Metal Reduction by *Geothrix fermentans*

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Misha Girish Mehta

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Daniel R. Bond

[August 2013]

Acknowledgements

First and foremost, I want to thank my advisor, Dr. Daniel Bond, for the opportunity to work in his laboratory, his guidance, and advice throughout this long and challenging journey. You truly are an exceptional mentor and a great friend. Any success I have had or will have in the future is due to the unwavering support I have received from you over the years. I am incredibly lucky to have had this opportunity to work for you. I look forward to harassing you more about science for years to come.

I would also like to thank the rest of my committee members. Dr. Sandy Armstrong, Dr. Jeff Gralnick, Dr. Mike Sadwosky, and Dr. Brandy Toner for their direction and constructive feedback over the years and for serving as my defense committee. Special thanks to Jeff for allowing me to pick his brain on every genetic experiment that I have done, for sharing his strain collection without which chapter 4 would not have been possible, and for inviting me to his home. I would also like to thank Sandy for all her help and guidance, from helping me move to Minnesota to answering any questions about iron, and for providing Chelex. I would also like to thank the Center for Mass Spectrometry and Proteomic facility at the University of Minnesota, and the BMGC Sequencing facility of University of Minnesota. Thank you, Zhenya Shelobolina for sharing your collection of *Geothrix* species.

Over these years, the lab members have become an integral part of my life and helped me shape as a scientist. I would like to give a shout out to the past and present members of the Bond lab - Enrico Marsili, Ying Lui, and Daniel Baron for teaching me all the electrochemistry that I know today. Lewis Warner, Edward LaBell, Dan Ross, Lori Zachroff, and Rachel Snider - it was always great to have scientific discussions with

each and every one of you over the years. Thank you Chi Ho Chan and Caleb Levar for reading this monstrosity of a thesis. I have been incredibly lucky to have exceptional undergraduate students working with me - Ben Roller, Joanne Lui, and Viki Ding. A big thanks to the Gralnick lab for help with everything related to *Shewanella*. In particular Evan Brutinel and Nick Kotloski for Tn-seq, electrode growth of *Shewanella,* and all computer related problems. Especially Zarath Summers, thank you for being my science fairy godmother!

Caleb Levar, Janet Rollesfon, Clint Remarcik, David Sukovick, Matt Hamilton, and Michelle Gleason - thank you for your friendship. I have been very fortunate to have been able to interact with each one of you both socially and scientifically. This experience would not have been the same without each one of you. Caleb, thank you for all the beer you have provided over these years. God knows I needed them! Janet you are my closest friend and my faithful confidant. I cherish our friendship and will miss our 8 am Jello Shots!

Lastly, but definably not the least my family, Ma and Daddy - thank you for always supporting and loving me, and telling me that I can do anything I set my mind to. You have to know that I love and miss you both dearly. Thank you, Aai. You are the reason that I could go back to school after having Rayaan. Di, you are my pillar of strength, my ‘iron man’. Thanks for making it all possible. Di, Joe, and Sandeep - thank you for encouraging me every single day. Even at times when I did not deserve it. I love you guys. You are the glue that has held me together over all these years. Thank you for your unconditional love! Finally my baby Rayaan – I love you more than life itself, thank you for making me smile every single day.

Dedication

This dissertation is dedicated to my parents, Anjani and Girish Mehta, for their unconditional love, support, and encouragement. My sister, Prachi Mehta, brother-in-law, Joseph Varghese, my loving husband, Sandeep Kolte, and my baby, Rayaan Kolte.

Abstract

Bacteria with an ability to transfer electrons beyond their outer surface can utilize a variety of insoluble metals in anaerobic respiration. The consequences of these electron transfer reactions directly affect global Fe and Mn biogeochemistry, hydrocarbon cycle and U(VI) bioremediation. The main objective of this work is to understand the mechanism of electron transfer and ecological niche of *Geothrix*-like Acidobacteria consistently found in subsurface metal-reducing and bioremediating environments. In this thesis a variety of independent approaches were used to examine the mechanism by which *Geothrix fermentans* reduces Fe(III)-oxides and electrodes was examined in this thesis. The genomes of two *Geothrix* species were sequenced to identify key functional genes involved in respiration and metabolism. Electrochemical tools, that are now standard methods for characterizing the multi-dimensional aspects of microbial electron transfer, were used to identify the high potential dependent, shuttle-based respiration of *G. fermentans*. Biochemical characterizations of membrane proteins were performed to understand how electrons generated during intracellular metabolism are relayed across membranes to an extracellular terminal electron acceptor. Decaheme *c*-type cytochromes were identified and heterologously expressed in mutant strains of the metal-reducing bacterium *S. oneidensis* MR-1, lacking key proteins required for metal respiration. This research also identified different microbial communities associated with current production in microbial fuel cells. Additionally a genetic technique was optimized in order to identify important genes required for electron transfer by *Geobacter sulfurreducens* under selective growth conditions.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Microbial metal reduction

Microorganisms use respiration to capture energy from the movement of electrons generated during central metabolism in a process involving a cascade of reactions through a series of electron carrier proteins and ultimately transferring electrons to terminal electron acceptors. This energy in most microorganisms is conserved as a proton motive force that can be used to make ATP for other cellular tasks. Most forms of respiration involve a soluble compound as the electron acceptor and this reduction occurs within the cell. However, some diverse microorganisms are capable of respiring insoluble acceptor like metal oxides (Lovley 1991; Kashefi & Lovley 2000; Lovley *et al* 2004; Weber *et al* 2006; Gralnick & Newman 2007)

Iron (Fe) is the fourth most abundant element on Earth and is an essential trace metal in nearly all organisms. The abundance of Fe in the Earth's crust and ability of Fe to transition between Fe(III) and Fe(II) redox states has resulted in Fe becoming a key metal in environmental metal-microbe interactions (Nealson 1992; Coates *et al* 1998; Lovley 2000; 2006). At circumneutral pH in natural anoxic zones, Fe exists primarily as insoluble, solid phase mineral oxides (Nevin & Lovley 2000; Cornell & Schwertmann 2003). These Fe(III) oxides are readily reduced and serve as an important electron sink for chemical and biological processes such as microbial metal respiration, making the reduction of Fe(III) one of the most geochemically significant processes that takes place under anoxic conditions such as the subsurface sediments and aquifers (Lovley *et al* 2004). Hence, Fe(III) reducing microorganisms have a great geological impact in terms of biomineralization as they reduce and solubilize different forms of Fe(III), as well as other minerals bound to Fe.

There are two physiologically distinct types of microbial Fe(III) reduction: assimilatory and dissimilatory. In assimilatory Fe(III) reduction, Fe(III) is converted into a soluble form that can be brought into the cell with specific transporters such as siderophores. This Fe is incorporated into Fe-containing proteins as cofactors that are required for important catalytic activities. In contrast, dissimilatory Fe(III) reduction or Fe(III) respiration refers to the process of generating energy to support cellular growth (Myers & Nealson 1990; Lovley 1991; Lovley *et al* 1991; Nealson 1992; Coates *et al* 1998; Lovley *et al* 2004). Microorganisms that are capable of conserving energy by Fe(III) respiration are phylogenetically diverse and belong to both Archaeal and Bacterial domains (Lovley & Goodwin 1988; Lovley & Chapelle 1995; Achtnich *et al* 1995; Kashefi & Lovley 2000; Weber *et al* 2006). Typically Fe(III) reducing microorganisms couple oxidation of organic compounds or even H_2 to Fe(III) reduction with energy transduction and ATP synthesis (Coates *et al* 1998).

The use of insoluble, extracellular Fe(III)-oxides represents a fundamental challenge for cells participating in their reduction. The mechanisms involving in extracellular respiration must be significantly different from those required for growth with soluble electron acceptors such as oxygen, nitrate or sulfate. Electrons, in the form of reducing equivalents generated through the oxidation of a carbon source, must cross at least one insulating membrane before leaving the cell, after which they must pass some distance further from the cell to the final terminal electron acceptor. Two well-characterized mechanisms for electron transfer involved in the final step between the cell surfaces and the terminal electron acceptors are described here. In the case of direct mechanism as shown in Figure 1.1, the redox-

active proteins associated with the cell or complex cell-associate extracellular matrix and the insoluble terminal acceptor are in physical contact (Nevin & Lovley 2000; Marsili, *et al* 2008b; Marsili *et al* 2010). Pilin-like filamentous structures ("nanowires") that are covered with redox-active proteins have also been shown to transfer electrons from cell surface and beyond to the terminal acceptor, facilitating long-range electron transfer (Reguera *et al* 2005; Mehta *et al* 2005; Leang *et al* 2010). In contrast, indirect mechanisms are facilitated by small redox-active molecules produced by the microorganisms and can be of one of two means as shown in Figure 1.1. One involves the secretion of small redox-active molecules called shuttles that facilitate electron transfer by cycling between the cell surface and the terminal electron acceptors. The second is by secretion of metal chelators or siderophores that solubilize the metal oxides thereby making it accessible.

The mechanism of microbial Fe(III) reduction is of interest due to the role it plays in various geochemical processes such as biomineralization and bioremediation. Fe(III) reducing bacteria have diverse metabolism including the ability to use variety of alternative electron acceptors such as Mn(IV), V(V), Cr(VI), Mo(VII), U(VI), Co(III), Tc(VII), As(V), nitrate, elemental S, fumarate, and thiosulfate (Lovley 1991; Lovley *et al* 1991; Nealson 1992; Coates *et al* 1998; Lloyd *et al* 2000; Kashefi & Lovley 2000; Liu *et al* 2002; Lloyd 2003; Lovley *et al* 2004; Islam *et al* 2004, 2005; Lovley 2006; McBeth *et al* 2007; Hau *et al* 2008). In unperturbed, stratified environments, it is the presence of more energetically favorable terminal electron acceptors that impacts the extent and rate of Fe(III) reduction. Terminal electron acceptors may be utilized sequentially from the highest

to lowest energy yield for the overall bioreduction reaction (Lovley & Goodwin 1988; Achtnich *et al* 1995; Lovley & Chapelle 1995). The utilization of these metals as acceptors has a major impact on contaminated environments. Biodegradation of organic pollutants such as benzene coupled to Fe(III) reduction is also frequently observed and is an important process for attenuation of anaerobic plumes in contaminated aquifers (Lovley 1991; Lovley & Chapelle 1995; Anderson *et al* 1998; Rooney-Varga *et al* 1999; Lovley *et al* 2004). These metal-reducing microorganisms also influence several other environmental processes such as groundwater quality, soil chemistry, formation of geologically important minerals like Precambrian banded iron formations (Weber *et al* 2006), and regulate the availability of trace nutrients and minerals. For example, release of Fe(II) due to microbial reduction increases dissolved metal concentrations in aquifers, affecting groundwater quality and causing corrosion or clogging of pipes (Lovley *et al* 2004; 2011). More recently, biotechnological applications of metal-reducing microorganisms is use these Fe(III) reducing organisms to donate electrons directly to an electrode acting as a the terminal acceptor in a fuel cell, thereby producing an electric current (Bond *et al* 2002; Bond & Lovley 2003; Lovley 2012).

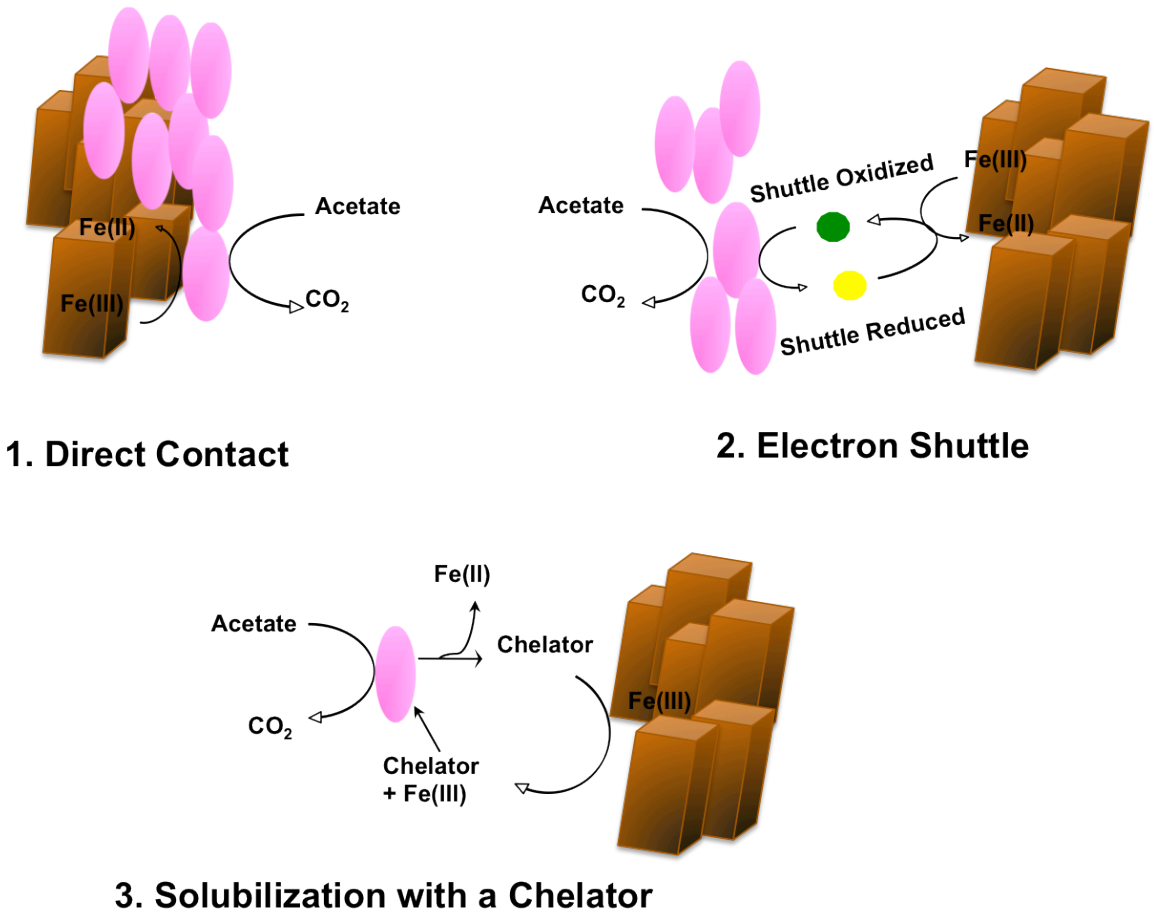

Figure 1.1 Mechanisms of electron transfer to insoluble Fe(III), showing (1) direct electron transfer, (2) indirect electron transfer using a shuttle, and (3) indirect electron transfer using a chelator.

1.2 Metal-reducing microorganisms

Most studies describing Fe(III) reducing microorganisms to date have focused on two model organisms, *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR-1. *S. oneidensis,* a γ-Proteobacterium, has been isolated from diverse oxic and anoxic environments and has been intensively studied. They are facultative organisms capable of using either O_2 or metal-oxides as acceptors coupled to incomplete oxidation of organic compounds (Myers & Nealson 1990; Hau & Gralnick 2007). *G. sulfurreducens* belonging to the *Geobacteraceae* family of the δ-

Proteobacteria can conserve energy from complete oxidation of organic compounds to CO_2 (Caccavo *et al* 1994; Lovley *et al* 1993). *Geobacter* species have been consistently detected in anaerobic sediments, such as subsurface and aquatic environments. Both model organisms have a wide physiological diversity regarding different soluble and insoluble electron acceptors used for respiration (Hau & Gralnick 2007; Marsili, *et al* 2008a; Lovley *et al* 2011). More recently, a gram positive thermophilic bacterium, *Thermincola potens* strain JR, was shown to be able to conserve energy by coupling acetate oxidation with the reduction of Fe(III)-oxides and anthraquinone disulfonate (AQDS) (Wrighton *et al* 2008; 2011; Marshall & May 2009).

S. oneidensis reduces insoluble terminal electron acceptors using flavin mononucleotide (FMN) as an electron shuttle. Oxidized FMN is reduced at the surface of the cell and diffuses to the terminal electron acceptor, where it is oxidized again and diffuses back to the cell (von Canstein *et al* 2008; Marsili, *et al* 2008a; Coursolle *et al* 2010). In contrast, in *G. sulfurreducens,* the reduction of insoluble acceptors requires direct contact between cell and the terminal electron acceptor through the use of surface and extracellular matrix-anchored redox active proteins such as *c*-type cytochromes (Nevin & Lovley 2000; Marsili, *et al* 2008b; Marsili *et al* 2010; Rollefson *et al* 2011). Similar to *Geobacter* species, *Geothrix fermentans* strain H-5*,* another metal-reducing bacterium, has also been consistently detected in a variety of subsurface environments that are undergoing active metal cycling and bioremediation. *G. fermentans* is the only known anaerobic metal reducing bacterium belonging to the Acidobacterial phylum (Coates *et al* 1999).

1.3 Acidobacteria

Acidobacteria represent one of the dominant bacterial phylum detected in soils. Pyrosequencing of 16S rRNA gene fragments across 87 diverse soils identified 31% of all classified sequences as acidobacterial (Ludwig *et al* 2006; Jones *et al* 2009). Metagenomic surveys have indicated as many as 26 deeply branching Acidobacterial subgroups that exist in various environments, such as deep-sea plankton and surface sediments (Quaiser *et al* 2008; Kouridaki *et al* 2010), hot springs (Bryant *et al* 2007; Weltzer & Miller 2013; Mackenzie *et al* 2013), arctic tundra (Männistö *et al* 2009), marine sponges (Karlińska-Batres & Wörheide 2013; Costa *et al* 2013), ancient cave wall paintings (Portillo *et al* 2008) and also from a variety of subsurface environments undergoing Fe(III) reduction, as well as from heavy metal and hydrocarbon contaminated sediments (Abed *et al* 2002; Barns *et al* 2007; McBeth *et al* 2007). To date, subdivision one, has nine genera; *Acidobacterium* (Kishimoto *et al* 1991), *Koribacter* Ellin 345 (Ward et al 2009), *Terriglobus* (Eichorst et al 2007), *Edaphobacter* (Koch *et al* 2008), *Granulicella* (Rawat *et al* 2012), *Acidipila* (Okamura *et al* 2011), *Telmatobacter* (Männistö *et al* 2013), *Acidicapsa* (Männistö *et al* 2013), and *Bryocella* (Dedysh *et al* 2012). Subdivision three has two genera *Solibacter* Ellin 6076 and *Bryobacter* (Foesel *et al* 2013). Recently, subdivision four representative *Blastocatella* was isolated (Foesel *et al* 2013). Subdivision eight includes genus *Holophaga* (Liesack *et al* 1994), *Geothrix* (Coates *et al* 1999), and *Acanthopleuribacter* (Fukunaga *et al* 2008). Lastly, subdivision ten has the genus *Thermotomaculum* (Izumi *et al* 2012).

1.4 Environmental relevance of *Geothrix*-like Acidobacteria

While *Geobacter* species are often one of the dominant organisms in pristine and contaminated subsurface aquifers undergoing metal reduction, *Geothrix*-like acidobacteria have also consistently been detected in these environments. However, consistent detection of *Geothrix* in these same environments raises a question about their ecological significance in Fe(III)-Fe(II) cycling and bioremediation of contaminants.

Fe(III) reduction is an important process coupled to degradation of various contaminants in groundwater and aquifers polluted by hydrocarbons such as petroleum and toxic heavy metal contaminants. Depletion of oxygen in such environments is primarily due to enhanced microbial activities which in general leads to Fe(III) becoming the most abundant alternate electron acceptor for the oxidation of these recalcitrant compounds. Microbial community analysis of various geographic locations associated with benzene oxidation in microcosms column studies as well as in *in situ* field trials have consistently detected both *Geobacter* and *Geothrix* like 16S rDNA sequences by denaturing gradient gel electrophoresis (DGGE) as well as by most probable number enumeration (MPN) and MPN-PCR using *Geobacter* and *Geothrix* specific primers (Anderson *et al* 1998; Rooney-Varga *et al* 1999; Snoeyenbos-West *et al* 2000; Abed *et al* 2002; Lin *et al* 2005). Recently, Klueglein *et al* isolated a new Fe(III) reducing and magnetite producing *Geothrix fermentans* strain HradG1 from heavily contaminated hydrocarbon surface sediment (Klueglein *et al* 2013).

Metals and radionuclides are common subsurface contaminants that are

difficult to remediate. Oxidized uranium U(VI) is soluble and mobile in these environments, but metal-reducers can reduce U(VI) to U(IV), which is insoluble and hence precipitates from groundwater (Lovley *et al* 1991). A successful field-tested strategy for containment of uranium is *in situ* stimulation of these metal-reducing bacteria by adding organic compounds. Many laboratory and field studies at the Oak Ridge Field Research Center in Oak Ridge, TN over the last decade have shown a very specific consortium of microorganisms that are involved in bioprecipitation of uranium. Although the most abundant sequences and cultured isolates involved in metal-reduction from this consortium have always been *Geobacter* species, there have been numerous studies that detect *Geothrix*-like species (Cardenas *et al* 2008; Vishnivetskaya *et al* 2010). For example, 16S microarray analysis and confirmatory qPCR showed that *Geothrix*-like Acidobacteria increase in population by three orders of magnitude following lactate stimulation during the uranium reduction phase (Brodie *et al* 2006). Clone libraries from ethanol injected field sites also showed *Geothrix*-like bacteria were enriched in uranium precipitation. Another microcosm study from the same site showed that *Geothrix* species comprised anywhere between 22% - 90% of 16S rRNA sequences after stimulation with acetate or ethanol with varying concentration of bicarbonate (Luo *et al* 2007). A recent study using pyrosequencing with strict indicator species statistical analysis was performed on the Oak Ridge Field Research site to specifically test uranium reduction upon stimulation with ethanol also showed *Geothrix* sequences to be present (Cardenas *et al* 2010). Altogether, these studies imply that *Geothrix* species are important members of the communities that are involved in bioreduction of uranium. However,

no reports have shown that *Geothrix* is capable of uranium reduction in pure cultures, it is possible that it can reduce U(VI) indirectly via reduced Fe(II) compounds or reduced humic acids (Lovley & Anderson 2000). In addition to uranium reduction, *Geothrix*-like Acidobacteria have also been detected during sediment technetium reduction (McBeth et al 2007), in microcosms that were involved in pertechnetate reduction (Xiangzhen Li & Krumholz 2008) and in subsurface release of arsenic from Bengal delta sediments (Islam *et al* 2004; 2005).

Abundant *G. fermentans* 16S rRNA sequences in addition to *Geobacter* sequences have also been enriched from current-producing freshwater anodes compared to non-current producing controls or fuel cells inoculated with marine sediments. All this is consistent with *Geothrix* species being the second most abundant Fe(III) reducer in subsurface environments in which Fe(III) reduction is a dominant process (Holmes *et al* 2004). Another microbial fuel cell study for wastewater treatment that was inoculated with synthetic waster water and rice paddy soil, enriched for two major groups. Along with *Geobacter*, *G. fermentans* was the second most dominant organism present in these current generating microbial fuel cells (Miyahara *et al* 2013). Moreover, *G. fermentans* in pure cultures have also been shown to colonize current-harvesting anodes by oxidizing organic compounds (Bond & Lovley 2005; Mehta-Kolte & Bond 2012).

In addition to contaminated environments *Geothrix* species have also been detected in pristine environments such as the glacial Mahomet aquifer. Along with *Geobacter* species, they represent more than 20% of the total attached communities compared to suspended communities (Flynn *et al* 2008). The microbial community

composition of the soil crust of sand dunes in Cape Cod National Seashore contained *Geothrix/Holophaga*/*Acidobacterium* like sequences based on DGGE profiles (Smith *et al* 2004).

1.5 General characteristics of *Geothrix fermentans*

G. fermentans strain H-5 was isolated from the Fe(III) reducing zone of a petroleum contaminated aquifer that had been adapted for toluene oxidation coupled to Fe(III) reduction (Coates *et al* 1999). *G. fermentans* was subsequently enriched and isolated using acetate as the electron donor and Fe(III) pyrophosphate as the terminal acceptor. *G. fermentans* is non-motile, non-spore forming rod, that has different cell morphologies, for example growth with soluble Fe(III) as an electron acceptor would yield single cells or short chains of two to three cells each, compared to long, intertwined, hair-like chains of several hundred cells when grown with fumarate as an acceptor (Coates *et al* 1999). *G. fermentans* can use variety of organic acids in addition to acetate such as lactate, propionate, succinate, and palmitate with Fe(III)-pyrophosphate as the terminal acceptor. In addition to Fe(III)-pyrophosphate, *G. fermentans* can use other forms of Fe(III), such as Fe(III)-oxide, Fe(III)-NTA, Fe(III)-citrate, nitrate, Mn(IV)-oxides, the humic acid analogue anthraquinone-2,6-disulfonate, poised electrodes, and O_2 at low partial pressure (Coates *et al* 1999; Thrash & Coates 2010; Mehta-Kolte & Bond 2012). *G. fermentans* can also ferment organic acids such as citrate and fumarate (Coates *et al* 1999). *G. fermentans* can grow optimally between 20°C - 25°C. Growth at 30°C resulted in cell lysis of over 50% of the population within 2 days of reaching stationary phase. Lower

temperatures slowed this process and at 20°C the cells remained stable (Mehta-Kolte & Bond 2012).

G. fermentans strain HradG1 was recently isolated from hydrocarbon contaminated surface sediments at Hradcany, Czech Republic (Klueglein *et al* 2013). Strain HradG1 can reduce Fe(III)-oxides by coupling oxidation of fumarate, succinate, lactate, acetate citrate, glucose, and glycerol. It can also use nitrate and fumarate as a terminal electron acceptor and can also grow fermentatively using fumarate or citrate, with temperature optima of 28°C. Similar to *G. fermentans* strain H-5, strain HradG1 can also grow under microoxic conditions (Klueglein *et al* 2013).

1.6 Insights into the *G. fermentans* genome

As part of this thesis the genome of *G. fermentans* was sequenced and assembled. It is 3,289,814 bp, with a GC content of 68%. It has 2922 protein coding genes with 2 rRNA operons. Similar to the other sequenced Acidobacteria *G. fermentans* also has abundant transporters, suggestive of a very diverse oligotrophic lifestyle (Ward *et al* 2009).

One feature about *G. fermentans* central metabolism is the citrate synthase gene, which is involved in acetate incorporation into the TCA cycle. This enzyme controls the flux into the TCA cycle by catalyzing the condensation of acetyl-CoA and oxaloacetate to produce citrate. Based on sequence similarity, the closest homolog to this citrate synthase is from *Symiobacterium thermophilum* and all *Geobacter* species. *G. fermentans* citrate synthase protein sequence is very different from all the other sequenced acidobacteria. This sequence similarity was interesting

as the *Geobacter* citrate synthase is more closely related to the eukaryotic one than to the prokaryotic protein sequence (Bond *et al* 2005) and has been suggested as a *Geobacter*-specific peptide-based biomarker to trace the activity of *Geobacteraceae* during biostimulation processes in contaminated subsurface environments (Wilkins et al 2009; 2010). This similarity between the proteins sequences of *Geobacter* and *Geothrix* citrate synthase, suggests a re-examination of the proteogenomic monitoring of microbial community stimulated during uranium bioprecipitation (Wilkins *et al* 2010; Yun *et al* 2011).

Similar to other metal-reducing bacteria, *G. fermentans* also has abundant multi-heme *c*-type cytochromes. Chapter two also describes the ability of *G. fermentans* to couple oxidation of complex carbohydrates like chitin and xylan with the reduction of Fe(III), unlike any other metal-reducer. The *G. fermentans* genome has a three different putative glycosyl hydrolases; two putative glycosyl transferase; and four putative polysaccharide deacetylase genes, which may be involved in oxidation of these complex carbohydrates.

1.7 *G. fermentans* metal reduction strategy

Metal-reducing bacteria must solve at least three challenges in order to successfully transfer electrons from the cell interior to an insoluble terminal acceptor that is spatially separated from the cytoplasmic membrane. The cytoplasmic membrane-associated proteins, such as cytochromes, typically catalyzes the reduction of soluble acceptors like O_2, or nitrate, using electrons generated from intracellular metabolism. For insoluble extracellular acceptors, the electrons

generated must cross a distance of 80 Å - 100 Å, first crossing the inner membrane, cell wall, and if present, an outer membrane via a chain of interacting redox proteins. Then, the transfer from cell surface proteins to the extracellular terminal acceptor must also be accomplished. This transfer is done via proteins positioned within 10 Å of an acceptor, and/or through the use of a soluble mediator, which can diffuse across this gap and shuttle the electrons. Finally, to maintain the close distances required for direct or diffusion-based electron transfers, cells must be capable of locating and adhering to target surfaces. As cells replicate, layering on top of each other, they may require an additional level of complexity to relay the electrons across longer distances to sustain respiration.

An example for how metal reducing bacteria solve the first two challenges of electron transfer is the well understood case of *S. oneidensis* MR-1. Using combination of genetic, biochemical, and structural approaches, the Mtr respiratory pathway was identified (Richardson, *et al* 2012b; Richardson, Edwards, *et al* 2012a; Gralnick 2012). The Mtr pathway is comprised of two multi-heme *c*-type cytochromes, MtrA and MtrC along with an integral outer-membrane protein MtrB, that forms a transmembrane electron conduit through which electrons are able to move from the menaquinone pool, to the periplasmic space and to the outer-membrane (Richardson, *et al* 2012b). Once the electrons have reached the cell surface, the electrons are then subsequently transferred from MtrC to the terminal acceptor either directly or by using the electron shuttle FMN (Coursolle *et al* 2010). The analysis of structural MtrC models based on crystal structure of its paralog (MtrF) reveals FMN binding domains near two solvent-exposed heme groups

(Clarke *et al* 2011; Edwards, *et al* 2012a; Edwards, *et al* 2012b), further providing an example of how cells might be interacting with flavins to facilitate electron shuttling for extracellular respiration.

G. fermentans initially was shown to be able to reduce Fe(III)-oxide when Fe(III) was incorporated into micro-porous alginate beads that prevented direct contact between the cells and Fe(III)-oxide. The Fe(III)-oxide encodes in the beads was subsequently reduced, which is suggestive of an electron shuttling mechanism. Also, the addition of cell-free culture filtrates of *G. fermentans* to washed cell suspensions of *G. fermentans* stimulated Fe(III) reduction (Nevin & Lovley 2002). These results suggest that *G. fermentans* secretes an extracellular compound that can shuttle electrons between the cells and the poorly crystalline Fe(III) oxides. Thin-layer chromatography of the cell free supernatans indicated that this compound had characteristics similar to a water-soluble quinone and that it was not a protein, based on protease or heat treatment of the filtrate (Nevin & Lovley 2002).

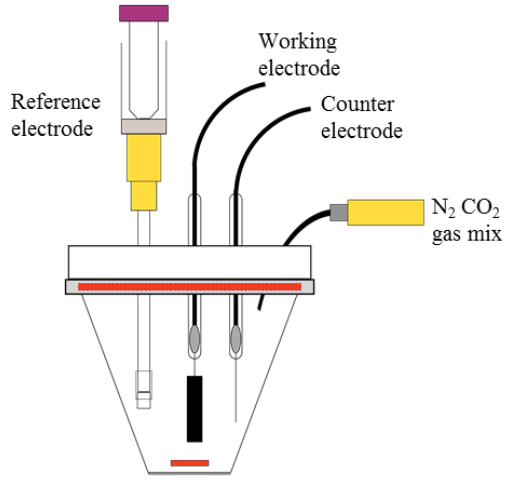

Figure 1.2 Three-electrode bioreactor, adapted from Marsili *et al,* AEM 2008 (Marsili, *et al* 2008b).

Metal reducing bacteria have also been shown to use poised electrode as a terminal electron acceptor. This use of electrodes to study extracellular respiration has proven to be an invaluable technique to the field of microbial metal respiration. Electrochemical tools provide the ability to monitor and probe proteins as they function on electrodes and in living cells by offering the capability to control and monitor electron transfer in real time. Figure 1.2 is a schematic of a three-electrode bioreactor, used for electrochemical growth and characterization of the metal-reducing bacteria. Traditionally used mineral oxides have inherent drawbacks that influence the potential of the acceptor and thereby the experimental results. These drawbacks include the difficulty to produce chemically homogenous Fe(III)-oxide particles, variable surface areas of the Fe(III)-oxide particles and redox potentials that change over time due to passivation and dehydration. In comparison, poised electrodes represent surfaces with known potentials that can remain a constant and inexhaustible source of electron acceptor for long periods of time. The response of electron transfer to electric potential allows investigations into kinetics between the cell surface (redox protein) and the electrode. Investigations into long-range kinetics throughout the biofilm and the electrode can also be performed (Marsili, *et al* 2008b; Marsili *et al* 2010; Bonanni *et al* 2012). Biofilms developed on the electrodes can be visualized by confocal and electron microscopy, allowing for identification of mutants that are deficient in one of the two stages. Additionally, anode-colonizing bacteria generate electric current, which has many potential biotechnological applications.

Similar to other metal reducing bacteria, *G. fermentans* can also grow in

electrochemical cells using a poised electrode as the sole terminal acceptor with organic acids as electron donors. A rapid increase in electron transfer upon inoculation was observed, followed by a slow increase over 400 hours. About 3 weeks were required to reach stable rates of electron transfer of 50-100 $\mu A/cm^2$. Visualization by scanning electron microscopy showed that *G. fermentans* cells were sparsely present as small clumps throughout the electrode, in contrast to *Geobacter* colonized electrode that are densely populated as a uniform biofilm that is usually about 20 μm thick. These observations are described in chapter three.

A slow scan rate cyclic voltameteric analysis performed by sweeping between low and high potentials showed that *G. fermentans* transferred electrons at two separate characteristic potential windows, one centered at - 0.2 V versus standard hydrogen electrode (SHE) and the second centered at 0.3 V versus SHE. This unique two potential dependent response of *G. fermentans* was different from previously studied metal reducing isolates and microbial fuel cell enrichments. *G. fermentans* reaches its maximum rate of electron transfer at a potential of 0.55 V versus SHE, which is much higher when compared to *G. sulfurreducens*, which reaches its maximum rate at 0 V versus SHE. Electron transfer for *G. fermentans* initiates at potentials as low as – 0.2V versus SHE, which is similar to *G. sulfurreducens*, but cells do not reach their maximum rates of electron transfer unless a very positive acceptor near potentials of 0.55 V versus SHE is applied, which is over half a volt higher than required for maximum rates for *G. sulfurreducens* (Mehta-Kolte & Bond 2012).

Previously, Nevin and Lovley used indirect techniques, such as encapsulated

alginate beads were used to detect the presence of an electron shuttles involved in *G. fermentans* metal reduction strategy (Nevin & Lovley 2002). Newer, more confirmatory electrochemical techniques, such as medium replacement experiments, can conclusively detect the involvement of redox active shuttles. During a medium replacement experiment on *G. fermentans* culture grown in an electrochemical cell showed an immediate drop of approximately 70% in the rate of electron transfer. The drop in rate of electron transfer could be recovered by subsequent readdition of the original spent medium (Mehta-Kolte & Bond 2012). The medium replacement experiment confirmed the presence of small soluble compounds facilitating electron transfer from the cell surface to the acceptor (electrode) and that this accounted for the majority of electron transfer by *G. fermentans*, similar to what has been observed for *S. oneidensis* MR-1 (Marsili, *et al* 2008a).

G. fermentans medium replacement experiments consistently showed a decrease in electron transfer rates at two discrete potential windows. The compound associated with the lower potential at -0.2 V, which could be selectively removed by octadecyl-functionalized silica gel (C_{18}), without affecting the higher potential compound and by fluorescence emission profile in HLPC analysis was identified as riboflavin. The higher potential compound centered at +0.3 V, had no affinity for hydrophobic columns, or the graphite electrode, but it had a characteristic UV fluorescence with an excitation maximum of 229nm, similar to quinone-like molecules (Mehta-Kolte & Bond 2012). Both these redox compounds are also present when *G. fermentans* is grown with insoluble acceptors such as Fe(III)-oxide and not with soluble acceptors like fumarate, suggesting that this shuttling

mechanism of electron transfer is not an artifact of electrode growth but is produced under all insoluble electron acceptor conditions tested.

1.8 Molecular mechanism of electron transfer in *G. fermentans*

Cytochromes are electron carriers that play a vital role in mediating electron transfer reaction associated with respiration. All cytochromes have heme as the prosthetic group and in *c*-type cytochromes the heme ion is covalently bound to the protein by two thioether linkages and coordinated with a proximal cysteine residue in the protein. Both *Shewanella* and *Geobacter* species contain numerous *c*-type cytochromes and various studies over the last decade have shown that these cytochromes play an essential role in anaerobic respiration (Shi *et al* 2007; 2009; 2012; Richardson, *et al* 2012a). Similarly, the genome of *G. fermentans* also encodes 69 predicted multi-heme *c*-type cytochromes, leading to the hypothesis that these proteins may also be involved in electron transfer mechanisms.

Biochemical analysis of membrane proteins resolved on a denaturing sodium dodecyl sulfate-polyacrylamide gel electrphonersis (SDS-PAGE) from detergent solubilized membrane fraction of Fe(III)-citrate grown cells resolved multiple heme containing proteins. The most abundant heme containing protein was identified as mixture of two decaheme *c*-type cytochromes, which we named as GxcA and GxcB. GxcA and GxcB are predicate to be outer membrane-associated proteins based on Psortb and LipoP prediction softwares. These proteins had 59% and 54% similarity to the decaheme *c*-type cytochromes of *Rhodoferax ferrireducens* T118 and about 30% similarity to the decaheme *c*-type cytochrome paralog, MtrF of *S. oneidensis*.

Despite low sequence similarity of *G. fermentans* decaheme *c*-type cytochromes to MtrF, GxcA and GxcB are predicted to have a similar three-dimensional conformation as the MtrF protein based on computer generated one on one threading onto the published MtrF structure (Clarke *et al* 2011; Edwards, *et al* 2012a). A mutant strain of *S. oneidensis* lacking all the outer membrane-associated decaheme *c*-type cytochromes (Mtr pathway), essential for electron transfer could be complemented with the heterologous expression of *gxcA*. Suggesting that GxcA is a functional homolog of *S. oneidensis* outer membrane decaheme *c*-type cytochromes.

1.9 Thesis rationale and goals

Bacterial respiration of Fe(III) plays an important role in the degradation of natural and contaminant organic matter in aquatic sediments and the subsurface and also influences the mobility of a variety of trace metals in these environments. Given the diversity of metal-reducing organisms, the primary focus of the last decade has been only on two well-characterized model organisms: *Geobacter* and *Shewanella* species. This was at the cost of ignoring other environmentally relevant metal-reducers, such as *Geothrix*-like Acidobacteria. The broad goal of this research was to characterize the molecular mechanisms of metal-reduction by *G. fermentans*, belonging to the Acidobacterial phylum. A variety of complementary approaches were used to study how *G. fermentans* respires insoluble electron acceptors. Key redox active proteins involved in electron transfer were identified and characterized.

1.10 Summary of thesis

The focus of the research described in this thesis is to understand the molecular mechanism of metal reduction by *G. fermentans*. Chapter 2 describes the genome sequencing and assembly of *G. fermentans* and another *Geothrix* species 1M1. This chapter provides an insight into *G. fermentans* metabolism such as the unique TCA cycle genes, which is different from all of the Acidobacterial genomes sequenced to date. It also highlights the possiblility of *G. fermentans* to couple oxidization of complex carbohydrates, based on presence of putative glycosyl transferases, glycosyl hydrolases and deacetylase. Chapter 3 identifies the higher potential requirement for *G. fermentans* to grow at its maximum rate and describes its potential dependent environmental niche. Also, this chapter identifies and describes the role of two different soluble redox-active electron shuttles required for *G. fermentans* extracellular respiration. Chapter 4 describes the proteins involved in the electron transfer mechanism by *G. fermentans*. Work presented in this chapter focused on two multi-heme *c*-type cytochromes that are abundant outer membrane proteins with predicted structural similarities to *S. oneidensis* WT outer membrane decaheme *c*-type cytochromes. These proteins were heterologously expressed in mutant *S. oneidensis* MR-1 strains unable to reduce metals, to test if the *G. fermentans* proteins are functional homologs.

Chapter 2: Draft Genomes of Two Metal-Reducing Bacteria *Geothrix; G. fermentans* and *G.* 1M1

2.1 Overview

Geothrix fermentans is a dissimilatory metal-reducing bacterium that was isolated from a Fe(III)-reducing zone of a petroleum contaminated aquifer at Hanahan, South Carolina. Another *Geothrix* species, 1M1, was isolated by Zhenya Shelobolina. The genomes of *G. fermentans* and *Geothrix* species 1M1 were sequenced using the MiSeq paired-end Illumina technology. The draft genomes provided insights into the oligotrophic lifestyle and environmental niche of *Geothrix*-like Acidobacteria. This genomic analysis will assist in understanding the electron transfer mechanism(s) by *G. fermentans* and other *Geothrix*-like Acidobacteria that are phylogenetically different from the more commonly studied metal-reducers, thereby contributing to and diversifying the field of extracellular metal-respiration.

2.2 Introduction

The phylum Acidobacteria represents one of the most abundant, ubiquitous and phylogenetically diverse bacterial phyla found in global soil environments (Barns *et al* 2007; Janssen 2006; Eichorst *et al* 2007; Jones *et al* 2009), such as acid mine drainages (Kishimoto *et al* 1991), plant roots (Li *et al* 2011), forest soils (Koch *et al* 2008), subsurface aquifers (Coates *et al* 1999; Klueglein *et al* 2013), the deep sea (Quaiser *et al* 2008), caves (Meisinger *et al* 2007), artic and boreal soils (Männistö *et al* 2007; 2009) and hot spring microbial mats (Bryant *et al* 2007). Acidobacteria to date have been divided in 26 phylogenetic subdivisions based on 16S rRNA. However, in spite of their diverse distribution and abundance, very little is known about their metabolic capabilities and ecological roles in these bacteria play

in diverse environments. There are only a few subdivisions that have been cultivated and described, as members of the Acidobacteria are generally difficult to culture due to stringent growth requirements (Joseph *et al* 2003; Eichorst *et al* 2007). *Holophaga foetida* and *Geothrix fermentans* are the only known anaerobic Acidobacteria that belong to the subdivision 8. *H. foetida* is a homoacetogenic acidobacterium that ferments aromatic compounds, and was isolated from an anoxic hydrocarbon-rich sediment (Liesack *et al* 1994; Anderson *et al* 2012).

G. fermentans is the only known dissimilatory Fe(III)-reducing bacteria belonging to the Acidobacterial phylum. It conserves energy to support growth by oxidizing fatty acids and other organic compounds to carbon dioxide, with Fe(III) serving as the sole terminal electron acceptor. *G. fermentans* cells are rod-shaped, non-motile, non-spore-forming, and are chemo-organotrophs. *G. fermentans* can also grow fermentatively on citrate or fumarate in the absence of an alternate electron acceptor (Coates et al 1999). *G. fermentans* can also colonize and use a poised electrode as an acceptor in an electrochemical reactor (Bond & Lovley 2005; Mehta-Kolte & Bond 2012).

This strain and another species, 1M1, were selected for genome sequencing for the following reasons. 1) They belong to the diverse but understudied Acidobacterial phylum and the only representative members identified to date that can respire insoluble metals. 2) *Geothrix*-like Acidobacteria have been consistently detected from various aquifers and subsurface environments that are undergoing bioremediation and bioprecipitation, implying that these metal-reducers are active members of the subsurface community. Yet very little is known about their

ecological significance in these processes. 3) Phylogenetically diverse microorganisms such as *Geothrix* may have novel metabolic capabilities and/or mechanisms for electron transfer.

2.3 Materials and Methods

2.3.1 Growth condition and DNA isolation

Genomic DNA was extracted from *G. fermentans* and *G.* 1M1 cultures grown under anaerobic conditions in minimal medium with fumarate as the electron acceptor and acetate as the electron donor at 20°C, as previously described (Mehta-Kolte & Bond 2012) using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). DNA was quantified by Qubit DS DNA broad range Assay Kit (Invitrogen, CA).

2.3.2 Genome sequencing, assembly, and annotation

The sequencing was performed at the University of Minnesota Genomic Center Core Facility using MiSeq (Illumina), and 454 GS (Roche) sequencing technologies. The Illumina paired-end sequences were quality filtered, yielding 4,996,233 reads of 250 bp in length, representing a 1000 X fold coverage of the genome. Various hash lengths between 31 and 89 were tested and an optimal assembly was achieved with a k-mer size of 61. Contigs less than 500 bp long were removed from the final assembly. Final genome coverage was 200 X. The reads were then assembled by Velvet 1.0.0 (version) into a draft genome using the optimal k-mer size of 61 bp to generate 431 contigs, with N_{50} of 38,450 bp, and the maximum

length of 132,653 bp. The mean G+C content of the genome was 68%. The 454 GS sequencing generated 271,539 reads. The output data were processed and assembled using the Newbler software producing 69 contigs resulting in approximately 3.28 Mb genome. This was annotated using the Rapid Annotation Using Subsystem Technology (RAST) server. A total of 2,900 coding sequences with 2 16S rRNAs and 51 tRNAs were predicted. Around the same time, DOE and Joint Genome Institute released a draft genome sequence of *G. fermentans* DMZ 14018, with the following NCBI taxon ID 1121920 and Gold ID 11528. This draft genome was 3.3 Mb with a mean G+C content of 68.85% and had 36 contigs, with 2,922 coding sequences with 3 16S rRNAs and 51 tRNAs.

The *G.* 1M1 genome was also sequenced at the University of Minnesota Genomic Center Core Facility using MiSeq sequencing technology. 4,895,391 250 bp paired-end illumina sequences were obtained, quality filtered using Galaxy tools, and assembled using Vague and A5 pipeline. Briefly, the sequences were trimmed and groomed to 150 bp paired interleaved fastqsanger format with a quality score of greater than 30 using Galaxy tools and the middle 50% of the trimmed sequences were used for assembly. Vague- a graphical user interface for the Velvet assembler was used (Powell & Seemann 2013) with the following parameters: Velveth: 63, -shortPaired and Velvetg: -very_clean yes, -exportFiltered yes, -min_contig-lgth 500, -scaffolding no, and -cov_cutoff 10 was used. This assembly gave 143 contigs with N_{50} of 48,567 bp and the maximum length of 142,915 bp.

Alternatively, the groomed and trimmed sequences with a final length of 120 bp, paired-end interleaved in fastqsanger format were assembled using the a5

pipeline (Tritt et al 2012). A5 pipeline automates data cleaning, error correction, contig assembly, and scaffolding. The default parameters were used as it auto-detects the best kmer size and other values for genome assembly. This assembly had 117 X coverage with 94 contigs greater than 500 bp. The N_{50} was 65,363 bp with the maximum length of 215,591 bp. This draft genome sequence was uploaded and annotated using Integrated Microbal Genomes-Expert Review (IMG-ER) of Joint Genome Institute (JGI) with Gold ID 49244. This assembly resulted in a genome size of 3.28 Mb containing 2,996 coding sequences, 2 16S rRNAs, and 47 tRNAs.

2.3.3 Complex carbohydrate utilization

G. fermentans was tested for the ability to couple oxidation of complex carbohydrates such chitin, birchwood and cellulose with the reduction of Fe(III)-oxide. *G. fermentans* was grown with acetate and fumarate as electron donor and acceptor respectively. Cells were then transferred (1% inoculation) to mineral medium containing 100 mM Fe(III)-oxide as the electron acceptor with 5 mM acetate as the electron donor. This culture was subsequently transferred (1% inoculation) to a mineral medium containing 100 mM Fe(III)-oxide with 0.05% of either chitin, birchwood or cellulose in triplicates. Samples were taken every 7 days, diluted in 1N HCl, and a modified ferrozine assay was used to measure Fe(II) production (Phillips & Lovley 1987). Ferrozine reagent (2 g ferrozine/L in 100 mM HEPES, pH 7) was combined with samples (dissolved in 1 N HCl) and absorbance at 625 nm was determined over time. Also, mineral medium containing 100 mM Fe(III)-oxide with varing concentrations of chitin (0.01%, 0.05%, 0.1%, 0.25%) were

used for measuring Fe(III) reduction rates of *G. fermentans* coupled to chitin oxidation in triplicates. *G. sulfurreducens* was used as a control in triplicates in the above experiment.

2.4 Results

2.4.1 Insights into the genomes of *G. fermentans* and *G.* 1M1

Similar to the sequenced aerobic Acidobacteria, *G. fermentans* and *G.*1M1 harbors two copies of rRNAs. This feature is consistent and fits with the slow growth lifestyle of Acidobacteria (Ward *et al* 2009). Genome statistics for both *G. fermentans* and *G.* 1M1 are reported in Table 2.1 based on IMG JGI annotation. Bidirectional best-hit analyses of all protein sequences were done to compare the two species. About 15% of the protein-coding sequences were unique to one of the two species. These unique genes were, for the most part, hypothetical proteins or proteins of unknown function. Only the *G.*1M1 genome encodes for putative genes that are associated with phage replication, phage major capsid, phage single stranded DNA synthesis, minor capsid and DNA pilot proteins that are similar to coliphage like-proteins. Unlike the other sequenced aerobic Acidobacteria, *Geothrix* species does not contain any flagella genes (Ward *et al* 2009).

Both genomes contain genes for nitrogen metabolism such as the respiratory nitrate reductase gene sequences for nitrate to nitrite reduction (*narG, narY,* and *napA*), these were similar to the genes found in *Thermus thermophilus*, and nitrite reductase (*nrfA* and *nrfH*), for nitrite to ammonia reduction, which were similar to the genes found in aerobic Acidobacteria subdivision 1, and 3 (Ward *et al* 2009). The

genomes also encode a polysulphite reductase related to *Desulfovibrio* species, presumably involved in sulfate reduction.

Geothrix species have putative genes involved in arsenic reduction/respiration (arsenate reductase ArsC). It also has the arsenical resistant protein (Acr3), and an arsenical resistance operon repressor. *G. fermentans* has one copy of each of these genes, whereas *G.* 1M1 has two copies of these genes. These genes may serve primarily in detoxification rather than in dissimilatory As(V) reduction. In fact, a study by Islam *et al* (Islam *et al* 2005) demonstrated that *G. fermentans* was unable to conserve energy using As(V) as a terminal electron acceptor. Moreover, when cells grown with Fe(III)-citrate as an electron acceptor in presence of As(V) resulted in precipitation of Fe(II)-As. A mineral complex that is analogous to vivianite was formed, suggesting an indirect removal of As from the solution.

The *G. fermentans* and *G.* 1M1 genomes have numerous genes encoding oxygen-utilization and tolerance functions, such as cytochrome *c* and *d* ubiquinone oxidases, putatively for detoxification purposes. Preliminary experiments show that *G. fermentans* can couple growth using molecular oxygen as a sole terminal acceptor (5% concentration). This supports the hypothesis that *G. fermentans* environmental niche could be the oxic-anoxic interface, instead of a strict anaerobic subsurface niche. Another significant finding in context to its respiration is the presence of a *Geobacter*-like central metabolism. *Geothrix* species tricarboxylic acid (TCA) cycle proteins such as the citrate synthase and isocitrate dehydrogenase, were found to be similar to the *Geobacteraceae* than to the sequenced Acidobacteria. Interestingly the

Geobacter citrate synthase is eukaryotic-like and was originally thought to be unique to the *Geobacteraceae* (Bond *et al* 2005). This finding led to the development of the *Geobacter* citrate synthase peptides as biomarkers or "fingerprints" for the detection of *Geobacter* activity during subsurface bioremediation of uranium (Holmes *et al* 2005; Wilkins *et al* 2009; 2010). The *Geothrix* species citrate synthase protein sequence has 87% identity to the *Geobacter* species citrate synthase protein sequence. In a study by Wilkins *et al* (Wilkins *et al* 2010), a proteomic database was generated using the citrate synthase protein sequences obtained from metagenomic data collected from the subsurface environments undergoing active bioprecipitation of uranium, along all sequenced *Geobacter* genomes. Unique multiple peptides that were highly conserved within the *Geobacter* species but absent from other prokaryotic and eukaryotic citrate synthase sequences were generated and used as *Geobacter* specific "biomarkers". Two of the peptides used for *Geobacter* community "fingerprinting" are also conserved within the two *Geothrix* species. Given the fact that both *Geothrix* and *Geobacter* species have been detected from the same subsurface environments, suggest that these "*Geobacter* specific" biomarkers could also be detecting *Geothrix*-like Acidobacteria in these environments.

Similar to the sequenced Acidobacteria from subdivision 1 and 3, the *G. fermentans* and *G.* 1M1 genomes contained abundant glycoside hydrolase, glycosyl transferases encoding genes and candidate chitin utilization genes (Table 2.2). Based on genomic information, *G. fermentans* was tested for its ability to use complex carbohydrates as electron donors with Fe(III)-oxide serving as an electron acceptor. Accumulation of Fe(II) overtime was observed with chitin as an electron donor and

Table- 2.1 Genome statistics of the two *Geothrix* species

	G. fermentans H-5		*G.* 1M1	
Properties	Number	% of Total	Number	% of Total
DNA total number of bases	3289813	100	3286281	100
DNA coding number of bases	3013658	91.61	3016127	91.78
DNA G+C number of bases	2265042	68.85	2233384	67.96
DNA scaffolds	36	100	99	100
Genes total number	2986	100	3051	100
Protein coding genes	2922	97.86	2996	98.20
RNA genes	64	2.14	55	1.80
rRNA genes	10	0.33	5	0.16
5S rRNA	4	0.13	2	0.07
16S rRNA	3	0.10	2	0.07
23S rRNA	3	0.10	1	0.03
tRNA genes	51	1.71	47	1.54
Other RNA genes	3	0.10	3	0.10
Protein coding genes with function	2370	79.37	2375	77.84
Protein coding genes without function	552	18.49	621	20.35
Protein coding genes with enzymes	818	27.39	818	26.81
Protein coding genes-Transporter Classification	321	10.75	324	10.62
Protein coding genes-KEGG pathways	842	69.99	822	26.94
Protein coding genes-KEGG Orthology	1424	47.69	1373	45.00
Protein coding genes-MetaCyc pathways	794	26.59	794	26.02
Protein coding genes with COGs	2311	77.39	2311	75.75
with KOGs	1106	37.04	1108	36.32
with Pfam	2442	81.78	2416	79.19
with TIGRfam	934	31.28	916	30.02
with IMG terms	709	23.74	689	22.58
with IMG pathways	254	8.51	262	8.59
with IMG parts list	265	8.87	242	7.93
in paralogs clusters	2389	80.01	2471	80.99
in Chromosomal Cassette	2989	100	3051	100
Chromosomal Cassette	167		206	
Fused protein coding genes	28	0.94	30	0.98
Protein coding genes-signal peptides	314	10.52	323	10.59
Protein coding genes-transmembrane proteins	629	21.06	620	20.32
COG cluster	1422	0.01	1411	0.01
KOG cluster	670	0.00	661	0.00
Pfam cluster	1633	0.01	1614	0.01
TIGRfam cluster	796	0.01	786	0.01

also increasing the chitin concentration led to an increase in total Fe(II) (Figure 2.2 and 2.3). However, *G. fermentans* failed to use other complex carbohydrates such as cellulose and birchwood (Figure 2.2). The ability to oxidize complex carbohydrates

such as chitin coupled to Fe(III)-oxide reduction is an exclusive metabolism that has not yet been observed in any other known metal-reducers.

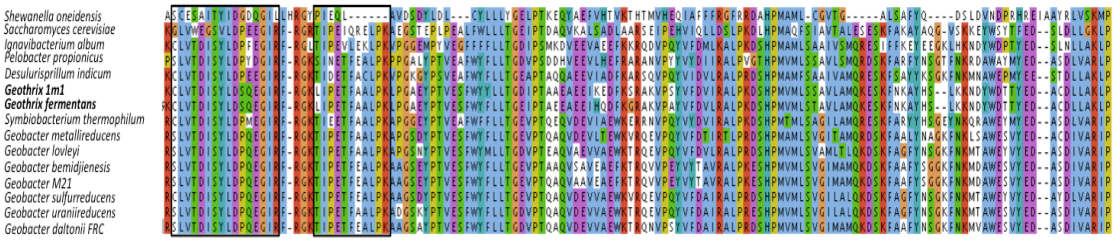

Figure 2.1 Alignment of eukaryotic and prokaryotic citrate synthase protein sequences showing the conserved regions associated with *Geobacter* copies of this protein that are also present in *Geothrix* copies (bold). Boxed regions correspond to two of the conserved peptides that were used as indicators "fingerprints" of overall *Geobacter* activity during uranium bioprecipitation in the Wilkin *et al* 2010 study. *Shewanella oneidensis* citrate synthase is used as an out-group in this alignment.

Both the *Geothrix* sequenced genomes had more than 50 genes that encode lower-specificity sugar and amino acid transporters, as well as high-affinity ABC transporters, and different types of secondary porters. This is a common trend seen throughout the Acidobacterial phylum and has been proposed to be a growth advantage under low-nutrient environments that they are typically found in (Ward *et al* 2009). *Geothrix* species have *feoAB* genes for assimilatory iron uptake, enabling them for low-affinity ferrous iron transporter that is similar to its closest 16S rRNA relative, *H. foetida*.

Table- 2.2 Putative genes predicated to be involved in complex carbohydrate utilization

Locus Tag G398DRAFT [a]	Class	Putative Activity [b]	% Similarity to Amino Acid Sequence
00805	GH3 [c]	β-N-acetyl hexosaminidase	47
02418	GH2	β-galactosidase	62
01767	GH5	Cellulase	47
02605	DE1 [d]	Polysaccharide deacetylase	51
02412	DE1	Polysaccharide deacetylase	51
02411	GT1 [e]	Glycosyl transferases	56
	DE1	Polysaccharide deacetylase	
02410	GT1	Glycosyl transferases	63
	DE1	Polysaccharide deacetylase	

[a] G398DRAFT locus designation assigned by JGI
[b] Based on annotation assigned by JGI
[c] GH= Glycoside hydrolases, [d] DE= Deacetylase, [e] GT= Glycosyl transferases

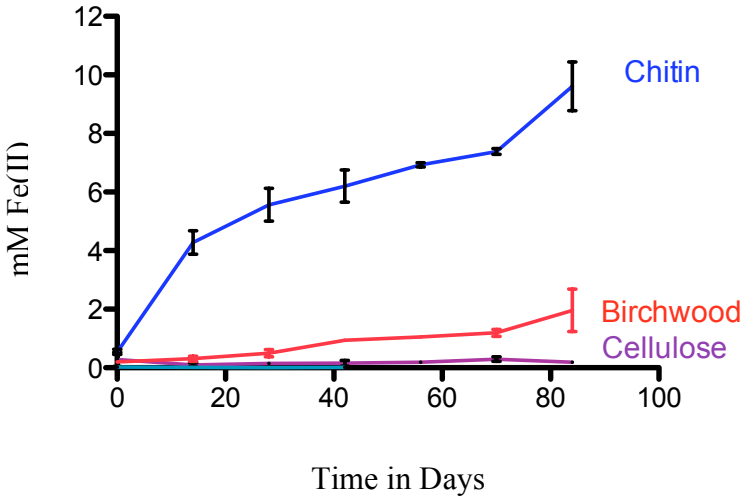

Figure 2.2 Fe(III) reduction rates of *G. fermentans* with different complex carbohydrates as electron donors with Fe(III)-oxide as the terminal electron acceptor

The *G.*1M1 genome contained genes that encode a putative toxin-antitoxin system (for example the YeoB-YefM pair), which were not found in the *G. fermentans* genome. The YeoB-YefM toxin-antitoxin pair is predicted to be an mRNA interferase and also an inhibitor of translation initiation. The presence of toxin-antitoxin system has also been identified in the other aerobic sequenced Acidobacteria (Ward *et al* 2009).

Many metal-reducing bacteria such as *Geobacter* and *Shewanella* species contain numerous *c*-type cytochromes that have been shown to be essential for metal respiration (Shi *et al* 2007; 2009). These metal-reducing organisms have developed electron transfer strategies that require interaction of many *c*-type cytochromes to facilitate electron transfer to the extracellular acceptor, especially the role of multiheme *c*-type cytochromes. The genomes of *G. fermentans* and *G.*1M1 also encode for numerous multiheme *c*-type cytochromes. One interesting gene arrangement in the genomes is a putative open reading frame consisting of a cluster of decaheme *c*-type cytochrome genes flanked by *c*-type cytochrome maturation genes as shown in Figure 2.4. *G. fermentans* has four putative decaheme *c*-type cytochromes and *G.* 1M1 has five putative decaheme *c*-type cytochromes in that cluster. The closest protein based sequence similarity is to the outer membrane decaheme *c*-type cytochrome MtrF of *Rhodoferax ferrireducens* (54%). These decaheme *c*-type cytochrome genes were named as *gxcA*, *gxcB*, *gxcC* and *gxcD* for G398DRAFT_1334, 1335, 1336 and 1338 genes respectively from the IMG/JGI sequenced strain. These *c*-type cytochromes had 45% or greater similarity with each other or between species.

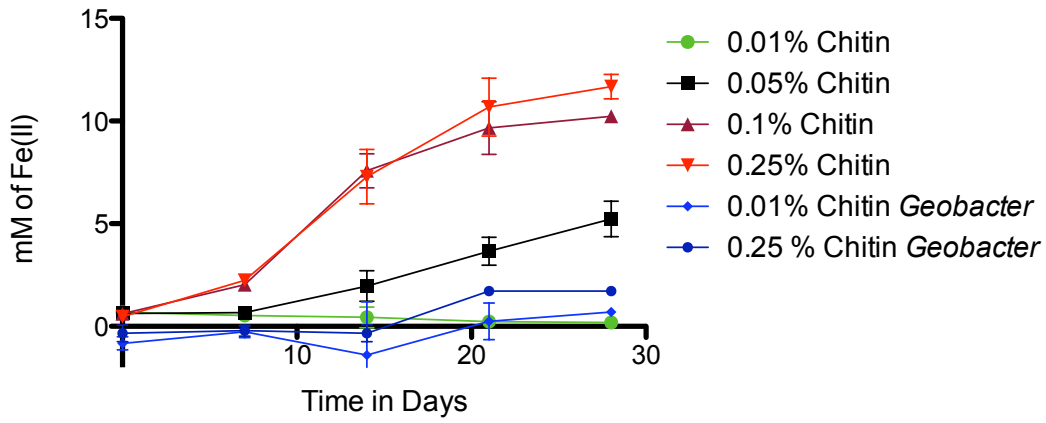

Figure 2.3 Increasing the chitin concentration leads to an increase in Fe(III) reduction by *G. fermentans*. Also, *G. sulfurreducens* fails to couple oxidation of chitin with the reduction of Fe(III)-oxide.

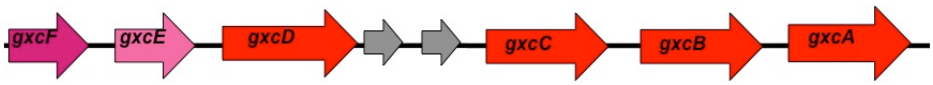

Figure 2.4 A representation of the putative open reading frame of a genomic region showing the cluster of four decaheme *c*-type cytochromes homologues to the *mtrF/C* genes of *Shewanella* species.

The decaheme *c*-type cytochrome MtrF belongs to the *Shewanella* MtrC/MtrF family of decaheme cytochromes. The MtrC/MtrF cytochromes have been characterized and shown to be a part of the Mtr electron transfer conduit of *S. oneidensis* MR-1 (Ross *et al* 2007; Jensen *et al* 2010). This electron conduit (Mtr CAB) is formed by two decaheme *c*-type cytochromes (MtrA, and MtrC) with an integral outer membrane protein (MtrB). MtrCAB has been shown to be essential for transfer of electrons from quinone pools, across membranes and to either soluble or insoluble electron acceptors. Interestingly, neither of the *Geothrix* genomes encodes

any of the other key components of the Mtr conduit based on BLAST searches, even at as low as 15% sequence identity. Suggesting a different mechanism or a different role for these decaheme *c*-type cytochromes in electron transfer.

2.5 Summary

Genome sequencing has augmented our understanding of microorganisms and the diverse roles they play in various ecological processes. The advent of next generation sequencing has made bacterial genome sequencing inexpensive and easily accessible. Also, multiple software platforms are available that assist in sequence data processing and genome assembly. Genome sequencing of an organism helps in identifying novel metabolic capabilities and also providing helpful information regarding its growth requirements. In many cases it is possible to link genomic information with novel physiological characteristics with testable hypothesis. For example, genome analysis identified putative genes associated with complex carbohydrate utilization. The presence of polysaccharide deacetylase and glycosyl transferase genes lead to testing the ability of *G. fermentans* to couple oxidation of chitin with the reduction of Fe(III)-oxides. Genomes of *Geothrix* species facilitated in identification of TCA cycle genes such as citrate synthase, and isocitrate dehydrogenase that were found to be similar to the *Geobacteraceae*. Implying that *Geothrix*-like Acidobacteria maybe closely related to the δ-Proteobacteria or horizontal gene transfer could have occurred, allowing *Geothrix*-like Acidobacteria to coexist in the subsurface environments. Also, the presence of a variety of different putative genes such as the cytochrome oxidases allowed speculation of the potential

environmental niche of *Geothrix*. Presence of abundant multiheme *c*-type cytochromes, suggest that electron transfer in *Geothrix* species could be mediated via these *c*-type cytochromes.

Chapter 3: *Geothrix fermentans* Secretes Two Different Redox-Active Compounds to Utilize Electron Acceptors across a Wide Range of Redox Potentials

***Geothrix fermentans* Secretes Two Different Redox-Active Compounds To Utilize Electron Acceptors Across a Wide Range of Redox Potentials**

Mehta-Kolte G. M., and D.R. Bond. Applied and Environmental Microbiology, October 2012, volume 78, p. 6987-6995.

doi: 10.1128/AEM.01460-12

Text and figures in Chapter 3 were previously published and are reproduced here with the permission of the authors and the publisher.

3.1 Overview

Research in the field of environmental microbiology over the last decade has highlighted the key roles of microorganisms in biodegradation of recalcitrant organic pollutants and in reduction of transition metals and radionuclides. A variety of approaches have been used to understand microbial metal-reduction coupled to biodegradation and have led to the discovery of the diversity of microorganisms involved in these processes. *Geothrix*-like Acidobacteria have been consistently detected from various subsurface environments, but yet very little is known about the mechanism of metal-reduction and their role in subsurface bioremediation. Exploiting the ability of these bacteria to use poised electrodes as electron acceptors has enabled us to use electrochemistry as a new tool to understand multidimensional aspects of microbial electron transfer. For example, this method allows growth of metal-reducing bacteria at specific redox potentials. These electrochemical analyses have provided evidence for a high potential requirement for *G. fermentans.* Additionally, electrochemistry allows for careful and controlled experimentation to determine the presence of a phylsiologically relevant extracellular electron shuttle.

3.2 Introduction

While most understanding regarding electron transfer to metals is derived from a small number of *Shewanella* and *Geobacter* species, a wide diversity of *Bacteria* and *Archaea* are known to use insoluble Fe(III)-oxides as terminal electron acceptors (Hernandez *et al* 2004; Lovley *et al* 2004; Shi *et al* 2007; Paquete & Louro 2010). In particular, sequences similar to an Fe(III) reducing isolate from the Acidobacterial phylum (sometimes referred to as 'Group 8 Acidobacteria') are consistently detected in 16S rRNA surveys of aquifers undergoing Fe(III) reduction, as well as in Fe(III)-rich sediments undergoing uranium bioreduction (Barns *et al* 2007; Wu *et al* 2007; Cardenas *et al* 2008), petroleum oxidation (Abed *et al* 2002), technetium reduction (McBeth *et al* 2007), and arsenic reduction (Islam *et al* 2004; 2005). In many of these environments, such Acidobacterial sequences are more abundant than *Geobacter* sequences, yet the basis for their competiveness with *Geobacter* is not known.

In general, members of the Acidobacteria are ubiquitous in both 16S and metagenomic surveys of soils, often representing over 50% of detected bacterial sequences (Sait *et al* 2002; Kalyuzhnaya *et al* 2008; Ward *et al* 2009; Kielak *et al* 2010; Rodríguez-Celma *et al* 2011; Garcia Costas *et al* 2011; Fierer *et al* 2011) which are classified into 26 Acidobacterial subdivisions (Coates *et al* 1999; Barns *et al* 2007; Bryant *et al* 2007; Eichorst *et al* 2007; Ward *et al* 2009; George *et al* 2011; Garcia Costas *et al* 2011). Cultivated Acidobacteria are slow growing, nutritionally versatile oligotrophs able to use a variety of carbon sources, that typically prefer microaerophilic conditions. The exception is the Fe(III)-reducing anaerobic isolate

G. fermentans, originally obtained from the Fe(III)-reducing zone of a petroleum-contaminated aquifer (Coates *et al* 1999).

To transfer electrons to extracellular compounds, bacteria must make contact with electron acceptors, and/or secrete redox active mediators. Both strategies have their advantages; flavin mononucleotide actively secreted by *Shewanella* accelerates metal reduction (Coursolle *et al* 2010; Covington *et al* 2010; Marsili, *et al* 2008a; Ross *et al* 2009), and phenazines secreted by *Pseudomonas* facilitate movement of electrons to aerobic edges of a biofilm (Dietrich *et al* 2008; Hernandez *et al* 2004; Wang *et al* 2010; Wang & Newman 2008). Direct mechanisms, such as those used by *Geobacter* (Lovley *et al* 2004; Nevin & Lovley 2000; Torres *et al* 2010), benefit only cells in contact with the acceptor, and may also electrically link daughter cells to the metal, often allowing electron transfer over longer distances than soluble shuttle (Torres *et al* 2010). Each strategy has potential tradeoffs; a contact-based strategy may requires a metabolic burden in terms of multiple structural and extracellular redox proteins in order to interface with unpredictable metal surfaces in the environment, while secreted molecules could be expensive to produce and be limited by diffusion.

G. fermentans will reduce Fe(III) trapped within porous beads (Nevin & Lovley 2002), and supernatants from *G. fermentans* cultures accelerate Fe(III) reduction by other bacteria (Bond & Lovley 2005), providing evidence that this bacterium may secrete a soluble compound as part of its metal reduction strategy. Both quinone-like compounds and metal chelators have been reported in *G. fermentans* supernatants (Nevin & Lovley 2002). However, the identity of any

soluble agents, their redox potentials, and their relative importance to electron transfer under conditions relevant to subsurface growth remain unknown (Nevin & Lovley 2002).

Within the last five years, a new set of electrochemical tools has emerged that allows monitoring of electron transfer from living cells. Compared to growth with insoluble metals, electrode potentials can be precisely controlled, allowing rates of electron transfer from cells to be measured without accumulation of reduced end-products confounding results (Marsili, *et al* 2008b; Marsili *et al* 2010). Such analyses show that *Geobacter* strains will transfer electrons beyond the cell membrane at potentials as low as -0.2 V vs. the standard hydrogen electrode (SHE), and reach their maximal rate of respiration at -0.1 V. These low values help explain the competitiveness of *Geobacter* in permanently anoxic environments. In *Shewanella* strains, direct electron transfer to electrodes can be measured, but the ability to easily remove the medium surrounding attached cells reveals that an indirect redox shuttling mechanism based on secreted flavin mononucleotide (FMN) is responsible for nearly ~80% of respiratory activity by biofilms under these conditions (Marsili, *et al* 2008a).

In this work, the electron transfer strategy of *G. fermentans* was studied for the first time using poised electrodes, and was found to be unique from any previously described organism. Specifically, *G. fermentans* electron transfer reached less than 30% of maximum rates between the commonly studied redox potential window of -0.2 and 0 V, and instead required potentials above +0.55 V to support maximal rates. Removal of supernatants from electrode-grown biofilms reduced the

rate of electron transfer, and cell-free supernatants from both Fe(III)-grown and electrode-grown *G. fermentans* cultures showed evidence of two independent soluble electron shuttles. Neither of these activities appeared to be due to metal chelator. One of the two redox shuttles, active in the low potential window of -0.2 V, was identified as riboflavin, while the higher potential compound remains unidentified. This dual redox potential strategy reveals that flavin-based electron shuttles are used by Fe(III)-reducing bacteria outside of the Proteobacteria, and suggests that anaerobic Acidobacteria may be more competitive in higher potential environments, such as near oxic-anoxic interfaces.

3.3 Materials and Methods

3.3.1 Bacterial strains, culture media, and conditions

Geothrix fermentans (ATCC 700665) was grown anaerobically at 20°C unless otherwise indicated in minimal media containing the following (per liter): 0.38g KCl, 0.2g NH_4Cl, 0.069g $NaH_2PO_4.H_2O$, 0.04g $CaCl_2.2H_2O$, and 0.2g $MgSO_4.7H_2O$. 10 ml of mineral mix containing 0.1g $MnCl_2.4H_2O$, 0.3g $FeSO_4.7H_2O$, 0.17g $CoCl_2.6H_2O$, 0.1g $ZnCl_2$, 0.04g $CuSO_4.5H_2O$, 0.005g $AlK(SO_4)2.\ 12H_2O$, 0.005g H_3BO_3, 0.09g Na_2MoO_4, 0.12g $NiCl_2$, 0.02g $NaWO_4.\ 2H_2O$, and 0.10g Na_2SeO_4 per 1 liter were added before autoclaving. 20 mM lactate was supplied as the electron donor and 40 mM fumarate as the soluble electron acceptor for routine growth, and 100 mM ferrihydrite (FeOOH) was added for metal reducing conditions. 20 mM lactate as an electron donor and a poised electrode as an electron acceptor was used for all electrochemical experiments. All media were

adjusted to pH 6.8, buffered with 2 g/liter $NaHCO_3$, made anaerobic by flushing with oxygen free N_2:CO_2 (80:20 vol/vol), and sealed with butyl rubber stoppers prior to autoclaving. 1 ml of a filter-sterilized anaerobic vitamin mixture was added to all cultures before inoculation. The vitamin mixture contained; 0.002 g biotin, 0.002 g folic acid, 0.01 g pyridoxine HCl, 0.005 g thiamine, 0.005 g nicotinic acid, 0.005 g pantothenic acid, 0.0001 g of vitamin B-12, 0.005 g p-aminobenzoic acid, and 0.005 g thioctic acid per 1 liter.

3.3.2 Electrode preparation and assembly

Carbon cloth (B-1/B WO W000179 E-TER, PEMEAS Fuel Cell Technologies, USA) was cut into 2 x 2 cm electrodes, (geometric surface area 8 cm^2). Carbon cloth electrodes were soaked sequentially in deionized water, 0.1 N NaOH, deionized water, 0.1 N HCl, and ethanol to remove contaminants. This treatment was repeated, and electrodes were stored in deionized water. Electrodes were attached to 0.1 mm platinum wire. A bioreactor containing a carbon cloth-working electrode, platinum counter electrode, and a saturated calomel reference electrode were prepared as described previously (Marsili, et al 2008b). Sterile anaerobic minimal medium (5 ml) lacking electron donors or acceptors was added to autoclaved bioreactors, and anaerobic conditions were maintained by a constant flow of humidified oxygen free N_2:CO_2 (80:20 vol/vol) at 20°C and connected to a potentiostat (VMP; Bio-Logic, Knoxville, TN, USA) (Marsili, *et al* 2008a; Marsili, *et al* 2008b; Baron *et al* 2009). After equilibration, 5 ml of a 0.5 OD^{600} culture entering stationary phase was added to the electrochemical cell (a 50% inoculum).

3.3.3 Electrochemical analysis

The parameters for cyclic voltammetry of attached biofilms were as follows; equilibrium time = 5 s, scan rate = 1 mV/s, E_i = -0.645 V vs. SHE; E_f = +0.745 V vs SHE, current was averaged over last 80% of each poised voltage as described previously by Marsili *et al* (Marsili, *et al* 2008a). Two sweeps were performed, with data from the second sweep reported. By changing the potential slowly (1 mV/s), data was collected across a range of applied redox potentials, reflecting steady-state electron transfer from cells to electrodes.

Analytical electrochemistry was also performed on the supernatants in duplicates between E_i = -0.645 V vs. SHE; E_f = +0.745 V vs. SHE at a scan rate of 100 mV/s. This faster scan rate was chosen to rapidly oxidize or reduce redox active compounds near the electrode and enhance the sensitivity of detection. Data collected from the second sweeps were analyzed and processed using SOAS (Fourmond *et al* 2009).

3.3.4 Biomass measurements

Biomass was determined using the bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL, USA). After electrochemical analysis, electrodes were harvested and gently washed in minimal media to remove loosely attached planktonic cells. The electrodes were disassembled and incubated in 1 ml of 0.2 M NaOH solution at 96°C for 20 minutes to remove attached biomass. Electrodes were also harvested and fixed in 3% glutaraldehyde, and dehydrated in increased concentration of ethanol (25%, 50%, 75%, 95% and 100%) for 10 minutes. The

electrodes were critical point dried, sputter coated, and imaged using a S3500N scanning electron microscope (Hitachi, Japan).

3.3.5 Collection of culture supernatants

Electrode-grown supernatants were harvested by carefully removing the surrounding medium into a sterile anaerobic tube with an 80% N_2, 20% CO_2 headspace, centrifuged anaerobically to remove planktonic cells, and stored at -20°C after flushing the head space with 80% N_2, 20% CO_2. Supernatants collected from Fe(III)-oxide grown cultures were centrifuged anaerobically at 1500 rpm to remove insoluble Fe(III)-oxide. The supernatant was anaerobically mixed with 0.5 g of Chelex® 100 resin (BIO-RAD, Hercules, CA, USA) per 10 ml of supernatant in a sterile anaerobic tube overnight at 4°C on a shaker. The supernatant was centrifuged to remove resin, and stored in a sterile anaerobic tube, or passed through an octadecyl-functionalized silica gel (C^{18}) column anaerobically in a glove box. The effluent was stored at -20°C in a sterile anaerobic tube. Hydrophobic compounds bound to the C^{18} column were collected by adding 100% methanol and stored at -20°C for further analysis.

3.3.6 High-performance liquid chromatography

Cell-free electrode and Fe(III)-oxide -grown supernatants were analyzed by HPLC using a protocol previously described (Marsili, *et al* 2008a). An Eclipse XDB-C18 column (4.6 mm x 525 mm and 5 μm particle size) from Agilent Technologies, Santa Clara, CA, USA was used. A fluorescence detector with an excitation

wavelength of 440 nm and an emission wavelength of 525 nm was used to detect fluorescent compounds.

3.4 Results

3.4.1 Conditions to minimize cell lysis

G. fermentans culture supernatants have previously been reported to contain soluble factors that accelerate reduction of Fe(III)-oxide (Nevin & Lovley 2002). While some bacteria actively excrete redox shuttles (Dietrich *et al* 2008; Marsili, *et al* 2008a; Wang & Newman 2008; Wang *et al* 2010), an alternative explanation is always the possibility of cell lysis releasing intracellular compounds. When *G. fermentans* cultures were grown at 30°C, over 50% of cells lysed within 2 days of reaching stationary phase. Reducing the growth temperature to 25°C slowed this process, and when cells were grown at 20°C, OD_{600} remained stable after over 200 hours of incubation in stationary phase (Figure 3.8). While cell turnover during prolonged incubations is a complex process, growth at 20°C was chosen for all further electrochemical studies to minimize the possibility of compounds being rapidly released that could create significant artifacts (Lloyd *et al* 1999).

3.4.2 Colonization of electrodes by *G. fermentans* requires higher potentials

Experiments were initially conducted using carbon fiber electrodes poised at +0.2 V vs. SHE, as these redox potentials are typically used to study metal-reducing *Geobacter* and *Shewanella* (Marsili, *et al* 2008a; Marsili, *et al* 2008b; Baron *et al* 2009; Marsili *et al* 2010). After 2 weeks of incubation, a small increase in electron

transfer was detected, but it rarely exceeded ~5 μA/cm^2 under these conditions. Voltammetry of these partially colonized electrodes revealed that the rate of electron transfer increased significantly when the electrode was raised to higher potentials. Based on these data, new growth experiments were initiated with electrodes poised at +0.55 V vs. SHE.

When the electrode was poised at a higher potential, a 50% inoculum of cells demonstrated a rapid initial rise in electron transfer rate, (Figure 3.1A) followed by a slow increase over the next 300-400 hours. Inoculated bioreactors required >2 weeks to reach stable rates of electron transfer to electrodes. For this report, 15 independent biofilms were cultivated at higher potential (+0.55 V), analyzed electrochemically, and used to produce supernatants for further analysis.

During colonization, data collection was paused at regular intervals for electrochemical analysis. For example, sweeping slowly between low and high potentials at 1 mV/s allowed measurement of electron transfer rates from bacteria to the electrode across a wide window of electron acceptor redox potentials. After the electrodes were returned to the standard potential for growth (+0.55 V), temporary surges of electron transfer from the biofilm were typically observed. In younger cultures, this surge decayed within than 5 h, but up to 30 h was needed to fully discharge older cultures. Representative data from early and late stages of growth, illustrating this 'capacitance' phenomenon where excess electrons were stored during pauses in current collection, are shown in Figure 3.1A.

Routine *G. fermentans* biofilm experiments allowed to grow over 640 h at 0.55 V achieved a density of 50-100 μA/cm^2, a value 10-fold lower than what is

observed for *Geobacter* strains, even when electrode roughness is considered. When attached biomass was measured, rates of electron transfer averaged 0.6 mA/mg protein (range = 0.3 to 1.1 mA/mg, higher values from older cultures), a range also an order of magnitude lower than *G. sulfurreducens,* but similar to rates achieved by *S. oneidensis* (3, 38). Scanning electron microscopy of fully colonized electrodes revealed clusters of microcolonies distributed between fibers, with exposed electrode still visible even after 1 month of growth (Figure. 3.1B).

Glassy carbon, polished graphite, gold, Indium Tin Oxide (ITO), and graphitic paper electrodes were also screened in triplicate reactors for attachment and colonization of *G. fermentans* using the new higher-potential conditions. None of these materials significantly improved colonization, or exceeded current densities of 5 $\mu A/cm^2$. None of these materials altered the potential required for growth, or the two different potential windows observed during voltammetry (such as those described in Figure 3.2). Standard high-temperature cleaning treatments also did not significantly alter colonization or growth rates on carbon fiber electrodes (Nevin & Lovley 2002).

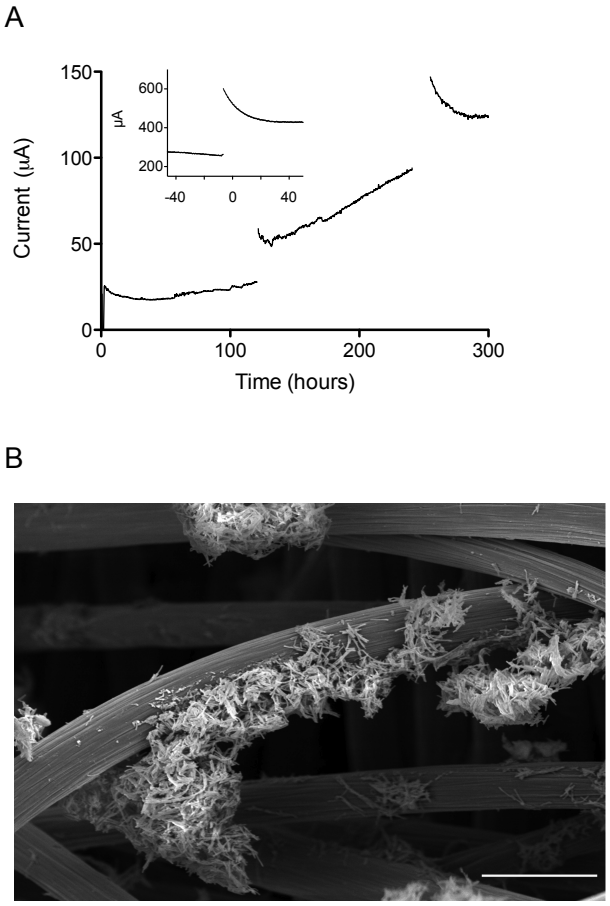

Figure 3.1 Typical increase in electron transfer rate by electrode-attached *G. fermentans* (A) Carbon fiber electrodes (8 cm^2 geometric surface area) were poised at +0.55V vs. SHE, and paused every 120 h for voltammetry analysis. Inset shows the increase in 'capacitance' behavior after pausing current collection of a 500 h biofilm. (B) SEM image of a *G. fermentans* biofilm on a carbon fiber electrode, one month after inoculation, showing sparse coverage and microcolony formation. The scale bar represents 25 μm.

3.4.3 Cyclic voltammetry reveals two different potentials that stimulate electron transfer

Slow scan rate cyclic voltammetry performed on biofilms of electrode-reducing bacteria such as *Geobacter* (Marsili, *et al* 2008b; Marsili *et al* 2010; Ross *et al* 2011), *Rhodopseudomonas palustris* DX-1(Xing *et al* 2008), and *Thermincola ferriacetica* (Wrighton *et al* 2011; Marshall & May 2009), always produces a sigmoidal response that increases steeply above voltages of -0.2 V vs. SHE, and reaches a maximum by 0 V vs. SHE. The response of *G. fermentans* differed from previously studied isolates and microbial fuel cell enrichments (Torres *et al* 2008; 2009; Chae *et al* 2009; Parameswaran *et al* 2010;). Most notable was how electron transfer from *G. fermentans* responded at two separate redox potentials. In addition, the majority of the increase in electron transfer rate occurred well above potentials of +0.2 V vs. SHE. A representative time series is shown in Figure 3.2A

At all time points, these two characteristic windows were observed; one centered at approximately -0.2 V, and the second centered at +0.3 V. First derivative analysis of data (Figure 3.2B) often revealed a third inflection point at +0.4 V, which increased in intensity in older biofilms. Together, this series of *G. fermentans* electrode-grown biofilms showed that while *G. fermentans* could initiate electron transfer at potentials as low as -0.2 V, cells could not reach their maximum rates of sustained electron transfer unless provided with an electron acceptor near potentials of +0.5 V vs. SHE, over half a volt higher than any previously characterized organism. This data also revealed an unusual pattern of multiple independent features within this wide potential window.

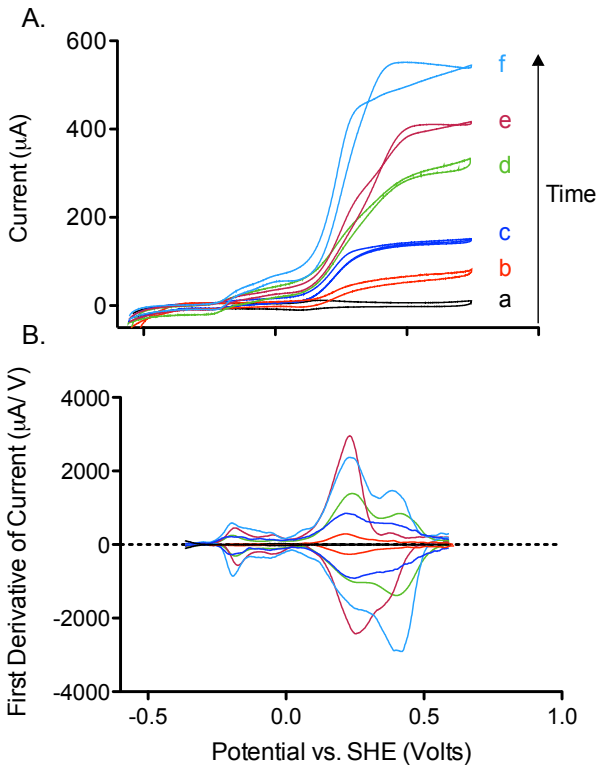

Figure 3.2 (A) Cyclic voltammetry (1 mV/s) of *G. fermentans* biofilms over twenty-eight days (b to f) of growth on carbon fiber electrodes. Voltamogram of the uninoculated carbon fiber electrode is shown in black (a). (B) First derivatives of cyclic voltammograms from (A), facilitating visualization of midpoint potentials in catalytic waves.

3.4.4 Soluble mediators are involved at both low and high potentials

Earlier work with indirect assays based on acceleration of Fe(III) reduction implied that *G. fermentans* releases at least one soluble compound (Bond & Lovley 2005; Nevin & Lovley 2002). To test for the presence of soluble electron shuttles, the medium surrounding electrode-attached biofilms was removed and replaced with sterile anaerobic medium containing only the electron donor. In all cases, an

immediate drop in current production was detected. Electron transfer could always be recovered by re-addition of the original medium, even after anaerobic centrifugation or filtration to remove planktonic cells.

The absolute magnitude of the current drop after fresh medium was added increased with the age of the culture, varying from as low as 50% to as high as 75%. Data from a 3 week old biofilm grown at +0.55 V is shown in Figure 3.3A. These experiments verified that soluble compounds that facilitate electron transfer between the cell surface and the electron acceptor, accounting for the majority of electron transfer. Surprisingly, these voltammetry experiments revealed changes in both redox potential windows in all experiments. This indicated that soluble compounds were involved in both low and high potential electron transfer mechanisms (Figure 3.3C and D).

3.4.5 Evidence for two independent redox-active compounds

As medium replacement always decreased electron transfer by *G. fermentans* in both potential windows, this raised two possibilities. Either a single soluble compound was produced, with two distinct redox potentials, or two independent compounds were accumulating. As the relative amount of electron transfer in each window appeared to vary independently with culture age, growth data supported the hypothesis of two independent compounds.

An electrochemical assay was used to detect electrochemically active compounds in filtered supernatants. Voltammetry of sterile supernatants obtained from biofilms of *G. fermentans* always revealed two distinct redox peaks, at the two

potential windows observed for electron transfer by whole cells (Figure 3.4A). These assays verified the presence of redox mediators in culture supernatants.

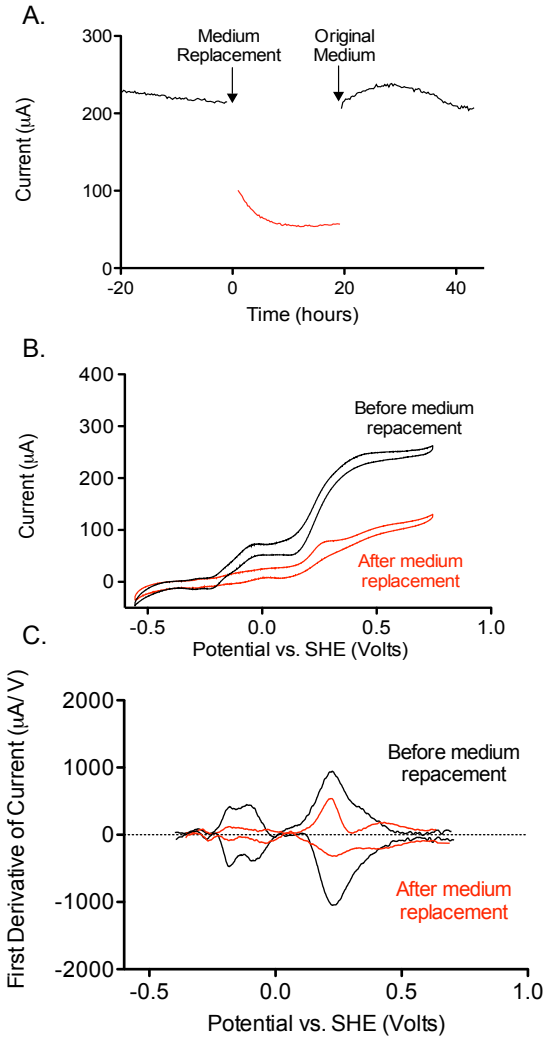

Figure 3.3 Evidence for two soluble mediators (A) Rate of electron transfer by an established *G. fermentans* biofilm (black trace), subsequent drop in electron transfer upon medium replacement (red trace), and recovery of electron transfer to the original level after re-addition of original medium following centrifugation (black trace). (B) Cyclic voltammograms performed before addition (black), and after addition of fresh medium (red) show changes in the catalytic waves in both low- and high-potential regions. (C) First derivative analysis of voltamograms from (B) used to identify potential windows affected by medium replacement.

Differential chromotographic separation was able to selectively remove one of these compounds, proving that two different mediators were present. Hydrophobic affinity columns, such as octadecyl-functionalized silica gel (C^{18}), removed the compound associated with the lower potential window (-0.2 V vs. SHE) without altering the higher potential peak (Figure 3.4B). When analyzed by HPLC, a single compound was detected in all supernatants that eluted at exactly the same time as authentic riboflavin standards, and this compound was not present in C^{18}-treated samples.

Unlike *Shewanella*, which first secretes flavin adenine dinucleotide (FAD), and enzymatically processes it to FMN, no FMN or FAD could be detected in cell-free supernatants of *G. fermentans* cultures. Elution of C^{18} columns with methanol detected only a compound that eluted in HPLC as riboflavin, and the fluorescence emission profile of this eluted compound also was identical to riboflavin. When pure riboflavin was added to back to C^{18}-treated supernatants, the low-potential redox peak returned in electrochemical assays (Figure 3.4C).

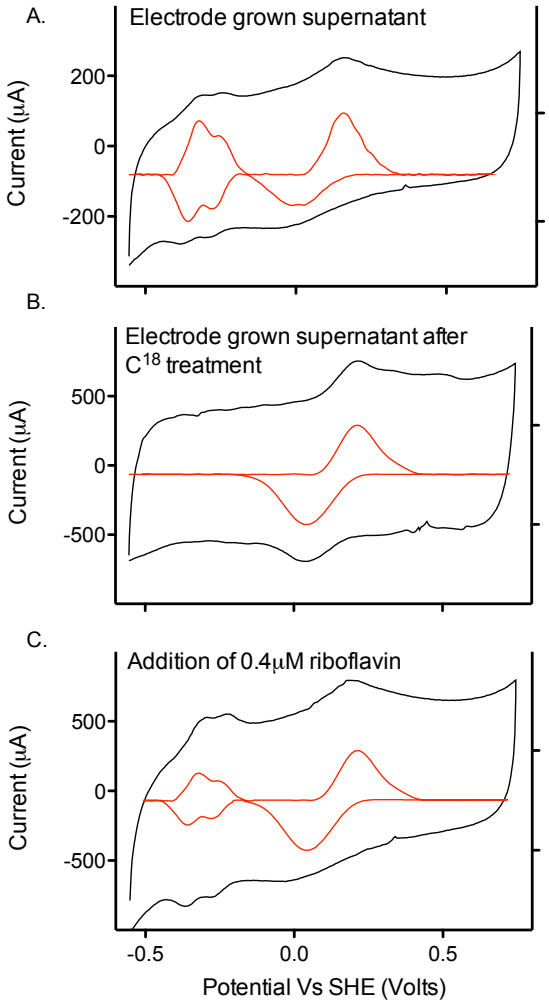

Figure 3.4 Analytical electrochemistry evidence for two independent mediators Supernatants collected from electrode-grown biofilms were centrifuged anaerobically to remove planktonic cells, and analyzed using graphite electrodes. (A) Supernatant containing both the low and high potential redox active compounds. (B) Same supernatant after elution from a C^{18} column, showing presence of only the higher potential redox active compound. (C) Supernatant from B, after addition of 0.4 μM riboflavin. Both compounds were detected in electrode-grown and FeOOH-grown cultures, but not in Fumarate-grown cultures. All scan rates were 100 mV/s. Insets (red traces) are baseline subtracted. All data were normalized to the highest peak.

Throughout these studies, the higher redox potential compound showed no affinity for binding to graphite electrodes, hydrophobic matrices (e.g. compare high potential peak in Figure 4A and 4B) or filters used to sterilize supernatants. Treatment with high concentrations of commercial proteases had no effect on the size or location of this peak, suggesting this mediator was not protein-based. Treatment with metal-binding resins also had no effect on the size or location of this residual redox activity, suggesting that this redox peak was not due to a chelator or metal.

When riboflavin was depleted from these samples by C^{18}-treatment, the supernatant containing the remaining high potential compound demonstrated a characteristic UV fluorescence with an excitation maximum of 229 nm. Emission by this fluorescent compound shifted to lower wavelengths after chemical reduction of samples. This redox activity and fluorescence was not detected in supernatants from fumarate-grown cultures. These observations were consistent with a hydrophilic fluorescent compound reported previously in *G. fermentans* supernatants (Nevin & Lovley 2002).

3.4.6 Both redox shuttles are also produced in Fe(III)-grown *G. fermentans* cultures

All electrode-grown and Fe(III)-oxide-grown supernatants were re-analyzed using HPLC for the presence of riboflavin. All electrode-grown cultures contained riboflavin, although the concentration varied widely, from ~200 nM (after 1-2 weeks of incubation) to ~ 600 nM, (after 3-4 weeks of incubation) (Figure. 5). Supernatants

from Fe(III)-oxide grown cultures also contained a range of riboflavin concentrations with levels between 75 nM and 300 nM detected. Since riboflavin is known to bind Fe(III)-oxide, and spontaneously degrade upon exposure to light, these values represent a lower boundary of riboflavin concentrations. In contrast, *G. fermentans* cultures grown using soluble electron acceptors always had less than 20 nM riboflavin, a level at the limit of detection, and similar to trace levels found in *G. sulfurreducens* supernatants from fumarate, Fe(III)-oxide, and electrode-grown cultures. Fe(III)-oxide grown supernatants collected from four recently isolated *Geothrix spp*. (all within 95% 16S rRNA similar to *G. fermentans*) kindly provided by Z. Shelobolina) also contained riboflavin in the range of 100-150 nM, providing preliminary evidence that secretion of riboflavin may be conserved in related strains.

Sterile supernatants from Fe(III)-oxide grown *G. fermentans* cultures were also analyzed to determine if the high redox potential compound was also secreted under these conditions. As previous reports detected significant levels of soluble Fe(III) in *G. fermentans* cultures reducing Fe(III)-oxide, it was possible that chelators maintaining trace minerals in a soluble state could create shuttling-like activity in our assays. To eliminate this possibility, all supernatants analyzed in this study were treated with metal binding resins to remove soluble metals. Electrochemistry of sterile supernatants from Fe(III)-oxide grown cultures revealed that Fe(III)-grown cultures always produced both riboflavin and the fluorescent high-potential compound. When these sterile, metal-depleted supernatants from Fe(III)-oxide grown cultures were added back to washed biofilms of *G. fermentans*, electron transfer to electrodes was stimulated in the same two potential windows as electrode-

grown supernatants (data not shown). This confirmed that redox active compounds produced during growth with electrodes were also produced during more environmentally relevant conditions, such as growth with Fe(III)-oxide.

3.4.7 Stimulation of one pathway is at the expense of the other

The presence of two redox-active compounds raised the question of whether the two mediators competed for electrons from the same supply, or if they were part of independent additive pathways. The experiments where riboflavin was depleted from supernatants and added back to growing biofilms required medium exchanges, elution of medium through columns, and extensive reactor manipulation to execute. At the very least, switching respiration on and off introduced the artifact of the biofilm 'capacitance' described in Figure 3.1A, and the requirement for discharging the biofilm before additional electrochemical measurements could be obtained. These manipulations limited the sensitivity of the assay.

To alter redox shuttle concentrations under more controlled conditions, riboflavin was added stepwise to biofilms, allowing the concentration of the high potential mediator and attached biomass to remain constant. Addition of riboflavin in this manner only increased the rate of electron transfer at the redox potential of riboflavin, without altering the total rate of electron transfer by the biofilm. Thus, when riboflavin was present, electrons could be transferred at a lower potential, but at the expense of flux available to flow through the higher potential pathway.

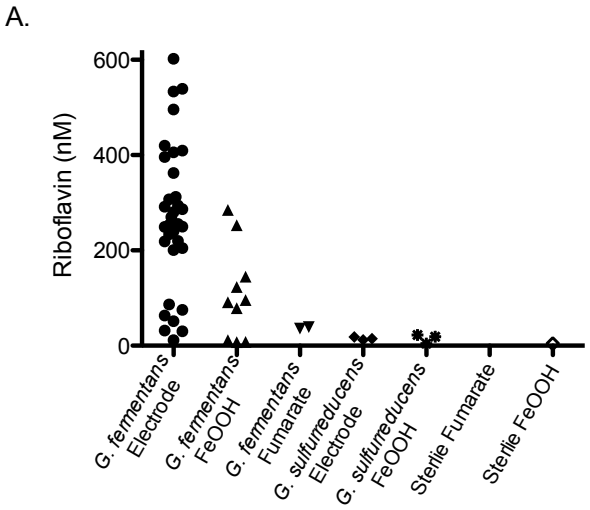

Figure 3.5 Concentrations of riboflavin in cultures as detected by HPLC. Electrode-grown *G. fermentans* cultures consistently accumulated riboflavin to between 200- 600 nM, depending on the age of the culture, and riboflavin was always detected in Fe(III)-oxide grown cultures. Fumarate grown *G. fermentans* cultures were always below 20 nM, similar to *Geobacter* supernatants collected from electrode and FeOOH grown cultures.

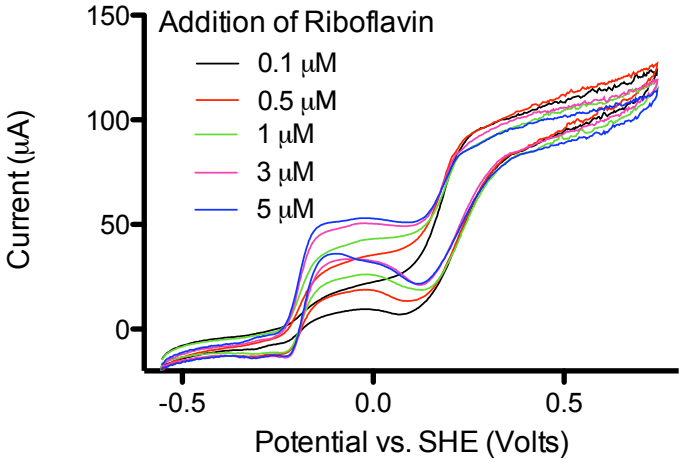

Figure 3.6 Dose response of an electrode-grown biofilm of *G. fermentans.* Addition of riboflavin, up to concentrations of 5 μM, affected electron transfer only at the potential of -0.2 V vs SHE, without stimulating overall current production.

3.5 Discussion

Acidobacterial sequences related to *Geothrix* are regularly detected in Fe(III)-reducing environments, particularly in zones recently stimulated to cause bioprecipitation of U(VI) (Wu *et al* 2007; Cardenas *et al* 2008). For example, *Geothrix*-like Acidobacteria were present in unamended Oak Ridge, TN Field Research Site sediments, and increased after lactate was injected to wells (Brodie *et al* 2006). Microcosm experiments from the same site also found *Geothrix*-like Acidobacteria comprising between 22-90% of 16S rRNA sequences after stimulation, depending on electron donor (Luo *et al* 2007). While such experiments also consistently detect *Geobacter*-like organisms, there is little information to address how these Fe(III)-reducing bacteria might coexist in the environment. These electrochemical data show that these organisms have fundamentally different approaches, in terms of respiration rates, need for direct contact, and the redox potentials at which they can operate.

3.5.1 A two-mediator, two redox-potential window strategy

To characterize the Fe(III)-reduction strategy of *G. fermentans*, electrochemistry was used to control the redox potential of its electron acceptor during growth. These experiments revealed a dependence on higher redox potentials not previously observed in other Fe(III)-reducing bacteria (Figure 3.2 and 3.3). Sterile filtered supernatants from both electrode-grown and Fe(III)-oxide grown cultures confirmed that two separate redox active compounds were secreted that stimulated electron transfer of *G. fermentans* (Figure 3.4). The low potential

mediator demonstrated a high affinity for hydrophobic resins, facilitating separation and identification of the mediator as riboflavin. Both of these compounds were also secreted during growth with environmentally relevant insoluble Fe(III) electron acceptors, but were not secreted during growth with fumarate. The other electron transfer mediator secreted by *Geothrix* had a much higher potential (~0.3 V vs. SHE) than natural phenazine or isoalloxazine ring-based flavin compounds, and was much more hydrophilic. Unlike flavins that bind tightly to graphite electrodes (Marsili, *et al* 2008a), the high-potential compound was easily washed from electrodes. Nevin and Lovley (Nevin & Lovley 2002) reported that supernatants from *G. fermentans* supernatants contained a water-soluble compound which fluoresced under UV light, that migrated in thin layer chromatography similar to model quinones. We also observed UV fluorescence in fractions from both electrode biofilms, and Fe(III) grown cultures. Even after riboflavin was removed, a hydrophilic high potential redox active compound remained. This behavior is consistent with substituted hydrophillic quinones, but as over 1,500 naturally produced quinones exist (Thomson 1991), the exact identity of this compound remains speculative. Purification of this compound will aid in study of its reactivity with Fe(III) or other possible terminal acceptors under environmental conditions.

Because the low- and high-potential compounds could be easily separated, it was also possible to alter the concentration of each mediator in supernatants, and correlate their presence with electron transfer at their characteristic redox potential. These experiments indicated that the effects of the two mediators were not additive. When additional riboflavin was added to biofilms, electron transfer rates at lower

potentials increased, but at the expense of electron transfer at higher potentials, resulting in the same total flux.

3.5.2 Comparisons with other redox shuttles

Some mediators appear to be secreted by bacteria to increase electron transfer to oxygen. Examples include phenazines ($E^{\circ\prime}$= -0.040 to -0.170 vs. SHE) secreted by *Pseudomonas spp.,* which improve survival of biofilm cells in anaerobic zones (Wang *et al* 2010). Strains of *Bifidobacterium* grow to higher yields in presence of 2-amino-3-carboxy-1,4-naphthoquinone ($E^{\circ\prime}$= -0.071 V), (a substituted hydrophilic quinone secreted by *Propionibacteria*), by shuttling electrons to oxygen and increasing NADH oxidation (Yamazaki *et al* 2002) .

In contrast, flavins have been shown to participate in reactions related to reduction of metals. FMN secreted by *Shewanella spp.* (FMN, $E^{\circ\prime}$= -0.21 V) enhance reduction of many external metals and azo dyes, and account for approximately 80% of electron transfer to extracellular acceptors (Marsili, *et al* 2008a; von Canstein *et al* 2008; Covington *et al* 2010). Gradients of oxidized flavins allow motile *Shewanella* strains to locate insoluble acceptors in space via energy taxis (Li *et al* 2012). Flavins secreted by plant roots are hypothesized to increase microbial rhizosphere availability of Fe(III) (Rodríguez-Celma *et al* 2011), and flavins in both yeast extract and intestinal contents promote electron transfer to oxygen (Khan *et al* 2012) and electrodes (Masuda *et al* 2010). The finding that members of this phylogentically distant Acidobacterial group secrete riboflavin to similar concentrations as does *Shewanella*, specifically under Fe(III)-reducing conditions, shows that use of this

versatile electron shuttle may be more widely distributed throughout the Bacterial domain than previously recognized.

3.5.3 What is the benefit of using higher potential electron acceptors?

A final question is the ecological implication of electron transfer in the higher potential domain. One benefit to higher potential electron acceptors in general is the opportunity to capture more ATP. Based on the lowest potential at which *Geobacter's* respiratory chain operates (-0.22 V), and the redox potential of acetate (-0.28 V), the poor ΔG of this redox couple (-46 kJ/mol acetate) explains why *Geobacter* has a growth yield consistent with only capturing < 1 ATP/acetate (Mahadevan *et al* 2006; Marsili *et al* 2010). However, this low yield also means that in low redox potential environments, such as permanently anoxic zones, *Geobacter* can still respire and compete even when environmental conditions lower the ΔG of acetate oxidation to values in this range (Figure 3.7A, B).

If *Geothrix* were to capture additional ATP via electron flow to higher potential electron acceptors (such as above an E°' = +0.2 V), a nearly 600% higher yield would be expected. In practical terms, *Geothrix* should reach OD_{600} densities of at least 0.3 (instead of the ~0.05 observed) when respiring Fe(III)-citrate. On electrodes, instead of producing ~100 μg of protein/cm^2 from oxidation of 30 mM acetate (standard for *Geobacter*), one would expect over 600 μg/cm^2 from the equivalent amount of lactate. Yet, in all of our experiments, and in all reported incubations with *Geothrix* and Fe(III) (Coates *et al* 1999; Nevin & Lovley 2002),

there is no evidence *Geothrix* achieves significantly higher ATP yields from respiration.

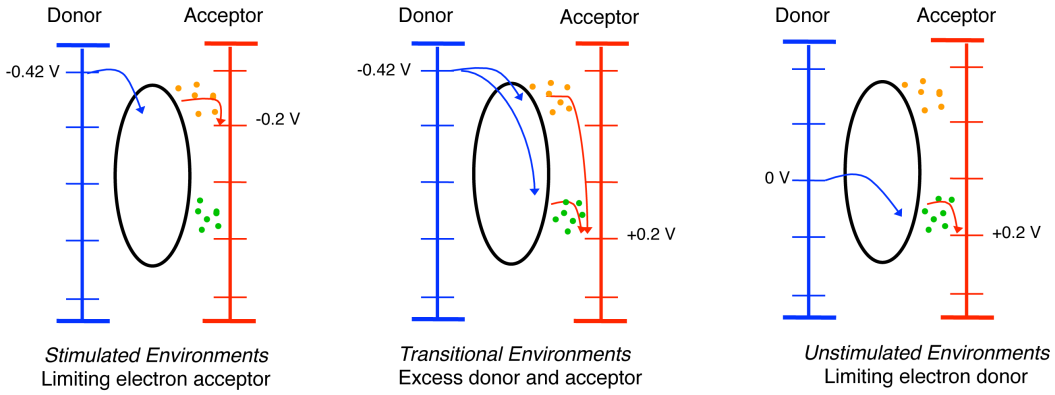

Figure 3.7 Examples of environmental niches that could exist as a function of varying electron donor and acceptor concentrations if an organism can take advantage of multiple redox potentials (A) Environments where donor is available, but acceptor concentrations (or metal redox state) limits the acceptor redox potential to -0.2 V. (B) Environments with excess electron donors and acceptors, such as during laboratory growth, shortly after stimulation of an oxidized habitat with electron donor, or with electrodes poised at high potentials. (C) Environments where donor concentrations are limiting, but the environment is oxidized, such as in low-carbon sediments or near oxic-anoxic interfaces.

In the absence of any evidence for increased ATP production, and based also on the fact that this slow-growing organism persists in unstimulated habitats where electron donor concentrations are low, another option is that a strategy to enable survival under conditions where a low ΔG exists. Oligotrophic sediments lacking a constant input of electron donor are likely higher in potential and interspersed with oxygenated zones. An organism able to shuttle electrons to freshly precipitated Fe(III) or Mn(IV) oxides, or low levels of oxygen, could grow at very low donor

concentrations, but only if that organism conserved a small amount of energy from the process. By keeping a only small amount of energy for itself, an organism like *Geothrix* could position its metabolic strategy to respire at very low electron donor concentrations than *Geobacter,* as shown in Figure 3.7C.

It was coupling of hydrogen partial pressure measurements with thermodynamic calculations that led Lovley and Goodwin (Lovley & Goodwin 1988) to explain zones where respiration was dominated by the presence of a single electron acceptor. In the typical version of this model, electron donors such as H_2 are released from fermentation of organic matter. Strong electron acceptors such as NO_3 provide a sufficiently negative ΔG to support growth at H_2 levels below 1 nM, a concentration unfavorable for reduction of Fe(III) and SO_4. This concept of 'threshold' donor concentrations was confirmed by measurements of pure cultures, typically under conditions of excess electron acceptor (Lovley 1985; Loffler *et al* 1999; Brown *et al* 2005).

The observation that *G. fermentans* can utilize higher-potential acceptors, and actively releases a compound that would only be beneficial at higher redox potentials, provides an example of an organism which may gain a competitive advantage, under different conditions than typical permanently anoxic Fe(III)-reducing zones. Whether this high potential window evolved for oxidized metals, shuttling to oxygen, or other acceptors remains to be discovered. Future enrichments targeting bacteria that have evolved to survive in such thermodynamic windows may need to control both redox potential and donor concentrations, to avoid organisms like *Geobacter* shifting conditions to where they can become dominant. In addition,

work characterizing communities at oxic-anoxic interfaces, or in subsurface environments where this niche could exist, should consider the possibility that not all metal-reducing bacteria function best at low environmental redox potentials.

3.6 Supplementary data

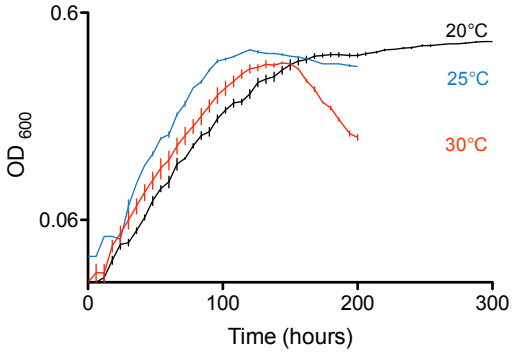

Figure 3.8 Temperature effect on *G. fermentans* growth. Slow growth was seen at all three temperatures with 25°C (blue) growing the fastest. Rapid growth and cell lysis was seen when *G. fermentans* was grown at 30°C (red). Sustained growth over time without any cell lysis was seen in cultures grown at 20°C (black) and hence used for all the subsequent experiments.

Chapter 4: Characterization of Two Outer Membrane Decaheme *c*-Type Cytochromes from the Metal-Reducing Acidobacterium *Geothrix fermentans*

4.1 Overview

G. fermentans is an environmentally ubiquitous metal-reducing microorganism. However, its mechanism of metal-reduction and key components involved in electron transport are unknown. The role of *c*-type cytochromes in metal-reduction has been extensively characterized in *Geobacter* and *Shewanella* species, both of which have abundant *c*-type cytochromes that are known to facilitate electron transfer. The *G. fermentans* genome encodes 63 putative *c*-type cytochromes some of which may be involved in reduction of insoluble metals. Two of these cytochromes were identified from detergent solubilization of membrane-associated proteins that were extracted from Fe(III)-citrate grown cells. Based on protein sequence and redox spectral analysis, these *c*-type cytochromes were identified as decaheme *c*-type cytochromes, belonging to the *Shewanella* MtrF/C family of decaheme *c*-type cytochromes. This chapter describes the investigation of the role of these cytochromes in electron transfer, by heterologous expression in *Shewanella oneidensis* mutant strains lacking the outer membrane *c*-type cytochromes.

4.2 Introduction

Fe(III)-respiring microorganisms couple the oxidation of organic carbon or H_2 to the reduction of Fe(III). Fe(III)-respiring organisms, use Fe(III)-oxide as the sole terminal electron acceptor in an energy conserving bacterial respiratory electron transport chain. One anomaly to this coupling of reduction of Fe(III)-oxide for energy conservation, is the example of *S.oneidensis*. In *S.oneidensis,* ATP generation under anaerobic conditions is primarily from substrate-level phosphorylation (Hunt

et al 2010). This Fe(III) respiration strategy is utilized by many phylogenetically, physiologically and morphologically diverse bacteria as well as archaea. (Lovley 1991; Nealson 1992; Tor *et al* 2001; Weber *et al* 2006).

Fe(III)-reducing microorganisms play a significant role in the environment. For example, they are involved in the degradation of contaminated organic compounds and act as natural agents for bioprecipitation of toxic heavy metals. These Fe(III)-reducing bacteria can generate an electrical current when a poised electrode is used as a sole electron acceptor thereby expanding the potential biotechnological applications. For instance, members of *Geobacteraceae* are able to power small electric devices (Bond *et al* 2002; Tender *et al* 2008) and also are known to generate electricity during wastewater treatment (Cervantes *et al* 2003).

Unlike soluble electron acceptors such as oxygen, nitrate, fumarate or sulfate that can diffuse freely through the cell membrane, Fe(III)-oxides in most natural environments are insoluble and thus have to be reduced outside of the cell. Therefore, in order to transfer electrons to external surfaces, metal-reducing bacteria possess mechanisms to overcome the challenges of transferring electrons generated by central metabolism to the exterior of the cell. Once the electrons reach the surface via membrane-associated redox proteins, these redox proteins can interact with extracellular matrix-anchored redox proteins and/or redox shuttles, ultimately reducing the mineral oxides. The inner membrane components of this electron transport chain generate a proton motive force, but the periplasmic and outer membrane processes are thought only to be involved in electron transfer to the

terminal acceptor (Leang *et al* 2003; Lloyd *et al* 2003; Mehta *et al* 2005; Butler *et al* 2010).

Biochemical analysis showed that the Fe(III) reduction activity in Fe(III)-reducing bacteria was predominantly localized in the membrane fraction (Gorby & Lovley 1991; Myers & Myers 1993; Magnuson *et al* 2001; Lloyd *et al* 2003;). In *S. oneidensis*, about 54% to 56% of this activity was localized in the outer membrane under anaerobic conditions (Myers & Myers 1993). The same was subsequently confirmed in studies with *G. sulfurreducens*; where 75% to 79% of the Fe(III) reduction was also localized in the outer membrane fraction (Gaspard *et al* 1998).

Several studies suggested that the Fe(III) reduction is cytochrome-associated in *S. oneidensis* (Myers & Myers 1992; Myers & Myers 1997; Shi *et al* 2007; Jensen et al 2010; Coursolle & Gralnick 2010; 2012; Gralnick 2012). In *G. sulfurreducens*, a genome study revealed that there are 111 genes encoding putative *c*-type cytochromes, many of which are predicted to be outer membrane-associated (Methe 2003) and also have been found to be outer membrane-associated proteins by various proteomic approaches (Ding *et al* 2006; Aklujkar *et al* 2013). The apparent abundance of *c*-type cytochromes in the outer membrane highlights the importance of electron transfer to *G. sulfurreducens*. Also, this redundancy and flexibility in the electron transfer network could be for the reduction of diverse electron acceptors in the environment (Methe 2003; Butler *et al* 2010).

The genome of the Gram-positive thermophilic dissimilatory metal-reducing bacterium, *Thermincola potens,* encodes 32 multiheme *c*-type cytochromes, with one predicted to have as many as 58 heme motif (Byrne-Bailey *et al* 2010). Biochemical

and biophysical experiments on *T. potens* provided evidence for cell wall or surface-associated cytochromes that are predictated to be involved in electron transfer (Carlson *et al* 2012). The presence of genes encoding membrane-associated multiheme *c*-type cytochromes in the genomes of diverse Fe(III)-reducing bacteria suggest that these cytochromes are used as a common strategy for extracellular electron transfer.

Fe(III)-reducers respire Fe(III)-oxides by different mechanisms; for example *Geobacter* species express *c*-type cytochrome that are involved in electron transfer to the cell surface and then subsequently to the Fe(III)-oxide via direct contact with terminal acceptor. Whereas, *Shewanella* species transfer electrons to the cell surface via a transmembrane electron conduit known as the Mtr respiratory pathway. The core Mtr electron transfer conduit contains a periplasmic decaheme *c*-type cytochrome (MtrA), an outer membrane integral β–barrel porin (MtrB), and an outer membrane-anchored decaheme *c*-type cytochrome (MtrC). Together this complex facilitates electron transfer from a cytoplasmic membrane-anchored quinone oxidase, a tetraheme *c*-type cytochrome, CymA. Once on the outside, the majority of electrons (about 80%) are then indirectly transferred to the insoluble oxide by redox active flavins that act as electron shuttles to achieve the final cell-acceptor exchange, obviating the need for direct cellular contact with the insoluble oxides (Coursolle *et al* 2010; Richardson *et al* 2013; Kotloski & Gralnick 2013).

The core Mtr components have multiple paralogs in *Shewanella* species, that form discrete functional modules for Fe(III) or dimethyl sulfoxide (DMSO) reduction (Coursolle & Gralnick 2010). There are three paralogs of MtrA, the

periplasmic electron carriers (PEC)-MtrD, DmsE, and SO4360 (Coursolle & Gralnick 2010). The β–barrel protein MtrB also has three paralogs-MtrE, DmsF and SO4359. The outer membrane-anchored decaheme *c*-type cytochrome (OMC) MtrC of the Mtr pathway has two paralogs-MtrF and OmcA. Extensive work done by Coursolle and Gralnick have shown different paralog combinations can form functional Fe(III)-reducing modules in *S. oneidensis* (Coursolle & Gralnick 2010; 2012).

G. fermentans is a phylogenetically diverse Fe(III)-reducing bacterium belonging to the ubiquitous Acidobacterial phylum. However, none of the redox proteins involved in the transfer of electrons to the cell surface or to soluble shuttles have been identified. The mechanism of electron transfer in *G. fermentans* is of interest because *Geothrix*-like Acidobacteria are ecologically important as they have been consistently detected in microbial communities that are involved in a variety of subsurface environments, as well as in anoxic environments where Fe(III)-reduction is the dominant terminal electron acceptor (Coates *et al* 1999; Klueglein *et al* 2013). Initial oxidation versus reduction spectra of *G. fermentans* showed characteristic *c*-type cytochrome absorbance maxima (Coates *et al* 1999). A search of *G. fermentans* genome revealed 63 *c*-type cytochromes. It is possible that some of these annotated *c*-type cytochromes are involved in electron transfer processes similar to *Geobacter* and *Shewanella* species.

The goal of this study was to identify the proteins involved in Fe(III)-reduction in *G. fermentans*. Separation of membrane-associated proteins of *G. fermentans* grown under fumarate and Fe(III)-reducing conditions was developed.

Membrane-associated proteins were solubilized using a variety of different detergents. The detergent solubilized membrane-associated protein fraction contained *c*-type cytochromes. This *c*-type cytochrome band was identified by mass spectrometric anaylsis as a mixture of two decaheme *c*-type cytochromes GxcA and GxcB, both of which have low sequence identity but similar predicted structural conformation to the MtrF/C family of outer membrane decaheme *c*-type cytochromes of *Shewanella* species. GxcA was further characterized and identified by heterologous expression in a mutant strain of *S. oneidensis* that was missing all of the outer membrane decaheme *c*-type cytochromes required for metal-reduction.

4.3 Materials and Methods

4.3.1 Bacterial strains and culture conditions

Geothrix fermentans (ATCC 700665) was grown anaerobically at 20°C in minimal medium as described previously (Mehta-Kolte & Bond 2012). Anaerobic minimal medium contained 20 mM acetate as the electron donor and 40 mM fumarate or 55 mM Fe(III)-citrate as the electron acceptor, and adjusted to pH 6.8, buffered with 2 g/liter $NaHCO_3$, made anaerobic by flushing with oxygen free N_2:CO_2 (80:20 vol/vol), and sealed with butyl rubber stoppers prior to autoclaving. One ml of a filter-sterilized anaerobic vitamin mixture was added to all cultures before inoculation. *Escherichia coli* strain WM3064 and *S. oneidensis* MR-1 wild type JG274, JG 1511, JG 1486, and JG 564 were generously provided by Dr. Jeffrey Gralnick and are listed in Table 4.1. Overnight cultures were made from single colonies isolated from a frozen stock inoculated into 10 mL of Luria-Bertani (LB)

medium and grown aerobically for 12 hours at 30°C for *S. oneidensis* strains and 37°C for *E. coli* strain. Kanamycin was used at a final concentration of 50 μg/mL, ampicillin was used at a final concentration of 100 μg/mL when necessary and diaminopimelic acid (DAP) was used at a concentration of 360 μM for all growth of *E. coli* WM3064. *Shewanella* basal minimal medium (SBM) was used for growth under anaerobic conditions as previously described by Baron *et al* 2009 (Baron *et al* 2009).

4.3.2 Isolation and enrichment of membrane-associated proteins

Cells for membrane protein extraction were grown in 1 L bottles with minimal anaerobic medium using acetate as an electron donor and fumarate or Fe(III)-citrate as the electron acceptor. Membranes were isolated following a modified protocol outlined in Afkar *et al* (Afkar *et al* 2005) to optimize for *G. fermentans*. Briefly, cells were harvested at late exponential phase (OD_{600} of 0.45) by centrifugation at 6000 x g for 10 min at 8°C. Cells were washed and re-suspended in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA with 10 μM phenol methyl-sulfonyl fluoride (PMSF) to prevent proteolytic degradation. A few crystals of DNase and RNase were added to minimize DNA and RNA contamination. Cells were then centrifuged at 12,000 x g for 10 min at 4 °C and re-suspended in 10 mM Tris-HCl (pH 7.5) with 10 μM PMSF and few crystals of DNase and RNase and subjected to three passes through a French press cell at 15,000 psi to lyse the cells. Cell-free extracts obtained after Frech pressing were centrifuged at 12,000 x g for 20 min at 4°C to separate out intact cells and cell debris. This cell-free extract was

centrifuged for 1 h at 100,000 x g at 4°C to separate the soluble fraction (periplasmic and cytoplasmic) from the total membrane-associated protein fraction. The total membrane-associated protein fraction was then subjected to further separation to solubilize membrane-associated proteins using different detergents. Eight different detergents were tested for their ability to differentially solubilize membrane-associated proteins. The following detergents were tested- Glycosidic (β pyranosidase and β maltosidase), Zwitterionic (CHAPS), Cationic (ABS, anionic-SDS, and lauryol sarcosine), and Nonionic (Briji 58 and Triton X-100). The membrane protein fractions were incubated for 30 min at 20°C with 1% solution of different detergents in Tris-HCl (pH 7.5) followed by centrifugation at 35,000 x g for 1 h at 4°C for selective enrichment of membrane proteins from *G. fermentans*.

4.3.3 SDS polyacrylamide gel electrophoresis and *c*-type cytochrome staining

Protein concentrations from *G. fermentans* total, and detergent solubilized membrane fractions were determined using the Pierce bicinchoninic Protein Assay Kit (Thermo Scientific, Rockford, IL). 20 μg of proteins were loaded onto a 12% Tris-HCl sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels. The Colloidal Blue Staining Kit (Invitrogen Corp., Carlsbad, CA) was used to visualize total protein. To detect *c*-type cytochromes via peroxidase activity, gels were incubated in 6.3 mM 3,3',5,5'– tetramethylbenzidine (TMBZ) in methanol (3 parts TMBZ mixed with 7 parts 0.25 M sodium acetate, pH 5.0) for 2 hours as previously described, after which 30 mM hydrogen peroxide was added (Thomas *et al* 1976; Francis & Becker 1984).

Various *S. oneidensis* mutant strains were grown overnight in SBM medium with 40 mM fumarate and 20 mM lactate as the electron acceptor and electron donor respectively. Cells were centrifuged at 4000 x g for 8 minutes and the resuspended in 500 µL of SBM medium lacking electron donor and acceptor and sonicated as described in Coursolle & Gralnick 2012. These cell lysate were then separated on 12% Tris-HCl gel by SDS-PAGE and total protein and *c*-type cytochromes were visualized as described above.

4.3.4 Phylogenetic analysis of *G. fermentans* membrane-associated proteins

Sequences with > 30 % identity with GxcA/GxcB from *G. fermentans* and > 50% identity with MtrC/MtrF/OmcA from *S. oneidensis* MR-1 and UndA from *Shewanella* sp. HCR-6 were collected using BLAST. Sequences were processed using "A La Carte Mode in Phylogeny.fr" (www.phylogeny.fr). Briefly, sequences were aligned using ClustalW. Gblocks was used for alignment curation and a neighbor-joining bootstrap phylogenetic tree was calculated using Jones-Taylor-Thornton substitution matrix. The tree generated was viewed in TreeDyn. The tree in newick format was then uploaded to give a non-rooted circular tree alignment in Itol (http://itol.embl.de).

4.3.5 Expression of *G. fermentans* cytochrome *gxcA* in *S. oneidensis*

G. fermentans genomic DNA was extracted from cultures at 0.45 OD_{600} using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Purified DNA was resuspended in nuclease free water. *gxcA* was cloned into a pBBAD22K

arabinose inducible vector using the primers described in Table 4.1. 500 μM of arabinose was used for overnight induction in LB medium for expression of *gxcA*. Two different mutant strains JG 654 and JG 1486 were used to test the ability of GxcA to facilitate electron transfer in *S. oneidensis.*

4.3.6 Fe(III) reduction assay

S. oneidensis strains expressing either the native Mtr components or *G. fermentans* outer membrane protein GxcA were tested for their ability to reduce Fe(III)-citrate. Isolated colonies of all strains used were inoculated in LB for 12 h and normalized to 1 OD_{600} in SBM, after washing twice in 10 mM HEPES buffer pH 7. 1 mL of these cultures were then added to 9 mL of SBM containing 20 mM lactate as electron donor with 10 mM Fe(III)-citrate. Fe(III)-reduction activity was measured by the accumulation of Fe(II) formation using a modified colorimetric ferrozine assay (Phillips & Lovley 1987). Ferrozine reagent (2 g ferrozine/L in 100 mM HEPES, pH 7) was combined with sample (dissolved in 1 N HCl) and absorbance at 625 nm was determined over time.

Table 4.1. Strains, plasmids, and primers used in this study

Strains	Relevant characteristics and genotype		Reference
G. fermentans	ATCC 700665		Coates *et al* 1999
E. coli			
WM3064	Donor strain for conjugation: *thr*B1004		Saltikov and Newman 2003
S. oneidensis			
JG564	*ΔmtrC/ΔomcA/ΔmtrF*		Coursolle Gralnick 2010
JG1486	*ΔmtrB/ ΔmtrE/ ΔmtrC/ ΔomcA/ΔmtrF/ ΔmtrA/ ΔmtrD, ΔdmsE, ΔS04360/ΔcctA/ΔrecA*		Coursolle and Gralnick 2012
JG1536	JG1486 with pBBR-BB, Km^r		Coursolle and Gralnick 2012
JG1511	JG1486 with *pmtrB/mtrC/mtrA*, Km^r		Coursolle and Gralnick 2012
DBMM001	JG1486 with *pBADgxcA*, Km^r		This work
DBMM002	JG564 with *pBADgxcA*, Km^r		This work
Plasmids			
pBBAD22K	Arabinose inducible plasmid, Km^r		
pMGM1	pBBAD-*gxcA* Km^r		This work
Primers[a]		Restriction Sites	
MGM01	ATTGAATTCATGCAACAACGACCTTTGAAACATC	EcoRI	Upstream *gxcA* pBBAD
MGM02	ATTGAGCTCCTAGTGCACCTTGGCGGGAT	XbaI	Downstream gxcA pBBAD

4.4 Results and Discussions

4.4.1 Expression of *c*-type cytochromes and other proteins

Protein and heme stains of soluble and total membrane fractions from fumarate and Fe(III)-citrate grown cells with acetate as the electron donor, separated by SDS-PAGE are shown in Figure 4.1A and 4.1B respectively. Greater intensity of the peroxidase activity was observed in the total membrane fraction compared to the soluble fraction. The most obvious peroxidase positive band is a high-molecular weight putative cytochrome(s) of approximately 85-90 kDa and is more prominent in the membrane-associated protein fraction, compared to the soluble protein fraction. However, there was no significant differences observed in protein profiles separated on the SDS-PAGE of fumarate versus Fe(III)-citrate grown *G. fermentans* cells.

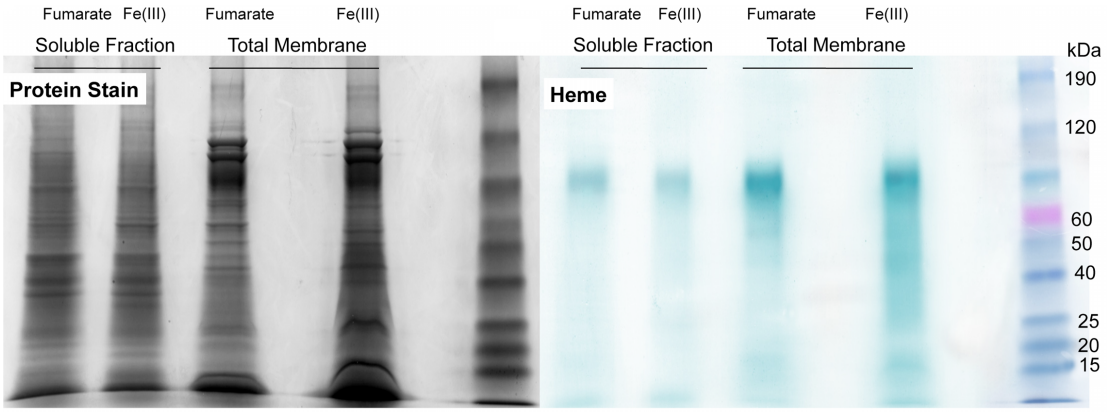

Figure 4.1 (A) Coomassie stain and (B) Heme stain of total soluble and membrane proteins of *G. fermentans* cells grown anaerobically on fumarate or Fe(III)-citrate with acetate as the electron donor.

4.4.2 Membrane-associated protein separation by differential detergent extraction

To identify the heme containing proteins that are associated with the membranes, different approaches such as sucrose gradients (ranges tried 20% to 70% with either 10% or 20% intervals) and detergent solublization of membrane-associated proteins were used for membrane protein solubilization. Different concentrations of sucrose gradients failed to separate the membrane-associated proteins (data not shown). Detergents representing each class were used for separation of membrane-associated proteins. Figure 4.2 shows heme and coomassie stained gels after differential detergent solublization. The treatment with nonionic detergent, Triton X-100 resulted in differential separation of membrane-associated proteins into Triton X-100 solubilized and insolubilized fractions. This observation was based on the separation of the proteins, by comparing the total membrane-associated proteins to the Triton X-100 solubilized proteins as shown in the total protein gel (Figure 4.2B). Also, most of the peroxidase activity is associated at about 85-90 kDa in the Triton X-100 solubilized fraction. No similar separation of membrane proteins was observed when other types of detergents were used.

UV/Visible absorption of oxidized and dithionite-reduced Triton X-100 soluble fraction is shown in Figure 4.2E. The oxidized fraction showed the Soret (γ) peak at 410 nm. The reduced spectrum showed characteristic *c*-type cytochromes specific shifts. A sharper and more intense Soret maximum at 420 nm, along with pronounced α and β peaks at 552 nm and 525 nm respectively were observed in the reduced fraction. This observation implies that some of the peroxidase activity

observed in the Triton X-100 soluble protein fraction, as seen on the heme stained gels, is due to the presence of *c*-type cytochromes.

4.4.3 Membrane protein identification by Liquid Chromatograhy-Mass Spectrometry/Mass Spectrometry

The Triton X-100 solubilized membrane proteins were separated on a SDS-PAGE gel and identified by LC-MS/MS trypsin digested peptide sequencing. A homolog of the *Myxococcus xanthus* outer membrane-associated protein Oar was identified in the Triton X-100 insoluble fraction. This Oar protein has been shown to be required for development in *M. xanthus* (Martinez-Canamero et al 1993; Kahnt et al 2010). Other proteins identified in this fraction were homologous to porins and efflux proteins.

Band number 4 in the Triton X-100 solubilized membrane-associated protein fraction in Figure 4.3 is of interest as majority of the peroxidase activity is associated with this band. The peroxidase positive band contains peptides that mapped to two proteins present next to each other in the genome. 19 unique peptides mapped to one protein and 18 unique peptides mapped to the other protein. These proteins were identified as two decaheme *c*-type cytochromes, which were named GxcA and GxcB. *gxcA* and *gxcB* are a part of a four-decaheme *c*-type cytochrome gene cluster. The closest amino acid sequence similarity in the protein database to both GxcA and GxcB was to the MtrF decaheme *c*-type cytochrome of another Proteobacterial metal-reducer *Rhodoferax ferrieducens* (Finneran et al 2003). This MtrF decaheme *c*-type cytochrome is similar to the MtrC / MtrF family of decaheme *c*-type

cytochromes of the *Shewanella* genus. As mentioned before *Shewanella* species use the Mtr multiheme module to transport electrons across the periplasm, the outer membrane and on to the surface of the cell.

4.4.4 Characteristics and phylogeny of GxcA and GxcB

Topology prediction programs such as SIGNALP, HMMTOP and PSORT indicate that GxcA and GxcB are likely to be extracellular/outer membrane-associated decaheme *c*-type cytochromes present in *G. fermentans*. Both GxcA and GxcB have a predicated lipoprotein signal peptide, based on LipoP. Based on the amino acid sequence similarity to MtrF and also due to the predicted localization, GxcA and GxcB were hypothesized to be involved in electron transfer by *G. fermentans*.

A comparison between the amino acid sequences of MtrC, MtrF, OmcA, and UndA of *Shewanella* species with Gxc-like decaheme *c*-type cytochromes (all four decaheme cytochromes of *G. fermentans* and its closest relatives) reveal that in addition to the four currently characterized cell-surface decaheme *c*-type cytochromes (Edwards, *et al* 2012a), the Gxc proteins with their closest neighbours grouped into a separate cluster (Figure 4.4). Sequences similar to Gxc-type decaheme *c*-type cytochromes cluster together forming a separate independent branch. This Gxc branch includes homologs of decaheme *c*-type cytochromes found in known metal-reducers such as *Geobacter* and *Rhodoferax* species.

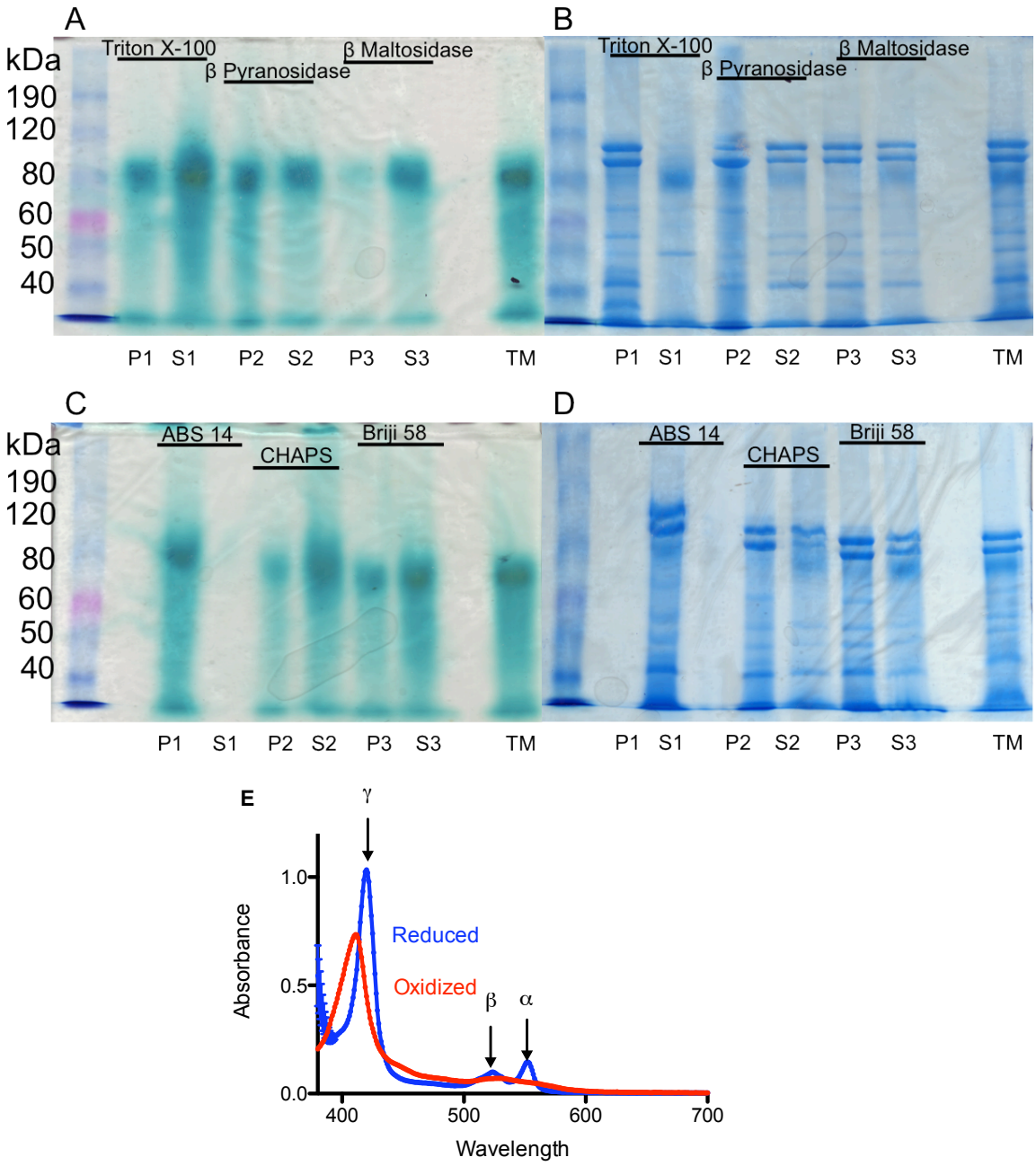

Figure 4.2 Panels **A** and **C**. Heme stain of soluble (S), insoluble (P) and total membranes (TM) fractions extracted from *G. fermentans* cells grow on Fe(III)-citrate and acetate and separated by SDS-PAGE. Different detergents used are labeled above the lanes. Triton X-100 detergent solublization led to the highest intensity of peroxidase activity. Panels **B** and **D**. Coomassie stain of the detergent solubilized fractions. (**E**) Absorption spectrum of oxidized and reduces Triton X-100 soluble fraction, showing the characteristic cytochrome *c* peaks (arrow).

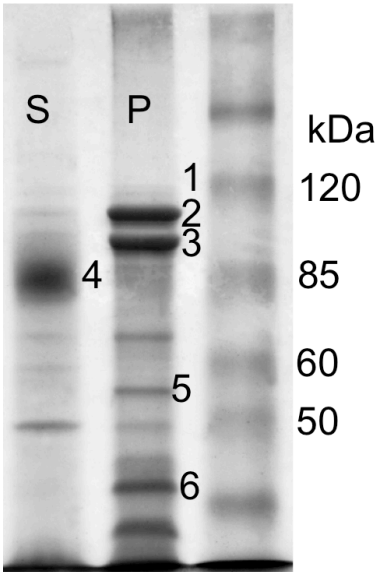

Figure 4.3 Separation of soluble proteins from Triton X-100 solubilized total membranes extracted from Fe(III)-citrate grown cells. The numbered proteins were identified by LC-MS/MS. Protein identified are listed in the table. (S) Soluble fraction and (P) insoluble fraction.

Table 4.2 Protein identification by LC-MS/MS

Band	Locus Tag G398DRAFT [a]	Putative Identification	Closest Relative in Database	Percent Identity
1	00016	Tol B protein precursor	*Solibacter usitatus* Ellin6076	56
2	00001	Oar protein	*Alteromonas macleodii*	43
3	00023	Oar protein	*Stigmatella aurantiaca*	39
4	01334/ 01335	Decaheme cytochrome MtrF	*Rhodoferax ferrireducens* T118	57
5	00006.6	Outer membrane efflux protein	*Geobacter* sp. FRC-32	49
6	00004	Porin signal peptide protein/ Porin channel	*Ralstonia pickettii*	61

[a] G398DRAFT locus designation assigned by JGI

Minimum 95% protein identification probability, 9 peptides identified

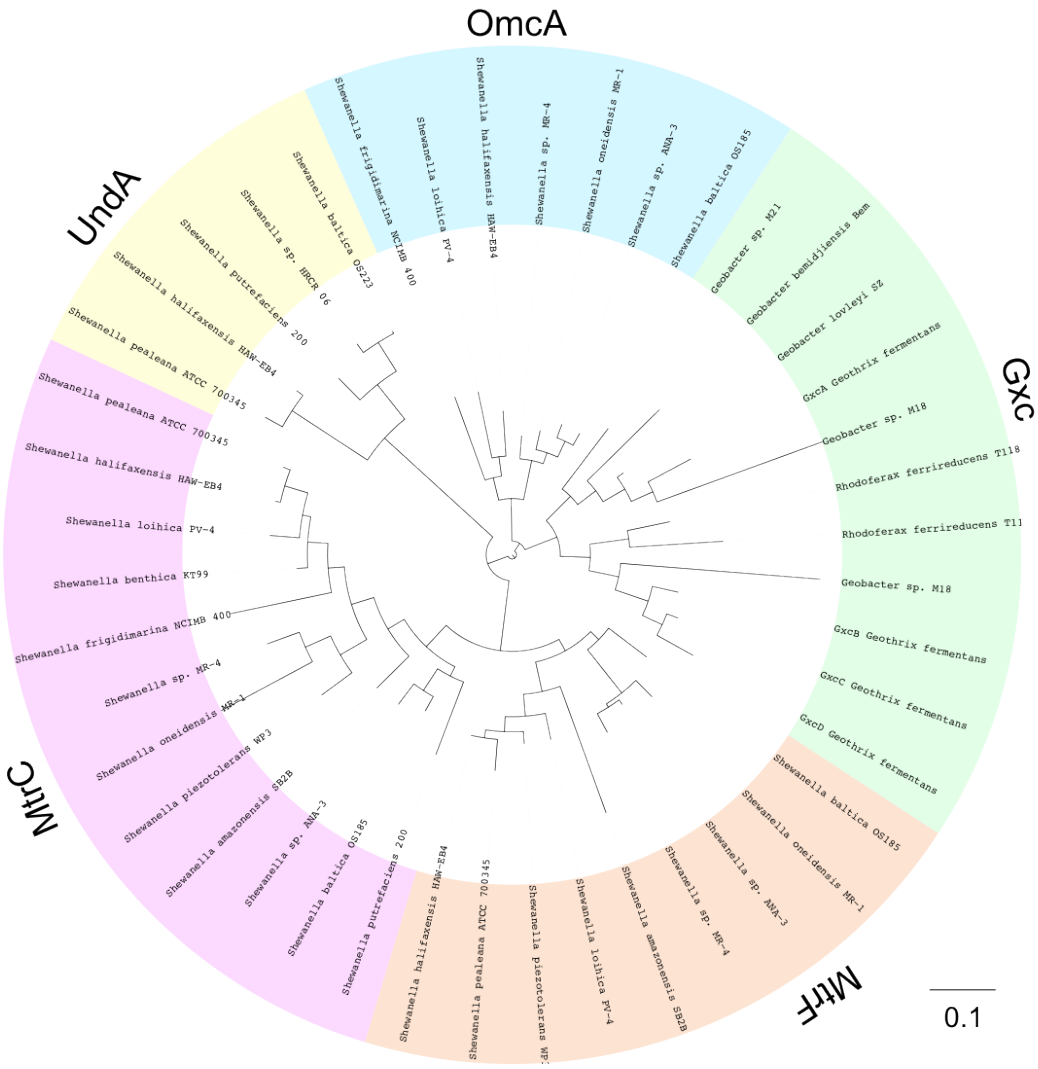

Figure 4.4 Phylogenetic alignment of MtrC, MtrF, OmcA, UndA and Gxc decaheme cytochromes. Sequences with greater than 50 % identity with MtrC, MtrF, OmcA from *S. oneidensis* or UndA from *Shewanella* species HRCR-6 or GxcA and GxcB from *G. fermentans* were collected using BLAST. Sequences were aligned in ClustalW. A neighbor-joining phylogenetic tree was calculated using the PAM substitution matrix using (http://www.phylogeny.fr/). The phylogenetic tree was then uploaded in newick format and an unrooted circular tree alignment was performed using ITOL (http://itol.embl.de). The scale bar represents 0.1 expected amino acid residue substitutions per site.

The crystal structures of MtrF of *S. oneidensis* and UndA of *Shewanella* HRCR-6 have been determined. The structure revealed the 10 or 11 hemes of the decaheme *c*-type cytochrome to be in a "staggered cross" arrangement, with four potential electron exchange sites (Clarke *et al* 2011; Edwards, *et al* 2012b). Based on the similarity to MtrF-like proteins, the amino acid sequences of GxcA and GxcB were threaded onto the MtrF crystal structure, to see if GxcA and GxcB were structural homologs with similar arrangement of the heme ions. Phyre as well as NCBI structure tools were used to align GxcA and GxcB sequences onto the crystal structure of MtrF (PDB code-3PMQ).

The amino acid sequence identity and similarity of GxcA to MtrF is 24 % and 30 % and of GxcB to MtrF is 22 % and 26 % respectively. In spite of the weak amino acid sequence homology, the model of GxcA and GxcB revealed a similar arrangement of the decaheme in a "staggered cross" conformation found in the MtrF structure (Figure 4.5). However, the only homology between GxcB and MtrF is in the arrangement of ten hemes (not shown), whereas GxcA has similar secondary structure as shown in Figure 4.5. Leading to the hypothesis that GxcA decaheme cytochrome could be involved in electron transport to the cell surface in a similar mechanism as MtrF in *Shewanella* species.

A

```
MtrF      ------------------------------------------------------------
GxcA      MQQRPLKHLCGAIAATAVLLGLIACDGKTGPAGLNGTNGTNGTNGTNGTNGTNATPKLDV

MtrF      ------------------------------------------------------------
GxcA      SKLSAEQWTALKPTGTITSVTMGAAPVVNFKLTDAAGTPIVGLGAVTSKSTVPDRSGNLA

MtrF      ------SATFIAAQLLPQGATGAGNSSEWQHFTSET-----------------CAASCP
GxcA      TLSSYPNLAFAIAKLVPEDATSK-APSKWVSYIVTSVPAIKASTGAAVPAGPTTPTTDNT

MtrF      GTFVDHKNGHYSYRFSATFNGM-----------------NGVTFLSDATQRLVIKIGGD
GxcA      GTLVDNGDGTYKYTFYRDITKTQAALDAATYTAPSVKADLGDVAYAPTLVHRITLQFSGN

MtrF      ALADGTVLP-------------ITNQHYDWQSSGN----MLAYTRNLVSIDTCNSCHSNL
GxcA      APGTGSNTPDGVTLTPGVAMENPINVIYDFVPSTGAPVAAGTDIRDVVSINRCNECHGKL

MtrF      AFHGGRYNQVETCVTCHNSKKVSN--------------------AADIFPQMIHSKHLT
GxcA      AFHGGGRVEARYCVVCHTDQRKFGRAEATTTATGFSGSTYKINGKAAGDFPTMVHQIHMG

MtrF      G------------------F---PQSISNCQTCHADNPDLADRQNWYRVPTMEACGACHT
GxcA      KELTKTGYNYANVLYNNIGYSILDGGQKMCAKCHSD---VPQAANWSVKPTRLACGSCHD

MtrF      QINFPAGQGHPA-----QTDNSNCVACHNADWTANVHSNAAQTSALAQFNA-------SI
GxcA      GVDFATGINHMPGTNVPLTDDQACTICHTSDAIKTYHMANNVTPNNPAIPAGLKNVTYEI

MtrF      SSA-SMDANGTITVAV-------------SLTNPTTGT--------AYADSADKLKFIS
GxcA      KSAAATDTTATVVFKVKVDGADATFLAPAAAMANPLDGFTGSPSFLLAYAMPQDGVAAP-

MtrF      DLRIYANWGTS---FDYSSRSARSIRLPEST----PIA--GSNGTYSYNISGLTV-PAGT
GxcA      --ADYNNLGSGSSNFQPISVSIANLLDPNYAATRGTLSGPDASGYYTANLVGTKIFPVGA

MtrF      ESDRGGLAIQGRVCAKDSVLVDCSTELAEVLVIKSSHSYFNMSALTTTGRREVISNAKCA
GxcA      KMR--SVSLQGYFTQ---VIATGNVARHAISVI--------KPVTGDSVRRTVIDSAKCA

MtrF      SCHGDQQLNIHGARNDLAGQCQLCHNPNMLADATAT-----------------------
GxcA      KCHEWFEG--HGNRVYEVQVCVQCHVPGLTTSGRGIADTALQAYAFTAADQAILTAWKFD

MtrF      --------NPSMTSFDFKQLIHGLHSSQF-----------------AGFEDLNYPGNIG
GxcA      KTLPNAALAFPQVTNNFKDMIHGLHAGKDRTTSIKIARDRTPSAIALIDGANIGFPGILS

MtrF      NCAQCHINDSTGISTVALPLNAAVQ-----------------------PLALNNGTFTS
GxcA      NCQSCHTPTGYSA----VPANALASRQEADNGVFLNGVSRTPADAKAALTTNNANDLVTT

MtrF      PIAAVCSNCHSSDATQNHMRQQGAVFAGTKADATAGTETCAFCHGQGTVADVLKVHPIN
GxcA      PFTAACVSCHDSTIAKAHMNQNGGQVLALRSTLNLAGESCGICHGAGATYDPAKVH---
```

B

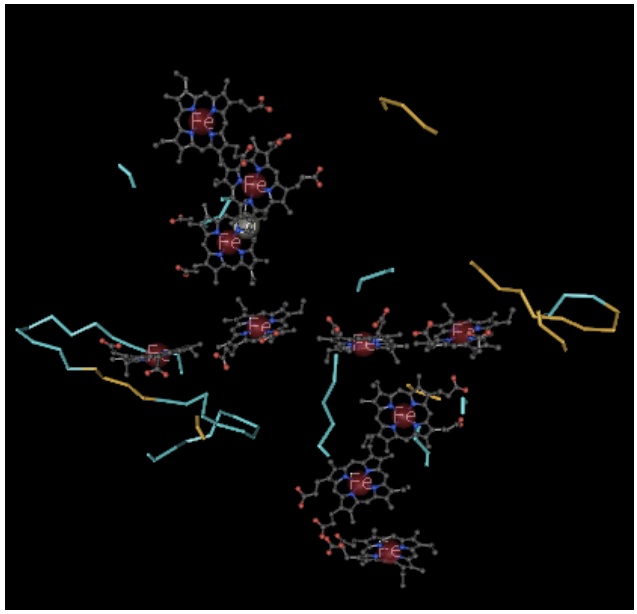

Figure 4.5 Comparison of GxcA with MtrF amino acid sequence and crystal structure. (**A**) Sequence alignment of GxcA and MtrF using ClustalW, highlighting CXXCH motifs and the distal histidine residues. (**B**) Cartoon diagram of GxcA amino acid sequence threaded onto the crystal structure of MtrF. The ten-hemes of GxcA are arranged in a similar manner of "staggered cross" identical to MtrF arrangement.

4.4.5 Expression of *gxcA* in *S. oneidensis* mutant strains

In order to test if GxcA can facilitate electron transport and is a functional homolog of the outer membrane-associated decaheme *c*-type cytochromes (MtrF/MtrC/OmcA) of *Shewanella* species, *gxcA* was heterologously expressed in two different mutant strains of *S. oneidensis*. *S. oneidensis* mutant strains that are either lacking all of the Mtr paralogs (Δ Mtr) or only lacking the outer membrane components (Δ OMC) (MtrC and its paralogs) were selected for characterizing the functional role of *gxcA* in electron transfer (Coursolle and Gralnick 2010; Coursolle and Gralnick 2012).

Plasmid containing *gxcA* under an arabinose inducible promoter was constructed and expressed in two mutant strains of *S. oneidensis,* ΔMtr or ΔOMC (Table 4.1). To confirm expression of *gxcA*, overnight cultures of ΔMtr expressing the following plasmids *pgxcA*, or *pmtrC* were lysed by sonication to extract total proteins and were separated by SDS-PAGE and stained for *c*-type cytochromes as described. Figure 4.6 shows presence of a 90 kDa heme positive band in lanes labled MtrC and GxcA induced. This observation shows that GxcA can be successfully expressed in *S. oneidensis* mutant strains upon addition of arabinose. This is indicated by detecting a heme positive band at about 90 kDa, which is missing from the GxcA uninduced and vector control lanes.

To determine if GxcA can form a functional electron transfer conduit, the ability to reduce Fe(III)-citrate was tested. *gxcA* was expressed in *S. oneidensis* mutant strain ΔMtr, which is missing all of the Mtr components that have been previously shown to be essential for electron transfer. GxcA in ΔMtr failed to reduce Fe(III)-citrate above background levels (Figure 4.8). As expected, MtrC/MtrA/MtrB in this same ΔMtr background could reduce Fe(III)-citrate.

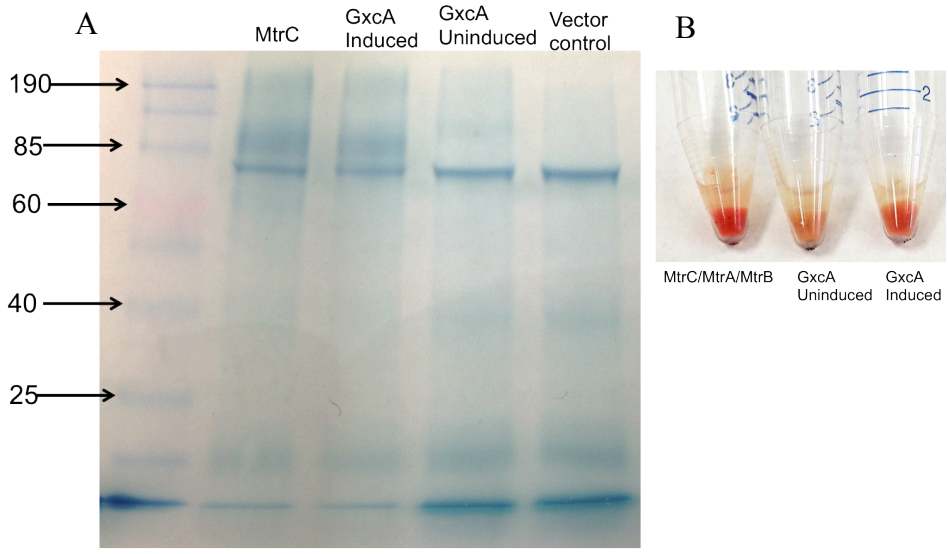

Figure 4.6 Expression of *G. fermentans* decaheme cytochrome *gxcA* in *S. oneidensis* ΔMtr mutant strain. (**A**) 20 µg of whole-cell lysate were separated by SDS-PAGE under denaturing conditions and stained for peroxidase activity. Lanes- (1) Ladder (2) ΔMtr+ pBBR-BB *mtrC*, (3) ΔMtr+pBBAD-22K+*gxcA* induced, (4) ΔMtr+pBBAD-22K+*gxcA* uninduced, and (5) empty vector control. (**B**) Overnight cell pellets of ΔMtr+ pBBR-BB *mtrC/mtrA/mtrB,* ΔMtr+pBBAD-22K+*gxcA* uninduced, ΔMtr+pBBAD-22K+*gxcA* induced.

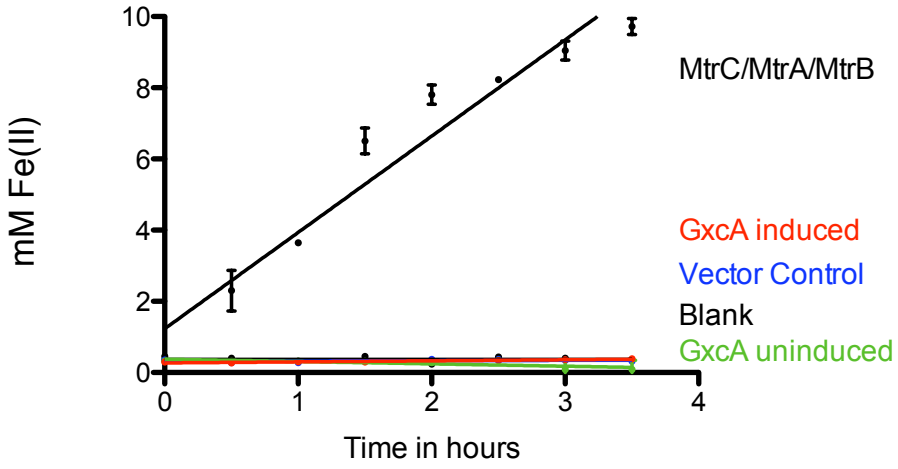

Figure 4.7 Ferric citrate reduction of GxcA in ΔMtr. GxcA in ΔMtr background failed to reduce Fe(III)-citrate above vector control levels. MtrC/MtrA/MtrB in ΔMtr background can reduce Fe(III)-citrate at 7.02 mM/h/mg protein.

The above results (Figure 4.8) suggest that GxcA may require the periplasmic (MtrA) and the integral outer-membrane β-barrel protein (MtrB) to facilitate electron transfer, as these components have been shown to be required for MtrC activity (Richardson *et al* 2012b). *gxcA* was expressed in a ΔOMC mutant strain, that has the native periplasmic cytochrome paralogs and the integral membrane β-barrel protein paralogs present. Figure 4.9 shows that GxcA interacts with the periplasmic cytochrome and the β-barrel protein paralogs to give *S. oneidensis* the ability to reduce Fe(III)-citrate. GxcA Fe(III)-citrate reduction was 2.15 mM/h/mg protein. These results suggest that GxcA with MtrA and MtrB can form a functional conduit and give *S. oneidensis* the ability to reduce Fe(III)-citrate. GxcA/MtrA/MtrB complex had one third Fe(III)-citrate reduction rates compared to the native MtrC/MtrA/MtrB. These rates are low but not suprising as GxcA and MtrC belong to phylogenetically diverse bacterial species.

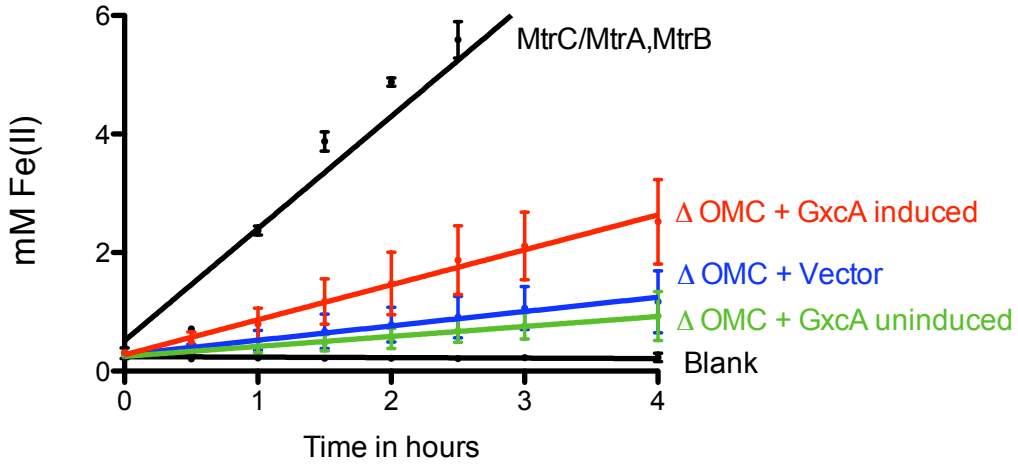

Figure 4.8 Fe(III)-citrate reduction assay of GxcA in ΔOMC background. Upon induction GxcA can form a functional conduit with MtrA and MtrB, giving *S. oneidensis* the ability to reduce Fe(III)-citrate.

4.6 Summary

Heterologous expression is an alternative strategy for characterization of proteins from organisms that are not genetically tractable. The use of *S. oneidensis* for heterologous expression of *c*-type cytochrome genes, in particular, is advantageous as it has all the machinery required for expression and maturation of *c*-type cytochromes. Also, especially for validation of cytochromes involved in electron transfer as *S. oneidensis* has a well-characterized electron transfer pathway comprising of modules that facilitate electron transfer.

In this chapter, decaheme *c*-type cytochromes, GxcA and GxcB were identified by detergent solubilization of membrane-associated proteins from *G. fermentans* Fe(III)-citrate grown cells. Oxidized and reduced UV/Visible spectrum

confirmed these enriched fractions to be *c*-type cytochromes. Based on amino acid sequence similarity, GxcA and GxcB were identified as decaheme cytochromes belonging to the *Shewanella* MtrF/MtrC family of cytochromes. Additionally, based on structural predictions, GxcA and GxcB also have the conserved "staggered cross" arrangement of the ten-heme molecules, similar to MtrF/UndA. Due to the inability to genetically manipulate *G. fermentans*, heterologous expression of *gxcA* was performed in mutant strains of *S. oneidensis*. Fe(III)-citrate reduction assays were performed to test whether GxcA can facilitate electron transfer with or without the periplasmic decaheme cytochrome MtrA and β-barrel outer membrane porin protein MtrB. GxcA was characterized to be a functional homolog of MtrC-like proteins as GxcA can facilitate electron transfer only as a part of the Mtr transmembrane conduit.

Chapter 5: Thesis conclusions and future work

5.1 Conclusions

The significance of microorganisms in the biogeochemical cycling of Fe has been recognized since the early 1990s (Lovley 1991; Nealson 1992). Dissimilatory Fe(III)-reducing bacteria couple the oxidation of organic matter or H_2 with the reduction of Fe(III)-oxides in order to derive energy for growth. Additionally, these Fe(III)-reducing organisms occupy varying environmental niches; For instance, *Geobacteraceae* are strict anaerobes found in subsurface environments, while the *Shewanella* species are facultative anaerobes which thrive at redox boundaries (Lovley *et al* 2004; Lovley 2012; Hau & Gralnick 2007). Dissimilatory Fe(III)-reducing bacteria are considered the dominant players of Fe(III)-reduction within these environments, thus accounting for global Fe as well as C cycling.

Most of the current knowledge about the mechanisms and ecological significance of microbial Fe(III)-respiration stems from two model organisms belonging to the Proteobacterial phylum, although Fe(III)-respiration is much more phylogenetically diverse in nature (Weber *et al* 2006). One of the most abundant metal-reducers present besides the members of the *Geobacteraceae* family in many subsurface environments are the *Geothrix*-like Acidobacteria. The goal of this thesis was to identify the mechanism of electron transfer by the metal-reducing bacterium *G. fermentans*. The research work described in this thesis identified a high potential acceptor requirement for *G. fermentans*. Also, it highlights another example of a metal-reducing bacterium using flavins as an electron shuttles, thereby implying a putative role of flavins in electron transfer by metal-reducing bacteria that are phylogenetically diverse.

The complete genome sequencing of *G. fermentans* and 1M1 species provided insights into its oligotrophic lifestyle. As described in Chapter 2, *Geothrix* species represent the only known metal-reducing members belonging to the diverse Acidobacterial phylum. Phylogenetic analysis with 31 proteins obtained from the sequenced aerobic Acidobacteria in combination with the 16S rRNA gene analysis place the Acidobacteria adjacent to the Proteobacteria, suggesting a deep relationship (Ciccarelli 2006; Ward *et al* 2009; Garcia Costas *et al* 2011). Similar to the aerobic Acidobacteria, the *Geothrix* genomes described in this thesis also supported the hypothesis that the δ-Proteobacteria is a sister clade as they share many core central metabolic genes for instance the citrate synthase (Bond *et al* 2005; Wilkins *et al* 2010).

The presence of glysoside hydrolase, glysoside transferase, and chitinase-encoding genes suggest the ability to utilize complex substrates such as xylan, cellulose and chitin. While complex carbohydrates have been successfully used to cultivate many of the aerobic Acidobacteria, the ability to couple oxidation of a complex carbon compound with the reduction of metal oxide as a terminal electron acceptor had not been demonstrated previously. *G. fermentans* can conserve energy by coupling the oxidation of complex carbohydrate such as chitin with Fe(III)-oxide reduction.

In Chapter 3, the electrochemical profile of *G. fermentans* was studied using poised electrodes as terminal electron acceptor in a three-electrode bioreactor. Electrochemical experiments showed that *G. fermentans* requires higher potential acceptors to grow at its maximum respiration rate, compared to other well-studied

bacteria like *Geobacter* species. While *Geobacter* will consistently transfer electrons near its maximum rate below potentials of 0 V versus SHE, *G. fermentans* requires potentials of nearly +0.55 V to achieve maximum rates of electron transfer (Figure 7.1). This observation implies that *Geothrix* species would be more competitive in environments where the electron acceptor is at a potential greater than +0.2 V. Chelated metals and Mn(IV)-oxides or possibly even microaerobic environments may have more oxidizing potentials, when compared to Fe(III)-oxides such as ferrihydrite or hematite that have lower redox potentials.

Electrochemical analysis also identified a shuttle based electron transfer mechanism for *G. fermentans* metal-respiration. Supernatants from Fe(III)-oxide grown and electrode-grown cultures showed evidence of two independent soluble electron shuttles, which stimulated electron transfer at low and high potential windows. One of these two redox shuttles was identified as riboflavin. The identity of the second higher potential redox shuttle is still unknown, but based on preliminary analysis it has quinone like characteristics.

The environmental role/niche of *G. fermentans* can be predicted based on the following observations-

1. High potential requirement of *G. fermentans* for maximum electron transfer rate
2. The secretion of a high potential (+0.3 V) redox shuttle
3. Ability to use complex carbohydrates as electron donors

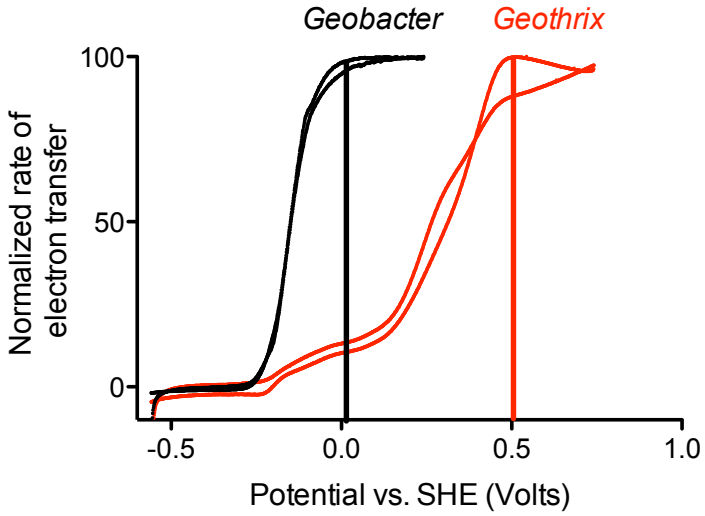

Figure 5.1 Cyclic voltammetry comparing *G. sulfurreducens* (black trace) with *G. fermentans* (red trace). At lower potential, below -0.25 V, neither organism can transfer electrons to the acceptor. However, when the potential is raised to 0 V, *Geobacter* reaches a maximal rate of electron transfer (black line), while *Geothrix* is only at 10% of its maximal rate at that potential. Changing the potential further, to +0.5 V, brings *Geothrix* to its maximal electron transfer rate (red line).

Taken together, these observations imply that *G. fermentans* may have a competitive growth advantage at the oxic / anoxic interface of some environments. The availability of higher potential terminal acceptors such as O_2, Mn(IV)-oxides, chelated-metals and even freshly precipitated ferrihydrite present at this interface, could help explain the effective use of the high potential shuttle secreted by *G. fermentans*. Additionally, complex organic matter is more often found in oxidized environments compared to the subsurface environments that are generally more reduced, perhaps providing *Geothrix* species a competitive advantage in such environments.

A biochemical approach was taken to identify the components required for the electron transfer across inner membrane, the periplasmic space, the outer membrane, and to the cell surface, where it can interact with the terminal mineral oxides. Abundant multiheme *c*-type cytochromes present in the genome along with heme positive bands in membrane fractions lead to the hypothesis that *c*-type cytochromes may be involved in electron transfer. Genome architecture of four decaheme cytochromes next to one another, flanked by cytochrome maturation genes, in a manner similar to the *mtr* gene cluster of *S. oneidensis*, further bolstered the probable role of these proteins in electron transfer. Membrane separation coupled with mass spectrometric characterization of heme positive bands, led to the identification of two decaheme cytochromes GxcA and GxcB. GxcA and GxcB were similar to the decaheme cytochromes belonging to the MtrF/C family of cytochromes. Additional GxcA and GxcB were tested in mutant strains of *S. oneidensis* lacking the outer membrane cytochromes to see if these were functional homologs.

5.2 Future work

There are many questions that still remain unanswered regarding the physiology and mechanism of metal reduction by *G. fermentans*. In order to understand better the mechanism of electron transfer and to conclusively demonstrate the role of proteins involved in the process, gene deletion and complementation experiments are essential. Many of the future goals depend on making *Geothrix* a genetically tractable organism.

5.2.1 Examining higher potential behavior

An unexplored question that arises from voltammetry studies of *Geothrix* growth on poised electrodes is the possibility that this organism can use external metals or electrodes as electron donors. The high-potential environments would ideally be the oxic-anoxic interface, where freshly precipitated reduced metals and oxygen overlap. One way to directly test this hypothesis is by using electrodes poised at lower potentials, under controlled atmospheres of low (<1%) oxygen, to mimic natural environments where Fe(II) oxidation could occur. It would be interesting if *Geothrix* can use lower potential poised electrodes as electron donor, as it would indicate that in the environments *Geothrix* might also gain energy as an Fe(II) oxidizing microorganism.

5.2.2 Regulation and export of riboflavin

The regulation of the production and export of riboflavin, the low potential shuttle, secreted by *G. fermentans* needs to be characterized. The electron shuttle

riboflavin is only secreted when *G. fermentans* is grown in presence of insoluble electron acceptors (Fe(III)-oxide and electrodes), suggesting that the expression and secretion of riboflavin is regulated under metal-reducing condition, as the production of the electron shuttle would only be beneficial if the organism gets multiple rounds of reduction and oxidation cycles. Recently Kotloski and Gralnick (Kotloski & Gralnick 2013) identified the flavin exporter of *S. oneidensis* MR-1: Bfe (Bacterial Flavin adenine dinucleotide exporter). Interestingly, the sequenced genomes of *Geothrix* species lack Bfe homologs, suggesting that flavin export mechanism maybe different in these Acidobacteria.

5.2.3 Identification of high potential shuttle

The higher potential (+0.3 V vs SHE) shuttle is also produced only in presence of insoluble acceptors (Fe(III)-oxides and poised electrodes). It is hydrophilic with no affinity for the graphite electrodes and has quinone like characteristics. The identification of this compound is important as to date no redox active shuttling molecules that have potential of +0.3 V vs SHE are known. One way to identify this quinone shuttle would be to extract supernatants from Fe(III)-oxide grown cells, treat these supernatants with C_{18} column to remove riboflavin and perform NMR spectroscopy to get the physical, chemical and structural information. Another way for selective enrichment of this hydrophilic quinone would be by using hydrophilic interaction liquid chromatography (Hsieh 2008; Cubbon *et al* 2010).

Second, it would be interesting to study its mechanism of regulation, production and secretion, in order to determine how *Geothrix* senses higher potential

acceptors such as oxygen to regulate the expression of the quinone-like shuttle. Identification of interacting partners proteins present on the cell surface, involved in donating electrons to this higher potential shuttle would be interesting to see if these have quinone-like binding pockets, similar to the decaheme *c*-type cytochromes MtrF/C that have putative flavin binding pockets.

5.2.4 Identification of interacting proteins involved in electron transfer

The role of *c*-type cytochromes in the electron transport across the membranes and to the terminal acceptor have been identified to be essential from studies in the two model organisms (Shi *et al* 2007; Myers & Myers 2003; Mehta *et al* 2005; Richardson, *et al* 2012b). As mentioned in Chapters 2 and 3 *Geothrix* genomes also encoded multiheme *c*-type cytochromes - GxcA and GxcB that are distant homologs of MtrF/C. Protein cross-linking studies can be used to identify the interacting partner proteins with GxcA and GxcB that facilitate electron transfer. The cross-linking experiments will help in identifying the protein complexes that are involved in the formation of an extended respiratory chain spanning the membranes in *Geothrix*. This would be interesting if the mechanism is similar to that of *S. oneidensis*, as different proteins that interact with these distantly related decaheme cytochromes would be identified. Additionally, antibody labeling and electron microscopy will also provide vital information about the function and localization of these decaheme *c*- type cytochromes.

Many questions remain to be addressed to further our understanding about the physiology and ecological significance of *Geothrix* species. A combination of

geochemical and microbiological approaches is required to address the role of *Geothrix*-like Acidobacteria in the "microbial ferrous wheel" and the global carbon cycling. The research presented in this thesis, serves as a foundation for understanding the environmental importance and the mechanism of electron transfer in *G. fermentans*.

References

Abed RM, Safi NM, Köster J, de Beer D, El-Nahhal Y, Rullkötter J, *et al* (2002). Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. Appl. Environ. Microbiol. 68:1674–1683.

Achtnich C, Bak F, Conrad R. (1995). Competition for electron donors among nitrate reducers, ferric iron reducers, sulfate reducers, and methanogens in anoxic paddy soil. Biol. Fertil. Soils 19:65–72.

Afkar E, Reguera G, Schiffer M, Lovley DR. (2005). A novel *Geobacteraceae*-specific outer membrane protein J (OmpJ) is essential for electron transport to Fe(III) and Mn(IV) oxides in *Geobacter sulfurreducens*. BMC Microbiol. 5:1–11.

Aklujkar M, Coppi MV, Leang C, Kim BC, Chavan MA, Perpetua LA, *et al* (2013). Proteins involved in electron transfer to Fe(III) and Mn(IV) oxides by *Geobacter sulfurreducens* and *Geobacter uraniireducens*. Microbiol. 159:515–535.

Anderson I, Held B, Lapidus A, Nolan M, Lucas S, Tice H, *et al* (2012). Genome sequence of the homoacetogenic bacterium *Holophaga foetida* type strain (TMBS4^{T}). Stand Genomic Sci 6:174–184.

Anderson RT, Rooney-Varga JN, Gaw CV, Lovley DR. (1998). Anaerobic benzene oxidation in the fe(III) reduction zone of petroleum-contaminated aquifers. Environ. Sci. Technol. 32:1222–1229.

Barns SM, Cain EC, Sommerville L, Kuske CR. (2007). *Acidobacteria* phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. Appl. Environ. Microbiol. 73:3113–3116.

Baron D, LaBelle E, Coursolle D, Gralnick JA, Bond DR. (2009). Electrochemical measurement of electron transfer kinetics by *Shewanella oneidensis* MR-1. The J Biological Chemistry 284:28865–28873.

Bonanni PS, Schrott GD, Busalmen JP. (2012). A long way to the electrode: how do *Geobacter* cells transport their electrons? Biochem. Soc. Trans. 40:1274–1279.

Bond DR, Holmes DE, Tender LM, Lovley DR. (2002). Electrode-reducing microorganisms that harvest energy from marine sediments. Science 295:483–485.

Bond DR, Lovley DR. (2003). Electricity production by *Geobacter sulfurreducens* attached to electrodes. Appl. Environ. Microbiol. 69:1548–1555.

Bond DR, Lovley DR. (2005). Evidence for involvement of an electron shuttle in electricity generation by *Geothrix fermentans*. Appl. Environ. Microbiol. 71:2186–2189.

Bond DR, Mester T, Nesbø CL, Izquierdo-Lopez A, Collart FL, Lovley DR. (2005). Characterization of citrate synthase from *Geobacter sulfurreducens* and evidence for a family of citrate synthases similar to those of eukaryotes throughout the *Geobacteraceae*. Appl. Environ. Microbiol. 71:3858–3865.

Brodie EL, DeSantis TZ, Joyner DCD, Baek SM, Larsen JT, Andersen GL, *et al* (2006). Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl. Environ. Microbiol. 72:6288–6298.

Brown DG, Komlos J, Jaffe PR. (2005). Simultaneous utilization of acetate and hydrogen by *Geobacter sulfurreducens* and implications for use of hydrogen as an indicator of redox conditions. Environ. Sci. Technol. 39:3069–3076.

Brutinel ED, Gralnick JA. (2012). Anomalies of the anaerobic tricarboxylic acid cycle in *Shewanella oneidensis* revealed by Tn-seq. Mol. Microbiol. 86:273–283.

Bryant DA, Costas AMG, Maresca JA, Chew AGM, Klatt CG, Bateson MM, *et al* (2007). *Candidatus* Chloracidobacterium thermophilum: an aerobic phototrophic Acidobacterium. Science 317:523–526.

Butler JE, Young ND, Lovley DR. (2010). Evolution of electron transfer out of the cell: comparative genomics of six *Geobacter* genomes. BMC Genomics 11:40–40.

Bücking C, Popp F, Kerzenmacher S, Gescher J. (2010). Involvement and specificity of *Shewanella oneidensis* outer membrane cytochromes in the reduction of soluble and solid-phase terminal electron acceptors. FEMS Microbiol. Lett. 306:144–151.

Byrne-Bailey KG, Wrighton KC, Melnyk RA, Agbo P, Hazen TC, Coates JD. (2010). Complete genome sequence of the electricity-producing *Thermincola potens* strain JR. J Bacteriol 192:4078–4079.

Caccavo F, Lonergan DJ, Lovley DR, Davis M, Stolz JF, McInerney MJ. (1994). *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol. 60:3752–3759.

Canstein von H, Ogawa J, Shimizu S, Lloyd JR. (2008). Secretion of flavins by *Shewanella* species and their role in extracellular electron transfer. Appl. Environ. Microbiol. 74:615–623.

Cardenas E, Wu W-M, Leigh MB, Carley J, Carroll S, Gentry T, *et al* (2008). Microbial communities in contaminated sediments, associated with bioremediation of uranium to submicromolar levels. Appl. Environ. Microbiol. 74:3718–3729.

Cardenas E, Wu W-M, Leigh MB, Carley J, Carroll S, Gentry T, *et al* (2010). Significant association between sulfate-reducing bacteria and uranium-reducing microbial communities as revealed by a combined massively parallel sequencing-

indicator species approach. Appl. Environ. Microbiol. 76:6778–6786.

Carlson HK, Iavarone AT, Gorur A, Yeo BS, Tran R, Melnyk RA, *et al* (2012). Surface multiheme *c*-type cytochromes from *Thermincola potens* and implications for respiratory metal reduction by Gram-positive bacteria. Proc. Natl. Acad. Sci. U S A 109:1702–1707.

Cervantes FJ, Duong-Dac T, Ivanova AE, Roest K, Akkermans AD, Lettinga G, *et al* (2003). Selective enrichment of *Geobacter sulfurreducens* from anaerobic granular sludge with quinones as terminal electron acceptors. Biotechno.l Lett. 25:39–45.

Chae K-J, Choi M-J, Lee J-W, Kim K-Y, Kim IS. (2009). Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells. Bioresource Technol. 100:3518–3525.

Ciccarelli FD. (2006). Toward automatic reconstruction of a highly resolved tree of life. Science 311:1283–1287.

Clarke TA, Edwards MJ, Gates AJ, Hall A, White GF, Bradley J, *et al* (2011). Structure of a bacterial cell surface decaheme electron conduit. Proc. Natl. Acad. Sci. U S A.108:9384–9389.

Coates JD, Councell T, Ellis DJ, Lovley DR. (1998). Carbohydrate oxidation coupled to Fe(III) reduction, a novel form of anaerobic metabolism. Anaerobe 4: 6–6.

Coates JD, Ellis DJ, Gaw CV, Lovley DR. (1999). *Geothrix fermentans* gen. nov., sp. nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer. Int. J. Syst. Bacteriol. 49 4:1615–1622.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:141–145.

Coppi MV, Leang C, Sandler SJ, Lovley DR. (2001). Development of a genetic System for *Geobacter sulfurreducens*. Appl. Environ. Microbiol. 67:3180–3187.

Cornell RM, Schwertmann U. (2003). The Iron Oxides: Structure, Properties, Reactions, Occurrences and Uses.

Costa R, Keller-Costa T, Gomes NCM, da Rocha UN, van Overbeek L, van Elsas JD. (2013). Evidence for selective bacterial community structuring in the freshwater sponge *Ephydatia fluviatilis*. Microb. Ecol. 65:232–244.

Coursolle D, Baron DB, Bond DR, Gralnick JA. (2010). The Mtr respiratory pathway is essential for reducing flavins and electrodes in *Shewanella oneidensis*. J Bacteriol. 192:467–474.

Coursolle D, Gralnick JA. (2010). Modularity of the mtr respiratory pathway of *Shewanella oneidensis* strain MR-1. Mol. Microbiol. 77:995–1008

Coursolle DD, Gralnick JAJ. (2012). Reconstruction of extracellular respiratory pathways for iron(iii) reduction in *Shewanella oneidensis* strain MR-1. Front Microbiol. 3:56–56.

Covington ED, Gelbmann CB, Kotloski NJ, Gralnick JA. (2010). An essential role for UshA in processing of extracellular flavin electron shuttles by *Shewanella oneidensis*. Mol. Microbiol. 78:519–532.

Cubbon S, Antonio C, Wilson J, Thomas-Oates J. (2010). Metabolomic applications of HILIC-LC-MS. Mass Spectrom. Rev. 29:671–684.

Dedysh S, Kulichevskaya I, Serkebaeva Y, Mityaeva M, Sorokin V, Suzina N, *et al* (2012). *Bryocella elongata* gen. nov., sp. nov., a member of subdivision 1 of the *Acidobacteria* isolated from a methanotrophic enrichment culture, and emended description of *Edaphobacter aggregans*. Int. J Syst. Evol. Microbiol. 62:654–664.

Deutschbauer A, Price MN, Wetmore KM, Shao W, Baumohl JK, Xu Z, et al (2011). Evidence-based annotation of gene function in *Shewanella oneidensis* MR-1 using genome-wide fitness profiling across 121 conditions. PLoS Genet. 7:e1002385–e1002385.

Dietrich LE, Teal TK, Price-Whelan A, Newman DK. (2008). Redox-active antibiotics control gene expression and community behavior in divergent bacteria. Science 321:1203–1206.

Ding Y-HR, Hixson KK, Giometti CS, Stanley A, Esteve-Núñez A, Khare T, *et al* (2006). The proteome of dissimilatory metal-reducing microorganism *Geobacter sulfurreducens* under various growth conditions. BBA-Proteins and Proteom. 1764:1198–1206.

Edwards MJ, Fredrickson JK, Zachara JM, Richardson DJ, Clarke TA. (2012a). Analysis of structural MtrC models based on homology with the crystal structure of MtrF. Biochem. Soc. Trans. 40:1181–1185.

Edwards MJ, Hall A, Shi L, Fredrickson JK, Zachara JM, Butt JN, *et al* (2012b). The crystal structure of the extracellular 11-heme cytochrome UndA reveals a conserved 10-heme motif and defined binding site for soluble iron chelates. Structure 20:1275–1284.

Eichorst SAS, Kuske CRC, Schmidt TMT. (2007). Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the phylum *Acidobacteria*. Appl. Environ. Microbiol. 73:2708–2717.

Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA, Knight R. (2011).

Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J 5:1565–1567.

Finneran KT, Johnsen CV, Lovley DR. (2003). *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). Int. J Syst. Evol. Microbiol. 53:669–673.

Flynn TM, Sanford RA, Bethke CM. (2008). Attached and suspended microbial communities in a pristine confined aquifer. Water Resour. Res. 44.

Foesel BU, Rohde M, Overmann J. (2013). *Blastocatella fastidiosa* gen. nov., sp. nov., isolated from semiarid savanna soil - The first described species of *Acidobacteria* subdivision 4. Syst. Appl. Microbiol. 36:82–89.

Fourmond V, Hoke K, Heering HA, Baffert C, Leroux F, Bertrand P, *et al* (2009). SOAS: a free program to analyze electrochemical data and other one-dimensional signals. Bioelectrochemistry 76:141–147.

Francis RT Jr., Becker RR. (1984). Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. Analyt. Biochem. 136:509–514.

Fukunaga Y, Kurahashi M, Yanagi K, Yokota A, Harayama S. (2008). *Acanthopleuribacter pedis* gen. nov., sp. nov., a marine bacterium isolated from a chiton, and description of *Acanthopleuribacteraceae* fam. nov., *Acanthopleuribacterales* ord. nov., *Holophagaceae* fam. nov., *Holophagales* ord. nov. and *Holophagae* classis nov. in the phylum '*Acidobacteria*'. Int. J. Syst. Bacteriol. 58:2597–2601.

Garcia Costas AM, Liu Z, Tomsho LP, Schuster SC, Ward DM, Bryant DA. (2011). Complete genome of *Candidatus* Chloracidobacterium thermophilum, a chlorophyll-based photoheterotroph belonging to the phylum *Acidobacteria*. Environ. Microbiol. 14:177–190.

Gaspard S, Vazquez F, Holliger C. (1998). Localization and solubilization of the Iron(III) reductase of *Geobacter sulfurreducens*. Appl. Environ. Microbiol. 64:3188–3194.

Gault, Ibrahim, Langley, Renaud, Takahashi, Boothman, *et al* (2011). Microbial and geochemical features suggest iron redox cycling within bacteriogenic iron oxide-rich sediments. Chem. Geol. 281:11–11.

George IF, Hartmann M, Liles MR, Agathos SN. (2011). Recovery of as-yet-uncultured soil acidobacteria on dilute solid media. Appl. Environ. Microbiol. 77:8184–8188.

Giardine B. (2005). Galaxy: A platform for interactive large-scale genome analysis.

Genome Research 15:1451–1455.

Gorby YA, Lovley DR. (1991). Electron transport in the dissimilatory iron reducer, GS-15. Appl. Environ. Microbiol. 57:867–870.

Gralnick JA. (2012). On conducting electron traffic across the periplasm. Biochem. Soc. Trans. 40:1178–1180.

Gralnick JA, Newman DK. (2007). Extracellular respiration. Mol. Microbiol. 65:1–11.

Hau HH, Gilbert A, Coursolle D, Gralnick JA. (2008). Mechanism and consequences of anaerobic respiration of cobalt by *Shewanella oneidensis* strain MR-1. Appl. Environ. Microbiol. 74:6880–6886.

Hau HH, Gralnick JA. (2007). Ecology and biotechnology of the genus *Shewanella*. Annu. Rev. Microbiol. 61:237–258.

Hernandez M, Kappler A, Newman D. (2004). Phenazines and other redox-active antibiotics promote microbial mineral reduction. Appl. Environ. Microbiol. 70:921–928.

Holmes DE, Bond DR, O'Neil RA, Reimers CE, Tender LR, Lovley DR. (2004). Microbial communities associated with electrodes harvesting electricity from a variety of aquatic sediments. Microb. Ecol. 48:178–190.

Holmes DE, Nevin KP, O'Neil RA, Ward JE, Adams LA, Woodard TL, *et al* (2005). Potential for quantifying expression of the *Geobacteraceae* citrate synthase gene to assess the activity of *Geobacteraceae* in the subsurface and on current-harvesting electrodes. Appl. Environ. Microbiol. 71:6870–6877.

Hsieh Y. (2008). Potential of HILIC-MS in quantitative bioanalysis of drugs and drug metabolites. J. Sep. Sci. 31:1481–1491.

Hunt KA, Flynn JM, Naranjo B, Shikhare ID, Gralnick JA. (2010). Substrate-level phosphorylation is the primary source of energy conservation during anaerobic respiration of Shewanella oneidensis Strain MR-1. J Bacteriol. 192:3345-3351.

Islam FS, Boothman C, Gault AG, Polya DA, Lloyd JR. (2005). Potential role of the Fe(III)-reducing bacteria *Geobacter* and *Geothrix* in controlling arsenic solubility in Bengal delta sediments. Mineral. Mag. 69:865–875.

Islam FS, Gault AG, Boothman C, Polya DA, Charnock JM, Chatterjee D, *et al* (2004). Role of metal-reducing bacteria in arsenic release from Bengal delta sediments. Nature 430:68–71.

Izumi H, Nunoura T, Miyazaki M, Mino S, Toki T, Takai K, *et al* (2012).

Thermotomaculum hydrothermale gen. nov., sp. nov., a novel heterotrophic thermophile within the phylum *Acidobacteria* from a deep-sea hydrothermal vent chimney in the Southern Okinawa Trough. Extremophiles 16:245–253.

Janssen PH. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72:1719–1728.

Jensen H, Albers A, Malley K, Londer Y, Cohen B, Helms B, *et al* (2010). Engineering of a synthetic electron conduit in living cells. Proc. Natl. Acad. Sci. U S A 107:19213–19218.

Jones RT, Robeson MS, Lauber CL, Hamady M, Knight R, Fierer N. (2009). A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. ISME J 3:442–453.

Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH. (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl. Environ. Microbiol. 69:7210–7215.

Nealson KH, Myers CR. (1992). Microbial reduction of manganese and iron: new approaches to carbon cycling. Appl. Environ. Microbiol. 58:439.

Kahnt J, Aguiluz K, Koch J, Treuner-Lange A, Konovalova A, Huntley S, *et al* (2010). Profiling the outer membrane proteome during growth and development of the social bacterium *Myxococcus xanthus* by selective biotinylation and analyses of outer membrane vesicles. J. Proteome Res. 9:5197–5208.

Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L. (2008). Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in Lake Washington. ISME J 2:696–706.

Karlińska-Batres K, Wörheide G. (2013). Phylogenetic diversity and community structure of the symbionts associated with the coralline sponge *Astrosclera willeyana* of the great barrier reef. Microb. Ecol. 10.1007/s00248-013-0212-5.

Kashefi K, Lovley DR. (2000). Reduction of Fe(III), Mn(IV), and toxic metals at 100 degrees C by *Pyrobaculum islandicum*. Appl. Environ. Microbiol. 66:1050–1056.

Khan MT, Duncan SH, Stams AJM, van Dijl JM, Flint HJ, Harmsen HJM. (2012). The gut anaerobe *Faecalibacterium prausnitzii* uses an extracellular electron shuttle to grow at oxic-anoxic interphases. ISME J. 6:1578–1585.

Kielak AM, van Veen JA, Kowalchuk GA. (2010). Comparative analysis of Acidobacterial genomic fragments from terrestrial and aquatic metagenomic libraries, with emphasis on *Acidobacteria* subdivision 6. Appl. Environ. Microbiol. 76:6769–6777.

Kishimoto N, Kosako Y, Tano T. (1991). *Acidobacterium capsulatum* gen. nov., sp. nov.: An acidophilic chemoorganotrophic bacterium containing menaquinone from acidic mineral environment. Current Microbiol. 22:1–7.

Klueglein N, Lösekann-Behrens T, Obst M, Behrens S, Appel E, Kappler A. (2013). Magnetite formation by the novel Fe(III)-reducing *Geothrix fermentans* strain HradG1 isolated from a hydrocarbon-contaminated sediment with increased magnetic susceptibility. Geomicrobiol. J 130405141851006.

Koch IH, Gich F, Dunfield PF, Overmann J. (2008). *Edaphobacter modestus* gen. nov., sp. nov., and *Edaphobacter aggregans* sp. nov., acidobacteria isolated from alpine and forest soils. Int. J Syst. Evol. Microbiol. 58:1114–1122.

Kotloski NJ, Gralnick JA. (2013). Flavin electron shuttles dominate extracellular electron transfer by *Shewanella oneidensis*. MBio 4(1):e00553-12.doi:10.1128/mBio.00553-12

Kouridaki I, Polymenakou PN, Tselepides A, Mandalakis M, Smith KL. (2010). Phylogenetic diversity of sediment bacteria from the deep Northeastern Pacific Ocean: a comparison with the deep Eastern Mediterranean Sea. Int. Microbiol. 13:143–150.

Leang C, Qian X, Mester T, Lovley DR. (2010). Alignment of the *c*-type cytochrome OmcS along pili of *Geobacter sulfurreducens*. Appl. Environ. Microbiol. 76:4080–4084.

Leang CC, Coppi MV, Lovley DR. (2003). OmcB, a *c*-type polyheme cytochrome, involved in Fe(III) reduction in *Geobacter sulfurreducens*. J Bacteriol. 185:2096–2103.

Li R, Tiedje JM, Chiu C, Worden RM. (2012). Soluble electron shuttles can mediate energy taxis toward insoluble electron acceptors. Environ. Sci. Technol. 46:2813–2820.

Li X, Krumholz LR. (2008). Influence of nitrate on microbial reduction of pertechnetate. Environ. Sci. Technol. 42:1910–1915.

Li Y, Lee CG, Watanabe T, Murase J, Asakawa S. (2011). Identification of microbial communities that assimilate substrate from root cap cells in an aerobic soil using a DNA-SIP approach. Soil Biol. Biochem.43:1928-1935.

Liesack W, Bak F, Kreft JU, Stackebrandt E. (1994). *Holophaga foetida* gen. nov., sp. nov., a new, homoacetogenic bacterium degrading methoxylated aromatic compounds. Arch. Microbiol. 162:85–90.

Lin B, Braster M, van Breukelen BM, van Verseveld HW, Westerhoff HV, Röling WFM. (2005). *Geobacteraceae* community composition is related to hydrochemistry

and biodegradation in an iron-reducing aquifer polluted by a neighboring landfill. Appl. Environ. Microbiol. 71:5983–5991.

Liu C, Gorby YA, Zachara JM, Fredrickson JK, Brown CF. (2002). Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria. Biotechnol. Bioeng. 80:637–649.

Lloyd JR. (2003). Microbial reduction of metals and radionuclides. FEMS Microbiol. Reviews 27:411–425.

Lloyd JR, Blunt-Harris EL, Lovley DR. (1999). The periplasmic 9.6-kilodalton c-type cytochrome of *Geobacter sulfurreducens* is not an electron shuttle to Fe (III). J of Bacteriol 181:7647–7649.

Lloyd JR, Leang CC, Hodges-Myerson AL, Coppi MV, Cuifo S, Methe B, *et al* (2003). Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*. Biochemical J 369:153.

Lloyd JR, Sole VA, Van Praagh CV, Lovley DR. (2000). Direct and Fe(II)-mediated reduction of technetium by Fe(III)-reducing bacteria. Appl. Environ. Microbiol. 66:3743–3749.

Loffler FE, Tiedje JM, Sanford RA. (1999). Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. Appl. Environ. Microbiol. 65:4049–4056.

Logan BE. (2012). Essential data and techniques for conducting microbial fuel cell and other types of bioelectrochemical system experiments. Chem Sus Chem 5:988–994.

Logan BE. (2008). Microbial Fuel Cells. Wiley-Interscience. Chapter 2:12-28.

Lovley D. (2000). 'Chapter 1: Fe(III) and Mn(IV) Reduction' by Derek Lovley. Dissimilatory Fe(III)- and Mn(IV)-Reducing Prokaryotes 2-28.

Lovley DR. (2006). Dissimilatory Fe (III)-and Mn (IV)-reducing prokaryotes. The Prokaryotes. Chapter 1.21 2:635-658.

Lovley DR. (2012). Electromicrobiology. Annu. Rev. Microbiol. 66:391–409.

Lovley DR. (1985). Minimum threshold for hydrogen metabolism in methanogenic bacteria. Appl. Environ. Microbiol. 49:1530–1531.

Lovley DR, Anderson RT. (2000). Influence of dissimilatory metal reduction on fate of organic and metal contaminants in the subsurface. Hydrogeology Journal 8:77–88.

Lovley DR, Chapelle FH. (1995). Deep subsurface microbial processes. Rev.

Geophys. 33:365.

Lovley DR, Giovannoni SJ, White DC, Champine JE, Phillips EJ, Gorby YA, *et al* (1993). *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch. Microbiol. 159:336–344.

Lovley DR, Goodwin S. (1988). Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim Cosmochim Acta 52:2993–3003.

Lovley DR, Holmes DE, Nevin KP. (2004). Dissimilatory Fe(III) and Mn(IV) reduction. Adv Microb Physiol 49:219–286.

Lovley DR, Phillips EJ. (1988). Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. 54:1472–1480.

Lovley DR, Phillips EJ, Gorby YA, Landa ER. (1991). Microbial reduction of uranium. Nature 350:413–416.

Lovley DR, Ueki T, Zhang T, Malvankar NS, Shrestha PM, Flanagan KA, *et al* (2011). *Geobacter*: the microbe electric's physiology, ecology, and practical applications. Adv. Microb. Physiol. 59:1–100.

Lovley DRD. (1991). Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol Rev 55:259–287.

Ludwig W, Bauer SH, Bauer M, Held I, Kirchhof G, Schulze R, *et al* (2006). Detection and in situ identification of representatives of a widely distributed new bacterial phylum. FEMS Microbiol. Lett. 153:181–190.

Luo W, Wu W-M, Yan T, Criddle CS, Jardine PM, Zhou J, *et al* (2007). Influence of bicarbonate, sulfate, and electron donors on biological reduction of uranium and microbial community composition. Appl. Microbiol. Biotechnol. 77:713–721.

Mackenzie R, Pedrós-Alió C, Díez B. (2013). Bacterial composition of microbial mats in hot springs in Northern Patagonia: variations with seasons and temperature. Extremophiles 17:123–136.

Magnuson TS, Isoyama N, Hodges-Myerson AL, Davidson G, Maroney MJ, Geesey GG, *et al* (2001). Isolation, characterization and gene sequence analysis of a membrane-associated 89 kDa Fe(III) reducing cytochrome *c* from *Geobacter sulfurreducens.* Biochemical Journal 359:147–152.

Mahadevan R, Bond DR, Butler JE, Esteve-Núñez A, Coppi MV, Palsson BO, *et al* (2006). Characterization of metabolism in the Fe(III)-reducing organism *Geobacter*

sulfurreducens by constraint-based modeling. Appl. Environ. Microbiol. 72:1558–1568.

Marshall CW, May HD. (2009). Electrochemical evidence of direct electrode reduction by a thermophilic Gram-positive bacterium, *Thermincola ferriacetica*. Energy Environ. Sci. 2:699.

Marsili E, Baron DB, Shikhare ID, Coursolle D, Gralnick JA, Bond DR. (2008a). *Shewanella* Secretes flavins that mediate extracellular electron transfer. Proc. Natl. Acad. Sci. U S A 105:3968–3973.

Marsili E, Rollefson JB, Baron DB, Hozalski RM, Bond DR. (2008b). Microbial biofilm voltammetry: direct electrochemical characterization of catalytic electrode-attached biofilms. Appl. Environ. Microbiol. 74:7329–7337.

Marsili E, Sun J, Bond DR. (2010). Voltammetry and growth physiology of *Geobacter sulfurreducens* biofilms as a function of growth stage and imposed electrode potential. Electroanalysis 22:865–874.

Martinez-Canamero M, Munoz-Dorado J, Farez-Vidal E, Inouye M, Inouye S. (1993). Oar, a 115-kilodalton membrane protein required for development of *Myxococcus xanthus*. J Bacteriol 175:4756–4763.

Masuda M, Freguia S, Wang Y-F, Tsujimura S, Kano K. (2010). Flavins contained in yeast extract are exploited for anodic electron transfer by *Lactococcus lactis*. Bioelectrochemistry 78:173–175.

Männistö MK, Kurhela E, Tiirola M, Häggblom MM. (2013). Acidobacteria dominate the active bacterial communities of Arctic tundra with widely divergent winter-time snow accumulation and soil temperatures. FEMS Microbiol. Ecol. 84:47–59.

Männistö MK, Tiirola MM, Häggblom MM. (2009). Effect of freeze-thaw cycles on bacterial communities of arctic tundra soil. Microb. Ecol. 58:621–631.

Männistö MK, Tiirola MM, Häggblom MM. (2007). Bacterial communities in Arctic fjelds of Finnish Lapland are stable but highly pH-dependent. FEMS Microbiol. Ecol. 59:452–465.

McBeth JM, Lear G, Lloyd JR, Livens FR, Morris K, Burke IT. (2007). Technetium reduction and reoxidation in aquifer sediments. Geomicrobiol. J 24:189–197.

Mehta T, Coppi MV, Childers SE, Lovley DR. (2005). Outer membrane *c*-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. Appl. Environ. Microbiol. 71:8634–8641.

Mehta-Kolte MG, Bond DR. (2012). *Geothrix fermentans* secretes two different

redox-active compounds to utilize electron acceptors across a wide range of redox potentials. Appl. Environ. Microbiol. 78:6987–6995.

Meisinger DB, Zimmermann J, Ludwig W, Schleifer KH, Wanner G, Schmid M, et al (2007). In situ detection of novel Acidobacteria in microbial mats from a chemolithoautotrophically based cave ecosystem (Lower Kane Cave, WY, USA). Environ. Microbiol. 9:1523–1534.

Methe BA. (2003). Genome of *Geobacter sulfurreducens*: Metal reduction in subsurface environments. Science 302:1967–1969.

Miyahara M, Hashimoto K, Watanabe K. (2013). Use of cassette-electrode microbial fuel cell for wastewater treatment. J Biosci. Bioeng. 115:176–181.

Myers CR, Myers JM. (2003). Cell surface exposure of the outer membrane cytochromes of *Shewanella oneidensis* MR-1. Lett. Appl. Microbiol. 37:254–258.

Myers CR, Myers JM. (1993). Ferric reductase is associated with the membranes of anaerobically grown *Shewanella putrefaciens* MR-1. FEMS Microbiol. Lett. 108:15–22.

Myers CR, Myers JM. (1992). Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1. J Bacteriol 174:3429–3438.

Myers CR, Nealson KH. (1990). Respiration-linked proton translocation coupled to anaerobic reduction of manganese (IV) and iron (III) in *Shewanella putrefaciens* MR-1. J Bacteriol 172:6232–6238.

Myers CRC, Myers JMJ. (1997). Cloning and sequence of *cymA*, a gene encoding a tetraheme cytochrome c required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens* MR-1. J Bacteriol 179:1143–1152.

Nevin KP, Lovley DR. (2000). Lack of production of electron-shuttling compounds or solubilization of Fe (III) during reduction of insoluble Fe (III) oxide by *Geobacter metallireducens*. Appl. Environ. Microbiol. 66:2248–2251.

Nevin KP, Lovley DR. (2002). Mechanisms for accessing insoluble Fe (III) oxide during dissimilatory Fe (III) reduction by *Geothrix fermentans*. Appl. Environ. Microbiol. 68:2294–2299.

Okamura K, Kawai A, Yamada T, Hiraishi A. (2011). *Acidipila rosea* gen. nov., sp. nov., an acidophilic chemoorganotrophic bacterium belonging to the phylum *Acidobacteria*. FEMS Microbiol. Lett. 317:138–142.

Paquete CM, Louro RO. (2010). Molecular details of multielectron transfer: the case of multiheme cytochromes from metal respiring organisms. Dalton transactions 39:4259–4266.

Parameswaran P, Zhang H, Torres CI, Rittmann BE, Krajmalnik-Brown R. (2010). Microbial community structure in a biofilm anode fed with a fermentable substrate: the significance of hydrogen scavengers. Biotechnol. Bioeng. 105:69–78.

Phillips E, Lovley DR. (1987). Determination of Fe(III) and Fe(II) in oxalate extracts of sediments. Soil Science Society of America 51:938–941.

Portillo MC, Gonzalez JM, Saiz-Jimenez C. (2008). Metabolically active microbial communities of yellow and grey colonizations on the walls of Altamira Cave, Spain. J. Appl. Microbiol. 104:681–691.

Powell DR, Seemann T. (2013). VAGUE: a graphical user interface for the Velvet assembler. Bioinformatics 29:264–265.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, *et al* (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196.

Quaiser A, López-García P, Zivanovic Y, Henn MR, Rodriguez-Valera F, Moreira D. (2008). Comparative analysis of genome fragments of Acidobacteria from deep Mediterranean plankton. Environ. Microbiol. 10:2704–2717.

Rawat SR, Männistö MK, Bromberg Y, Häggblom MM. (2012). Comparative genomic and physiological analysis provides insights into the role of Acidobacteria in organic carbon utilization in Arctic tundra soils. FEMS Microbiol. Ecol. 82:341–355.

Reguera G, McCarthy KD, Mehta T, Nicoll JS, Tuominen MT, Lovley DR. (2005). Extracellular electron transfer via microbial nanowires. Nature 435:1098–1101.

Richardson DJ, Butt JN, Clarke TA. (2013). Controlling electron transfer at the microbe-mineral interface. Proc. Natl. Acad. Sci. U S A 110:7537–7538.

Richardson DJ, Edwards MJ, White GF, Baiden N, Hartshorne RS, Fredrickson J, *et al* (2012a). Exploring the biochemistry at the extracellular redox frontier of bacterial mineral Fe(III) respiration. Biochem. Soc. Trans. 40:493–500.

Richardson DJD, Butt JNJ, Fredrickson JKJ, Zachara JMJ, Shi LL, Edwards MJM, *et al* (2012b). The 'porin-cytochrome' model for microbe-to-mineral electron transfer. Mol. Microbiol. 85:201–212.

Rodríguez-Celma J, Vázquez-Reina S, Orduna J, Abadía A, Abadía J, Álvarez-Fernández A, *et al* (2011). Characterization of flavins in roots of Fe-deficient strategy I plants, with a focus on *Medicago truncatula*. Plant & Cell Physiol. 52:2173–2189.

Rollefson JB, Levar CE, Bond DR. (2009). Identification of genes involved in

biofilm formation and respiration via Mini-Himar transposon mutagenesis of *Geobacter sulfurreducens*. J Bacteriol 191:4207–4217.

Rollefson JB, Stephen CS, Tien M, Bond DR. (2011). Identification of an extracellular polysaccharide network essential for cytochrome anchoring and biofilm formation in *Geobacter sulfurreducens*. J Bacteriol 193:1023–1033.

Rooney-Varga JN, Anderson RT, Fraga JL, Ringelberg D, Lovley DR. (1999). Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. Appl. Environ. Microbiol. 65:3056–3063.

Ross DE, Brantley SL, Tien M. (2009). Kinetic characterization of OmcA and MtrC, terminal reductases involved in respiratory electron transfer for dissimilatory iron reduction in *Shewanella oneidensis* MR-1. Appl. Environ. Microbiol. 75:5218–5226.

Ross DE, Flynn JM, Baron DB, Gralnick JA, Bond DR. (2011). Towards electrosynthesis in *Shewanella*: energetics of reversing the mtr pathway for reductive metabolism. PLoS One 6:e16649.

Ross DE, Ruebush SS, Brantley SL, Hartshorne RS, Clarke TA, Richardson DJ, et al (2007). Characterization of protein-protein interactions involved in iron reduction by *Shewanella oneidensis* MR-1. Appl. Environ. Microbiol. 73:5797–5808.

Sait M, Hugenholtz P, Janssen PH. (2002). Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ. Microbiol. 4:654–666.

Saltikov CW, Newman DK. (2003). Genetic identification of a respiratory arsenate reductase. Proc. Natl. Acad. Sci. U S A. 100:10983-10988.

Schloss PD, Handelsman J. (2006). Introducing SONS, a tool for operational taxonomic unit-based comparisons of microbial community memberships and structures. Appl. Environ. Microbiol. 72:6773–6779.

Schloss PDP, Handelsman JJ. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. 71:1501–1506.

Shi L, Richardson DJ, Wang Z, Kerisit SN, Rosso KM, Zachara JM, et al (2009). The roles of outer membrane cytochromes of *Shewanella* and *Geobacter* extracellular electron transfer. Environ. Microbiol. Reports 1:220–227.

Shi L, Rosso KM, Zachara JM, Fredrickson JK. (2012). Mtr extracellular electron-transfer pathways in Fe(III)-reducing or Fe(II)-oxidizing bacteria: a genomic perspective. Biochem. Soc. Trans. 40:1261–1267.

Shi L, Squier TC, Zachara JM, Fredrickson JK. (2007). Respiration of metal

(hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem *c*-type cytochromes . Mol. Microbiol. 65:12–20.

Smith SM, Abed RMM, Gercia-Pichel F. (2004). Biological soil crusts of sand dunes in Cape Cod National Seashore, Massachusetts, USA. Microb. Ecol. 48:200–208.

Snoeyenbos-West OL, Nevin KP, Anderson RT. (2000). Enrichment of *Geobacter* Species in Response to Stimulation of Fe(III) Reduction in Sandy Aquifer Sediments - Springer. Microb. Ecol.39:153–167.

Tender LM, Gray SA, Groveman E, Lowy DA, Kauffman P, Melhado J, et al (2008). The first demonstration of a microbial fuel cell as a viable power supply: Powering a meteorological buoy. J Power Sources 179:5–5.

Thomas PE, Ryan D, Levin W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Analytical Biochemistry 75:168–176.

Thomson RH. (1991). Distribution of naturally occuring quinones. Pharmaceutisch Weekblad Scientific edition 13(2):70-73.

Thrash JC, Coates JD. (2010). Phylum XVII. Acidobacteria phyl. nov. In:Bergey's Manual® of Systematic Bacteriology, Springer New York: New York, NY, pp. 725–735.

Tor JM, Kashefi K, Lovley DR. (2001). Acetate oxidation coupled to Fe(III) reduction in hyperthermophilic microorganisms. Appl. Environ. Microbiol. 67:1363–1365.

Torres CI, Krajmalnik-Brown R, Parameswaran P, Marcus AK, Wanger G, Gorby YA, et al (2009). Selecting anode-respiring bacteria based on anode potential: phylogenetic, electrochemical, and microscopic characterization. Environ. Sci. Technol. 43:9519–9524.

Torres CI, Marcus AK, Lee H-S, Parameswaran P, Krajmalnik-Brown R, Rittmann BE. (2010). A kinetic perspective on extracellular electron transfer by anode-respiring bacteria. FEMS Microbiol. Reviews 34:3–17.

Torres CI, Marcus AK, Parameswaran P, Rittmann BE. (2008). Kinetic experiments for evaluating the Nernst-Monod model for anode-respiring bacteria (ARB) in a biofilm anode. Environ. Sci. Technol. 42:6593–6597.

Tritt AA, Eisen JAJ, Facciotti MTM, Darling AEA. (2012). An integrated pipeline for de novo assembly of microbial genomes. PLoS One 7:e42304–e42304.

van Opijnen T, Bodi KL, Camilli A. (2009). Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nat.

Methods 6:767–772.

Vick JE, Johnson ET, Choudhary S, Bloch SE, Lopez-Gallego F, Srivastava P, et al (2011). Optimized compatible set of BioBrick™ vectors for metabolic pathway engineering. Appl. Microbiol. Biotechnol. 92:1275–1286.

Vishnivetskaya TA, Brandt CC, Madden AS, Drake MM, Kostka JE, Akob DM, et al (2010). Microbial Community Changes in Response to Ethanol or Methanol Amendments for U(VI) Reduction. Appl. Environ. Microbiol. 76:5728–5735.

Wang X, Cheng S, Feng Y, Merrill MD, Saito T, Logan BE. (2009). Use of carbon mesh anodes and the effect of different pretreatment methods on power production in microbial fuel cells. Environ. Sci. Technol. 43:6870–6874.

Wang Y, Kern SE, Newman DK. (2010). Endogenous Phenazine Antibiotics Promote Anaerobic Survival of *Pseudomonas aeruginosa* via Extracellular Electron Transfer. J Bacteriol 192:365–369.

Wang Y, Newman DK. (2008). Redox reactions of phenazine antibiotics with ferric (hydr)oxides and molecular oxygen. Environ. Sci. Technol. 42:2380–2386.

Ward NL, Challacombe JF, Janssen PH, Henrissat B, Coutinho PM, Wu M, *et al* (2009). Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. Appl. Environ. Microbiol. l75:2046–2056.

Weber KA, Achenbach LA, Coates JD. (2006). Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. Nat. Rev. Micro. 4:752–764.

Weltzer ML, Miller SR. (2013). Division-Specific Differences in Bacterial Community Assembly of Two Alkaline Hot Spring Ecosystems from Yellowstone National Park. Microb. Ecol.DOI 10.1007/s00248-013-0214-3.

Wilkins MJ, Callister SJ, Miletto M, Williams KH, Nicora CD, Lovley DR, *et al* (2010). Development of a biomarker for *Geobacter* activity and strain composition; Proteogenomic analysis of the citrate synthase protein during bioremediation of U(VI). Microbial Biotechnol. 4:55–63.

Wilkins MJ, VerBerkmoes NC, Williams KH, Callister SJ, Mouser PJ, Elifantz H, et al (2009). Proteogenomic monitoring of *Geobacter* physiology during stimulated uranium bioremediation. Appl. Environ. Microbial.75:6591–6599.

Wrighton KC, Agbo P, Warnecke F, Weber KA, Brodie EL, DeSantis TZ, et al (2008). A novel ecological role of the Firmicutes identified in thermophilic microbial fuel cells. ISME J 2:1146–1156.

Wrighton KC, Thrash JC, Melnyk RA, Bigi JP, Byrne-Bailey KG, Remis JP, et al

(2011). Evidence for direct electron transfer by a gram-positive bacterium isolated from a microbial fuel cell. Appl. Environ. Microbiol. 77:7633–7639.

Wu W-M, Carley J, Luo J, Ginder-Vogel M, Cardenas E, Leigh MB, et al (2007). In situ bioreduction of uranium (VI) to submicromolar levels and reoxidation by dissolved oxygen. Environ. Sci. Technol. 41:5716–5723.

Xing D, Zuo Y, Cheng S, Regan JM, Logan BE. (2008). Electricity generation by *Rhodopseudomonas palustris* DX-1. Environ. Sci. Technol. 42:4146–4151.

Yamazaki S, Kaneko T, Taketomo N, Kano K, Ikeda T. (2002). 2-Amino-3-carboxy-1,4-naphthoquinone affects the end-product profile of bifidobacteria through the mediated oxidation of NAD(P)H. Appl. Microbiol. Biotechnol. 59:72–78.

Yun J, Ueki T, Miletto M, Lovley DR. (2011). Monitoring the metabolic status of *Geobacter* species in contaminated groundwater by quantifying key metabolic proteins with *Geobacter*-specific antibodies. Appl. Environ. Microbiol. 77:4597–4602.

Appendix I: The Effect of Inoculum Source on the Structure and Function of Bacterial Communities in Microbial Fuel Cells

I.1 Overview

This project was done as collaboration with a civil engineering student Chris Harrington. He was responsible for microbial fuel cell (MFC) design, assembling, operation, and volatile fatty acid measurement. This chapter describes the bacterial diversity studies perfomed on microbial fuel cells inoculated with sediments from three different freshwater sites and two different waste streams to test the hypothesis that the source of inoculum affects the performance of microbial fuel cells. Microbial fuel cells were operated for a month and at the end of each experiment the electrodes were harvested and the attached microbial communities were identified by 16S rDNA clone library analysis.

I.2 Introduction

A microbial fuel cell (MFC) is a device that converts chemical energy in to electric energy by using microorganisms as catalysts. The observation that microorganisms can produce electricity dates back to the early 1900. It has regained significant interest in the recent times as it helps run self-sufficient wastewater treatment plants and provides renewable power sources. In a MFC, the microorganism oxidizes organic compounds and transfers the electrons that are generated in metabolic reactions to the electrode that acts as a terminal electron acceptor, there by generating electrical energy (Logan 2012).

Bacteria capable of electron transfer to electrodes come from a variety of phylogenetic groups. Major phylogenetic classifications that contain electrode-reducing bacteria are the α-Proteobacteria (*Ochrobactrum* and *Rhodopseudomonas*),

β-Proteobacteria (*Rhodoferax*), γ-Proteobacteria (*Aeromonas, Escherichia, Pseudomonas* and *Shewanella*), δ-Proteobacteria (*Desulfobulbus, Desulfuromonas, Geobacter, Geoalkalibacter,* and *Geopsychrobacter*), Firmicutes (*Clostridium,* and *Thermincola*) and Acidobacteria (*Geothrix fermentans*) (Logan 2008).

The aim of this research was to examine the effect of different microbial consortia on the performance of microbial fuel cells. Therefore, sediments from five distinct environments were inoculated into MFCs run under identical conditions for one month. Current, potential, and power were monitored continuously in these reactors. Periodically, the volatile fatty acid concentrations were measured and the electron transfer process was analyzed via cyclic voltammetry. At the end of the experiments, the structures of the microbial communities were determined by PCR-cloning of 16S rDNA genes.

I.3 Materials and Methods

I.3.1 MFC inoculation

Samples used for MFCs inoculation were collected from three different wetlands and two waste streams. Wetlands such as Iron Springs Bog and Cedar Lake Bog were selected, as these sites are unique pristine evironments with high iron concentrations. The Minnesota River Valley sediment was choosen to represent a heavly-contaminated site. The waste stream inoculation samples come from an anaerobic digester sludge collected from Empire Wastewater Treatment Plant and the dairy cow waste was collected from the Dairy Barn at the University of Minnesota. MFCs were inoculated with the sediments and incubated for a month and

the electrochemical analyses were performed. Additionally, the microbial diversity of Iron Springs Bog sediment was also analyzed as the reference bog sediment; in order to compare the total microbial diversity of the sediment to MFC enriched population.

I.3.1 Bacterial community analysis, DNA extraction, and amplification

The microbial community composition of reference bog sediment and the electrode-attached community from each reactor was determined via cloning and sequencing of the small subunit rRNA. The electrode was removed with sterile forceps, cut into four ~6 cm^2 pieces using sterile scissors and immediately placed at -20^oC for storage. Genomic DNA was extracted from one section of electrode using the Mo Bio Power Soil DNA kit (Mo-Bio Laboratories, Carlsbad, CA). 0.5 grams of reference bog sediment was used for genomic DNA extraction using the same kit.

PCR of the nearly complete 16S rRNA genes was performed using universal bacterial primers 27 forward (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492 reverse (5′-TAC GGY TCA CTT GTT ACG ACT T-3′) at concentrations of 1 μM. DNA was amplified with GoTaq Green Master Mix (Promega, Madison, WI). PCR cycling conditions were as follows: 2 min, 95^oC, followed by 30 cycles of 1 min, 95^oC, 1 min, 52^oC, 1 min, 72^oC, and a final extension time of 10 min at 72^oC. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA).

I.3.2 Clone library preparation

DNA cloning was performed using purified PCR products using an Invitrogen TOPO TA Cloning Kit for sequencing with pCR 4-TOPO Vector (Invitrogen Corporation, Carlsbad, CA). Plasmids were introduced into Invitrogen TOP10 electrocompetent cells. Electroporation of *E. coli* cells was performed at 1.8 kV for 3.6 ms in an electroporator (Bio-Rad, Hercules, CA). *E. coli* cells were subsequently grown on LB plates supplemented with 50 μg/ml kanamycin. Individual colonies were picked randomly from each plate and placed into 96 well microtiter plates containing 1.5 mL LB with 50 μg/ml kanamycin. Plates were then incubated for 24 hours at 37^{o}C. Cells were then pelleted, plasmids were extracted and then sequenced at the University of Minnesota Genomic Center Core Facility. Plasmid DNA was extracted using a kit (Qiagen, Valencia, CA). Plasmid inserts were sequenced using a 96 capillary DNA analyzer (ABI 3730xl, Applied Biosystems, Foster City, CA) with big dye version 3.1. Primer 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′) was used as the sequencing primer, yielding sequences of approximately 880 base pairs for phylogenetic analysis.

I.3.3 Phylogenetic alignment and clustering of 16S rRNA gene fragments

The 16S rRNA gene fragments were aligned and classified using the Ribosomal Database Project (RDP) release 9 (Cole *et al* 2009). Vectors sequences were removed with LUCY and base calling was done with PHRED in RDP. RNACAD, secondary-structure based aligner was used to align the library sequences, and the sequences were classified using the RDP Naive Bayesian

Classifier. 16S sequence artifacts such as chimeric sequences were examined by using RDP Chimera Detection. High quality library sequences were obtained from RDP to perform the multiple sequence alignment using the ClustalX alignment software. Based on this alignment, a distance matrix was generated using DNAdist from PHYLIP version 3.68 using the Jukes Cantor distance correction method. The generated pairwise distances served as input for Distance-Based operational taxonomical unit (OTU) and Richness (DOTUR) for clustering the sequences into OTUs of defined sequence similarity, using the furthest-neighbor algorithm (Schloss & Handelsman 2005). The clusters ranged from 100% to 80% similarity, and served as OTUs for generating rarefaction curves. Community similarities were estimated using the SONS (Shared OTUs and similarity), which uses the OTU data generated from DOTUR. SONS calculates abundance distribution and richness of OTUs shared between communities using a non-parametric estimator (Schloss & Handelsman 2006).

Sequences were aligned using the ARB/SILVA rRNA aligner (http://www.arb-silva.de/aligner/) and inserted into a reference 16S small subunit guide tree (SSU Silva 96 release) using the ARB parsimony insertion tool (Pruesse *et al* 2007). Misalignments were manually corrected in ARB using the closest relative sequence as a reference. Neighbor-joining, and maximum likelihood phylogenetic trees using Jukes-Cantor-corrected distances and DNA parsimony trees were constructed using the ARB software. Phyml tree was generated using various distance correction methods to have a most robust/confident tree, irrespective of the correction method used.

I.4 Results

Libraries of PCR amplified 16S rDNA gene sequences were obtained from the electrode of the MFC from each inoculum and the reference bog wetland sediment. Estimated OTU richness and diversity index for the 16S rDNA libraries generated from the bog sediment or the MFC encrichements were calculated using DOTUR. Rarefaction curves generated at the 97% similarity indicated that the sample size of 79 to 91 clones was enough to cover almost all the OTUs in the enriched MFCs. As Figure I.1 shows the curves becoming flat, suggesting a reasonable number of individual sequences has been sampled and more sampling will probably yield only a small number of additional species. However, the sample size of 100 clones obtained from the wetland sediment was clearly not enough to detect all the OTUs present. As Figure I.1 shows a steep slope for the reference bog sediment, indicating that the species diversity is not fully sampled.

The relative abundance of each phylum from all the MFCs were analyzed and compared. Majority of the δ-Proteobacteria comprised anywhere from 19-52% of the clones in all of the high-powered reactors, most of which were *Geobacter* species. Most of the *Geobacter* species were from subsurface clade 2 and comprised 12 to 44% of the clones (Figure. I.2). The dairy cow waste-inoculated reactor did not contain any clones similar to *Geobacter* species and only 1% of the clones represented the δ-Proteobacteria.

In the current producing reactors, bacteria from the β-Proteobacteria (9-37% of clones) and *Bacteriodetes* (7-26% of clones) were also detected (Figure I.2), but no single OTU was abundant (> 1 clone) in three or more reactors. In the dairy cow

waste-inoculated reactor, β-Proteobacteria and *Bacteriodetes* comprised 62% and 16% of the clones, respectively (Figure I.2). Major OTUs of ε-Proteobacteria were also detected in the MFCs inoculated with Cedar Bog Lake sediment, Minnesota River Valley sediment and dairy cow waste. The fact that these bacteria were also present in the dairy cow waste-inoculated reactor suggests that bacteria in this group are not most likely responsible for current production. Bacteria from the phyla *Firmicutes* were detected at low numbers (2-11% of the clones) in all of the MFC clone libraries except the MFC inoculated with Minnesota River Valley sediment, where it comprised 33% of the clone library (Figure I.2).

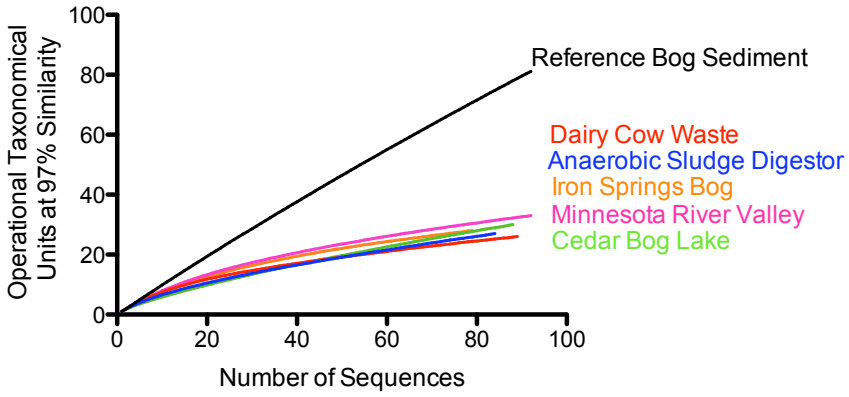

Figure I.1 Rarefaction curve of annotated species richness as a function of the number of sequences sampled for all MFC enrichments and the bog sediment.

Several other groups of bacteria were identified at low numbers in the clone libraries. γ-Proteobacteria were detected (< 6% of the clones) in all but the MFC inoculated with Minnesota River Valley sediment. Bacteria from the phyla

Acidobacteria were detected in three of the MFCs (1-8% of the clones), and bacteria from the phyla *Chlorobia*, *Lentisphaerae*, and *Verrucomicrobia* were found in some libraries (< 3 % of the clones).

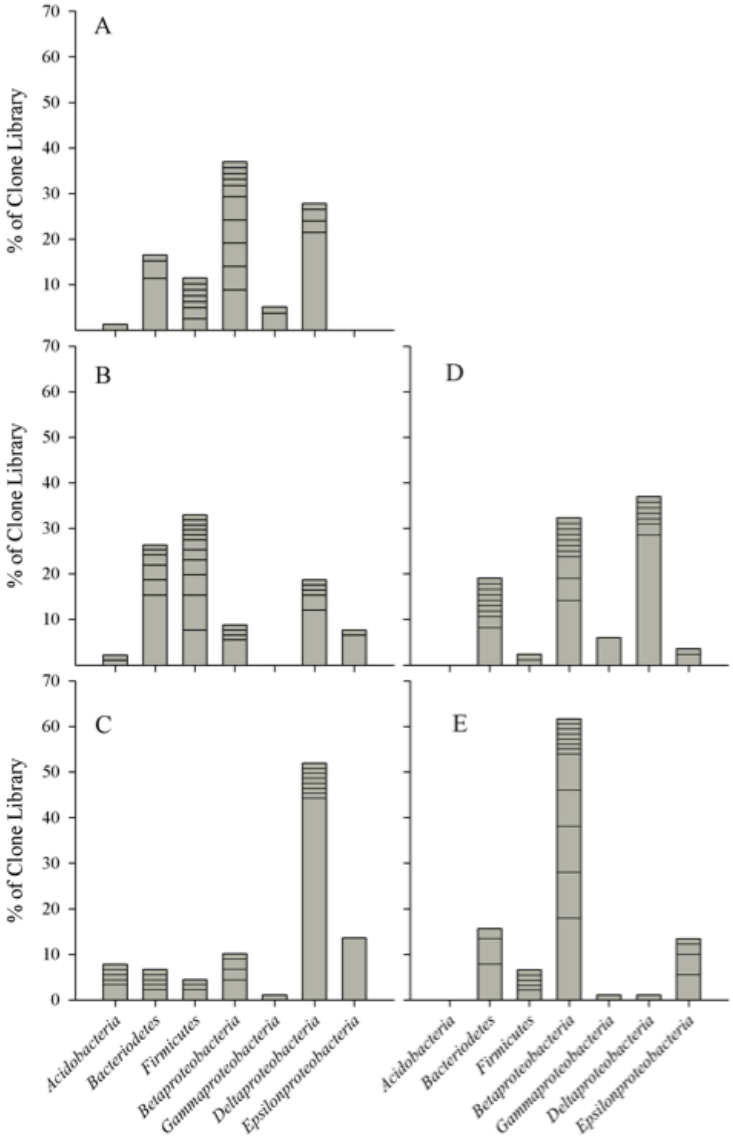

Figure I.2 Microbial community distribution for each MFC at 97% sequence similarity. (A) Iron Springs Bog wetland sediment, (B) Minnesota River Valley wetland sediment, (C) Cedar Bog Lake wetland sediment, (D) Anaerobic digester sludge, (E) Dairy cow waste

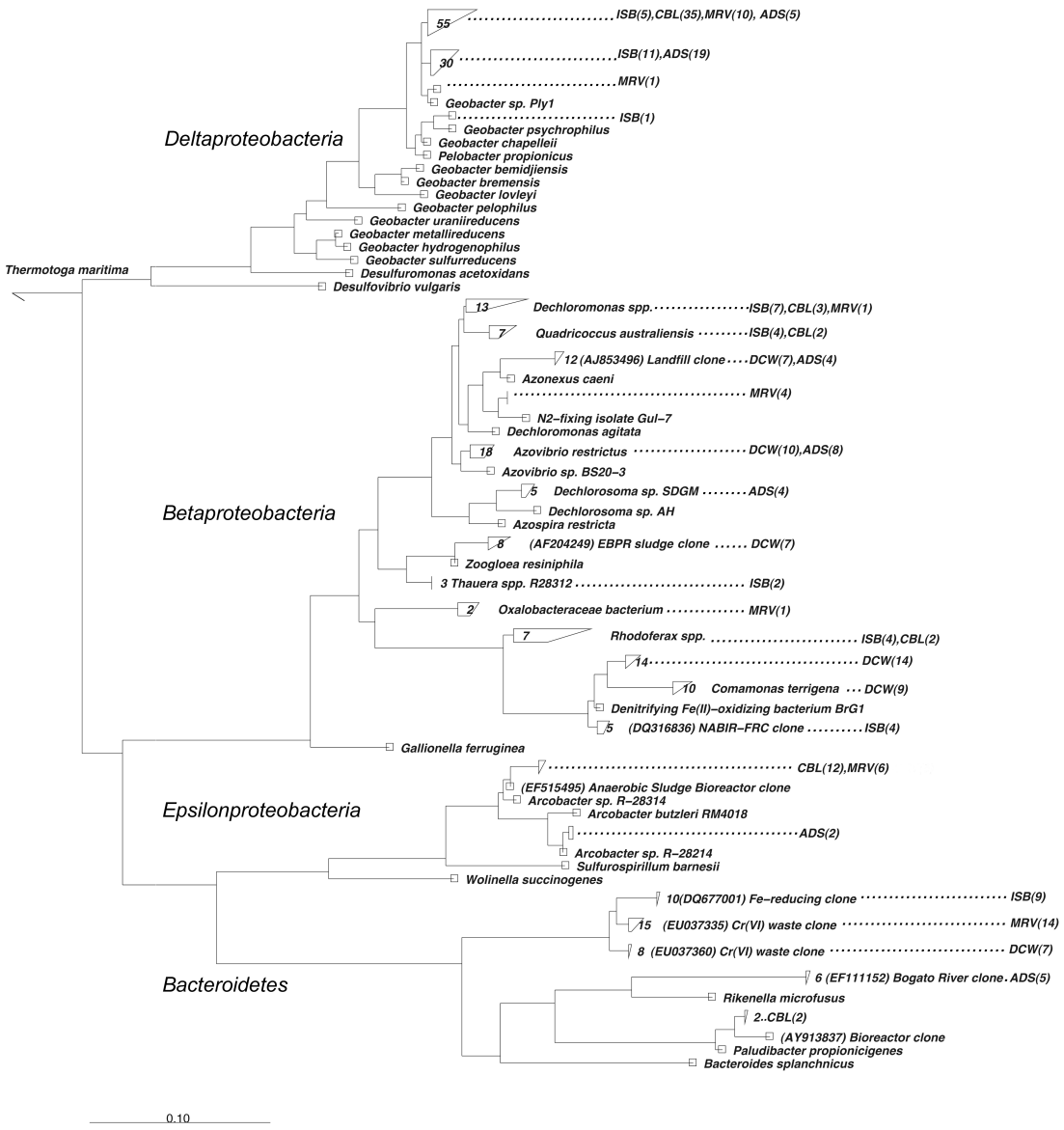

Figure I.3 Phylogenetic tree of the dominant OTUs present among three current producing libraries. Nodes were collapsed at the 95% similarity level.

I.5 Summary

Constructed clone libraries from the reactors had reached saturation in coverage for species richness and diversity based on rarefaction curves. The microbial consortium enriched in this study was selective and dominated by *Geobacter* species belonging to the subsurface clade 2. Bacteria from this clade were observed in high numbers in all of the current-producing reactors, but absent from the non-current producing reactor. Diversity indices like the Shannon index suggested that the diversity was the highest in the bog sediment sample and lower in the enriched reactors. Lowest Shannon index was observed for reactors inoculated with Cedar bog lake sediment, in which *Geobacter* sequences represented more than half of all the sequenced clones for that library. These results indicate that further study of *Geobacter* species from subsurface clade 2 is warranted as most of our knowledge about *Geobacter* species comes from laboratory research performed with bacteria from the *G. metallireducens* clade, more specifically *G. sulfurreducens*.

Appendix II: Development of Transposon Mutagenesis Combined with High-throughput Sequencing (Tn-Seq) in *Geobacter sulfurreducens*

II.1 Overview

A standard approach to identify genes that are involved for survival under certain conditions is to screen large pools of mutants under those conditions. One way to generate mutants is by using genetic elements such as transposons, which can insert within genomes by a cut and paste or via a replicative mechanism. Traditional transposon mutagenesis techniques coupled with high-throughput sequencing is referred to as transposon sequencing (Tn-Seq) (van Opijnen *et al* 2009). Tn-Seq allows large-scale quantitative phenotypic profiling of any microbial species in which transposon mutagenesis can be applied. Tn-Seq has been successfully used to identify gene function in a high-throughput manner (Deutschbauer *et al* 2011) and to identify essential genes for growth under specific conditions, for example: identification of a novel dynamic branching of the anaerobic tricarboxylic acid cycle in *S. oneidensis* (Brutinel & Gralnick 2012).

II.2 Introduction

G. sulfurreducens is a highly abundant Fe(III)-reducing organism found in many anaerobic, neutral pH subsurface environments. It can grow by complete oxidation of a carbon source coupled to the reduction of a variety of different electron acceptors such organic acids, chelated forms of Fe(III) as well as insoluble metal oxides like, Fe(III)-oxides and also poised electrodes (Lovley 2012).

The molecular mechanisms for metal-reduction by *Geobacter* species have been extensively studied over the last decade. Gene products required for attachment, biofilm development, type IV pili, and also redox active proteins such as *c*-type

cytochromes have been identified to be required for metal reduction. A variety of different approaches have been used to understand this process of electron transfer to the extracellular acceptors. These include targeted mutagenesis (Coppi *et al* 2001), transposon mutagenesis (Rollefson *et al* 2009), microarray (Aklujkar *et al* 2013), as well as biochemical and proteomic analysis (Ding *et al* 2006). These traditional genetic screens based on the growth/condition selected give rise to different mutant phenotypes, but are laborious to perform and have many limitations.

The Tn-Seq approach is a high throughput method for determining the fitness of an individual mutant in a population on a genome-wide scale. This method is based on the construction of a saturated transposon library in which the resulting mutant population is pooled and subjected to different growth conditions. Genomic DNA from the parent pooled mutant population and from the different growth conditions tested are extracted and fragmented by restriction digest or by physical shearing. Sequencing adaptors are then ligated and the resulting fragments are subsequently enriched by PCR with transposon and adaptor specific primers. The location and frequency of each transposon insertion is then determined by sequencing the flanking regions and mapping the fragments back to the reference genome to identify the unique insertion sites. For example, the number of sequence reads for a particular insertion corresponds to the frequency of that insertion mutant in the population. The change in insertion frequency after selection correlates with the fitness effect of the insertion mutation during the selection (van Opijnen *et al* 2009). Therefore, if the frequency of a particular insertion does not change, then it indicates that the gene with the transposon insertion is neutral and hence does not

have a fitness cost associated under the condition tested. Decrease in the insertions frequency of a gene indicates that it is essential under those conditions. Conversely, increase in the insertion frequency indicates that disruption of the gene is beneficial for the conditions tested.

In this project, the Tn-Seq technique was optimized for *G. sulfurreducens* using a modified pMiniHimar RB1-pEB001 (Brutinel & Gralnick 2012) to identify genes that are required for growth with soluble iron, such as Fe(III)-citrate as the terminal electron acceptor, compared to growth with an organic acid such as fumarate. This technique can also be used to identify genes that are essential for *G. sulfurreducens* growth under any given condition, for example: genes required for electrode growth which would involve electrode colonization (attachment), biofilm development and long range electron transfer/respiration.

II.3 Materials and Methods

II.3.1 Library preparation

Wild type *G. sulfurreducens* was used in filter mating experiments with *E. coli* WM3064 carrying pEB001, obtained from the Gralnick lab (Brutinel & Gralnick 2012) following the standard *Geobacter*/*E.coli* mating procedure (Rollefson *et al* 2009). After growth the resulting transposon mutants were scraped off the plates and collected in minimal medium with 40 mM fumarate and 20 mM acetate with 200 μg/ml of kanamycin and immediately frozen at -80°C in 1 ml aliquots with 10% DMSO (This step of making a saturating transposon library was performed by Caleb Levar).

II.3.2 Library outgrowth under selective conditions

One vial of the library from the -80°C stock was thawed and inoculated into 10 ml of pre-warmed minimal medium with acetate and fumarate (NBFA) as electron donor and acceptor containing kanamycin (200 μg/ml) after 8 hours, transfer all 10 ml to a 100 ml NBFA bottle containing kanamycin. The culture was grown until a 0.2 optical density was achieved, this is the parent population named -T1. This culture was then transferred by back dilution so as to get at least 4-5 doublings (1:10 dilution for fumarate and 1:20 dilution for ferric citrate) into 100 ml of pre-warmed minimal medium containing acetate as an electron donor with either fumarate (T2F) or ferric citrate (T2FC) as the terminal electron acceptor. Cells from T1 were collected by centrifugation and the pellets were store at -20°C.

Growth was monitored by OD_{600} measurements for fumarate cultures (T2F), and Fe(II) accumulation, a proxy for growth, was monitored for ferric citrate cultures (T2FC) by performing a ferrozine assay every 8 hours. Once the cultures achieved 4 doubling (about ~0.32-0.35 OD and~ 40 mM Fe(II)) as in Figure II.1. The cells were harvested by centrifugation and genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega), as described in Rollefson *et al* 2009 (Rollefson et al 2009). DNA was quantified by the Qubit assay, and the yield obtained was any where between 560 μg/ml to 990 μg/ml for ferric citrate and fumarate grown cells respectively from 100 ml of culture.

II.3.3 Library preparation for sequencing

Library preparation for sequencing was prepared by first digesting the genomic DNA with MmeI restriction enzyme. MmeI is a type IIS restriction endonuclease that generates a 2 bp staggered cut 20 bases downstream of its recognition site, thereby generating fragments of transposon plus 16 bp of flaking genomic DNA. This 16 bp is unique enough to accurately identify the location of the transposon insertion. After digestion, a unique 4 bp barcoded adaptors are ligated to the transposon-genomic DNA fragments. The addition of a unique barcode allows many different samples to be mixed into a single lane for sequencing. These fragments are then PCR amplified using transposon and adaptor-specific primers, followed by isolation of the 120 bp PCR product by agarose gel purification. This purified product is then sent for single read 50 bp Illumina sequencing. II.5 describes a detailed protocol for the library preparation and II.6 explains the primers used for amplification and Illumina sequencing.

II.4 Results

After Illumina sequencing, all raw sequence reads were processed using Galaxy (Giardine 2005). All original unprocessed data were stored on the Drobo under “Misha Sequences” in TNSEQ folder. For Galaxy processing of the data, perform the following steps- (1) Groom the data to fastqsanger format, (2) Trim the transposon, (3) Reverse complement the library, (4) Sort the library for barcodes, (5) Map with Bowtie for Illumina, (6) Assign insertion locations, and (7) Parse the genome for insertion location within genes.

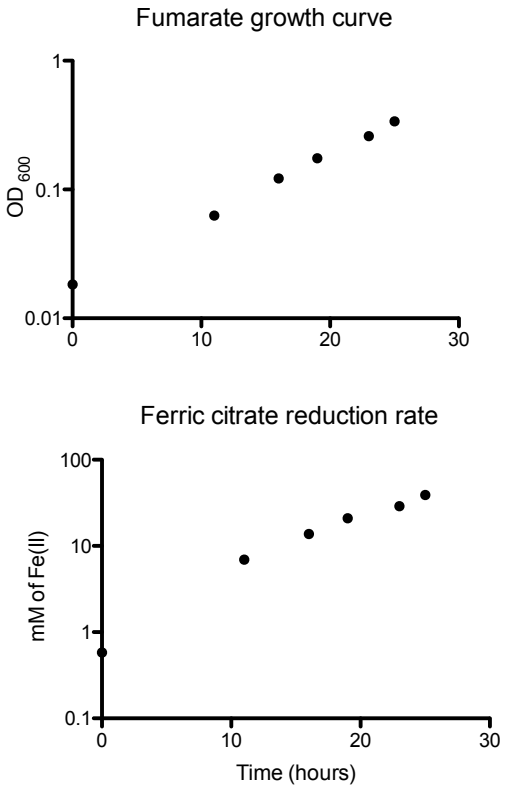

Figure II.1 An example of outgrowth of the parent library under the two conditions tested-fumarate and Fe(III)-citrate. Under fumarate reducing conditions the growth rate (μ) was 0.165 h^{-1} and for Fe(III)-citrate reducing conditions the μ was 0.17 h^{-1}.

Table II.1 Primers and adaptor oligonucleotide sequences used for Tn-Seq

Primer/Barcode	Sequence 5' to 3'
P1_M6_Mme1	CAAGCAGAAGACGGCATACGAAGACCGGGGACTTATCATCCA ACCTGT
Gex PCR	AATGATACGGCGACCACCGACAGGTTCAGAGTTCCTACAAGTC CGA
AP-A-bc-ACGC[a]	GTTCAGAGTTCTACAGTCCGACGATCACGCNN

AP-B-bc-ACGC[b]	5'Pho/GCGTGATCGTCGGACTGTAGAACTCTGAACCTGTC/3'Pho
AP-A-bc-ACTG	GTTCAGAGTTCTACAGTCCGACGATCACTGNN
AP-B-bc-ACTG	5'Pho/CAGTGATCGTCGGACTGTAGAACTCTGAACCTGTC/3'Pho
AP-A-bc-AGTC	GTTCAGAGTTCTACAGTCCGACGATCAGTCNN
AP-B-bc-AGTC	5'Pho/GACTGATCGTCGGACTGTAGAACTCTGAACCTGTC/3'Pho
AP-A-bc-ACAC	GTTCAGAGTTCTACAGTCCGACGATCACACNN
AP-B-bc-ACAC	5'Pho/GTGTGATCGTCGGACTGTAGAACTCTGAACCTGTC/3'Pho
AP-A-bc-ATGC	GTTCAGAGTTCTACAGTCCGACGATCATGCNN
AP-B-bc-ATGC	5'Pho/GCATGATCGTCGGACTGTAGAACTCTGAACCTGTC/3'Pho

[a] The underline sequence is the barcode sequence (ACGC). The "NN" two-base random nucleotide overhangs allows for ligation to the ends of Mme1-digested fragments.
[b] The underline sequence here represents the reverse complement of the barcode sequence (GCGT). The phosphorylated 5'end allows for ligation to the dephosphorylated genomic DNA fragments. The longer length of AP-B-bc-XXXX is five nucleotides longer on the 3' end than the AP-A-bc-XXXX, which prevents adaptor-adaptor ligation. 3' phosphorylation of AP-B-bc-XXXX prevents the formation of adaptor-adaptor dimers.

To identify the genes required for growth under fumarate or ferric citrate reducing condition, Tn-Seq experiment was performed in triplicate 2MTnseq, 3MTnseq and 4MTnseq. The 2M and 3MTnseq was performed using Janet Rollefson's transposon library that had 17,000 unique insertions from an approximately 30,000 mutant library. The 4MTnseq was performed using Caleb Levar's transposon library that had 42,898 unique insertions from a 50,000 mutant library. The following barcodes as listed in Table II.2 were used for each of the samples in these experiments. The raw sequences were processed in Galaxy using

the Tn-Seq Bond lab workflow, as diagramed in Figure II.2 below. Each individual experiment (2M, 3M, and 4M) gave roughly equal amount of mapped reads for given conditions (parent, fumarate and ferric citrate). The number of reads ranged between 23,000,000 to 40, 000,000 reads.

Table II.2 Barcodes used for Tn-Seq

Experiment	Barcode	Reverse Complement
2MTnSeq		
2MT1F	ACTG	CAGT
2MT2F	AGTC	GACT
2MT2FC	ACAC	GTGT
3MTnSeq		
3MT1F	ACAC	GTGT
3MT2F	AGTC	GACT
3MT2FC	ACTG	CAGT
4MTnseq		
4MT1F	ACTG	CAGT
4MT2F	ACAC	GTGT
4MT2FC	AGTC	GACT

Log ratios were calculated for each experiment and stored in the lab computer under the folder "Misha sequences". To reduce variability, fitness values were calculated for the parent population (T1F) for only those genes that had at least a minimum of 3 independent insertion sites. Genes that had a log ratio of $\leq$ -0.38 under Fe(III)-citrate reducing condition but had $\geq$ 0 values for fumarate growth were considered to have a defect in their ability to use Fe(III)-citrate as a terminal electron acceptor, compared to the parent population. Table II.3 represents a list of genes that are required for growth with Fe(III)-citrate as the terminal acceptor.

The most consistent and significant defect for growth under Fe(III)-citrate conditions was observed in a previously uncharacterized multi-heme *c*-type

cytochrome, GSU 3259. However, no growth defect was observed with fumarate as a terminal electron acceptor, thus suggesting that GSU 3259 is required for ferric citrate growth conditions. Based on previous proteomic analysis, GSU 3259 is most likely an inner membrane *c*- type cytochrome that interacts with the quinone pool based on its similarity to NapC /NirT (Ding *et al* 2006). Functional validation of Tn-Seq and further characterization of GSU 3259 is currently under investigation by Caleb Levar.

Overall a successful Tn-Seq approach was developed for the metal-reducing bacterium *G. sulfurreducens*. This Tn-Seq technique generated a massive dataset of genes that are either essential for *G. sulfurreducens* growth under any conditions (genes that have no insertion in the parent T1F population) or that are required for growth under metal reducing conditions (fumarate vs Fe(III)-citrate out growth). This technique can be applied to identify genes that are important under a variety of different physiologically relevant growth conditions.

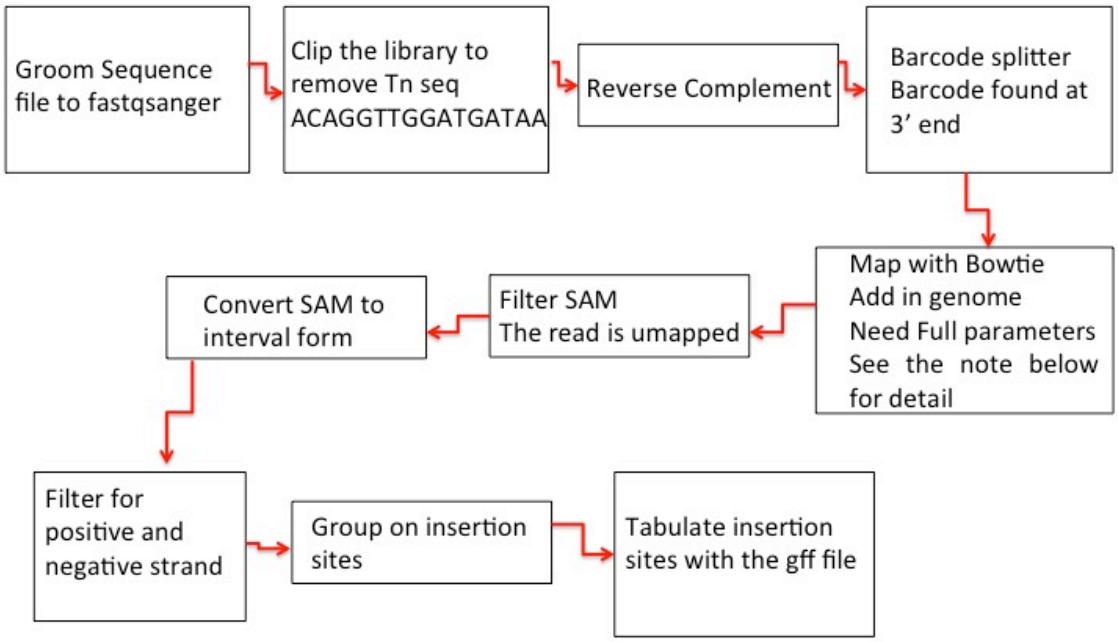

Figure II.2 Workflow for processing Tn-Seq illumina reads.
Note- Parameters for Mapping using Bowtie: (1) reference genome used is NC_002939 for *G. sulfurreducens* (Lab computer under Tn-Seq file), (2) Single-end library, (3) Full parameter list, (4) skip the first n reads: 0, (5) Trim n bases from left: 0, (6) Trim n bases from right: 4, Maximum number of mismatch permitted: 0, (7) Maximum permitted total quality values at mismatched read positions: 70, (8) Seed length: 28, (9) Whether or not to round to the nearest 10: Do not round to the nearest 10, (10) Number of mismatches for SOAP like alignment policy: -1, (11) Whether or not to try as hard as possible to find valid alignments: Try hard, (12) Report up to n valid alignments per read: 1, (13) Whether or not to report all valid alignments per read: Report all valid alignments, (14) Suppress all alignments for a read if more than n reportable alignments exist: 1, (15) Write all reads with a number of valid alignments: yes, (16) Write all reads that could not be aligned: yes, (17) Whether or not to make Bowtie guarantee that reported singleton alignments are best: Use best, (18) Maximum number of backtracks permitted when aligning a read: 800, (19) Whether or not to repot only those alignments that fall in the best stratum: Do not use strata option, (20) Override the off rate of the index to n:-1, Seed for pseudo-ransom number generator: -1 and (21) Suppress the header in the output SAM files: yes.

Table II.3 Genes that had fitness defect under ferric citrate reducing conditions

GSU locus[a]	Annotation[b]
0014	DnaJ-related molecular chaperone
0155	conserved hypothetical protein
0290	3-oxoacyl-(acyl-carrier-protein) synthase III
0584	conserved hypothetical protein
0591	cytochrome c, 1-2 heme-binding sites
0667	membrane protein, putative
0674	iron-sulfur-oxygen hybrid cluster protein (prismane)
0785	periplasmically oriented, membrane bound [NiFe]-hydrogenase
0881	sensor histidine kinase (HAMP, HisKA, HATPase_c)
0882	periplasmic/secreted protein DUF534
0883	ligand-gated TonB-dependent porin
1105	Xaa-Pro dipeptidase
1122	metal-dependent phosphohydrolase, HD superfamily
1419	helix-turn-helix DNA-binding protein,
1443	sensor histidine kinase response regulator (HAMP, HisKA)
1445	TonB-dependent receptor, putative
1501	ABC transporter, ATP-binding protein
1601	3-oxoacyl-(acyl-carrier-protein) synthase III
1802	putative carbohydrate kinase
1899	conserved hypothetical protein
2074	PPIC-type PPIASE domain protein
2758	excinuclease ABC family protein
2935	cytochrome c, 12 heme-binding sites
2989	cobyric acid aminotransferase/decarboxylase, putative
3257	glycogen synthase
3259	cytochrome c, 6-7 heme-binding sites
3349	putative cyclase/hydrolase
3380	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase,
3401	branched-chain amino acid ABC transporter,
3427	lipoprotein, putative

[a] GSU locus designation assigned by JGI

[b] Annotation assigned by JGI

II.5 Tn-Seq Protocol

At least 3 μg of DNA from each growth condition is required to get sufficient product for sequencing. This is important as lower DNA yields may require further optimization of this protocol.

Digestion of genomic DNA with restriction enzyme MmeI

Digest genomic DNA with MmeI at 37°C for 2.5 hours:
20 μl 10x buffer 4
0.44 μl SAM
6 μl Mme1
3 μg genomic DNA
Bring up to 200 μl with ddH_2O

Phosphatase treatment

Phosphatase treat DNA at 37°C for 1 hour
22 μl 10x phosphatase buffer
2 μl phosphatase (Antarctic phosphatase, NEB)
Heat inactivate 65°, 15 min

DNA extraction using phenol chloroform

Use phenol:chloroform:isoamyl alcohol (25:24:1) and extract the aqueous phase (this works better if the phenol-chloroform has been bought to room temperature before use)
Add 2 μl of glycogen (20 mg/ml)
Add 1/10 volume sodium acetate 3M pH 5.5
Add 3X volume of 100% Ethanol (molecular biology grade 200 proof)
Incubate on ice 15 min/overnight (overnight works much better)
Centrifuge 14000g for 30 mins
Wash once with 70% ethanol,
Centrifuge for 15 mins
Re-suspend in 25 μl ddH_2O, rehydrate overnight at 4°C

Adaptor preparation and ligation

Preparation of adaptors:

Add 2 µl oligo (100 µM concentration) to 998 µl 1mM Tris pH 8 (Note- these are phosphorylated and hence need to be used carefully- don't freeze thaw multiple times)

There are 5 sets of 2 (A and B for each see below in table 5.1), mix equal volumes of these and place in a heat block at 96°C for 2 minutes

Let the heat block cool to room temperature on the bench (remove the block) then store adaptors at -20°C

Ligate adaptors overnight at 16°C:

3 µl 10X T4 ligase buffer

1 µl adaptor (keep track of which adaptor for which samples)

25 µl chromosomal DNA (digested and purified)

1 µl ligase

PCR Amplification

PCR amplify 120 bp fragment in exponential phase

Setup each reaction in 4 PCR tubes to pull one out at cycle 19, 21, 23, 25 (initially to see if the reaction is linear if needed go either less or more cycles)

PCR Reaction	1 X µl
10 µM Primer P1_M6_Mmel	2.5
10 µM Primer Gex PCR Primer 2	2.5
DMSO	1.5
dd H_20	16
2X Phusion Master Mix	25
DNA	2.5

Total	50

PCR Program: TNSEQ in PCR
95°C – 30 sec
95°C – 10 sec
55°C – 5 sec
72°C - 15 sec
Go to step 2 18-25 times
Run all PCR products on 2% agarose gel with 5 µl of xylene cyanol loading dye and take a picture and gel purify the fragments
Determine yield of DNA, around 3 ng/µl is required for sequencing

Verification for addition of barcodes

Sequence the purified PCR fragments with the P1_M6_Mme1 primer and the sequence should have transposon, then genomic region and then the barcode

Submit samples for Illumina sequencing

Submit all the samples in tubes for a HiSeq 50 bp cycle run for 160 million reads for one flow cell

II.6 Sequences and Primers for Tn-Seq

Transposon inverted repeat (red) and MmeI overhang

AGA**CCGGGGACTTATCATCCAACC**TGTNNNNNNNNNNNNNNN
TCTGGCCCCTGAATAGTAGGTTGGACANNNNNNNNNNNNNNN

Linker

GTGTGATCGTCGGACTGTAGAACTCTGAACCTGTC
NNCACACTAGCAGCCTGACATCTTGAGACTTG

MmeI Digest ligated to linker

AGA**CCGGGGACTTATCATCCAACC**TGTNNNNNNNNNNNNNNNNGTGTGATCGTCGGACTGTAGA
ACTCTGAACCTGTC*
TCTGGCCCCTGAATAGTAGGTTGGACANNNNNNNNNNNNNNNNCACACTAGCAGCCTGACATCTTG
AGACTTG

PCR Product

CAAGCAGAAGACGGCATACGAAGA**CCGGGGACTTATCATCCAACC**TGTNNNNNNNNNNNNNNN
NGTGTGATCGTCGGACTGTAGAACTCTGAACCTGTCGGTGGTCGCCGTATCATT
GTTCGTCTTCTGCCGTATGCTTCTGGCCCCTGAATAGTAGGTTGGACANNNNNNNNNNNNNNNNNCA
CACTAGCAGCCTGACATCTTGAGACTTGGACAGCCACCAGCGGCATAGTAA

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT
TCCGATCT

Oligonucleotide sequences for the v1 and v1.5 Small RNA Kits
RT Primer
5' CAAGCAGAAGACGGCATACGA
5' RNA Adapter
5' GUUCAGAGUUCUACAGUCCGACGAUC
3' RNA Adapter
5' P-UCGUAUGCCGUCUUCUGCUUGUidT
v1.5 Small RNA 3' Adapter
5' /5rApp/ATCTCGTATGCCGTCTTCTGCTTG/3ddC/
Small RNA PCR Primer 1
5' CAAGCAGAAGACGGCATACGA
Small RNA PCR Primer 2
5' AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA
Small RNA Sequencing Primer
5' CGACAGGTTCAGAGTTCTACAGTCCGACGATC

Oligonucleotide sequences for Genomic DNA

Adapters
5' P-GATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

PCR Primers
5'
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT
TCCGATCT
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT

Genomic DNA Sequencing Primer
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT