeric Substances (EPS), 3) *T.* AO3C Fe-bioreactor Outer Membrane (OM), 4) blank, 5) *T.* AO3C planktonic EPS, 6) *T.* AO3C planktonic OM. MS/MS tandem spectroscopy protein sequencing was performed on the outlined heme-stained bands.

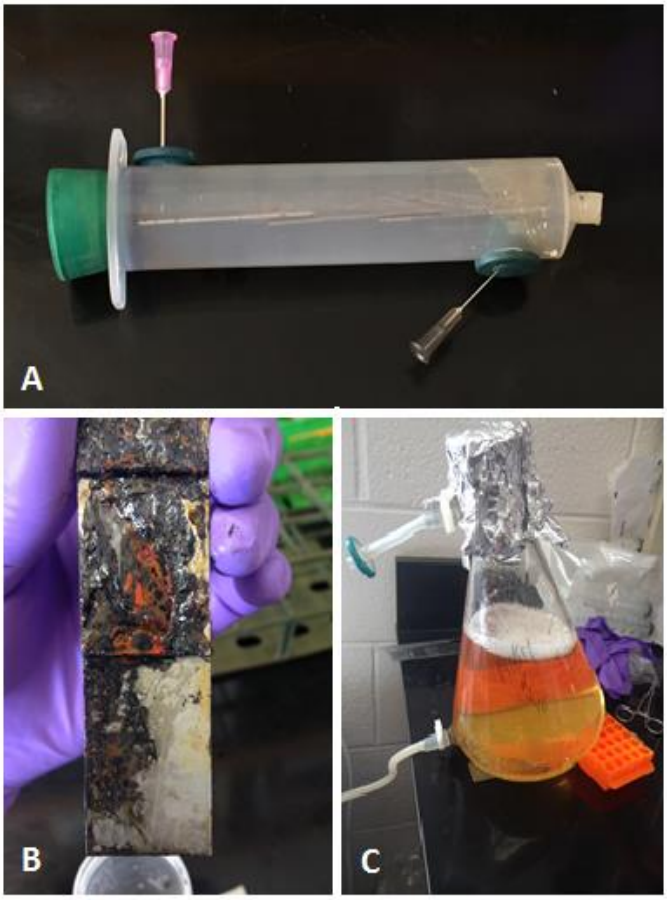


Figure 2.2. Custom flow-through bioreactor. (Panel A) 60 mL syringe with nozzle stoppered with silicon. A #7 stopper sealed the plunger port. Holes were cut and blue butyl stoppers were installed at opposite ends of the syringe body on opposing sides. An 18ga needle served as the inflow port and a 22ga needle served as the outflow port. Silicone hosing connected the inflow port on the bioreactor with the media feed reservoir. (Panel B) Fe coupons were housed within the bioreactor for biofilms to adhere to. Media continuously flowed over the coupons and then out into a waste Erlenmeyer flask. Media flowed at a rate of 1L/24hr for 10 days. The medium reservoir was replaced as necessary. (Panel C) The media reservoir was a double ported 2L Erlenmeyer flask sealed with a #7 stopper, or a 10L double ported glass carboy, and air allowed to enter through a .22µm filter. A Masterflex C/L ultra-low flow pump (model 77122-04, 10 rpm) regulated the flow of media into the bioreactor.

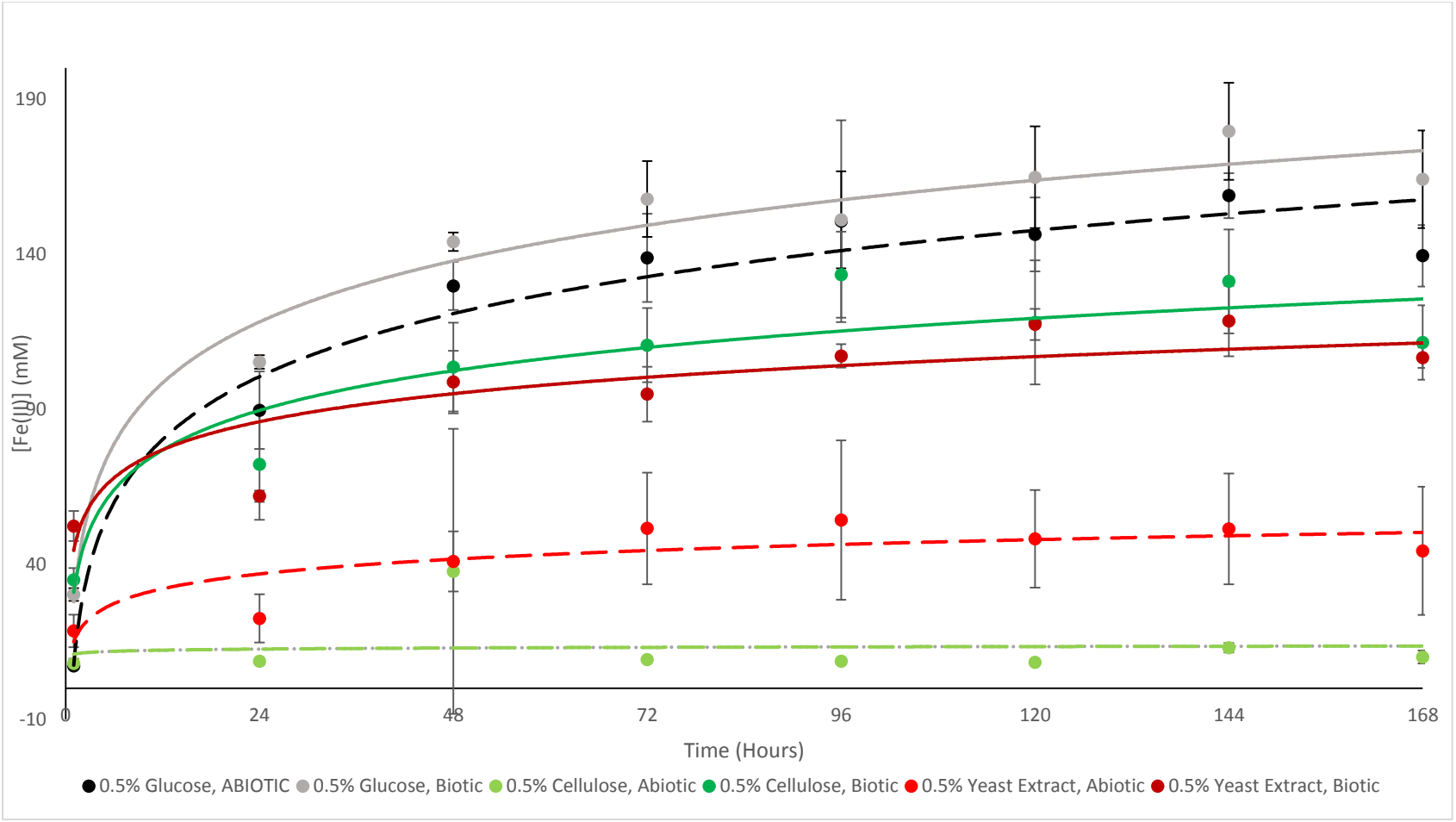
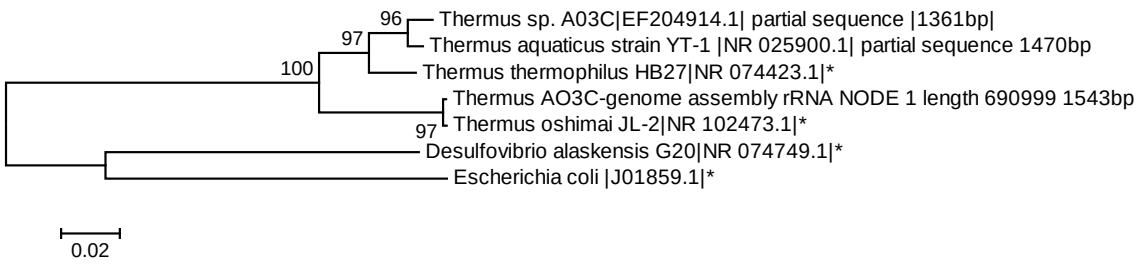
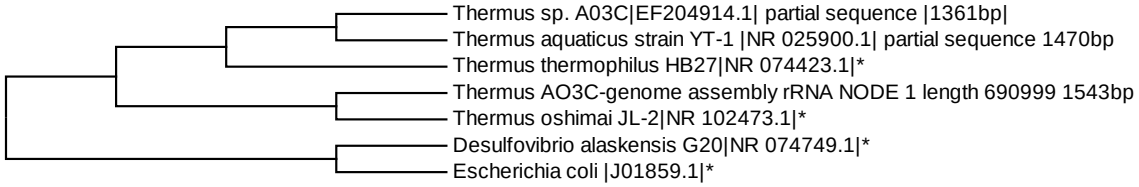


Figure 2.3. Ferrozine assay of *Thermus* and abiotic Fe reduction in the presence of three potential electron donors. As Fe(III) is bacterially-reduced to Fe(II), Fe(III) concentration will decrease and Fe(II) concentration will increase. All samples, inoculated or uninoculated, were grown anaerobically at 65℃ with MHSM, as the basal growth medium, supplemented with a carbon source and ferrihydrite,



Original tree



Bootstrap consensus tree

Figure 2.4. Phylogenetic trees based off of 16S rRNA gene sequences highlighting the position of *Thermus* sp. AO3C relative to two other members of *Thermaceae* with outgrouping to a mesophilic DMRB genus and non-DMRB *E.coli.* All sequences were at least 1500bp unless otherwise stated. *denotes completed reference genomes are available. The strains and their corresponding GenBank accession numbers (and, when applicable, draft sequence coordinates) for 16S rRNA genes are (type=^{T}): *Thermus* sp. AO3C from genome assembly; *Thermus* sp. A03C, EF204914.1; *Thermus oshimai* JL-2, NR_102473.1; *Thermus thermophilus* HB27, NR_074423.1; *Thermus aquaticus* YT-1, NR_025900.1; *Desulfovibrio alaskensis* G20, NR_074749.1, *Escherichia coli*, J01859.1. Evolutionary relationships of taxa: The **evolutionary history was inferred using the Neighbor-Joining method (Saitou N, 1987). The optimal tree with the sum of branch length = 0.47053362 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1335 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013)**.

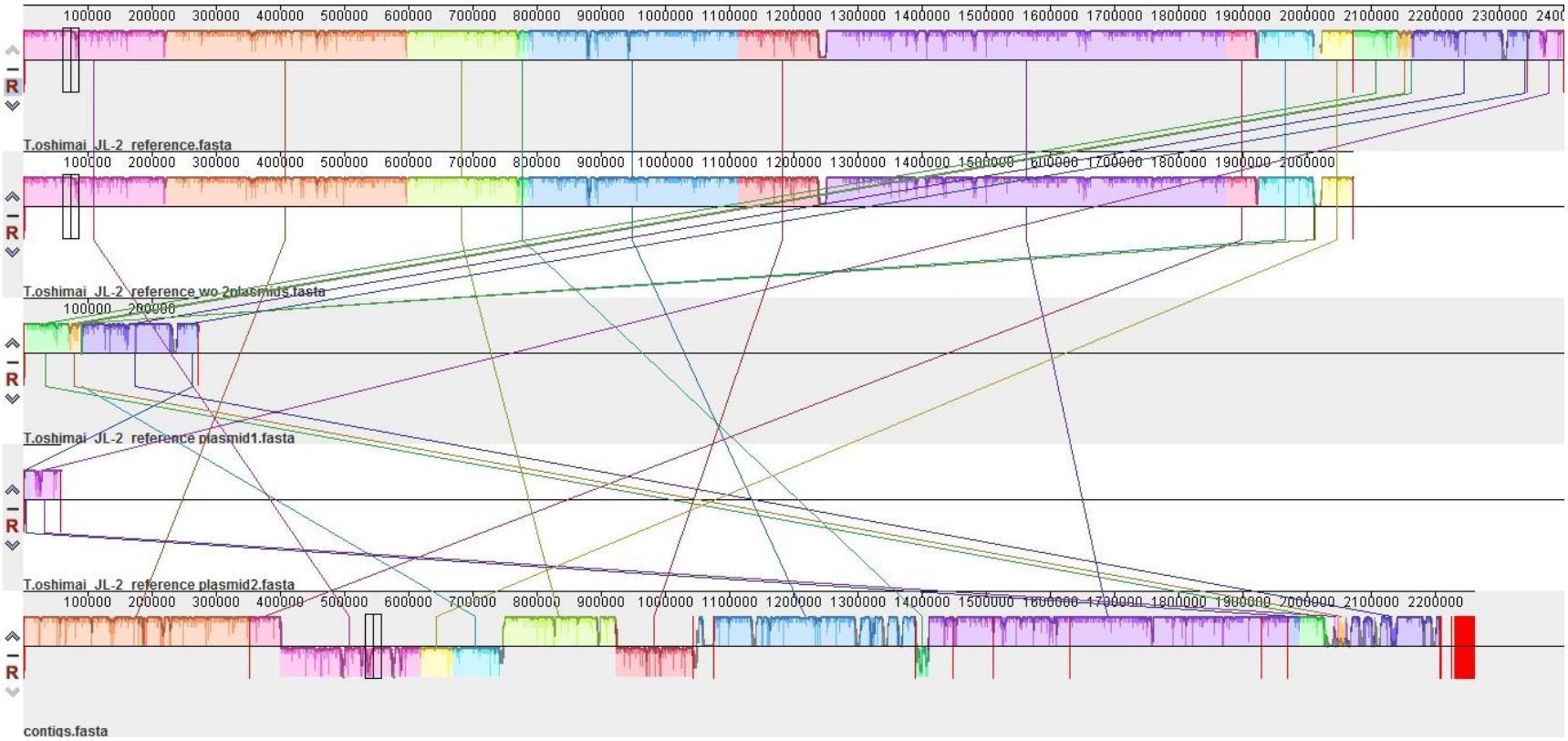


Fig 2.5. Mauve re-ordering of *T.*AO3C draft genome against the complied *T. oshimai* JL-2 reference genome, individual reference chromosome, and two reference plasmids.

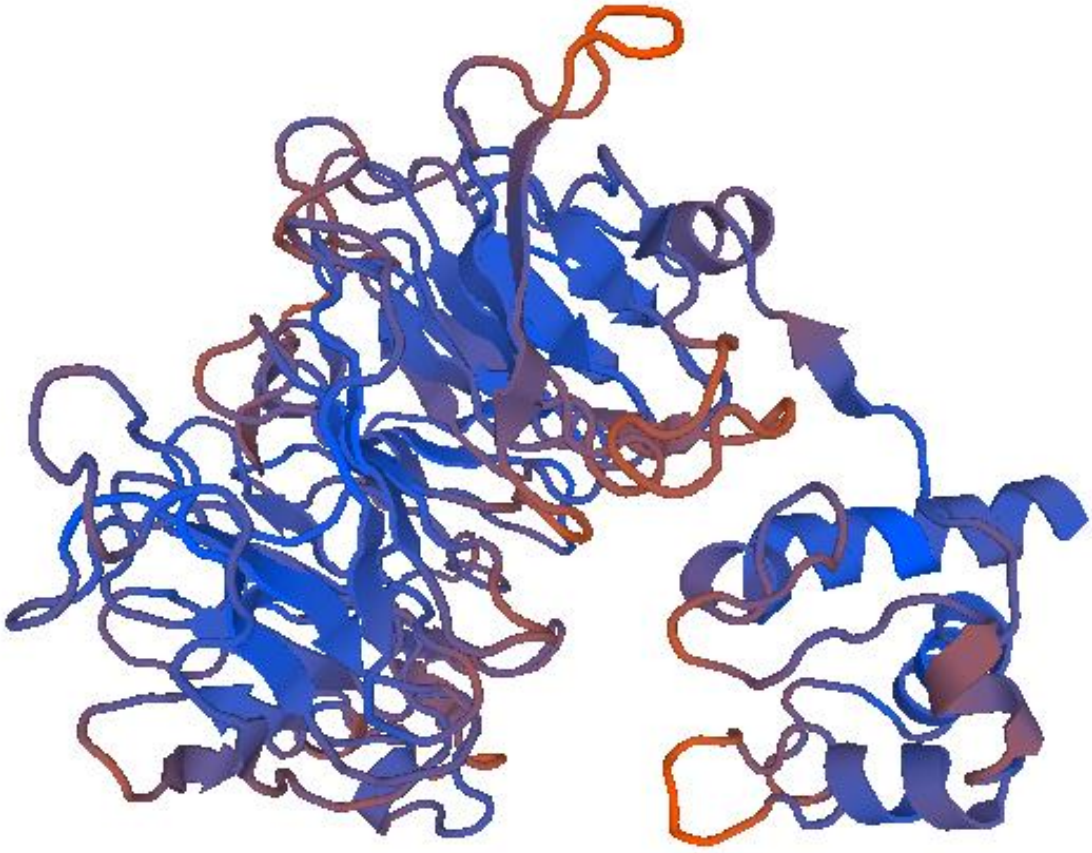


Figure 2.6. Swiss Model hypothetical secondary structural models for Cytochrome c, mono- and di-heme (Thermus oshimai JL-2 reference). Surface charge representations are positive (blue) and negative (red). Ascession gi|410697034. Isolated from Fe-grown biofilm EPS. Two short protein peptides fragments were identified and their locations were mapped to the reference sequence gi|410697034|gb|AFV76102.1).The short peptide sequence A is K.MVSTMSYTK.G and short peptide sequence B is R.VVAESKPCVDAR.S.

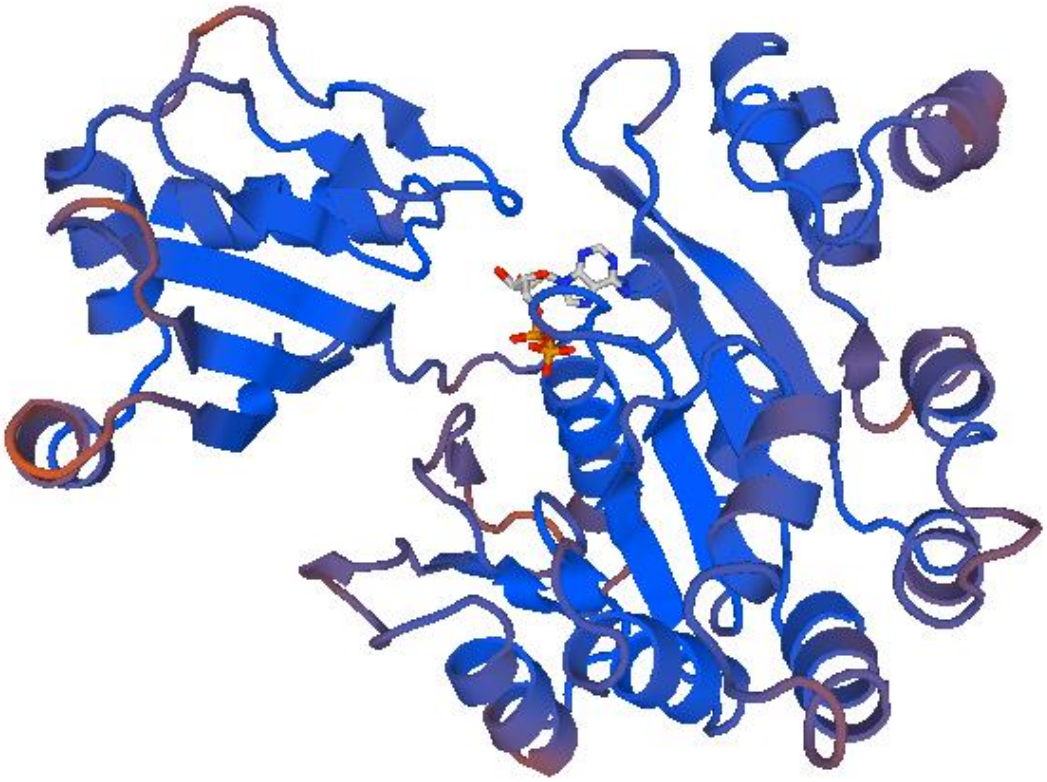


Figure 2.9.Swiss Model hypothetical secondary structural models for pilus retraction protein PilT [Thermus oshimai JL-2]. Surface charge representations are positive (blue) and negative (red). MS/MS asscession gi|410696272. Isolated from Fe-grown biofilm OM. A short protein peptide fragment was identified and its location was mapped to the reference sequence (gi|511098367|ref|WP_016328540.1).The short peptide sequence is R.ILLAESLLGILSQR.L.

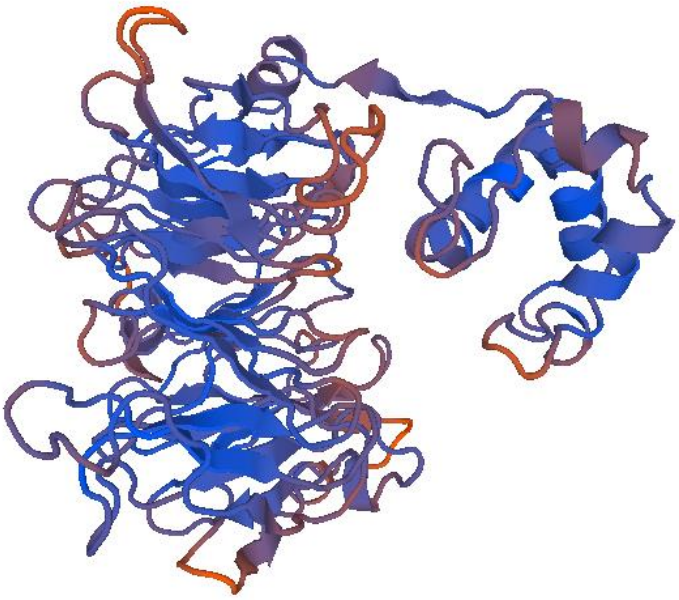


Figure 2.11.Swiss Model hypothetical secondary structural models for cytochrome c, mono- and diheme variants family [Thermus oshimai JL-2], surface charge representations are positive (blue) and negative (red). MS/MS asscession gi|410697034. Isolated from planktonic grown OM. A short protein peptide fragment was identified and its location was mapped to the reference sequence (gi|410697034|gb|AFV76102.1). The short peptide sequence is K.DTPTFIVVYDALTLK.E.

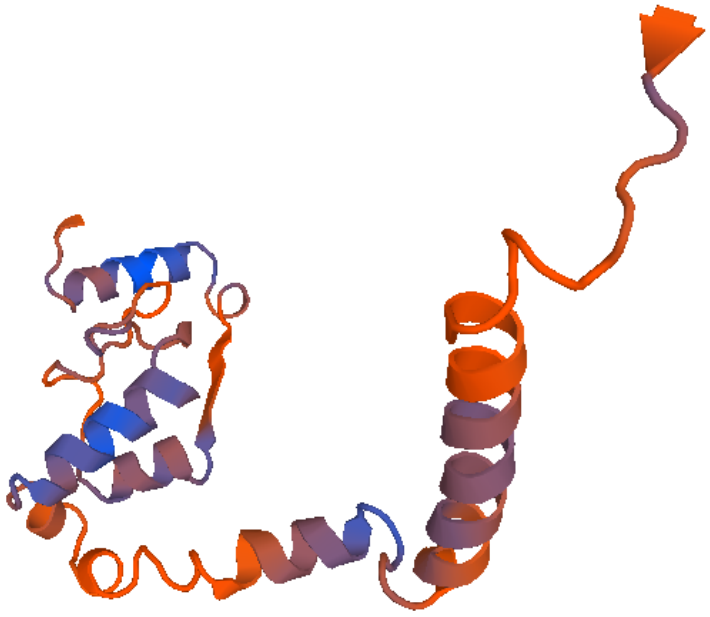


Figure 2.12. Swiss Model hypothetical secondary structural models for cytochrome c, mono- and diheme variants family [Thermus oshimai JL-2], surface charge representations are positive (blue) and negative (red). MS/MS sequence gi|410696121| Isolated from planktonic grown OM. Two short protein peptides fragments were identified and their locations were mapped to the reference sequence (gi|511098216|ref|WP_016328389.1). The short peptide sequence A is R. LSVGEPDLPVEPVR.V and short peptide sequence B is R.MEVYLDEAKEPLAVLK.E.

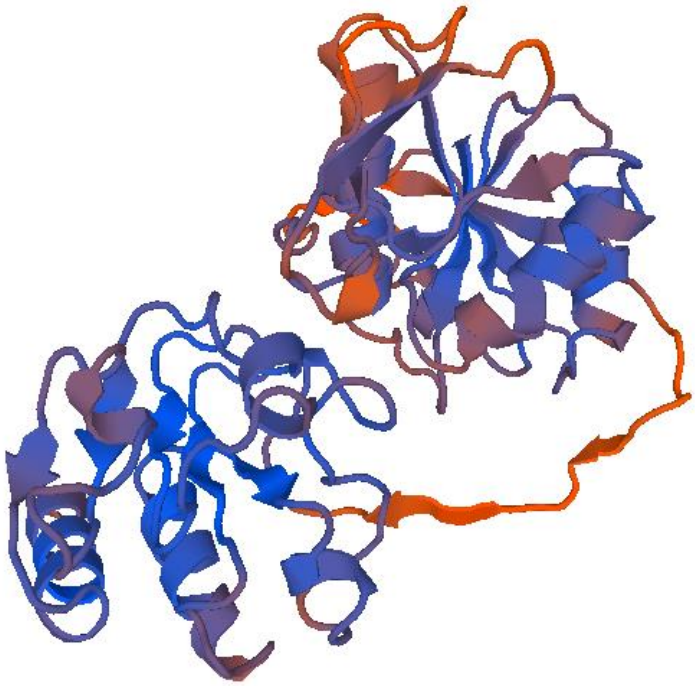


Figure 2.13. Swiss Model hypothetical secondary structural models for electron transfer flavoprotein subunit alpha [Thermus oshimai], surface charge representations are positive (blue) and negative (red). MS/MS sequence gi|517272632. Isolated from planktonic grown OM. Two short protein peptides fragments were identified and their locations were mapped to the reference sequence (gi|511099547|ref|WP_016329720.1). The short peptide sequence A is K. AIEELAALLGGAVGATR.A and short peptide sequence B is R.VAFALGAGLLEDTLESWAEGGEVYATR.Y.

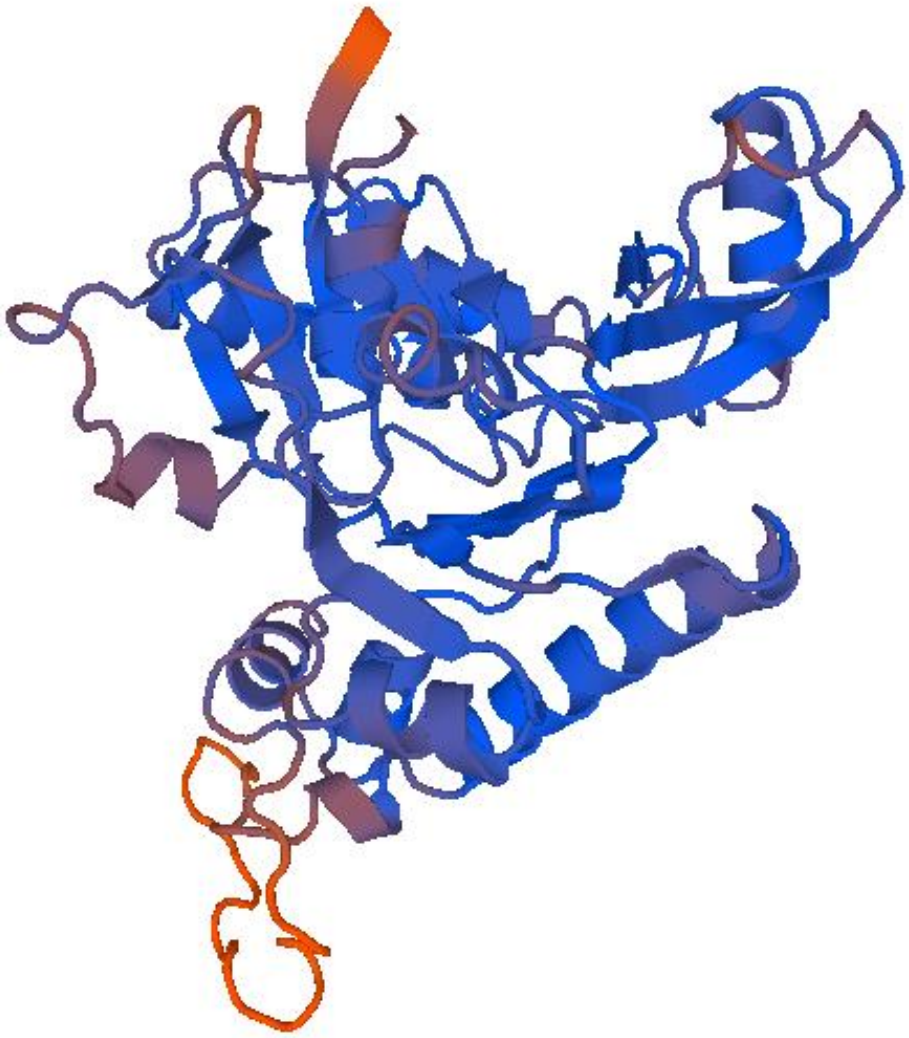


Figure 2.14. Swiss Model hypothetical secondary structural models for type IV pilus assembly protein PilM [Thermus oshimai JL-2], surface charge representations are positive (blue) and negative (red). MS/MS sequence accession gi|410696445 Isolated from planktonic grown OM A short protein peptide fragment was identified and its location was mapped to the reference sequence (gi|511098536|ref|WP_016328709.1). Short peptide sequence is R.GAGLIPVVVDVKPFAGLYPLEEK.L.

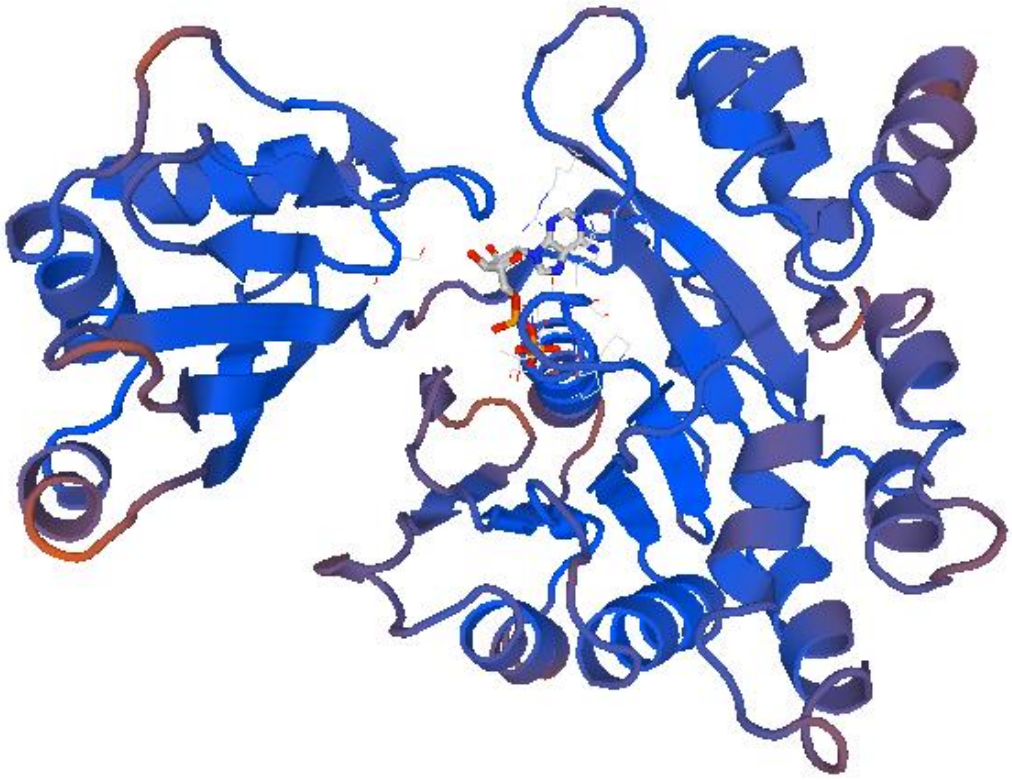


Figure 2.15. Swiss Model hypothetical secondary structural models for type pilus retraction protein PilT [Thermus oshimai JL-2],], surface charge representations are positive (blue) and negative (red). MS/MS sequence accession gi|410696272. Isolated from planktonic grown OM. A short protein peptide fragment was identified and its location was mapped to the reference sequence (gi|511098367|ref|WP_016328540.1). The short peptide sequence is R.ILLAESLLGILSQR.L.

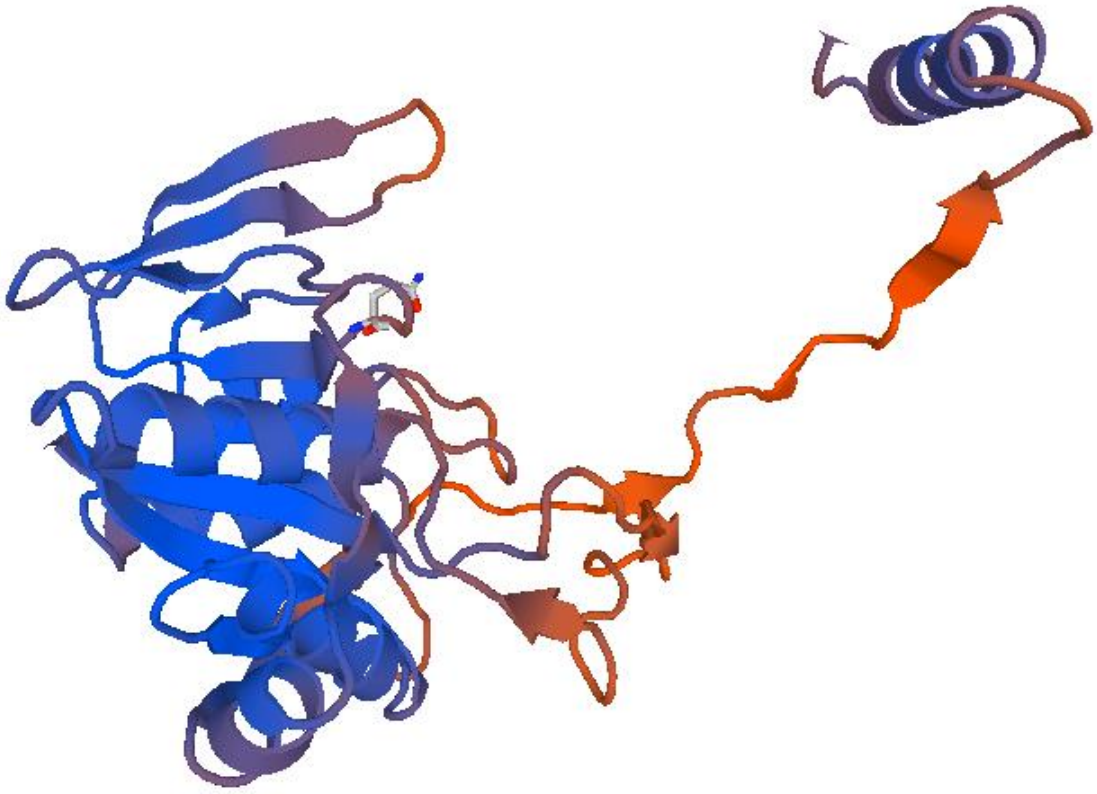


Figure 2.16. Swiss Model hypothetical secondary structural models for electron transfer flavoprotein, beta subunit [Thermus oshimai JL-2], surface charge representations are positive (blue) and negative (red).MS/MS sequence accession gi|410697470. Isolated from planktonic grown OM. A short protein peptide fragment was identified and its location was mapped to the reference sequence (gi|511099546|ref|WP_016329719.1) The short peptide sequence is R.LPAVFTTQQGLNEPR.Y.

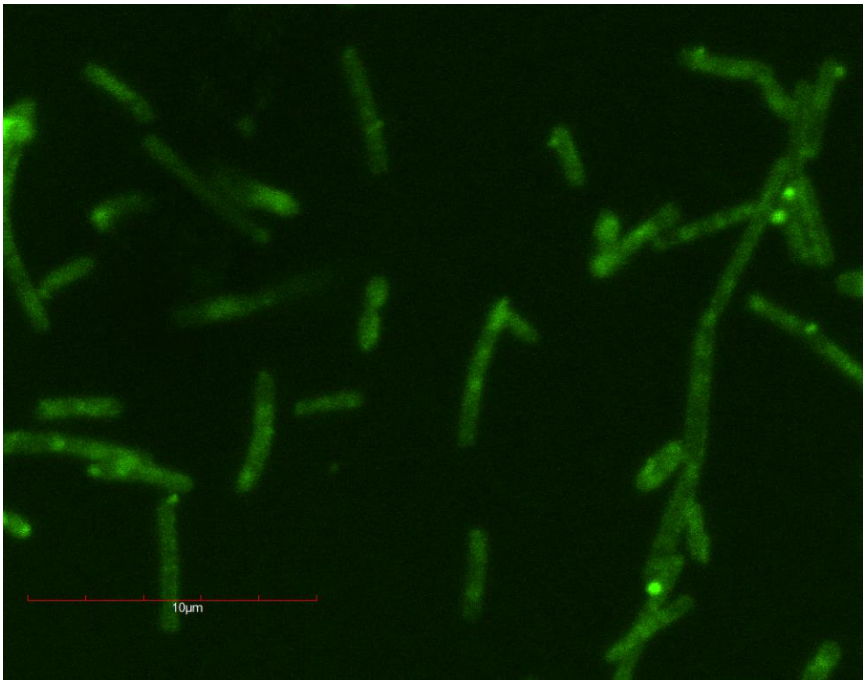


Figure 2.17 *Thermus* sp. AO3C stained with Nano-Orange (Life Technologies), a mercocyanine dye that undergoes large fluorescent enhancement upon binding to proteins. Imaged on an Olympus FV1000 inverted fluorescent confocal microscope housed with Idaho State Universities Advanced Imaging Core Facility.

Associated MIGS Record

Table S1. Associated MIGS record

MIGS-ID	field name	description
MIGS-1	Submit to INSDC/Trace archives	
1.1	PID	
1.2	Trace Archive	
MIGS-2	MIGS CHECK LIST TYPE	
MIGS-3	Project Name	Thermus sp. AO3C
MIGS-4	Geographic Location	Alvord Thermal Basin, Oregon, USA
4.1	Latitude	N42.5439
4.2	Longitude	W118.5531
4.3	Depth	-
4.4	Altitude	-
MIGS-5	Time of Sample collection	2/2006
MIGS-6	Habitat (EnvO)	Hot springs microbial mats
6.1	temperature	55-70C
6.2	pH	-
6.3	salinity	-
6.4	chlorophyll	-
6.5	conductivity	-

6.6	light intensity	-
6.7	dissolved organic carbon (DOC)	-
6.8	current	-
6.9	atmospheric data	-
6.10	density	-
6.11	alkalinity	-
6.12	dissolved oxygen	-
6.13	particulate organic carbon (POC)	-
6.14	phosphate	-
6.15	nitrate	-
6.16	sulfates	-
6.17	sulfides	-
6.18	primary production	-
MIGS-7	Subspecific genetic lineage	-
MIGS-9	Number of replicons	-
MIGS-10	Extrachromosomal elements	-
MIGS-11	Estimated Size	2.4Mbp
MIGS-12	Reference for biomaterial or Genome report	CP0003249.1, CP0003250.1, CP0003251.1
MIGS-13	Source material identifiers	
MIGS-14	Known Pathogenicity	

MIGS-15	Biotic Relationship	
MIGS-16	Specific Host	
MIGS-17	Host specificity or range (taxid)	
MIGS-18	Health status of Host	
MIGS-19	Trophic Level	
MIGS-22	Relationship to Oxygen	Facultative anaerobe
MIGS-23	Isolation and Growth conditions	(Connon et al., 2008)
MIGS-27	Nucleic acid preparation	
MIGS-28	Library construction	
28.1	Library size	540,544,526;454,470,460
28.2	Number of reads	5,036,652 ; 4,108,232
28.3	vector	
MIGS-29	Sequencing method	Miseq
MIGS-30	Assembly	SPAdes
30.1	Assembly method	
30.2	estimated error rate	
30.3	method of calculation	
MIGS-31	Finishing strategy	
31.1	Status	
31.2	coverage	150X

31.3	contigs	78
MIGS-32	Relevant SOPs	
MIGS-33	Relevant e-resources	

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