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Following Electron Flow: From a Gram-positive Community to Mechanisms of
Electron Transfer

by

Kelly Catherine Wrighton

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor John D. Coates, Chair

Professor Steven Lindow

Professor P. Buford Price

Spring 2010

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Electron Transfer

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by Kelly Catherine Wrighton

Abstract

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University of California, Berkeley
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Research endeavors are committed to the optimization and function of Microbial fuel cells (MFCs), with most efforts largely dedicated towards increasing power density by optimizing physical parameters. Knowledge of the microbial physiology intrinsic to MFCs is hindered by the limited number of current-producing isolates in pure culture and the confinement of most mechanistic studies to the model Fe(III)-reducing organisms *Geobacter* and *Shewanella*, members of the Delta- and Gamma- Proteobacteria respectively. This discrepancy between known physiology and organisms evaluated in the MFC suggests that the diversity of electricity-producing organisms remains unknown. For MFCs to achieve their potential, however, knowledge of the microbiological factors controlling current production is essential. The research within this dissertation characterizes the electrochemistry (Chapter 2), ecology (Chapter 3), and physiology (Chapter 4) of bacterial current production.

The thermophilic (55°C) fermentation of solid waste has higher reaction rates, lower biomass yields, a broader range of carbohydrate utilization, and tolerance to higher loading rates when compared to mesophilic counterparts. While research has begun to examine the efficiency and microbiology of mesophilic MFCs, the equivalent electrochemical, ecological, and physiological studies have not been performed in MFCs operated under thermophilic conditions. Microbial fuel cells operated under elevated temperatures show promise due to potentially higher energy yields resulting from higher rates of metabolic activity, easier maintenance of anaerobic reducing conditions because of the lower solubility of O₂ at elevated temperatures, and thermal removal of most known pathogens. Additionally, MFCs operated at 55°C exclude the growth of *Geobacter* and *Shewanella* species and thus may enrich for novel electricity-producing communities and organisms.

MFCs inoculated with thermophilic anaerobic digester were constructed and operated at 55°C for 100 days. Electrochemical performance was well replicated within the reactors (Chapter 2). Over the experimental period, current was continuous, averaging 0.57 mA (100 mA.m⁻²) with a mean electron recovery of 89% and maximum power output of 37 mW.m⁻². Relative to similarly constructed mesophilic MFCs thermophilic MFCs may offer increased electrochemical performance with elevated current production, coulombic efficiency, and power generation. Not only does this detailed electrochemical description demonstrate that MFC technology is compatible with elevated temperature waste streams, but also functions a benchmark for future comparative studies.

To date, the characterization of bacterial communities in electricity production is hampered by the lack of controls, the use of low-resolution DNA fingerprinting techniques, and the failure to discriminate between active and colonizing members of the anode biofilm. To

assess bacterial composition and function of anode biofilm communities we used two complementary approaches: a novel high-density oligonucleotide microarray (PhyloChip) and clone library sequencing (Chapter 3). Within the anode bacterial community, active members were distinguished from persistent members by monitoring 16S rRNA in addition to cataloging 16S rRNA gene presence. Nucleic acids from a no-acetate control (no electron donor), open circuit control (no electron acceptor), and the initial inoculum were extracted to verify community membership on current producing anodes.

Research presented in this dissertation revealed that current-producing anode communities were statistically different from control reactors and the initial inoculum. PhyloChip results indicated that Firmicutes were dominant in the persistent and active bacterial community on thermophilic anodes. To identify specific Firmicutes OTUs involved in current generation, 16S rRNA clone libraries from the initial inoculum and current producing reactors were constructed. Two Gram-positive phyla, Firmicutes (11 OTUs, 229 clones, 77% of clones) and Coprothermobacteria (2 OTUs, 48 clones, 16% of clones), represented 93% of the clone sequences. Within the Firmicutes, sequences belonging to the genera *Thermicanus*, *Alicyclobacillus*, *Thermincola*, and *Geobacillus* represented 27%, 25%, 22%, and 2% of the total clones respectively. These genera could not be detected in clone libraries from the initial inoculum (482 total clones), suggesting their enrichment corresponds to current production in this system. Members of these genera have not previously been identified in MFCs operated under mesophilic conditions.

To link the phylogeny with functional current production, we complemented 16S rRNA approaches with isolation of pure cultures. Several bacteria representing three genera, *Thermincola*, *Geobacillus*, and *Coprothermobacter* were isolated from the MFC anode (Chapter 3). These genera contain three of the five most dominant members of the anode community and collectively represent 39% of the clone library sequence diversity. Both Firmicutes isolates, *Thermincola potens* strain JR and *Geobacillus sp.* strain S2E, are of great interest given their enrichment from the initial inoculum and their ability to reduce solid phase iron, or hydrous ferric oxide (HFO). Interestingly, while both isolates reduced HFO coupled to acetate oxidation, only *Thermincola potens* strain JR could generate current independently with acetate as an electron donor. Strain JR generated an average of 0.42 mA in two separate experiments with a coulombic efficiency of 91%, similar to that observed for the original complex community (89%). In contrast to *Thermincola potens* strain JR, *Geobacillus sp.* strain S2E could not produce current in the absence of an exogenous electron shuttle (AQDS) and only produced small amounts of current in its presence (0.03 mA).

While it has been shown that Gram-negative bacteria use either endogenously produced electron shuttles (contact-independent) or direct electron transfer (contact-dependent) for exporting electrons to the anode of MFCs, there is limited data about the mechanisms employed by Gram-positive bacteria for transferring electrons to insoluble electron acceptors. A combination of physiological, electrochemical and imaging methods support the hypothesis that *Thermincola sp.* strain JR does not produce an electron shuttling compound but requires direct contact for current production. Medium replacement experiments and cyclic voltammetry (CV) failed to detect redox active components secreted into the surrounding medium when strain JR was grown on these electron acceptors. Confocal microscopy revealed highly stratified biofilms in which cells contacting the electrode surface were primarily responsible for current generation. These results, along with cryo-electron microscopy (cryo-EM), suggest that *Thermincola potens* strain JR directly transfers electrons from the cell membrane across the 37nm cell envelope to

the cell surface. Analogous to direct electron transfer by Gram-negative organisms, physiological and genomic evidence suggests that direct extracellular electron transfer in Gram-positive bacteria is mediated by periplasmic and cell wall associated *c*-type cytochromes. Together, these results are the first to implicate a role for *c*-type cytochromes in direct extracellular electron transfer by Gram-positive bacteria (Chapter 4).

This dissertation follows electron flow in MFCs operated at 55°C to reveal a novel physiological role for Gram-positive Firmicutes within current-producing anode communities. We confirmed the functional role for Firmicutes in this systems by demonstrating current-production from two novel bacteria from the anode surface, *Thermincola potens* strain JR and *Geobacillus sp.* strain S2E. Physiological investigation of Gram-positive extracellular respiration was carried out in strain JR demonstrating that this organism is capable of independent and direct electron transfer to insoluble electron acceptors like Fe(III) and anode surfaces. Further, genomic and physiological studies of strain JR provide evidence for multiheme *c*-type cytochromes in facilitating electron transfer across the Gram-positive cell envelope. As a result of this dissertation, an option now exists for efficient MFC current-production at elevated temperature, two novel anode-respiring bacteria have been isolated, independent electricity generation by Gram-positive bacteria has been demonstrated, the genome of one of these isolates has been sequenced, and the molecular mechanism of electron transfer by a Gram-positive anode respiring bacterium elucidated.

This dissertation is dedicated

*To my parents who
nurtured my curiosity and taught me to question*

&

*To Ethan Martini whose unending
support has made this possible*

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We have done it!

Chapter 1

Microbial Fuel Cells: Plug-in and Power-on Microbiology

Abstract

MFCs take advantage of the fact that all organisms require an electron donor and electron acceptor, and use bacteria as catalysts to convert electron donors into electricity using an anode as a surrogate electron acceptor. In this review we discuss limitations to high power production in these systems, as well as look to the future for technological applications. Beyond electricity generation, however, MFCs are important tools for microbiologists. Here we highlight the specific contributions of bacterial MFC research including, but not limited to, expanding the broad phylogenetic diversity of organisms capable of extracellular respiration and characterization of molecular mechanisms of electron transfer. In addition, MFCs also show promise as research tools used to explore biofilm dynamics as well as the effect of redox on structuring microbial communities.

Denizens of power: The role of bacteria in a MFC

If scientists have their way, “green” beer won’t be limited to St. Patrick’s day celebrations anymore. Breweries are taking their wastewater, which is rich in organic material, and turning it into electricity with a little help from bacteria in microbial fuel cells (MFCs). Like Rumpelstiltskin who spun straw into gold, bacteria in MFCs can generate fiscally important commodities from a wide variety of organic wastes that are abundant and essentially free. This technology is not limited to brewery wastes, bacteria have generated electricity from industrial wastewaters, sewage, and even sediment (1). In MFCs, bacteria act as living catalysts to convert organic substrates into electricity. While this technology may sound like mother natures answer to our energy crisis, these devices are not yet viable for most applications. Ongoing research is dedicated to optimizing the system performance with only recent attention being given to the microbial details of waste to wattage conversion.(2)

Similar to microbial metabolism, power generation in an MFC is dependent on oxidation-reduction (redox) chemistry. MFCs contain anodic and cathodic compartments that each holds an electrode separated by a cation permeable membrane (Figure 1A). In the anode chamber, microbial substrates such as acetate (electron donor) are oxidized in the absence of oxygen by respiratory bacteria to produce protons and electrons. The electrons are passed through an electron transport chain (ETC) and protons are translocated across the cell membrane to generate ATP. Generally electrons and protons exiting the ETC are passed onto a terminal electron acceptor such as oxygen, nitrate, or Fe(III), however, in the absence of these in an MFC, some microorganisms can pass the electrons onto the anode surface. The difference in redox potentials (i.e. the ability of a compound to donate or accept electrons, denoted E_o and measured in volts), between the electron donor and the electron acceptor is a gauge of the potential energy available to the microorganism for anabolic processes. In an MFC, this is determined by the electrochemical redox potential difference of the anode and cathode. The electrons produced in an MFC flow from the anode through an external electrical circuit to the cathode to generate electrical current. While electrons move externally, protons diffuse from the anode to the cathode via the cation membrane to complete the internal circuit. At the cathode, the electrons and protons combine to reduce the terminal electron acceptor, which in many applications is oxygen. Therefore bacteria in the anode are physically separated from their terminal electron acceptor in the cathode compartment.

Ultimately the electrical power (measured in watts) produced by an MFC is based on the rate of electrons moving through the circuit (denoted current and measured in amps) and electrochemical potential difference (volts) across the electrodes. Current production is affected by many factors including substrate concentration, bacterial substrate oxidation rate, presence of alternative electron acceptors, and microbial growth. Electrochemical potential, on the other hand, is dependent on the redox couple between the bacterial respiratory enzyme or electron carrier and the potential at the anode, which is determined by the terminal electron acceptor in the cathode and any system losses. (Figure 1B)

Although the ability to transfer electrons generated on the cell membrane to the cell surface is a requirement for bacteria to produce electricity in an MFC, very little is known about bacterial interactions with electrodes. While research has shown that both anodes and cathodes can function in bacterial respiration (3, 4), most research has been dedicated to understanding microbial anodic electron transfer. Anode-respiring bacteria catalyze the transfer of electrons in

organic substrates onto the anode as a surrogate for natural extracellular electron acceptor (e.g. ferric oxides or humic substances), employing a variety of mechanisms (Figure 2).

When bacteria transfer electrons to the anode in the absence of exogenous mediators, they use either direct or mediated mechanisms. In direct electron transfer, bacteria require physical contact with the electrode for current production. The contact point between the bacteria and the anode surface requires outer membrane bound cytochromes or putatively conductive pili known as nanowires. Although direct contact of an outer-membrane cytochrome to an anodic surface would require microorganisms to be situated upon the electrode itself, direct electron-transfer mechanisms are not necessarily limited to short-range interactions as nanowires produced by *Geobacter sulfurreducens* have been implicated in electron conduction through anode biofilms greater than 50 μm thick (5). In mediated electron transfer mechanisms, bacteria either produce or take advantage of indigenous soluble redox compounds (electron shuttles, e.g. quinones) to shuttle electrons between the terminal respiratory enzyme and the anode surface (6 2008).

Power Tools of Microbiology: MFC technology advances microbial research

Beyond energy generation, MFCs are also ideal tools for addressing questions about microbiology. With current acting as a real-time proxy for bacterial activity and their quantifiable and controllable redox potential, MFCs are experimentally controlled systems for evaluating the “who”, “how”, and “why” questions of extracellular electron transfer. The use of MFCs as a research tool has notably expanded scientific knowledge of bacterial diversity of extracellular electron transfer, the mechanisms used to transfer electrons, and biofilm ecology.

Identifying Novel Bacteria

Microbial research from MFCs has expanded the diversity of bacteria known to transfer electrons onto external electron acceptors. Until recently, knowledge of electricity generating bacteria was limited to bacteria that transfer electrons to solid metals, thus phylogenetically confining most MFC studies to the Proteobacteria. However, culture-independent studies from MFC anode biofilms indicates that diversity of MFC microbial communities far exceeds that of the available electricity producing isolates, suggesting that much of diversity of organisms with this capability remains to be discovered (1). This has spurred interest in using a variety of alternative inoculum sources, operating conditions, and isolation methods to increase the known diversity of electrode reducing organisms.

For example, our lab focused on MFCs operated at 55°C, conditions where model metal and anode reducing species, *Geobacter* and *Shewanella* spp., cannot survive. These studies demonstrated electricity production by anode communities dominated by Gram-positive species and resulted in the isolation of novel organisms from three of the five most dominant populations identified by 16S rRNA gene clone libraries. Characterization revealed that the isolates utilized species-specific mechanisms for electricity production, emphasizing not only the phylogenetic diversity that exists in active MFCs but also the phenotypic diversity to perform the same function, i.e. transfer electrons onto an electrode surface within a single community. One of the isolates, *Thermincola* strain JR, a member of the Firmicutes, produced current comparable to that of the original MFC community and greater than either *Geobacter* or *Shewanella* species in similarly designed MFCs. Furthermore, this was the first demonstration of power production by a Gram-positive bacterium without the addition of exogenous mediators. A second isolate,

Geobacillus strain S2E, also a member of the Firmicutes, was the first reported member of this genus capable of respiration with solid phase iron oxides.(7)

Our results indicate that microbial analyses from MFCs can result in the discovery of novel bacteria that are proficient at energy generation and have unique metabolic functions. The significant enrichment of Gram-positive bacteria in our systems illuminates a potentially new ecological role for these organisms in respiration of insoluble electron acceptors. Surprisingly, studies concerning the mechanisms of electron transfer to solid phase electron acceptors have not included Gram-positive bacteria, which unlike Gram-negative bacteria lack an outer membrane. Exactly how these bacteria transfer electrons extracellularly without an outer membrane is a mystery.

Knowledge of electrode reducing communities and bacteria is required to optimize power generation. Molecular approaches characterizing the microbial communities in these systems are needed to reveal the phylogenetic diversity as well as the activity of electrode respiring communities. Little is known about population level interactions within the anode community, but it is foreseeable that these types of interactions can be reinforced or controlled to increase substrate utilization or electron transfer efficiency. Research will not only be important to understanding power generation but also is relevant to the fate, transport, and bioremediation of metals in the environment. Future studies can use MFCs like a radio, tuning to different stations of reduction-oxidation potentials to better understand the effect of redox potential and electron donor or microbial community structure and activity.

Elucidating mechanisms of extracellular electron transfer

MFCs as a research tool have expanded knowledge of bacterial electron transfer mechanisms. Unlike studies using natural external electron acceptors such as Fe(III) or Mn(IV), anodes do not participate in mineral dissolution reactions and electron transfer rates can be quantified in real-time. Advantageously, anodes provide a stable source of electron acceptor and do not generate reduced products, which can interfere with downstream genomic or proteomic applications. Additionally, colonized anodes have also been adapted for electrochemical methods that reveal the presence, redox potential, and reversibility of electroactive components in biofilms (8-12). The power of MFCs to elucidate mechanisms of solid phase electron transfer was convincingly demonstrated where the application of cyclic voltammetry techniques to anode biofilms established that *Shewanella* spp. excrete flavins which function in anode electron transfer, metal chelation, and may aid in bacterial cell adhesion to anode surfaces (12).

In light of previous studies showing that *Shewanella* utilized outer membrane cytochromes and putatively electron transfer through conductive nanowires, this study exemplifies that extracellular electron transfer mechanisms are not mutually exclusive within a single species. These results also explained observed discrepancies in research findings by different laboratories (13-15). Looking towards the future, understanding how bacteria attach to anodes could result in design of more efficient electron transfer systems. Also genetic and metabolic engineering of electrode active bacteria, including the over expression of essential cytochromes or shuttling compounds, could increase current production.

Modeling and Framing Biofilm Ecology

Consistent with views from the natural world, it is well documented that bacteria in MFCs exist in biofilms on the anode surface. Because MFCs can measure bacterial activity in real-time and detect redox active components, they provide a platform for modeling and framing

relevant questions about biofilm ecology. To understand the biological and electrochemical phenomena that drive anode performance Kato Marcus *et al.* derived mathematical models to describe anode biofilms. This research revealed that the entire anode biofilm might be electrically conductive and that biofilm density and detachment is an important factor in electrochemical performance.(16)

With this as a springboard, recent efforts have been dedicated to increasing the active biomass capable of electron transfer to the anode surface without altering mass transfer events or the physical environment within the anode biofilm. Recent research identified that biofilms formed under increased shear contained a higher density of active bacteria resulting in a three fold increase in MFC performance (17). Future modeling studies could highlight discrepancies between predicted and observed power production in microbial fuel cells, suggesting abiotic and biotic areas of improvement. Beyond modeling, research on anode biofilms can explore the environmental and biological cues involved in biofilm formation and dissolution. The role and temporal dynamics of quorum sensing compounds on anode colonization and current production has not yet been evaluated in MFCs, which is unfortunate given that many signaling molecules may also function as electron shuttling components in mediated electron transfer (18). MFCs provide an ideal platform to gain a better understanding of the attachment, succession, dissolution and interspecies interactions that occur within biofilms.

A Current Affair: Ongoing Research and Challenges

Consistent with nonrenewable energy costs, MFC research endeavors are increasing exponentially each year. Much of the research attention is dedicated to the optimization of power. Limitations to MFC power densities can be attributed to hardware and operational constraints rather than microbial activity. Consequently, improvements in MFC design and materials have significantly contributed to improved reactor performance resulting in a 10,000-fold power increase since 1999. Despite this advancement, it is predicted that a further increase of 10-100 fold is required for MFCs to be considered for practical applications (19, 20).

Specifically, research dedicated to reducing the internal reactor resistance and increasing cathodic reaction efficiency is integral to power maximization. As of now, most MFC research has been performed in lab scale systems. However, in these studies the cost of component materials and operation far exceeds the value of energy generated. Pilot-scale applications are essential to evaluate the design, construction, operation, and microbial restrictions prior to performing economic feasibility studies regarding the large-scale implementation of MFC technology. In envisioning the transition from the bench to pilot-scale systems, associated costs become a primary concern (20, 21). There is still uncertainty regarding which aspects of the MFC will scale linearly and which will not. Furthermore, the design of cheaper and more durable electrodes and cation permeable membranes is required.

For the first time, MFCs are currently being put to the test in pilot-scale operations. Both Anheuser-Busch Inc. and Foster's breweries, of the United States and Australia respectively, are evaluating MFC technology with the aim of simultaneously treating organic-rich soluble wastewater and producing electricity (20). Researchers from the University of Queensland in collaboration with Foster's implemented the first pilot-scale (1000 L) MFC in September 2007 (Figure 3A). Such pilot-scale projects are a necessary first step if MFCs are to transition from the lab to real-world applications.

Full of Potential: Future applications of MFC technology

To satisfy their energy and resource demands, microbes participate in a vast array of biochemical reactions. As such, the microbial metabolism in MFCs can also be harnessed for bioremediative, industrial, and hydrogen production applications resulting in environmentally responsible and fiscally valuable end products.

Electricity production from waste

The conversion of biomass, especially organic waste, to energy is considered an essential part of a sustainable global energy portfolio. A variety of potentially valuable underutilized energy sources exist in the United States. For example, assuming all the organic material is completely oxidized to carbon dioxide, human waste alone contains 34 billion kWh of energy annually (22). This represents a nearly limitless energy source that is currently untapped. It has also been demonstrated that MFCs can generate electricity directly from cellulose (23, 24). Thus it is feasible that MFCs can treat wastewater from biofuel processing, removing waste material to recycle water as well as generate electricity. The coupling of these technologies can minimize production costs and increase energy recovery ensuring the profitability and sustainability of “green energy” (25).

Batteries for remote sensors

While the use of MFCs for wastewater treatment is in its infancy, MFCs as batteries for environmental sensors is nearing practical use. In contrast to traditional batteries, MFCs powered by organic matter in sediments offer many advantages as power sources because they can generate limitless amounts of energy without recharging. MFCs in this application are termed benthic unattended generators (BUGs) and have been used in inaccessible areas such as river and ocean sediments (26, 27). Operation is technically simple; a graphite plate is deployed into the anoxic sediment (anode) that is electrically connected to another graphite plate in the overlaying aerobic water (cathode) (Figure 3B and 3C). Although power density is minimal, recent developments have overcome this by incorporating a capacitor in the electrical circuit to store the produced BUG energy for use in short bursts. Using this approach, an MFC deployed in creek sediment powered an environmental sensor and a wireless data transmitter to monitor air and water temperature and transmit this data to a shore-based receiver (28).

Bioremediation of environmental contaminants

MFCs have applications beyond electricity production. The value of generating reducing equivalents from waste material cannot be ignored. In this case, MFCs are not used to produce electricity but rather to power cathodic reduction reactions of bioremediative or industrial importance. Since electricity is not being harvested, but rather biologically generated current is used to stimulate microbial metabolism on a cathode, these systems are not considered fuel cells, but are coined bioelectrical reactors (BERs). Studies to date have focused on BERs where an external power source provides the reducing equivalents in these systems, however, recently it has been demonstrated that a biological anode may alternatively be used. As reviewed by Thrash and Coates (2008) cathodes have served as electron donors for bacterial reduction of a range of bioremediation targets including uranium, perchlorate, chlorinated solvents, and nitrate (29). However, it is easy to envisage the application of this technology to a diversity of other contaminants including toxic metals, dyes, pesticides, and herbicides.

Synthesis of industrial products

It is foreseeable that reducing equivalents produced in the anode may also serve to produce industrially important chemicals such as hydrogen peroxide, sulfur, and perhaps even butanol. The production of potential biofuels like propanol and butanol from organic waste is very appealing. In this process, organic waste, which is too low in sugar content for ethanol production, is microbially fermented in the absence of an electron acceptor into volatile fatty acids (VFA). These VFA can be fed to the cathode compartment, where bacteria use the electrons supplied from the cathode to reduce VFA into alcohols like propanol and butanol. This research is largely conceptual, but the potential has been demonstrated using hydrogen as a source of reducing equivalents, rather than MFC cathodes (30). Specific research hurdles include evaluating the use of current rather than hydrogen for reducing equivalents, fine-tuning concentrations of VFA and electrons for favorable thermodynamic conditions, and developing a method for physically separating the desired end-products from the reactor liquor.

Hydrogen production

In addition to powering BERs, MFCs can also be modified to produce hydrogen gas. Given that transportation fuels are responsible for 20-25% of the global fossil fuel consumption, replacement of these with alternative sustainable fuel sources, is likely in the future energy portfolio (31). Thus, generation of renewable hydrogen from waste materials is highly desirable. This process can occur in a microbial electrolysis cell (MEC), which like an MFC, is based on bacterial oxidation of organic substrates occurring at the anode and electrons flowing to the cathode. Here electrochemical potential achieved in the anode is supplemented with an additional ~250 mV from an exogenous power source. At this elevated electrical potential electrolysis of water occurs at the cathode, resulting in hydrogen production. In the past two years research in this area has advanced significantly, with the amount of hydrogen generated per mol of oxidized glucose nearing the U.S. Department of Energy's target for technology viability. (32) While hydrogen can theoretically be produced from any biodegradable waste stream, more research is needed from actual feedstocks that contain microbially recalcitrant substances. Today, hydrogen production in reactors using existing technology is too low to make large-scale MECs likely in the immediate future. However, a combination of improved reactor design and treatment of organic rich wastewaters make this an attractive proposition for the future.

Concluding Sparks

It is plausible that in some form MFCs will play a role in the future energy paradigm; given the rising cost of energy and the abundance of affordable and sustainable fuel sources. In addition to electricity generation, the power generated in an MFC has applications relevant to bioremediation, industrial chemical, and hydrogen production. For MFCs to be considered more than a lab novelty, standardization of data expression is necessary to allow reliable and accurate comparison of results. Furthermore, advancements in the hardware, operation, and microbial components are also prerequisites. Regardless of the final manifestations of this technology, the microbial knowledge already gleaned from the research is unparalleled. MFCs with their real-time proxy for microbial activity and defined reduction potential are valuable research tools for characterizing the physiology and ecology of extracellular electron transfer, modeling electron flow in complex microbial ecosystems, as well as framing and testing ecology theory. Given the benefits of MFC technology, the future for MFC research is a green light.

Figure 1. A) Schematic of a microbial fuel cell illustrating oxidation of fuel by bacteria in the anode compartment to produce electrons and protons. Electrons (red arrow) resulting from microbial oxidation flow from the anode through an external connection to the cathode to generate electrical current, while protons (green arrow) travel through the cation membrane. Together both function to reduce the terminal electron acceptor, which in this case is oxygen, in the cathode. **B)** Redox tower for components that are significant to current production in a MFC. For a redox couple, the electron donor must have a greater negative potential than the electron acceptor, this difference in potential is proportional to the amount of energy generated from the reaction. Current generation in a MFC is based on sequential redox reactions. First, bacteria oxidize fuel (yellow) and transfer these electrons to an electron carrier at a more positive potential (red), thereby generating energy for the bacteria. The final power generated by an MFC is based on the current production and redox couple between the bacterial respiratory enzyme or electron shuttle and the potential at the anode, which is determined by the terminal electron acceptor in the cathode (purple) and any system losses.

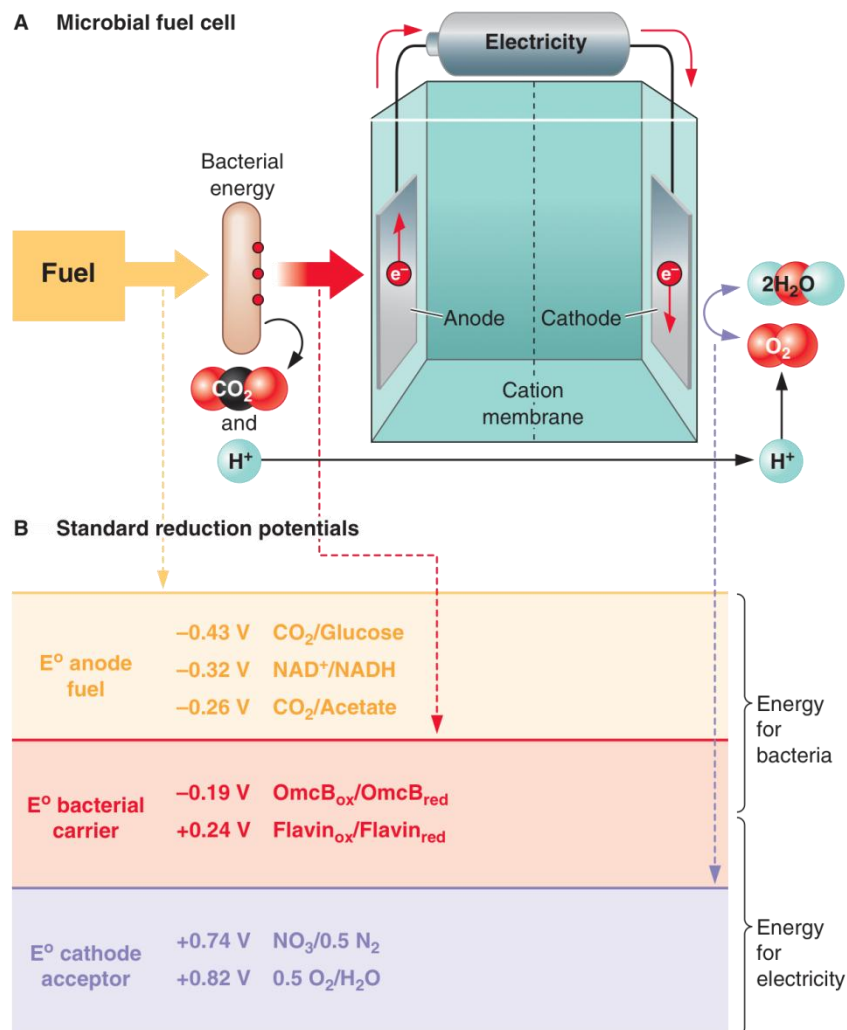


Figure 2. Bacteria use direct and mediated mechanisms to transfer electrons from the cell membrane to the anode surface. Electrons can be transferred from the cell or through a conductive biofilm using each method. References: Redox potential of *Shewanella* produced flavin (6) and a *Geobacter* (OmcB) outer membrane cytochrome (33).

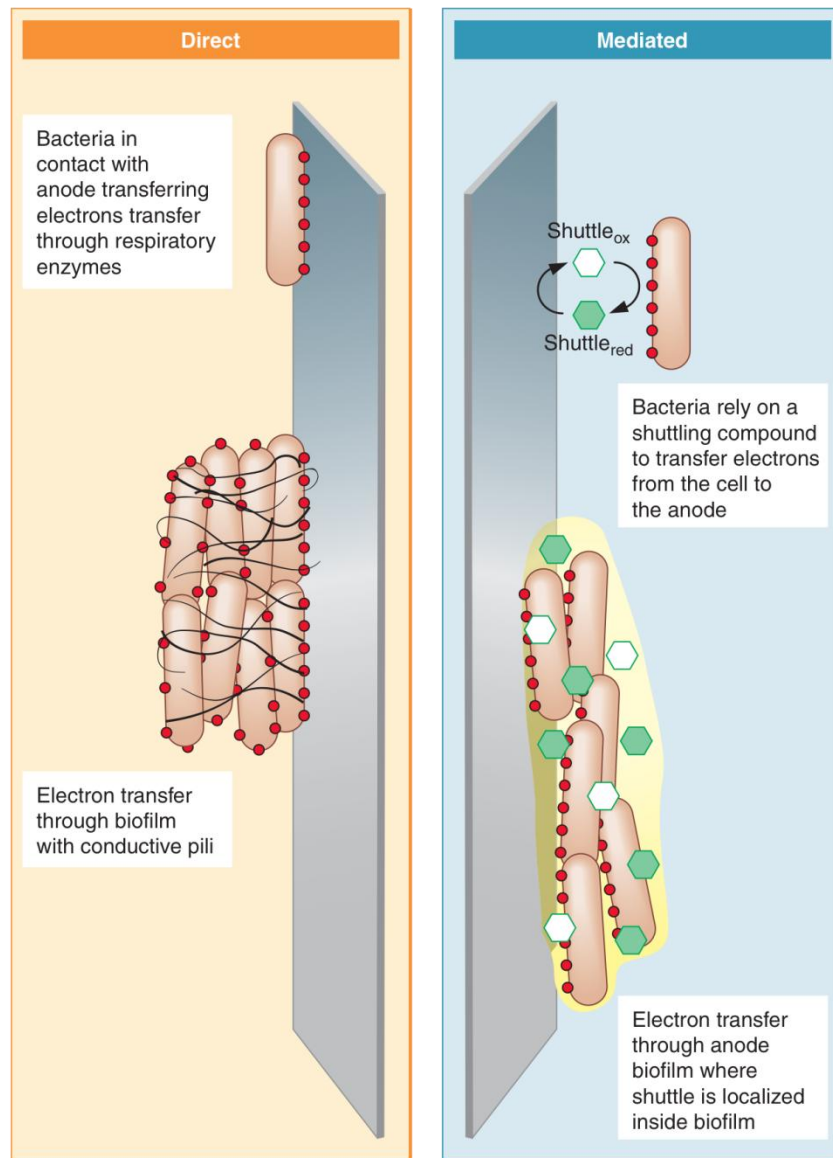
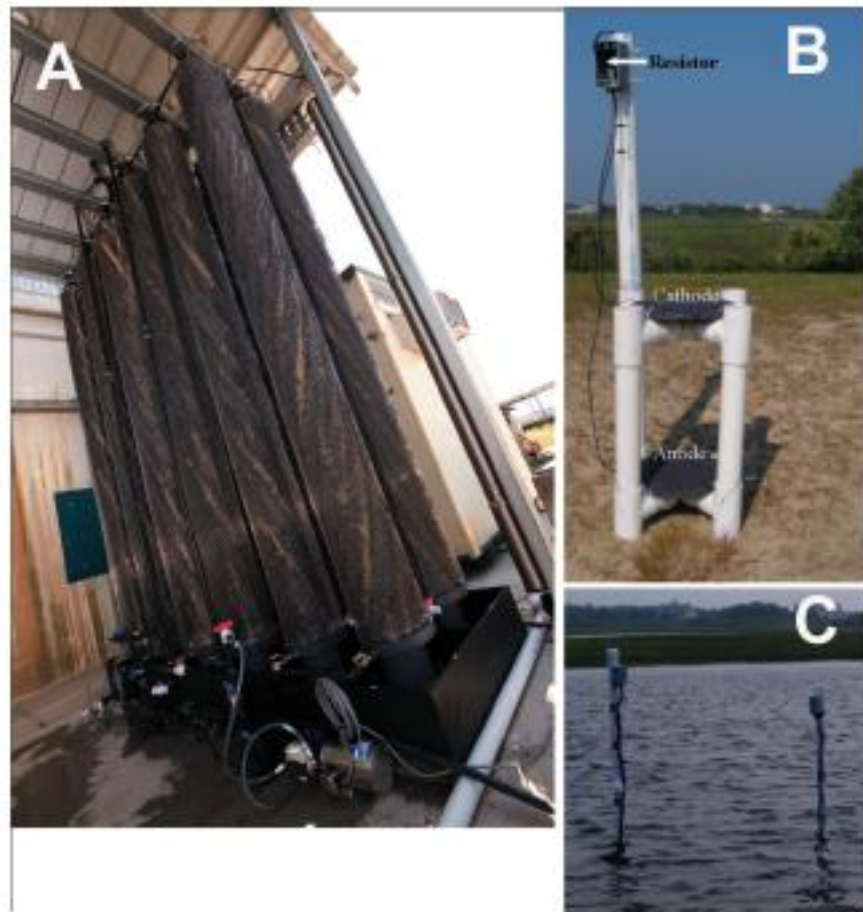


Figure 3. Applications for MFCs. **A)** Picture of the pilot scale microbial fuel cell constructed by the advanced water management centre at the University of Queensland and Foster's brewery, both in Australia. Image is of graphite electrodes, which are contained in 12 modules approximately 3 m in height. **B)** Sediment MFC prior to deployment in salt marsh sediments. **C)** Active sediment microbial fuel cells.



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Chapter 2

Electrochemical Performance of Thermophilic Microbial Fuel Cells

Abstract

Significant research effort is currently focused on microbial fuel cells (MFC) as a source of renewable energy. To date, most of these efforts have concentrated on MFCs operating at 20-30°C and the corresponding mesophilic organisms and communities. However, many previous studies have reported on the superiority of elevated temperatures in anaerobic digestion and demonstrated a greater energy yield, in terms of methane, relative to the increased energy requirements. Because of this, we focused on current generation from anode compartments maintained at 55°C. Here the electrochemical factors influencing system effectiveness at elevated temperatures are characterized. Current production from MFCs with anodes operated at 55°C for 100 days were stable and achieved a maximum power density of 37 mW.m⁻² with 89% coulombic efficiency.

Introduction

Microbial fuel cells (MFCs) offer an affordable and effective means of harvesting electrical energy from the oxidation of waste materials; with efficiencies as great as 80-96% (1). Contrasting this with the much lower efficiencies of internal combustion emphasizes that energy reserves in wastes can be effectively utilized by the direct biological conversion of chemical energy into current in a MFC (2, 3). An advantage of MFCs over traditional treatment methods is that they are not based on the combustibility of a compound; therefore energy can be readily harvested from any biodegradable material. For instance, annually in the United States, human waste alone contains 34 billion kWh of energy (3), while food processing industries produce an average of 1 million gallons of wastewater per day (4). If 80% of the energy can be recovered as electricity in a MFC, this would result in a savings of 2 billion dollars annually to treat human wastes and a savings of 20 billion dollars annually to treat food processing waste (3, 4). Moreover, MFC technology may have important implications for other bioenergy disciplines, as the generation of byproducts and energy from biofuel waste materials is the greatest challenge to ensure that this industry is profitable and sustainable (5). It is possible that MFCs could treat wastewater from cellulosic wastewaters, thereby removing waste material to recycle water as well as generate either electricity or reducing equivalents for desirable cathodic reactions.

Research endeavors are committed to MFC optimization and function, with most research efforts largely focused on increasing power densities by optimizing system engineering and architecture. To date, most MFCs are operated at temperatures from 20-30°C. This finding is surprising given that many industrial wastewaters are produced at elevated temperatures and that the thermophilic fermentation of solid waste has been reported to include higher reaction rates, lower biomass yields (which reduces sludge formation), a broader range of carbohydrate utilization, and tolerance to higher loading rates when compared to mesophilic counterparts (6-10). Additionally, bacteria isolated from MFCs operated at elevated temperatures will provide a source of thermostable enzymes with broad biotechnological applications (11, 12)

While extensive research has examined the efficiency and microbiology of thermophilic and mesophilic anaerobic digesters, these same comparative studies have yet to be performed in MFCs. Microbial fuel cells operated under elevated temperatures hold much promise due to potentially higher rates of metabolic activity, a reduced internal resistance, and an unexplored microbiology. Here we demonstrate that MFCs inoculated with a thermophilic anaerobic digester sludge and amended with acetate generate electricity at 55°C. Additionally we characterize the electrochemical parameters of thermophilic current production to provide a benchmark for future comparative analyses.

Materials and Methods

MFC construction

The MFCs used in this study are composed of two chambers, or H-shape design. Each MFC was constructed from two 50 mm diameter glass chambers with three sample ports, connected with a 30 mm pinch clamp assembly (Laboratory Glass Apparatus, Berkeley, CA). Chambers were separated with a cation exchange membrane (Nafion 117). Sample ports were sealed with butyl rubber stoppers and aluminum crimp seals. The electrodes were 2.5 x 1.25 x 7.5 cm unpolished graphite (G-10 Graphite Engineering and Sales, Greenville, MI) connected with watertight threaded fittings (Impulse, San Diego, Ca) to wires and sealed with conductive silver epoxy (Epoxy Technology, Billerica, MA). Wires were fed through stoppers on the top of each chamber. Prior to initial use and before each use thereafter, the electrodes were washed repeatedly in 1N HCL to remove residual materials and biomass, and rinsed and stored in sterile deionized water prior to assembly. (13)

When conducting experiments with bacterial inoculum, the MFCs were assembled, filed with deionized water, and autoclaved prior to addition of media or inoculum. After autoclaving, the chambers were drained and the anodic chamber was filled with 250 ml of pre-sterilized, anoxic 30 mM bicarbonate buffered fresh water basal medium (pH 6.8), which contained the following per liter: 0.1g KCl, 0.2g NH₄Cl, 0.6g of NaH₂PO₄, 10 ml vitamin mix, and 10 ml trace mineral mix and 2.5 g of NaHCO₃ (14). Standard anaerobic culturing techniques were used throughout (15). Where noted, certain reactors were amended with 10 mM acetate as the sole electron donor. In all cases the cathodic chamber was filled with 250 ml of 30 mM TRIS buffer (pH 6.8). The anode chamber was bubbled continuously with filtered (0.22 µm pore size) N₂:CO₂ (80:20; vol:vol) gas to ensure anaerobic operation and maintenance of pH throughout operation, while the cathode chamber was bubbled continuously with filtered air (0.22 µm pore size). With the exception of MFCs inoculated with *Geobacter sulfurreducens*, which were operated at room temperature, all other results were from MFCs with anodes maintained at 55°C.

Proton diffusion test

MFCs operated in three formats (mesophilic anode and cathode, thermophilic anode and cathode, and thermophilic anode and mesophilic cathode) assessed whether a temperature difference between chambers impacted proton diffusion from the anode to the cathode. MFCs were amended with sterile deionized water as the medium. The influence of chamber temperature on proton exchange rate between anode and cathode compartments was examined by lowering the anodic pH and subsequently monitoring the cathodic pH.

MFC experimental design and inoculation

For the thermophilic reactors acetate was chosen as the electron donor since it is not readily fermentable, it is the dominant electron donor in anaerobic systems, and our primary goal was to identify novel bacteria capable of anodic respiration (16). The experimental design included three treatments: 10 mM acetate amendment with circuit (two reactors), non-amended with circuit control (two reactors), and 10mM acetate amended without circuit, or open circuit, control (one reactor). The non-amended reactors control for redox active components in sludge slurry, confirmed the absence of electron donors in the sludge inoculum, and identified bacterial populations residing on the anode independent of acetate dependent current production. The open circuit control normalizes for microbial populations that oxidize acetate independent of current generation and also controls for organisms that colonize the anode surface without concomitant current production.

The inoculum for the thermophilic anode was collected from an operational thermophilic methanogenic anaerobic digester. In the laboratory, anaerobic digester sludge was centrifuged (10,000g; 15min.) and washed with bicarbonate buffer (30 mM, pH 6.8) under an N₂:CO₂ (80:20; vol:vol) headspace to remove soluble electron acceptors and electrochemically active compounds. The pellet was suspended in bicarbonate freshwater media lacking electron donor and acceptor to create a cell slurry under a headspace of N₂:CO₂ (80:20; vol:vol) and incubated at 55°C. Removal of endogenous electron donors was monitored by cessation of biogas production as well as a depletion of organic acids. Upon removal of endogenous electron donors, four MFCs were inoculated (10:90; vol:vol) with the laboratory acclimated anaerobic digester sludge.

As a comparison, MFCs were inoculated with *G. sulfurreducens* strain PCA (ATCC 51573) from laboratory culture collections. Cells were grown at room temperature in anoxic bicarbonate fresh water media amended with ferric citrate (80mM) or fumarate (40mM) as electron acceptors and acetate (10mM) as electron donor. When an OD of 0.3 was reached (fumarate) or 40 mM ferrous iron was produced (ferric citrate), cells were harvested by centrifugation (3,000 x g) and washed with anoxic bicarbonate buffer (30mM) under an N₂:CO₂ (80:20; vol:vol) headspace. Washed cells were resuspended in 1 ml anoxic anode media and inoculated into the anode chamber

Electrochemical characterization

Voltage (E_{cell} or cell potential) was measured across a fixed external resistor (R_{ext}) and logged using Chart 4.0 software (ADInstruments, CA). Data were recorded every ten minutes for 100 days. Current (I) was calculated from measured voltages using Ohm's law ($I=E_{\text{cell}}/R_{\text{ext}}$). Coulombic efficiency, or electrons in acetate that are recovered as current, was calculated in duplicate and the mean value reported. The actual coulombs transferred was determined by integrating current ($A=C/S$) vs. time (S) plots. The total amount of electrons available was calculated by [(concentration of added acetate, mM)*(1mol Ac/1000 mMol Ac)*(8 mol e⁻/1mol Ac)*(96485 C/mol e⁻)]. Open circuit voltage was measured by breaking the circuit and logging potential every 0.1 seconds until plateau. For polarization curves, resistors (10, 22, 100, 220, 470, 1000, 2200, 4700) were used to set variable external loads. Starting from open circuit and moving from low to high loadings and vice versa, the voltage was measured and current calculated using Ohms law for every resistor after a ten-minute equilibration period. The results recorded represent the mean of these values. A power curve was calculated from the polarization curve data, while the maximum power reported in this system is based on the value obtained over the 100-day operation. With the exception of polarization and power curves, external resistance was kept constant at 470 Ω , which was determined from polarization curves as the external load where power was maximized. Maximum overall power (P) was calculated as $P=E_{\text{cell}}^2 \text{Max}/ R_{\text{ext}}$.

Results and Discussion

MFCs were amended with sterile deionized water and operated with either a mesophilic anode and cathode, a thermophilic anode and cathode, or a thermophilic anode and mesophilic cathode to assess the impact of chamber temperature on proton diffusion from the anode to the cathode. For this experiment, the pH of the anode compartment was reduced to 2.11 ± 0.05 and the diffusion of protons to the cathode chamber, with its initial pH of 6.43 ± 0.12 , was monitored over time (Figure 1). Proton transfer was least impacted in the MFC operated with a thermophilic anode and mesophilic cathode, as cathode pH was reduced by 30% in approximately 0.5 hours. Alternatively, a 30% reduction in cathodic pH was observed in the thermophilic anode and cathode after 2.3 hours, while a 30% reduction in cathodic pH was not recorded in the mesophilic operated MFC after 7 hours. Based on these results, we concluded that MFC operation with a 55°C anode would not be detrimental to proton migration and thus thermophilic current-generation.

Current production was monitored in MFCs inoculated with thermophilic anaerobic digester sludge and operated with anode chamber temperature of 55°C for over 100 days (Figure 2). Our experimental design included five reactors: two acetate-fed reactors, two unfed (no acetate) reactors, and one acetate-fed open circuit control. Electrochemical performance was well replicated within the reactors; current harvesting was similar not only from replicates within this experiment but also to another independent experiment (data not shown). Over the course of the experiment, current production was continuous; averaging $100 \text{ mA} \cdot \text{m}^{-2}$ or 0.54 mA (Figure 3). As expected the control reactors receiving no exogenous carbon amendment failed to produce significant current after two days. To standardize thermophilic current production, two MFCs were inoculated with pure cultures of *G. sulfurreducens*, a model current-producing bacterium (1, 2) (Figure 4). Only the results from fumarate inoculum are shown for simplicity. MFCs inoculated with *G. sulfurreducens* produced an average of 0.25 mA. While not normalized for anode biomass or differences in current-production between a mixed-culture and pure culture, this result is promising and suggests that thermophilic operation may result in current increases of 46% relative to mesophilic operation.

In addition to the amount of electrons recovered, the ability of the microbial community to convert chemical energy into current, known as coulombic efficiency, is also an important consideration when assessing MFC functional performance. Coulombic efficiency is the ratio of total coulombs actually transferred to the anode from acetate to the maximum possible coulombs if all acetate removal produced current. Of the 82.3 C added as acetate, a mean of 73.1 C (69.4C and 76.8C) could be recovered as current (Figure 5). MFCs operated at elevated temperatures had a coulombic efficiency of 89% over more than 3 months of current harvesting for the two experimental reactors. This represents one of the highest electron recoveries reported to date for an MFC inoculated with a complex microbial community. We speculate that the increased efficiencies associated with thermophilic operation could be attributed to decreased oxygen contamination from the cathode chamber and attribute this to continued sparging of the anode with $\text{N}_2:\text{CO}_2$ gas, a practice not common in the MFC literature, as well as poor oxygen solubility at elevated temperatures.

In addition to the amount of current generated and coulombic efficiency, other performance characterizations include the maximum potential of the system, the internal resistance, and maximum power generation. Open cell voltage (OCV) is the difference in electrical potential between the cathode and anode when operated without an external load

connected, i.e. the circuit is broken, and represents the maximum potential difference in the system independent of current losses. As such, the OCV should approach the theoretical electrochemical MFC potential, which based on the difference in potentials between the terminal electron acceptor in the cathode (oxygen in this study) and the anode potential. However, typically the OCV is far less than the theoretical potential, due to system-wide potential losses (17). The thermophilic MFCs in this study had a maximum potential of 550 mV (Figure 6), a value consistent with other H-type fuel cells, which have an OCV less than 600 mV (18, 19). For comparison, the maximum MFC OCV of 800mV was reported was for a single chamber and still represented an approximate 25% reduction from theoretical values (18).

The discrepancy between OCV and theoretical potential summarizes anode and cathode associated losses but fails to capture losses associated current production in a MFC. These losses are proportional to the generated current (I) and internal resistance (R_{int}) and include current dependent losses at the electrodes as well losses attributed to the resistance of electrons or protons in the circuit. Polarization curves represent the voltage as a function of the current and are a powerful tool for electrochemical characterization of fuel cells.(20) Because the measured cell voltage is usually a linear function of current in a MFC, polarization curves can be used to estimate the internal resistance. For a linear polarization curve, the value of internal resistance is equal to the slope ($R_{int}=-\Delta E/\Delta I$). (21)

The slopes of the polarization curve for MFCs operated at 55°C were linear over the range of 0 – 1.2 mA, with an estimated internal resistance (slope) of 430 Ω (Figure 7). This range is well within reported values for dual chambered MFCs. Reported internal resistances range from 174- 925 Ω , with variations attributed to differences in cathodic electron acceptor (oxygen or ferric cyanide), the use of platinum catalyst in cathode chamber, distance and size of electrodes, and PEM size (21). Notably, MFCs with an identical design and electrode size as those examined here inoculated with *Geobacter* demonstrated a 24% increase (approximately 560 Ω) in internal resistance (22). This difference may signify that thermophilic operation relative to mesophilic operation results in minimization of internal losses; however, future studies dedicated to this hypothesis are warranted.

Power is one of the most important parameters for surmising MFC performance. Ultimately the electrical power (measured in watts; $P=IV$) produced by an MFC is based on the rate of electrons moving through the circuit (denoted current and measured in amps) and electrochemical potential difference (volts) across the electrodes. A power curve describes the power as a function of the current and is used to define the maximum power point (MPP) generated from a MFC. Figure 8 shows a power curve based on the polarization curve (Figure 7) with a 10 minute equilibration time. As no current flows in open circuit conditions, no power is produced. As the circuit is completed, power increases with current to a MPP of 0.15mW (24 mW.m⁻²). Beyond the MPP power decreases, due to increasing ohmic losses and electrode overpotentials, to the point where no more power is produced as the system is effectively short-circuited.

Placing an external load of 470 Ω , the load that maximized power in the polarization analysis (Figure 7), a maximum of 37 mW.m⁻² was generated over long-term operation. The power reported in this study is approximately twice that produced by similarly constructed MFCs operated at 30°C with either pure cultures or complex microbial community inocula. The next highest reported power, 16 mW.m⁻², was produced using a pure culture inoculum of *G. sulfurreducens* (22) in nearly identical MFCs to those evaluated in this study. Both of these values were considerably higher than mesophilic consortium of 8.3 mW.m⁻² (23), or pure

cultures of *Rhodoferrax ferrireducens* or *Shewanella putrefaciens*, which produced 8.2 mW.m^{-2} or 0.32 mW.m^{-2} respectively (24, 25).

Conclusion

MFCs are constructed using a variety of materials, configurations, and operations. Included in these parameters are differences in temperature, pH, electron acceptor, electron donor, electrode surface area, reactor size, and operation time. This range of conditions and the lack of reported performance data often make it difficult to compare performance results between systems. Here the physical construction and electrochemical operation of MFCs operated at 55°C are characterized. Altogether our findings demonstrate that thermophilic MFCs may offer increased electrochemical performance with elevated current production, coulombic efficiency, and power generation relative to similarly constructed and operated mesophilic counterparts. While promising, these results established a baseline for future comparative studies and demonstrated that MFC technology is compatible with elevated temperature waste streams.

Figure 1. Proton diffusion from acidified anodes results in pH reduction in the cathode chamber. Proton movement through the proton exchange membrane was evaluated in three operational MFCs: mesophilic anode and cathode (square, dashed line), thermophilic anode and cathode (triangle, solid line), and thermophilic anode and mesophilic cathode (circle, solid line).

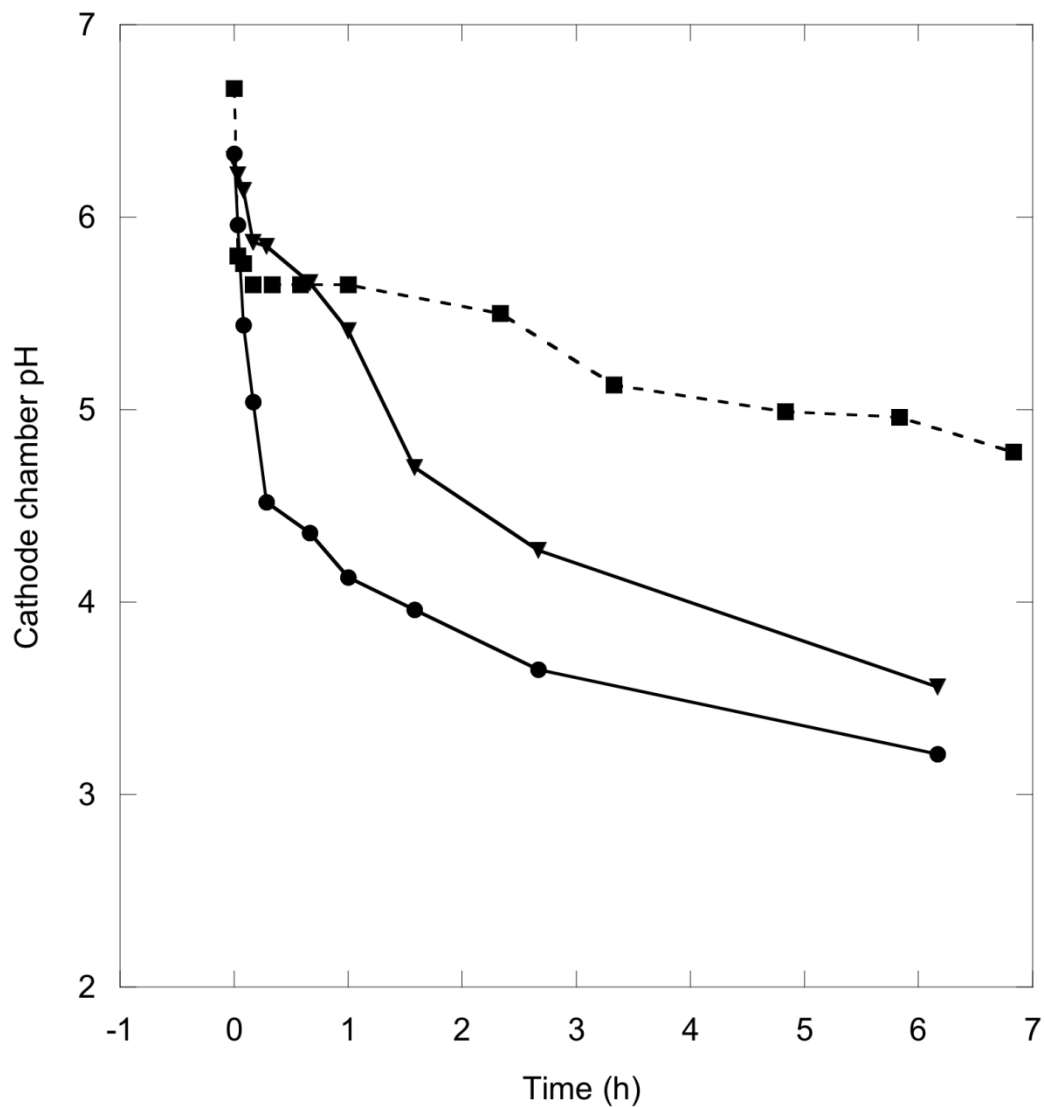


Figure 2. Photograph of the H-type (two-chambered) thermophilic MFCs used in this study. Sludge from a thermophilic digester was inoculated (10:90; vol:vol) into an anaerobic and heated 55°C anode chamber containing growth medium (10mM acetate) and a graphite block electrode. The circuit was completed by connecting the anodic electrode to another electrode in the aerobic and abiotic cathode chamber with a 470 Ω resistor.

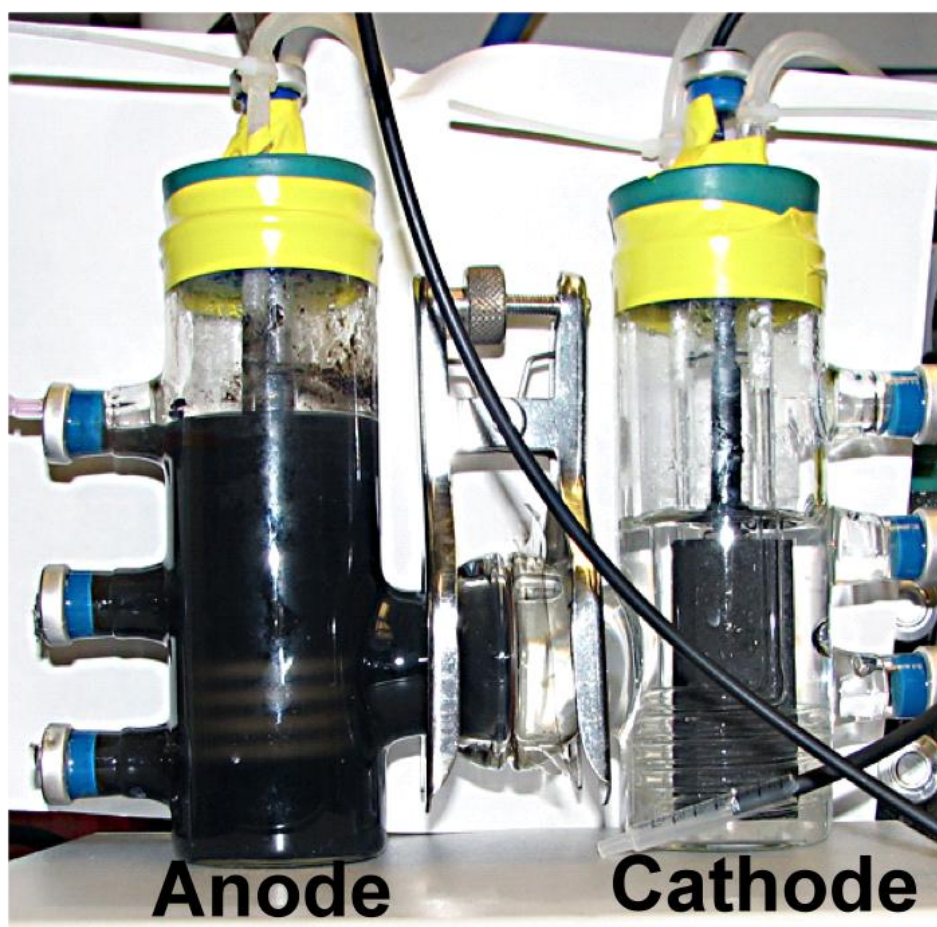


Figure 3. Current produced by the 55°C maintained anode bacterial community with (solid lines) and without amendment with 10mM acetate (dashed lines).

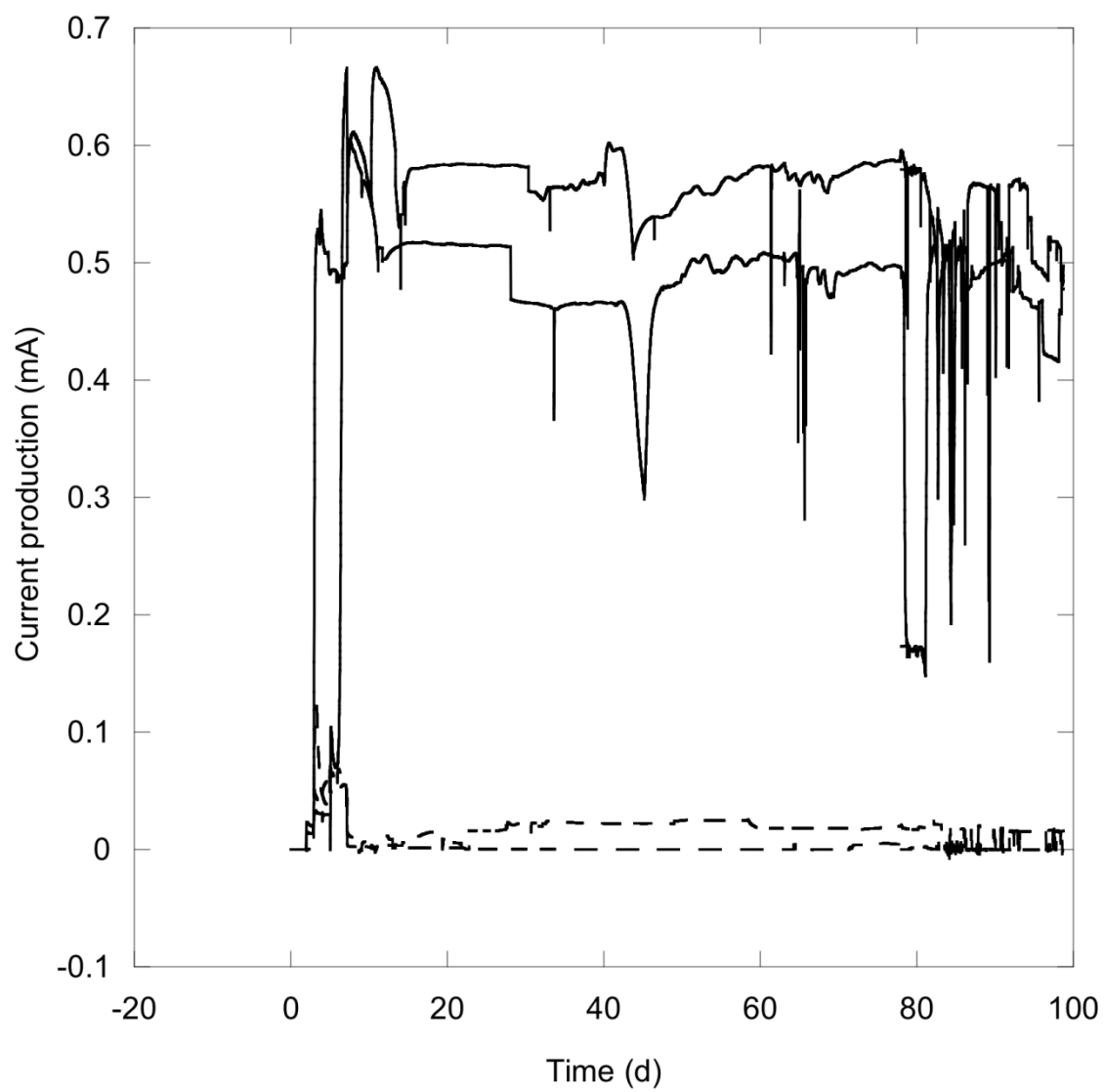


Figure 4. Current production by *Geobacter sulfurreducens* strain PCA in the H-type MFC used in this study. Cells were inoculated into anode containing growth medium (10mM acetate) and graphite block anode.

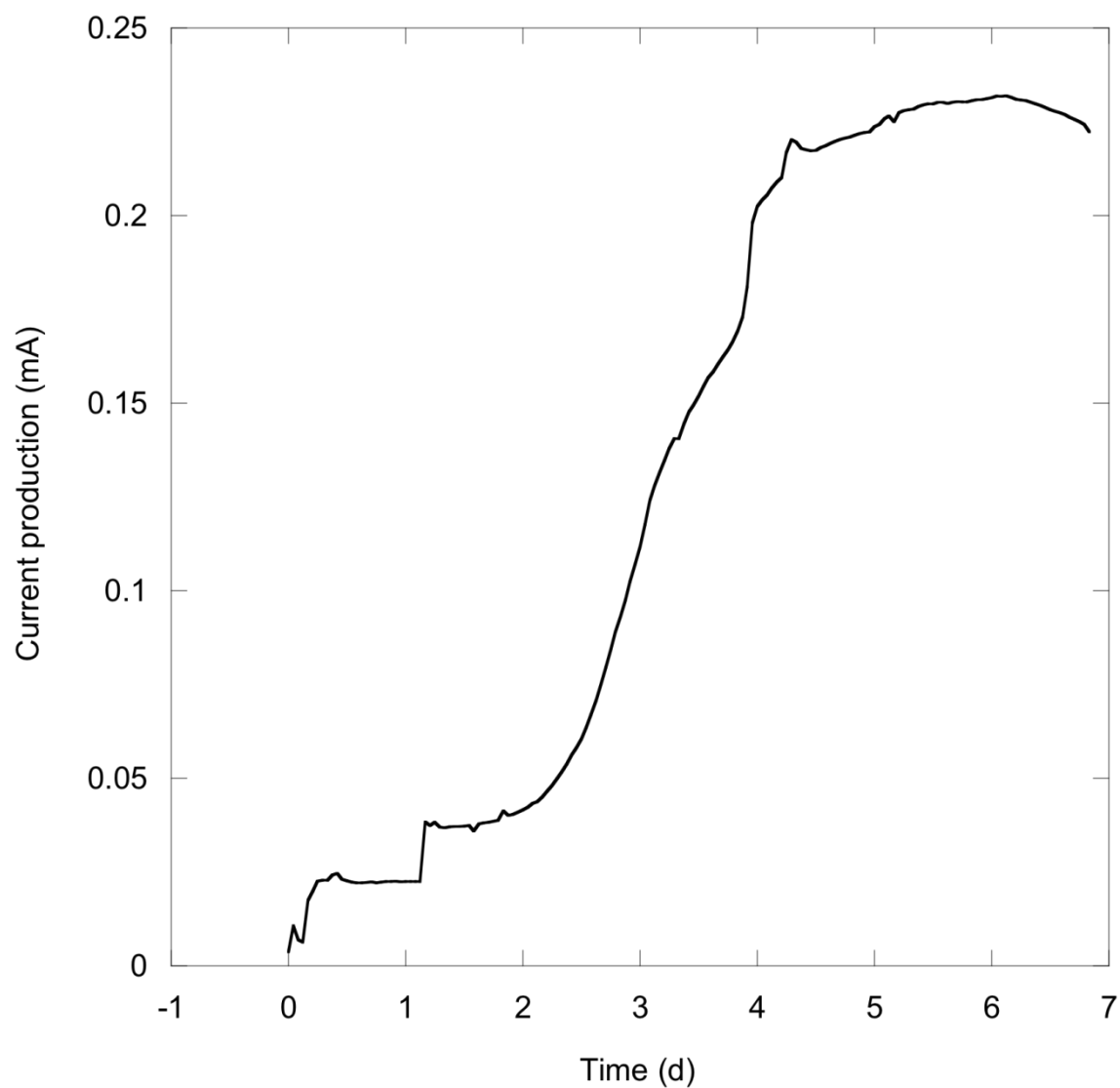


Figure 5. Coulombic efficiency is the ratio of total coulombs actually transferred to the anode from acetate to the maximum possible coulombs if all acetate removal produced current. The total coulombic yield from acetate oxidation is determined by integrating the current over time. Of the 82.3 C added as acetate, a mean of 73.1 C could be recovered as current, indicating a recovery of 89%.

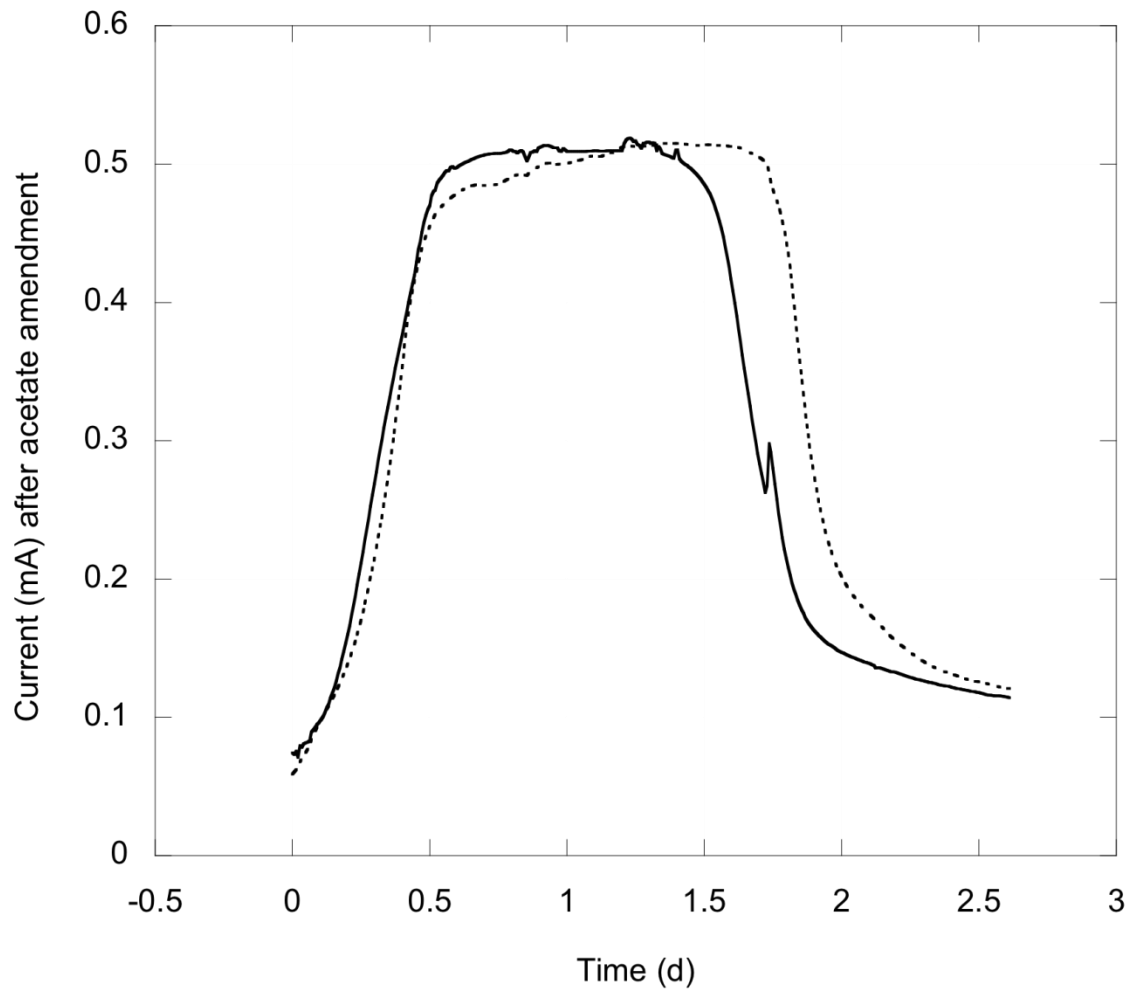


Figure 6. Open circuit voltage (OCV) is the potential that can be measured in the absence of current. In this system, the OCV approaches 550 mV.

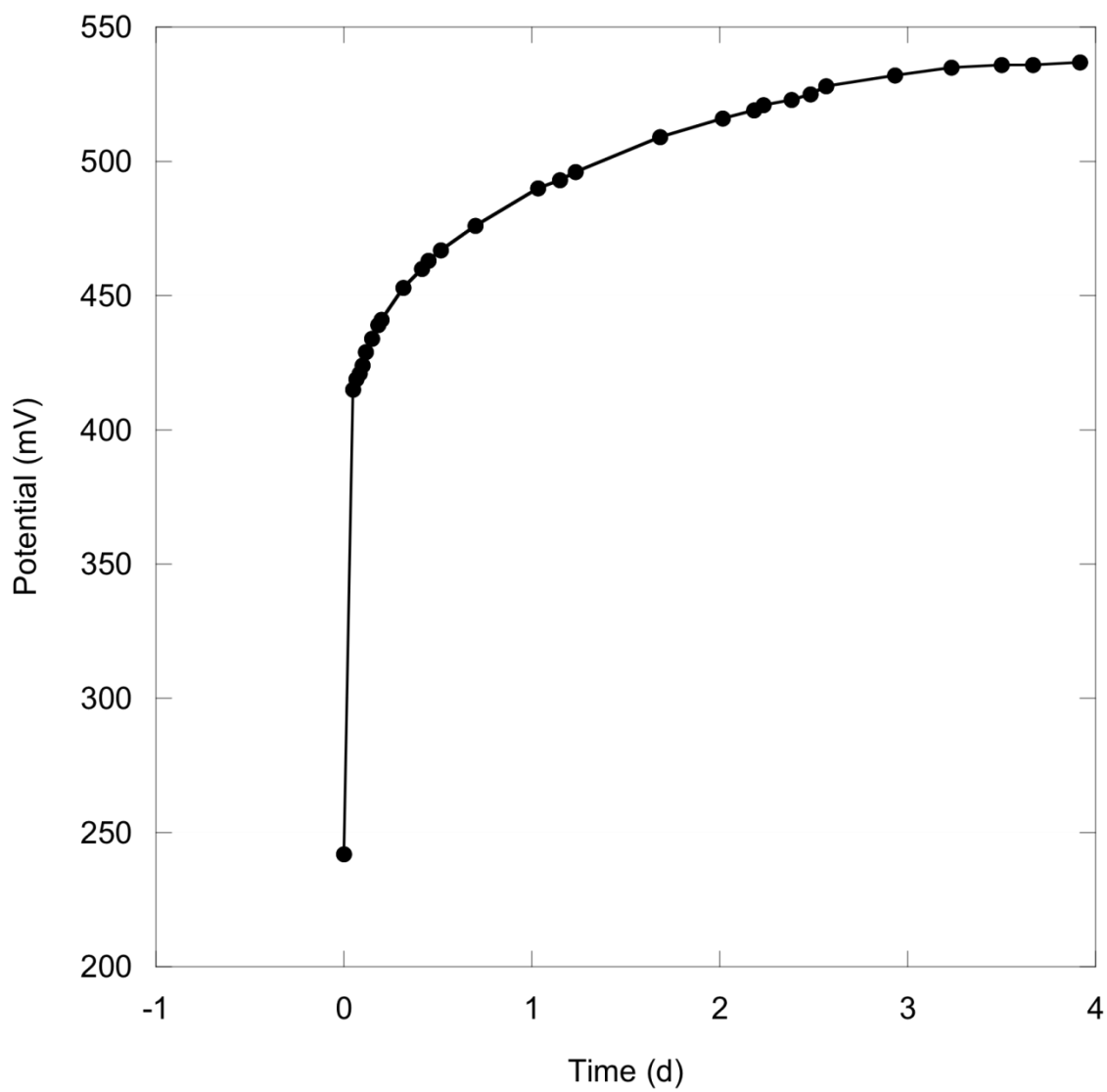


Figure 7. The current-voltage relationship is summarized in a polarization curve for thermophilic MFCs operated on acetate. A comparison of the mean observed data (circles) and calculated result (line) indicates a slope, or R_{int} , of 444.61Ω .

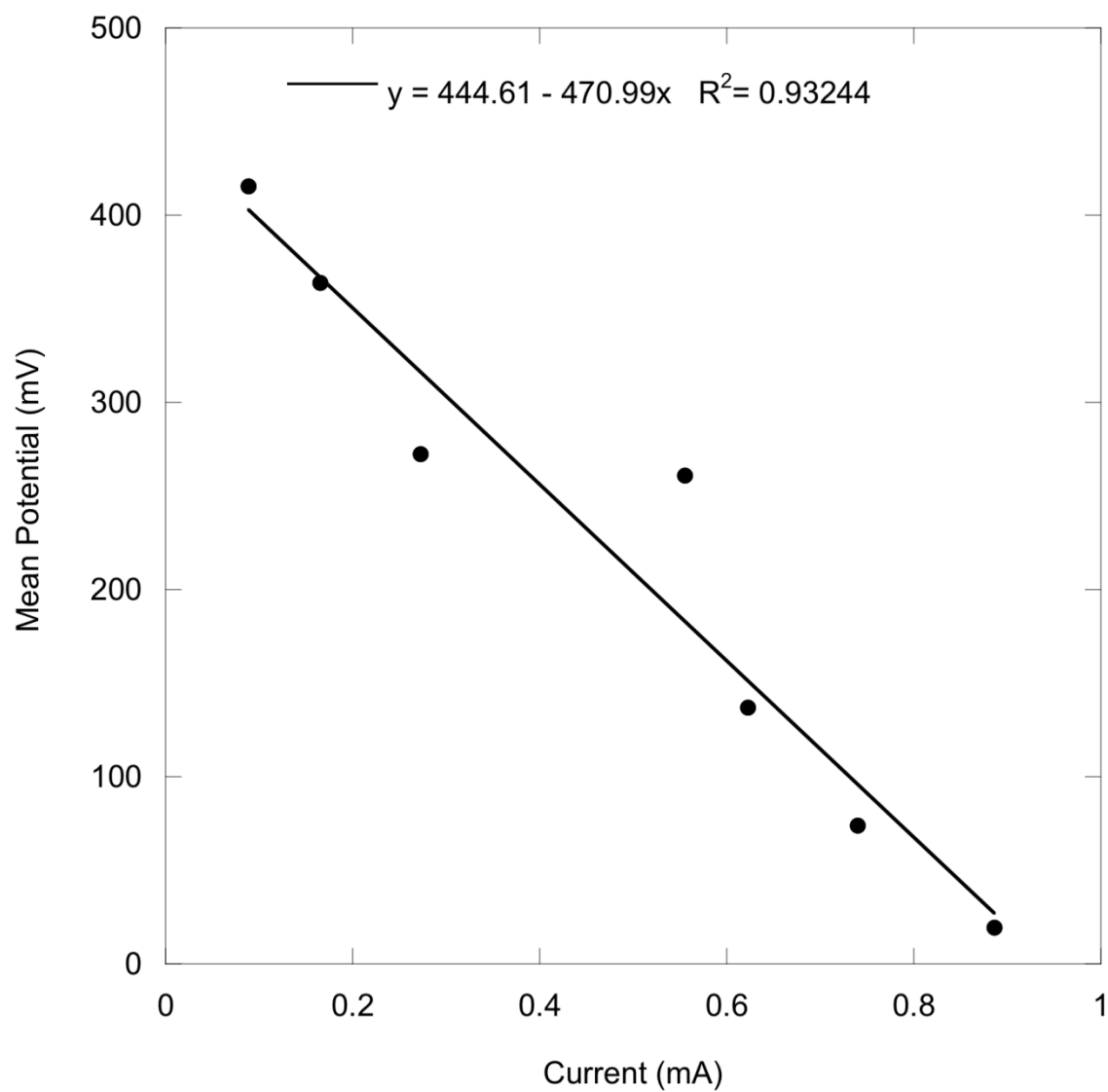
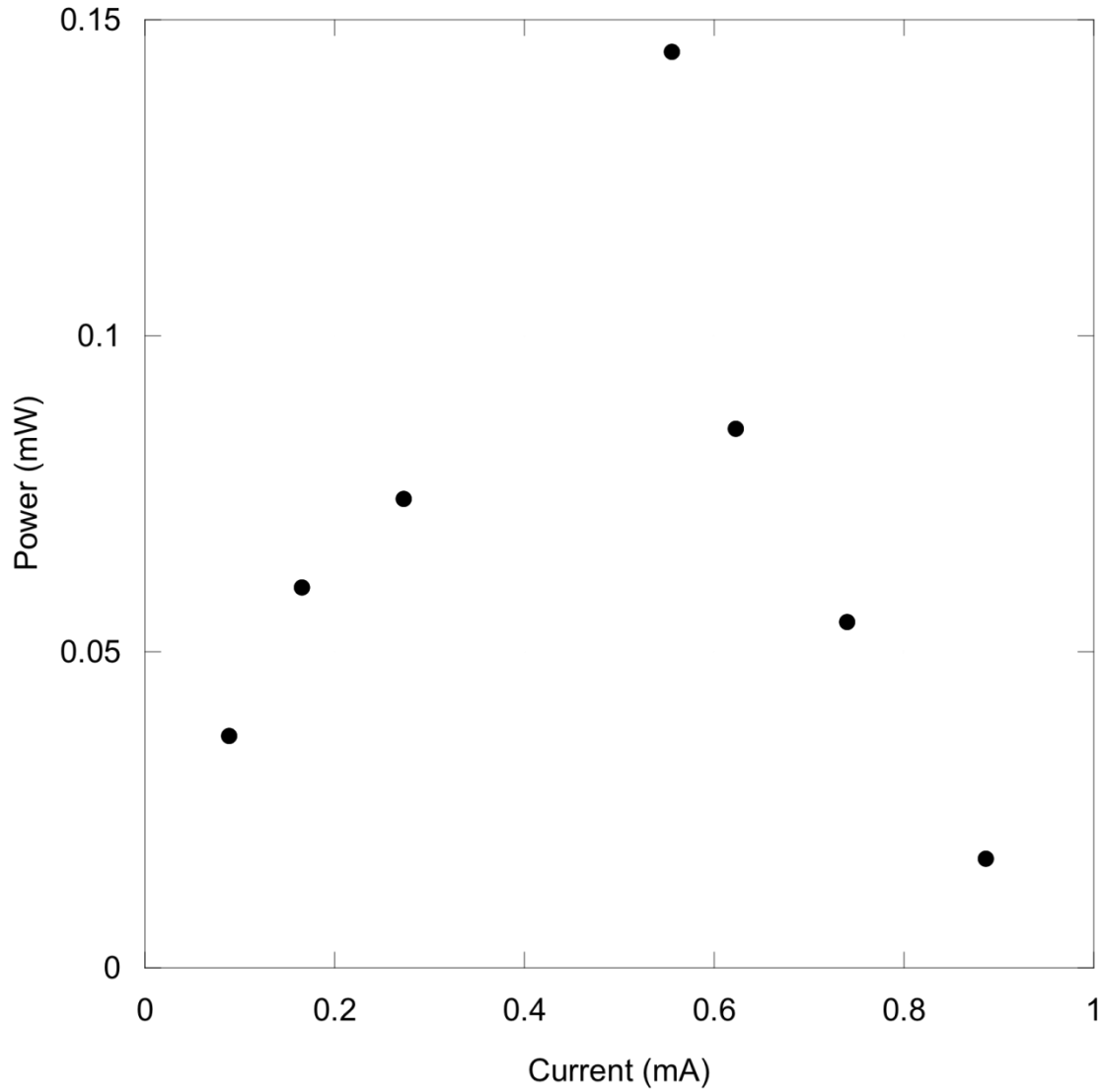


Figure 8. The current-power relationship is summarized in a power curve for thermophilic MFCs operated on acetate. The maximum short-term power (MPP) was approximately 0.15mW or 24 mW.m⁻², while the maximum power for the experiment was 37 mW.m⁻².



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Chapter 3

A Novel Ecological Role of the Firmicutes Identified in Thermophilic Microbial Fuel Cells

Abstract

Significant effort is currently focused on microbial fuel cells (MFCs) as a source of renewable energy. Most studies concentrate on operation at mesophilic temperatures. However, anaerobic digestion studies have reported on the superiority of thermophilic operation and demonstrated a net energy gain in terms of methane yield. As such, our studies focused on MFC operation and microbiology at 55°C. Over a 100-day operation, these MFCs were stable and achieved a power density of 37 mW.m⁻² with a coulombic efficiency of 89%. To infer activity and taxonomic identity of dominant members of the electricity producing community we performed phylogenetic microarray and clone library analysis with small subunit ribosomal RNA (16S rRNA) and ribosomal RNA gene (16S rDNA). The results illustrated the dominance (80% of clone library sequences) of the Firmicutes in electricity production. Similarly rRNA sequences from Firmicutes accounted for 50% of those taxa that increased in relative abundance from current producing MFCs; implying their functional role in current production. We complemented these analyses by isolating the first organisms from a thermophilic MFC. One of the isolates, a Firmicutes *Thermincola* strain JR, produced more current than known organisms (0.42 mA) in a H-cell system but also represented the first demonstration of direct anode reduction by a member of this phylum. Our research illustrates the importance of using a variety of molecular and culture based methods to reliably characterize bacterial communities. Consequently, we revealed a previously unidentified functional role for Gram-positive bacteria in MFC current generation.

Introduction

Microorganisms in a microbial fuel cell catalyze the conversion of organic matter into electrical energy. As a carbon neutral technology, MFCs represent a novel method for renewable energy production and wastewater treatment mitigating greenhouse gas emissions and producing electricity directly from biomass. While research endeavors are committed to MFC optimization, these are largely focused on increasing power densities through system design. In contrast, less attention has focused on the microbiology driving electricity production. Given that energy generation is governed by biological activity, understanding the relevant microbiology is crucial to increasing the power yield and applicability of these systems. (1)

To date, knowledge of the microbial physiology of electricity production is hindered by the limited number of current-producing isolates in pure culture. With the exception of *Geothrix fermentans*, an Acidobacteria, studied isolates capable of direct electricity production are confined to the Proteobacteria (2-7). Furthermore, despite the broad phylogenetic diversity of bacteria known to transfer electrons onto solid phase electron acceptors (8), studies exploring electron transfer in MFCs predominately use the Fe(III)-reducing *Geobacter* and *Shewanella* species, members of the delta and gamma Proteobacteria respectively (5, 9-12) suggesting that the true diversity of electricity-producing organisms remains to be discovered. This is further supported by culture-independent studies, which indicate that the phylogenetic diversity of MFC microbial communities far exceeds that of electrochemically active isolates. Unfortunately these community studies (2-4, 13-17) were hampered by the use of low resolution DNA fingerprinting techniques targeting the 16S ribosomal RNA gene (16S rDNA), the presence of which does not necessarily demonstrate electrochemical activity but may simply be an artifact of gene persistence in the environment (18). Furthermore, because these studies focused on gene presence (16S rDNA) rather than gene expression (16S rRNA) they could not elucidate active members of the anode biofilm. However, in spite of these limitations, the broader microbial diversity identified in MFCs by molecular approaches highlights our limited understanding of the species involved in power generation and emphasizes the need for an in-depth characterization of the functional microbiology of anode communities.

In this study we use several complementary approaches to assess microbial diversity and function of anode biofilm communities. To expand the known phylogenetic diversity of electricity producing organisms, we concentrated on MFCs operated at 55°C, under which conditions neither *Geobacter* nor *Shewanella* species can survive (19). Moreover, as Proteobacteria represent a minor constituent of thermophilic anaerobic digester microbiota (17, 20), MFCs inoculated with thermophilic digester sludge offer a high likelihood of isolating novel electricity producing bacteria. Additional benefits also exist for operating fuel cells at elevated temperatures including potentially higher energy yields, easier maintenance of anaerobic reducing conditions because of the lower solubility of O₂ at elevated temperatures and thermal removal of most known pathogens (17).

To define the microbial ecology of an undefined system, we utilized several approaches to minimize the individual bias of any one method. We used clone library sequencing and a novel high-density oligonucleotide microarray (PhyloChip) (21-23) to identify organisms enriched only in current producing reactors. As the first application of the PhyloChip to anode biofilm communities, we provide a higher resolution analysis of microbial community composition uncovering a far greater diversity than observed previously. Additionally, although previous MFC community analyses have included a temporal context, in contrast to results

presented here, they did not account for bacterial populations that were also enriched on non-current producing electrodes. Also, we distinguished active from persistent members of the electricity-producing community by monitoring 16S rRNA expression in addition to cataloging 16S rRNA gene presence (18, 24, 25). Finally, to connect metabolic function and taxonomic identity, we isolated dominant members of the current generating bacterial community and demonstrated their ability to produce electricity. By understanding the potential function of these organisms in pure culture we infer their function within the electrode community.

Materials and Methods

MFC inoculum

The inoculum was collected from an operational thermophilic methanogenic anaerobic digester. In the laboratory, anaerobic digester sludge was centrifuged (10,000g; 15min.) and washed with bicarbonate buffer (30 mM, pH 6.8) under an N₂:CO₂ (80:20; vol:vol) headspace to remove soluble electron acceptors and electrochemically active compounds. The pellet was suspended in bicarbonate freshwater media lacking electron donor and acceptor to create a cell slurry under a headspace of N₂:CO₂ (80:20; vol:vol) (26) and incubated at 56°C. Removal of endogenous electron donors was monitored by cessation of biogas production as well as a depletion of organic acids. Upon removal of endogenous electron donors, four MFCs were inoculated (10:90; vol:vol) with the laboratory acclimated anaerobic digester sludge.

To evaluate the electrochemical activity of isolates *Geobacillus sp.* strain S2E and *Thermincola sp.* strain JR pure cultures were washed and inoculated into the MFC. Cells were grown anaerobically at 56°C in 1L volumes using acetate (10mM) as the electron donor and AQDS (10mM) as the electron acceptor using standard anaerobic techniques and basal bicarbonate media. Bacterial cells were harvested upon reduction of AQDS by centrifugation under an anoxic atmosphere (80:20; N₂:CO₂ atmosphere).

MFC experimental design

Acetate was chosen as the electron donor since it is not readily fermentable, it is the dominant electron donor in anaerobic systems, and our primary goal was to identify novel bacteria capable of anodic respiration (1). The experimental design included three treatments: 10 mM acetate amendment with circuit (two reactors), non-amended with circuit control (two reactors), and 10mM acetate amended without circuit control (one reactor). The non-amended reactors control for redox active components in sludge slurry, confirmed the absence of electron donors in the sludge inoculum, and identify bacterial populations residing on the anode independent of acetate dependent current production. The open circuit control normalizes for microbial populations that oxidize acetate independent of electron transfer to the anode.

Electrochemical characterization

Voltage (E_{cell} or cell potential) was measured across a fixed external resistor (R_{ext}) and logged using Chart 4.0 software (ADInstruments, CA). Data were recorded every ten minutes for 100 days. Current (I) was calculated from measured voltages using Ohm's law ($I = E_{\text{cell}}/R_{\text{ext}}$). With the exception of polarization curve (supporting information), external resistance was kept constant at 470 Ω . Maximum overall power (P) was calculated as $P = E_{\text{cell}}^2 \text{ Max} / R_{\text{ext}}$. Coulombic efficiency, or electrons in acetate that are recovered as current, was calculated in duplicate and the mean value reported. The actual coulombs transferred was determined by integrating current ($A = C/S$) vs. time (S) plots. The total amount of electrons available was calculated by [(concentration of added acetate, mM)*(1mol Ac/1000 mMol Ac)*(8 mol e-/1mol Ac)*(96485 C/mol e-)].

Nucleic acid isolation

To characterize changes in the microbial community after 100 days of current production, anode electrodes were removed from the four reactors. The surface of the anode was rinsed to remove debris using sterile nuclease-free water. The graphite electrode was scraped into sterile nuclease-free 2ml centrifuge tubes with a sterile razor blade. Nucleic acids were extracted from the graphite using a modified CTAB extraction buffer (equal volumes of 10% CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer, pH 8). 0.3 g of liquid nitrogen frozen graphite

was added to Lysing Matrix E tubes (Bio101 Systems, California) containing 0.5 ml CTAB buffer, 0.1 mg/ml proteinase K (Ambion, TX). Samples were mechanically lysed by beadbeating for 20 seconds at 550 rpm. Nucleic acids were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) followed by a second extraction with chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated overnight with isopropanol at -20 degrees and pellets rinsed with 70% ethanol. Five extractions were performed for each anode sample; pellets from each sample were combined and resuspended in 100 ul TE buffer. RNA and DNA were purified using the All Prep DNA/RNA kit (Qiagen, California). Isolated RNA was purified from any potentially contaminating DNA using on-column DNase digestion with DNase-free RNase set (Qiagen, California). To confirm the purity of RNA, and lack of DNA contamination, PCR amplifications were performed using non-reverse transcribed DNase-treated RNA as a control. Only samples demonstrating negative results, no amplification, were reverse transcribed into cDNA using Superscript II reverse transcriptase per the manufacturers protocol (Invitrogen, California).

16S rRNA gene amplification for PhyloChip and isolate identification

The 16S rRNA gene was amplified from gDNA extracts using universal primers 27F (5' AGAGTTTGATCCTGGCTCAG) and 1492R (5' GGTTACCTTGTTACGACTT) for bacteria and 4Fa (5' TCCGGTTGATCCTGCCRG 3') combined with 1492R for archaea. PCR amplifications were set up according to protocol outlined previously (27). PCR products for each sample were combined, concentrated by precipitation, and resuspended in nuclease-free water.

16S rRNA amplicon analysis by PhyloChip hybridization

The pooled PCR product was spiked with known concentrations of synthetic 16S rRNA gene fragments and non-16S rRNA gene fragments as internal standards for normalization, with quantities ranging from 5.02×10^8 to 7.29×10^{10} molecules applied to the final hybridization mix. Target fragmentation, biotin labeling, PhyloChip hybridization, scanning, and staining, as well as background subtraction, noise calculation, and detection and quantification criteria were performed as reported (27). A taxon was considered present in the sample when 90% or more of its assigned probe pairs for its corresponding probe set were positive (positive fraction ≥ 0.90).

Identifying anode community membership and dynamic populations by PhyloChip

A total of 10 PhyloChip arrays were performed including 3 DNA arrays from time zero inoculum, 4 time final DNA arrays from each of 4 reactors (2 experimental, 1 open circuit control, and 1 no acetate control), and 3 time final cDNA arrays (2 experimental and 1 no acetate control). Three randomly chosen replicate samples from time zero were analyzed with PhyloChip microarrays to confirm that reactors were inoculated with bacterial communities containing a similar composition; verifying bacterial heterogeneity was not a factor contributing to differences in the anode communities.

Microbial bacterial communities were present on anodes of current producing reactors and non current producing reactors, cluster analyses were performed on PhyloChip outputs from RNA and DNA arrays. Sample clustering was also confirmed using the R statistical programming environment using the function "heatmap" within the package "made4". Both samples and OTUs were clustered with 1 Pearson's correlation as the distance metric and UPGMA as the linkage method.(22). Given the high similarity with replicate reactors in the current producing reactors and time zero treatments, overall hybridization intensity values for these treatments represent the average of the HybScores from the reactors within the treatment group.

Identifying dominant anode members by clone library sequencing

Clone libraries were generated for each treatment (time zero inoculum, experimental, open circuit control, no acetate control) based on from pooled DNA PCR products. Bacterial 16S rRNA amplicon pools amplified with universal primers 27F (5' AGAGTTTGATCCTGGCTCAG) and 1392R (5' ACGGGCGGTGTGTAC) were ligated into pCR4-TOPO vectors (Invitrogen, CA). Ligated plasmids were transformed into *E. coli* TOP10 chemically competent cells according to the manufacturer's recommended protocol (Invitrogen, CA). Clones were randomly selected by a robotic picker and inserts were sequenced bi-directionally using M13 vector specific primers. Sequences were primer and vector screened using cross_match, quality scored using Phred and assembled into contigs using Phrap. Sequences were trimmed to retain only bases Phred \geq 20 and high quality contigs were tested for chimeras (five of which were removed from further analysis) using Bellerophon version 3 (DeSantis, 2006). To generate libraries for each sample, the respective full-length 16S RNA PCR products were cloned into pCR4-TOPO vectors (Invitrogen) according to the manufacturer's instructions. One hundred ninety-two transformants from each library were picked randomly. Double-ended sequencing reactions of the entire 16S RNA sequence were carried out with PE BigDye terminator chemistry (Perkin Elmer) and resolved with an ABI PRISM 3730 (Applied Biosystems capillary DNA sequencer) integrated into a pipeline (E. Kirton, unpublished).

Bacterial isolation

All enrichments and isolates were incubated at 55°C. To obtain bacteria adapted to utilizing insoluble electron acceptors, electrode scrapings were inoculated into fresh water media (28) amended with poorly crystalline Fe(III) oxide (29) as the sole electron acceptor. *Geobacillus* sp. strain S2E was isolated from anaerobic enrichments with 10 mM acetate and 50 mM Fe(III) oxide following serial dilution to extinction five times. To obtain bacteria capable of using exogenous electron shuttles, electrode scrapings were inoculated into fresh water media with acetate (10mM) and AQDS (10mM). Isolation of *Thermincola* sp. strain JR and *Coprothermobacter* sp. strain COPO were performed by transferring scrapings of the anode biofilms into 9 ml AQDS media under an anaerobic (80:20 N₂:CO₂) headspace. Colonies were isolated using the agar shake-tube technique (28) with acetate serving as the electron donor and AQDS serving as the electron acceptor. Once pure cultures were identified, all isolated colonies were tested for ability to reduce poorly crystalline Fe(III) oxide. Strain JR and S2E were maintained on Fe(III) oxide, where COPO was maintained on AQDS.

Analytical methods

All experimental analyses were performed in triplicate to ensure reproducibility and the results are expressed as the mean of these determinations. The concentration of acetate and organic acids in the fuel cells were determined via high pressure liquid chromatography with a fast acid analysis column (Biorad, Hercules, CA), operated with an eluent of 0.02 N H₂SO₄ and UV detection @ 210nm.

Nucleotide sequence accession numbers.

The sequence data of 16S rRNA gene fragments have been submitted to the GenBank database with accession numbers EU638332 - EU639378. The accession number for *Thermincola* sp. strain JR is GU815244.

Results and Discussion

PhyloChip analysis of community membership and dynamics

MFCs were constructed, inoculated, operated, and electrochemically characterized as previously described (Chapter 2). After 100 days of current-production the composition of the microbial community was assessed by excising biofilms from the electrodes. Nucleic acids (RNA and DNA) were extracted from the graphite matrices and the time zero inoculum. Total RNA, extracted from the current generating replicates and from the unfed reactor, was reverse transcribed to cDNA and PCR amplified along with genomic DNA from all reactors and the time zero inoculum. PCR amplification bias was minimized by using non-degenerate primers, 25 amplification cycles, and pooling of PCR products from 8 different annealing temperatures (22). Archaea were concluded to be an insignificant portion of the current producing community as PCR amplification of extracted nucleic acids using specific primers failed to produce any products in the current producing reactors.

We performed a hierarchical cluster analysis on PhyloChip intensity data to compare the overall community composition within the current generating reactors and between the current and non-current reactors (Figure 1A). Three clusters were detected: no current controls, time zero inoculum, and current generating community. The no current controls formed a separate cluster from the samples of the initial inoculum and the final current producing reactors, with the latter two arising from a common node indicating a population divergence resulting from the treatments. PhyloChip profiles with 16S rDNA and 16S rRNA extracted from current generating electrodes clustered together and represented similarly structured communities, whereas 16S rDNA and 16S rRNA profiles from a control reactor failed to cluster (Figure 1A). Although some previous studies using anaerobic sludge bioreactors indicated a lack of phylogenetic reproducibility in functionally performing reactors (30, 31), the 16S rDNA and 16S rRNA described communities in our two current producing reactors were similar (>89%) to each other. The high similarity of 16S rDNA and 16S rRNA communities from current producing anodes is explained by the electrode enrichment process, with electrode colonization (16S rDNA) comprised of organisms that may grow by electron transfer to the anode or grow in association with active members of the active current generating community (16S rRNA).

When analyzing the DNA PhyloChip arrays, we calculated and plotted the hybridization intensity score (HybScore) differences between each treatment and the initial inoculum to determine bacterial taxa enriched in current producing reactors over time. Differences in HybScores are a direct measurement of changes in gene copy number with a 1000 fold change in HybScore proportional to a single log change in relative abundance (22). Despite the fact that overall bacterial richness decreased in all reactor samples with time, certain members of the bacterial electrode community increased in relative abundance from the initial inoculum (Figure 1B). Bacterial phyla increasing by at least one log in relative abundance in current producing reactors and decreasing in non-current controls comprised the dynamic subset. Members of the Firmicutes (16 taxa, 38%), Proteobacteria (11 taxa, 26%), and Chloroflexi (5 taxa, 12%) were enriched only in current producing reactors and constituted dominant members of the dynamic subgroup. Alternatively in both of our non-current control reactors taxa from the family Enterobacteriales of the gamma Proteobacteria increased significantly but did not increase in current producing reactors. This indicates a metabolic role independent of power generation for members of the Enterobacteriales.

Our plan for tracking both the 16S rDNA and 16S rRNA was to link bacterial community structure with metabolic activity. This distinction is theoretically possible because the number of ribosomes in a cell is correlated to metabolic activity (32). To identify functionally active taxa we performed a subtractive analysis using 16S rRNA Hybcores from current producing reactors and a non-current producing control (no circuit treatment). 12 of 14 taxa that increased significantly in PhyloChip RNA response increased significantly in PhyloChip DNA response outlined above. As a result the 16S rRNA analysis identified active populations within the dynamic 16S rDNA subset. We conclude that analysis of both the 16S rRNA and 16S rDNA profiles is necessary for discerning the active subpopulations within the electrode bacterial community. Like the 16S rDNA response, the 16S rRNA response was dominated by the Firmicutes (7 taxa, 50%) and Chloroflexi (3 taxa, 21%). Interestingly, even though the majority of known electricity producing organisms are members of the Gamma- and Delta subclasses of the Proteobacteria (6, 9, 33), this phylum was not well represented in the 16S rRNA subset. In fact, the relative abundance of Proteobacteria reduced from 26% in the DNA response to less than 7% of the RNA response (1 taxon); indicating that dominantly detected Proteobacteria taxa may not have high levels of metabolic activity in the final current-producing community in our fuel cells.

Together the 16S rDNA and rRNA PhyloChip results indicate that Firmicutes play a significant role in this MFC current generating community. While 16S rDNA sequences belonging to the Firmicutes have previously been detected in the anode communities of active fuel cells (13-16, 33-35), there are a limited number of publications where Firmicutes represent a dominant portion (>50%) of the anode community composition (15, 33-35). Previous studies of MFCs inoculated with Firmicute isolates only produced current in the presence of an exogenous electron shuttle (4, 34, 36, 37) or as a byproduct of glucose fermentation (38, 39). Therefore, it was recently concluded that the presence of Gram-positive bacteria in an MFC is an outcome of ecological interaction with Gram-negative electricity producing organisms rather than a functional interaction and electron transfer with the electrode surface (34).

Dominant bacterial species identified

To more specifically identify Firmicutes involved in current generation we constructed 16S rDNA clone libraries from the initial inoculum and current producing reactors. Clone sequences (297) were produced from the current generating samples and were classified into 21 OTUs spanning six major bacterial lineages (Figure 2). Two Gram-positive phyla, Firmicutes (11 OTUs, 229 clones, 77% of clones) and *Coprothermobacter* (2 OTUs, 48 clones, 16% of clones), represented 93% of the clone sequences. *Coprothermobacter* species were previously identified by culture-independent molecular techniques in an MFC study with acetate as an electron donor and a thermophilic anaerobic digester inoculum (17). In that study, *Coprothermobacter* sequences represented 15.1% of the community. However, the importance of monitoring bacterial community dynamics rather than assaying the final community was clearly illustrated by the fact that in our study *Coprothermobacter* sequences decreased in relative abundance from the initial inoculum (24%) to the final time point on current producing anodes (16%) (Table 1). This result implies that the presence of *Coprothermobacter* in thermophilic communities may be an artifact of their ability to use diverse electron donors independent of electron transfer to the anode rather than a selective enrichment in the MFC due to current production.

Within the Firmicutes, sequences belonging to the genera *Thermicanus*, *Alicyclobacillus*, and *Thermincola* represented 27%, 25%, and 22% of the total clones respectively. These 3

genera could not be detected in clone libraries from the initial inoculum (482 total clones) (Table 1), suggesting their enrichment is associated with current generation. Sequences related to *Thermincola* spp. have been detected in only one other electrode community (40). In this study, MFCs were inoculated with marine sediment and operated at 60°C with acetate serving as the electron donor. Similar to our system, Firmicutes represented dominant members of the microbial community (64 of 80 clones; 80%) with 7 distinct phylotypes closely related (88-99% similar) to *Thermincola carboxydiphila* (Mathis *et al.*, 2007). This, combined with our results suggests that *Thermincola* species may have some unknown selective advantage over other bacteria in thermophilic MFC systems.

Here we use two culture-independent methods in our analysis. Both demonstrate the significant role of Firmicutes in the anode community expanding the recognized diversity of known electricity producing organisms. While the microarray approach detected greater than 35 times more bacterial types than the clone library sequencing, the PhyloChip data mirrored the clone library data. Due to the limited number of clones that can feasibly be sampled from clone libraries, highly abundant species like the Firmicutes may mask the presence of less abundant but functionally significant species. For instance, members of the phylum Chloroflexi were enriched in both DNA and RNA dynamic subgroups in the PhyloChip analysis but were not identified in the clone library analysis. (21-23)

Bacterial isolates capable of anodic electron transfer

In contrast to observations made with MFCs operated at mesophilic temperatures our community analyses indicate a functional role for the Firmicute phylum in current production at 55°C. Despite the large amount of data concerning the molecular identification of 16S rRNA sequences in the environment, understanding the function of microbial communities is a major bottleneck in microbial ecology (18). In order to conclusively link taxonomic identification with physiological function, we isolated bacteria capable of transferring electrons to the anode using media amended with amorphous hydrous ferric oxide (HFO) or 2,6-anthraquinone disulfonic acid (AQDS). Similar to an anode, HFO is an insoluble electron acceptor that should enrich for bacteria that can transfer electrons extracellularly without a requirement of exogenous electron shuttle (41). In contrast, AQDS will select for bacteria that can utilize an exogenous electron shuttle to indirectly reduce the anode and produce current.

Here we report for the first time a culture-based approach to describe members of the thermophilic bacterial anode community. We isolated several bacteria representing three genera, *Thermincola*, *Geobacillus*, and *Coprothermobacter* species. These genera represent three of the five most dominant members of the anode community and collectively represent 39% of the clone library sequence diversity (Figure 2). Both Firmicutes isolates, *Thermincola* sp. strain JR and *Geobacillus* sp. strain S2E, are of great interest given their significant enrichment from the initial inoculum (Table 1) and ability to reduce the solid phase electron acceptor (HFO). Interestingly, while both isolates reduced solid phase iron coupled to acetate oxidation, only *Thermincola* sp. strain JR could generate current independent of an electron shuttle with acetate as an electron donor (Figure 3A). Strain JR generated an average of 0.42 mA (when normalized for background current) in two separate experiments with a coulombic efficiency of 91%, similar to that observed for the original complex community (89%). In comparison to similarly designed MFCs operated at 30°C, *Geobacter sulfurreducens* produced a mean current of 0.251 mA (n=3, ± 0.04) from acetate oxidation (Bond and Lovley, 2003; Richter *et al.*, 2007; J.D. Coates and K.C

Wrighton, unpublished data), while *Shewanella onedensis* strain MR-1 produced between 0.03-0.3 mA from lactate oxidation (42).

These studies show for the first time that Firmicutes can play a direct role in current production with acetate as the electron donor. Our results demonstrate that it is imperative that the role of Gram-positive bacteria in anodic communities as well as in the environment be redefined to include their ability to respire insoluble electron acceptors such as ferric iron or anodes. The data presented here demonstrates that the presence of Gram-positive bacteria in anodes is not an artifact of ecological interaction but a direct result of their ability to transfer electrons to the anode independent of exogenous electron shuttles. Future experiments exploring the mechanism of electron transfer will be an important next step in elucidating the role of *Thermincola sp.* strain JR within the anode biofilm.

In contrast to *Thermincola sp.* strain JR, *Geobacillus sp.* strain S2E could not produce current in the absence of an exogenous electron shuttle (AQDS) and only produced small amounts of current in its presence (0.03 mA) (Figure 3B). This data demonstrates that utilization of an insoluble electron acceptor does not directly translate to the ability of the bacterium to utilize an anode as an alternative electron acceptor (39). A similar discrepancy between HFO and anode reduction was recently observed by Richter et al. using the mesophilic Fe(III)-reducing organism *Pelobacter carbinolicus* (43). Reasons for this discrepancy demand further attention, and may be attributed to a unfavorable anode redox potential, or possibly the production of ligands by strain S2E similarly to the Fe(III)-reducing Acidobacteria *Geothrix fermentans* (44).

The results of our culture-based investigation demonstrate the novel role of the Firmicutes in electricity generation. Given the broader diversity of other organisms identified in culture-independent analyses, it is highly likely there are additional species that can similarly transfer electrons directly to the electrode. Although not normalized to individual cell numbers, current production by strain JR alone accounts for approximately 70% of the amount produced by the entire community. This supports the notion that the increased current generation by the community may be due to the additional activity of other bacteria not yet identified in pure culture or by the synergistic electron transfer activities within the biofilm. Additionally, the importance of synergistic activity in electrochemically active biofilms has been demonstrated in mesophilic MFCs. Here electrochemically inactive isolates were able to exploit the redox shuttle produced by electrochemically active isolates (4, 34, 36). It seems plausible that bacteria like *Geobacillus spp.* may act similarly in the thermophilic electrode community identified here.

Conclusion

Altogether our findings demonstrate that thermophilic MFCs offer increased current production as well as the opportunity to uncover hidden microbial diversity and function. We use both DNA- and RNA-based approaches as well as pure culture studies to link taxonomic identification of the community to a functional role in current production. Here we show the insight gained through a combined application of cultivation and cultivation-independent methods to comprehensively characterize the structure and function of microbial communities.

Our results demonstrate that methods like the PhyloChip can uncover 35 times the diversity previously identified using lower resolution techniques like DGGE and clone library analysis. Complementary to the PhyloChip method, clone libraries identified the dominance of Firmicutes in our anode biofilms. Culture based methods isolated dominant Firmicutes and confirmed a previously unidentified electricity producing role for members of this phylum. Independent anodic electron transfer by Firmicutes is consistent with physiological studies demonstrating that isolated members of this Phylum can transfer electrons to solid phase electron acceptors, including iron and manganese, as part of a respiratory metabolism (8, 45, 46). Future physiological studies show promise toward unraveling the complexity of interactions in the anode biofilm as well as exploring mechanism of electron transfer to solid phase electron acceptors by Gram-positive bacteria.

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Table 1. Total number of clones and relative abundance of the five most frequently encountered OTUs within clone libraries from the initial inoculum and electricity producing electrodes.

OTU Number	Genus	Initial Inoculum		Current Production	
		Total Clones	%	Total Clones	%
OTU 1	<i>Thermicanus</i>	0	0	81	27
OTU 2	<i>Alicylobacillus</i>	0	0	73	25
OTU 3	<i>Thermincola</i>	2	0	65	22
OTU 4	<i>Coprothermobacter</i>	118	24	47	16
OTU 6	<i>Geobacillus</i>	0	0	4	2

Figure 1. A) Bacterial communities measured by PhyloChip hybridization intensity cluster into 3 distinct groups: initial inoculum, current producing reactors, and control reactors. **B)** Changes in hybridization intensity between current producing reactors and the initial inoculum identify bacterial taxa enriched in current producing anodes. Bacteria are ordered alphabetically from left to right according to taxonomic affiliation (color coded). Bars above the zero line represent bacteria that increased in abundance relative to the initial inoculum; bars below represent those bacteria that decreased in abundance. An increase of 1000 hybridization units is equivalent to a single log increase in relative abundance.

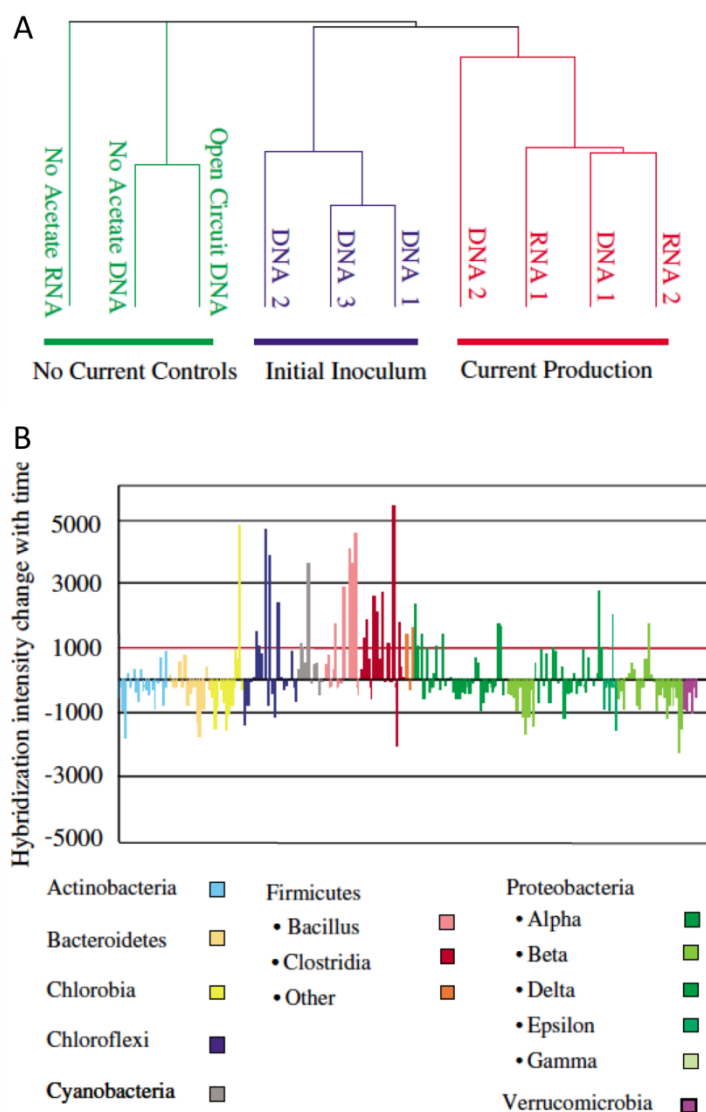


Figure 2. A maximum likelihood tree constructed from anode biofilm clone library sequences illustrating the phylogenetic position of 16S rRNA gene clones generated from current producing electrodes. A total of 297 clones were sequenced representing 21 OTUs (bold) with the total number of sequences for each OTU in parentheses. Bootstrap values are denoted with circles with values greater than 70% (○) and greater than 90% (●). Two dominant Phyla are denoted with colors; Firmicutes (mauve box) and Coprothermobacter (teal box). The three electrode biofilm isolates sequences are included on the tree (bold). The scale bar represents 0.1 inferred nucleotide changes per 100 nucleotides analyzed.

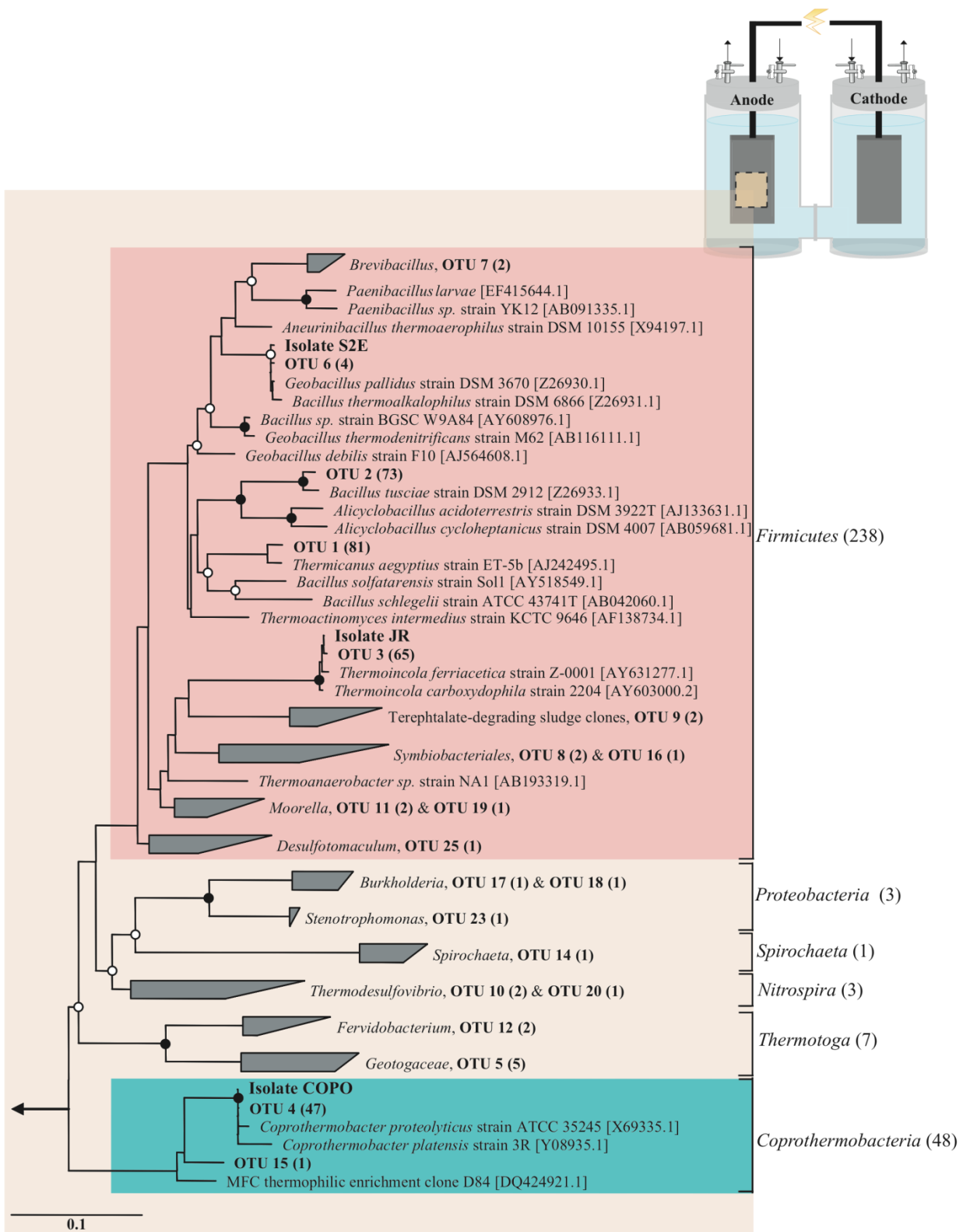
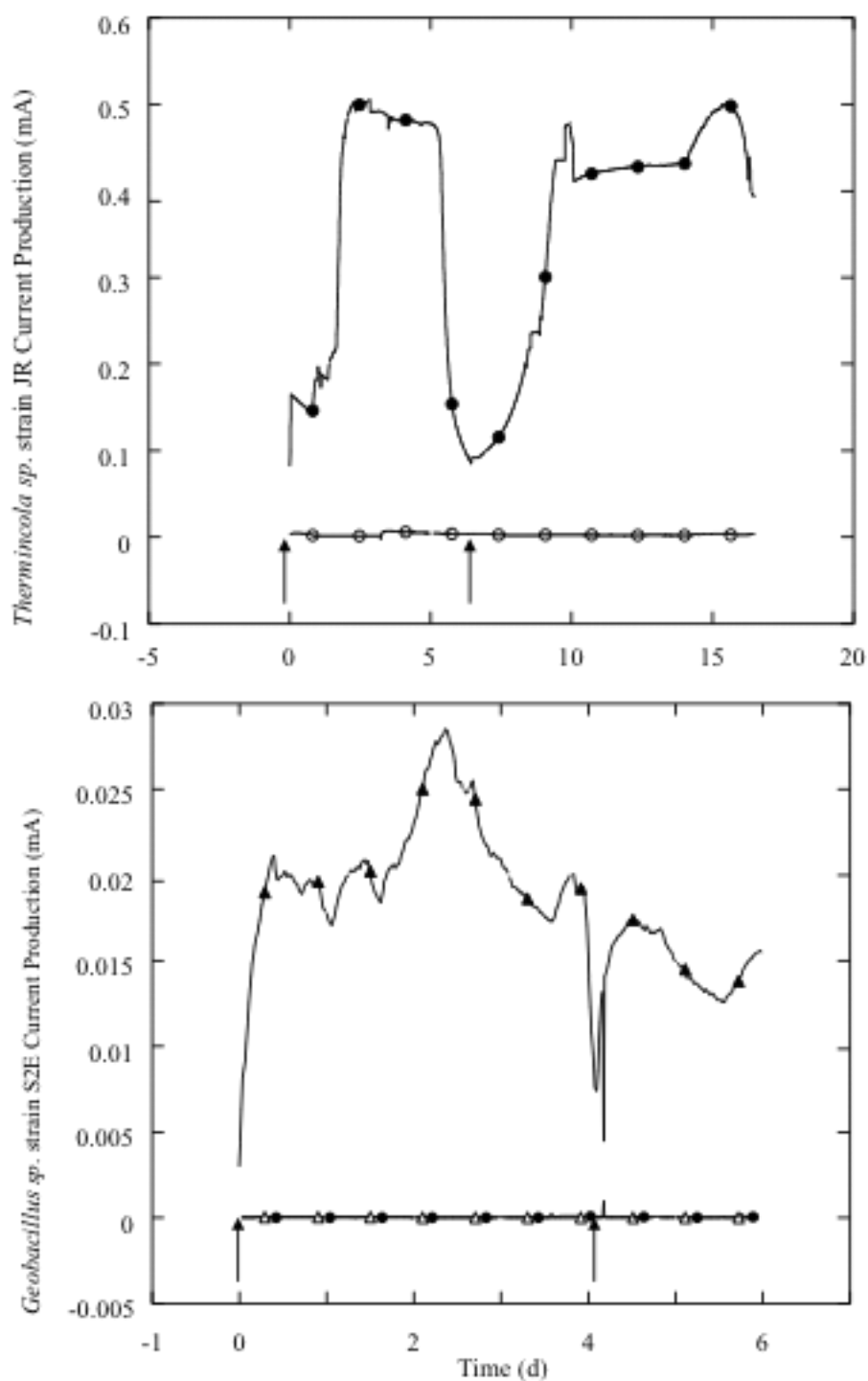


Figure 3. **A)** Current produced by a) *Thermincola sp.* strain JR with (●) and without (○) acetate in the absence of an exogenous electron shuttle. **B)** *Geobacillus* strain S2E with electron shuttle (AQDS) and acetate (▲), with electron shuttle without acetate (Δ), and without electron shuttle with acetate (●). Arrows indicate acetate amendment.



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Chapter 4

Evidence for cytochrome-mediated direct electron transfer by a Gram-positive bacterium isolated from a microbial fuel cell

Abstract

Despite their importance in environmental iron redox cycles and bioenergy production, the underlying physiological, biochemical, and genetic mechanisms of extracellular electron transfer by Gram-positive bacteria remain insufficiently understood. In this work, we investigated respiration of the insoluble electron acceptors Fe(III) oxyhydroxides and anode surfaces by *Thermincola potens* strain JR. This isolate, a Firmicutes belonging to the family Peptococcaceae in the order Clostridiales, was obtained from the electrode surface of a microbial fuel cell (MFC). Cyclic voltammetry (CV) measurements in conjunction with medium replacement experiments showed no evidence for soluble redox-active components secreted into the surrounding medium when strain JR was grown on these electron acceptors. Moreover, confocal microscopy revealed highly stratified biofilms in which cells contacting the electrode surface were disproportionately viable relative the rest of the biofilm. This together with the lack of correlation between biofilm thickness and power production suggests cells in contact with the electrode surface are primarily responsible for current generation. These data, along with cryo-electron microscopy experiments (cryo-EM), support direct electron transfer by *Thermincola potens* strain JR from the cell membrane across the 37-nm cell envelope to the cell surface. Furthermore, we present physiological and genomic evidence that direct extracellular electron transfer pathway is mediated by periplasmic and cell wall associated *c*-type cytochromes. Taken together, our findings provide the first study to implicate direct extracellular electron transfer by Gram-positive bacteria and identify *c*-type cytochromes as a potential molecular conduit for charge transport.

Introduction

Bacterial metal reduction is of broad global environmental, industrial, and medical significance. Assimilatory reduction of iron impacts pathogen physiology and disease virulence in phylogenetically diverse bacteria (1, 2), while dissimilatory metal reduction plays an critical role in a variety of biogeochemical cycles (3) and represents an essential metabolism for corrosion control in addition to the bioremediation of various organic and inorganic contaminants (4). Understanding mechanisms involved in microbial metal reduction are also of importance for harvesting electrical current from waste materials using microbial fuel cells (MFC) (5, 6).

Unlike most soluble electron acceptors used by microorganisms, Fe(III) and Mn(IV) exist primarily as insoluble oxyhydroxide minerals at circumneutral pH and must be reduced externally to the cell. As such, dissimilatory metal-reducing bacteria (DMRB) have evolved sophisticated mechanisms to transfer electrons from the electron transport chain on the cytoplasmic membrane to the electron acceptor on the cell surface (7). The two major types of extracellular electron transfer utilized by DMRB involve contact-dependent or contact-independent strategies for such extracellular electron transfer. Contact-dependent (or direct) mechanisms transfer electrons via multiheme *c*-type cytochromes (7, 8) or conductive pili (9, 10). In contrast, contact-independent mechanisms organisms utilize soluble redox-active mediators that shuttle electrons from the electron transport chain to the terminal electron acceptor (11, 12).

Despite the broad phylogenetic diversity of DMRB, current knowledge of electron transfer mechanisms is confined largely to two model Gram-negative species belonging to the *Geobacter* and *Shewanella* genera, which are members of the Delta- and Gammaproteobacteria, respectively. We now present a study on the reduction of insoluble electron acceptors by a novel Gram-positive bacterium, *Thermincola potens* strain JR, which is a dominant member of an anodic biofilm in a thermophilic MFC with over 90% conversion efficiency of acetate to electrical current (13). Using a combination of electrochemical and imaging measurements along with physiological and genomic data, we provide evidence for contact-dependency and the involvement of *c*-type cytochromes in direct electron transfer to anodes and insoluble ferric iron by this novel organism. Our data show that Gram-positive bacteria can participate in direct extracellular electron transfer, a finding that has broad implications for understanding environmental redox cycles and technological applications towards sustainable bioenergy.

Materials and Methods

Phylogenetic analysis

Genomic DNA of strain JR was isolated from pure cultures using the Power Soil Kit (MoBio) according to the manufacturer's protocol and PCR amplified as described previously (13). Sequences were aligned with MUSCLE 3.6 (14) and analyzed with MrBayes 3.2 (15, 16) using 4 chains until the standard deviation of the split frequencies was stabilized below 0.01, in this case for 1,087,000 generations, with a sample frequency of 1000. The first 25% of the samples were discarded for accurate estimation of the posterior probability distribution of the summary tree. To illustrate the phylogenetic placement of strain JR in the Firmicutes, *Geothrix fermentans* (U41563) and *Acidobacteria capsulatum* (D26171) were selected as representative Acidobacteria while *Geobacter metallireducens* strain GS-15 (L07834) and *Shewanella oneidensis* strain MR-1(AF005251) were chosen as representative Proteobacteria. Firmicutes accession numbers used for the analysis were: EU016427, EU016431, EU016410, EU016421, EU016442, EU016420, EU016424, EU016416, EU016422, EU016418, EU016409, EU016435, EU016425, EU016448, EU194832, EU194831, EU194830, EU638403, EU638851, EU638690, EU638872, EU638393, EU638902, EU638687, EU639376, EU638700, EU638698, EU638838, EU638794, EU638786, EU638783, EU638810, EU638644, AB159558, AB154390, AB232785, AB091323, X91169, AY631277, AY603000, EF542810, U76363, AJ621886, Y11575, DQ148942, AF516177, AJ276351, X61138, AJ575812, AB075768, X81021.

Microbiological methods

Unless otherwise noted, cultures were maintained at 60°C on fresh water media (17) amended with acetate as an electron donor and 100 mM poorly crystalline hydrous Fe(III) oxide (HFO) (18) as the sole electron acceptor. For cryo-EM microscopy and cytochrome scans cells grown in 1L AQDS and acetate (10mM respectively) were centrifuged (8000g for 10 minutes) and resuspended in 1ml PIPES buffer (pH 7.0). For inoculation into the MFC, cells grown in 1L AQDS and acetate (10mM respectively) were centrifuged (8000g for 10 minutes) and resuspended in 10 ml anode medium. Inocula (10%) for electron donor and acceptor characterization experiments were grown on basal medium supplemented with AQDS (10 mM) or acetate (10mM) for donor and acceptor characterizations respectively. To control for any electron donor or acceptor carry over, characterization results were not reported positive until after three successive positive transfers. We evaluated the use of the following electron acceptors: ferric citrate (10mM), ferric NTA (1, 5, or 10mM), ferric pyrophosphate (10mM), MnO₂ (5mM), fumarate (10mM), nitrate (10mM), nitrite (5mM), thiosulfate (5mM), sulfate (10mM), perchlorate (10mM), chlorate (10mM) and oxygen (4%).

To evaluate the ability of strain JR to grow by dissimilatory Fe(III) iron reduction, inocula (10%) from basal medium supplemented with HFO and acetate were inoculated into triplicate HFO and acetate tubes (treatment) and triplicate HFO lacking acetate (no donor control). At appropriate times samples were removed to measure cell concentration and iron reduction. Cell growth was determined by direct cell counts while Fe(III) reduction was monitored by measuring Fe(II) production using the ferrozine assay as previously described (19). Microbial fuel cells (MFC) were designed, constructed, and inoculated as previously described (13, 20) with a combined use of carbon fiber (Electrolytica) and graphite block anodes. MFCs were maintained at 60°C over the course of the experiments unless otherwise stated. Growth on the electrodes was measured by monitoring protein concentration using the bicinchoninic acid method in comparison against a standard curve prepared with bovine serum albumin (21).

Microscopy

Scanning electron microscopy (SEM) on the carbon cloth anode surface was performed. Aseptic and anoxic 1 cm² samples were collected in triplicate after current production stabilized (approximately 4 days after inoculation). Anode samples were immediately fixed in 2% (v/v) glutaraldehyde. Fixed samples were washed with 0.1M sodium cacodylate buffer, post fixed with 1% Osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hr, rinsed again with 0.1M sodium cacodylate buffer, ethanol series dehydrated (35%, 50%, 70%, 80%, 95%, 100% and 100%) and followed with critical point drying, mounting onto stubs and sputter coated with palladium/gold. Cells on the anode were viewed with a Hitachi S-5000 high resolution, cold field emission SEM with a secondary electron detector, under an accelerating voltage of 10kV.

For cryo-electron microscopy (cryo-EM) cells were placed onto lacey carbon grids (Ted Pella 01881) that were pre-treated by glow-discharge. The grids were blotted and plunged into liquid ethane using a Vitrobot (FEI automated vitrification device) before being transferred and stored in liquid nitrogen until imaged. Images were acquired at 20,000× magnification on a JEOL-3100 electron microscope with an underfocus value of 10µm. The microscope is equipped with an FEG electron source operating at 300 kV, an Omega energy filter, a Gatan 795 2K × 2K CCD camera and cryo-transfer stage. The stage and sample were cooled to 80 K for the duration of data collection.

To visually examine *Thermincola potens* strain JR biofilms three separate 2 cm² portions located on spatially distinct regions of the carbon cloth anode were aseptically and anoxically removed from two separate current-producing MFCs. Anode portions were dipped in freshwater medium to remove debris lightly associated with the biofilm and fixed in 3% glutaraldehyde. Anode biofilms were fluorescently stained with 50 µL each of SYTO BC and propidium iodide (also known as bacterial live/dead). After 15 minutes of staining, anodes were rinsed in freshwater medium for 2 minutes to remove excess dye. Biofilm structures were immediately examined via confocal laser scanning microscopy with a LSM710 (Carl Zeiss, Inc) microscope equipped with a 63X, and 100X objective lenses. Two and three dimensional images as well as quantification of red and green stained cells were calculated using Imaris 6.2 software (Bitplane, AG). Biofilms were monitored through time at 2, 22, and 42 days after current production stabilized. For each time point, biofilm thickness was reported from two current-producing electrodes. From each electrode, three samples were visualized, with three fields of view observed per sample.

Search for endogenous redox active shuttles

The role for free cells or soluble medium components (biofilm independent processes) in current production by strain JR was evaluated using media removal experiments. Growth medium in anodic chambers was removed (n=3) under aseptic anaerobic conditions. Anode biofilms were briefly rinsed and chambers were rinsed and drained with 30 mM bicarbonate buffer. Chambers were refilled with a sterile medium amended with 10 mM acetate that lacked vitamins and minerals, which may contain redox active components (22). Current production was recorded every 10 seconds over the course of the experiment.

The presence of excreted electron shuttle from current producing anodes was also evaluated. To process supernatants, media from duplicate current-producing anodes (operated for 8 weeks in batch format) was combined, centrifuged (8000g for 20 minutes) to remove cell debris, and degassed with N₂/CO₂ (80:20, vol:vol). Filters were not used to avoid losing organic molecules by adsorption to the filter (22). For experiments with supernatant additions, supernatants were processed and 8 ml aliquots (80% of the final working liquid volume) were

amended with HFO and acetate to obtain a final concentration of 100mM and 10 mM respectively. Three additional treatments (amended with either 10mM acetate, 10 mM acetate with 10 μ M AQDS, or without electron donor) were prepared in a similar fashion except spent medium was replaced with fresh FW media. AQDS is typically used as the “benchmark” exogenous shuttle and this treatment functions as a positive control. To ensure biomass loads were minimized, a 10% inoculum was added to each tube and Fe(II) production was monitored over time with the ferrozine assay in triplicate for each treatment.

Cyclic voltammetry (CV) performed on spent anode culture broth confirmed water-soluble redox components were not produced by strain JR. For the anode samples, 30 mL of anode medium were removed at appropriate intervals (T=0, T=4, and T=12 weeks from current stabilization) from duplicate MFCs operated in the dark. In addition, washed buffer from anode biofilms was also collected, degassed with N₂/CO₂ (80:20) in tin-foil wrapped bottles, and evaluated with CV to interrogate for redox active components contained within the biofilm. Cyclic voltammetry experiments were carried out using BASI’s Epsilon potentiostat and C-3 cell stand (West Lafayette, IN). The working electrode was a glassy carbon disk (3.0 mm diameter), the counter electrode was a platinum wire, and the solutions were referenced with an aqueous Ag/AgCl electrode purchased from BASI. The working electrode was cleaned prior to use with polishing alumina (0.05 μ m). Electrochemical cells were dried at 160°C in an oven and cooled to room temperature under a flow of N₂ prior to use. Cell-free aqueous samples (5 mL) were then introduced to the cell under a flow of N₂ and degassed with N₂. Cyclic voltammetry experiments were performed with scan rates ranging from 2 mV/sec to 1000 mV/sec. The temperature and pH of the media were measured immediately before and after each voltammogram to ensure the CV was measured under biologically relevant conditions. Cell-free samples with no identifiable redox active components were spiked with anoxic and light-protected stock solutions of riboflavin and AQDS as positive controls, thereby confirming that cyclic voltammetry could detect redox-active components at the appropriate mid-point redox potentials to biologically relevant concentrations.

Evidence for *c*-type cytochromes

To evaluate Fe(III) reduction by *c*-type cytochromes, spectrophotometric analyses were performed in triplicate in an anoxic glove bag using a Varian Cary 50 UV/Vis microplate spectrophotometer (Varian Inc., CA). Whole cells were diluted to approximately 0.11mg•mL⁻¹ total protein in phosphate buffer and titrated with small amounts of 0.5 M sodium dithionite until reduction of the *c*-type cytochromes was observed (1-3 μ L was typically required for reduction). Subsequently, aliquots of 1M Fe(III)-nitrilotriacetic acid (0.5-1.5 μ L) or 0.15M HFO suspension were added (3-6 μ L) and the spectrum was re-recorded.

Genome sequencing of *T. potens* strain JR was performed at the DOE Joint Genome Institute (23). Genes were identified using the publicly available gene modeling software Prodigal developed by the Oak Ridge National Laboratory (publication in progress). Analysis using blastp was performed against the entire database (24). Prediction of *c*-type cytochrome cluster gene product function was carried out by comparing gene models to the COGs and KEGG databases (25, 26). Domain structures of the gene models were assembled by searching against the InterPro database (27). Transmembrane domains and signal peptides were identified using the web-based programs TMHMM v2.0 and SignalP 3.0, respectively. The *c*-type cytochrome complement of strain JR was identified with the aid of the HMMER3.0b3 software package (28), using the doubled heme domain Pfam model PF09699 (29) as the input profile.

Results and Discussion

Phylogenetic and morphological characters

Thermincola potens strain JR was recently isolated from the anode surface of a functioning MFC operating at 55°C (13). Comparative analysis of the 16S rRNA gene sequence (1228 nucleotides) indicated that strain JR was a Firmicutes belonging to the family Peptococcaceae in the order Clostridiales (Figure 1A). As a member of the *Thermincola* genus, strain JR shared 99% 16S rRNA gene sequence identity with *Thermincola carboxdophila* and *Thermincola ferriacetica*, the two previously characterized members of this genus (30, 31). Although relatively little is known of these species or their environmental relevance, previous studies have revealed that clone library sequences from current-producing anodes inoculated with thermophilic anaerobic digester sludge (13) or marine sediment (32) were dominated by sequences similar (93-99% identity) to strain JR implying an important metabolic role for these organisms in MFCs. In addition, stable isotope probing and clone library studies also demonstrated that sequences related to strain JR (92% identity) were prevalent in benzene-oxidizing Fe(III)-reducing mesophilic mesocosms (33). Together these results demonstrate an enrichment of bacteria related to *Thermincola* in environments reliant on insoluble electron acceptors, suggesting this genus may have a selective advantage in systems defined by respiration requiring external electron transfer.

Cryo-EM images show that strain JR is a strict flagellated bacilli 1-2 µm in length and 0.5-1 µm in width and confirmed that strain JR was morphologically Gram-positive with a distinctive bipartite cell wall positioned above the plasma membrane that includes a 16nm low density inner wall zone (IWZ) surrounded by a 17nm high-density outer wall zone (OWZ) (Figure 1B). While our results are the first application of cryo-EM to thermophilic cell architecture, the images are consistent with cryo-EM images from previous studies of four mesophilic Gram-positive bacteria, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus gallinarum*, and *Streptococcus gordonii* (34-36). Recent cryo-EM studies have proposed that the previously identified IWZ represents a periplasmic space composed of soluble components while the OWZ represents the peptidoglycan cell wall network with its associated proteins (35, 36). This bipartite structure and physical distance has important ramifications in extracellular electron transfer by strain JR at the mechanistic and molecular level.

Preference for external electron acceptors

Reactors inoculated with strain JR have been producing current continuously for over three years. Current production by strain can be sustained at 90% of the original level over a 30°C difference in anode temperature (Figure 2A). Moreover current is dependent on temperature with a maxima between 60-70°C for one hour incubation and an optimum of 62°C for long-term operation. Below 55°C, current decreased linearly with temperature, while current decreased immediately at temperatures above 78°C. To visualize the organism on the anode, SEM of the electrode surfaces was performed after current production stabilized from two freshly inoculated MFCs. SEM revealed heterogeneous colonization with half the fields containing areas with a monolayer of cells and other areas of the electrode sparsely covered. Figure 2B was chosen to highlight individual bacterial attachment on the carbon fiber anode.

Many organisms capable of current generation in an MFC are also known Fe(III)-reducers (6). Given the ability of strain JR to couple acetate oxidation to current generation (13), we investigated the ability of this organism to alternatively respire metal ions. With acetate as an electron donor, strain JR readily reduced poorly crystalline hydrous ferric oxide (HFO) to a black magnetic precipitate, presumably magnetite, coupled to growth (Figure 3A). No Fe(III) reduction or growth occurred in the absence of acetate. Strain JR was also capable of reducing solid-phase Mn(IV) oxide with acetate or hydrogen as alternative electron donors. Importantly, apart from the humic substances analog 2,6-anthraquinone disulfonate (AQDS), the organism failed to grow using any of the alternative electron acceptors tested, including various soluble forms of iron (ferric citrate, ferric nitrilotriacetic acid, and ferric pyrophosphate) (Table 1). This preference for external electron acceptors is similar to findings with hyperthermophilic members of the Geobacteraceae, which were capable of reducing HFO but failed to reduce soluble forms of iron (8). To our knowledge, the obligate utilization of insoluble rather than soluble forms of ferric iron has not been reported for DMRB at thermophilic or mesophilic temperatures.

In addition to electron acceptors, putative electron donors were also evaluated with strain JR. Besides acetate, chemolithoautotrophy was possible using H₂ as an electron donor and AQDS as an electron acceptor. Sugars tested did not support growth; strain JR failed to reduce AQDS with other more complex electron donors and also failed to ferment casamino acids, yeast extract, and glucose. Isolate JR was able to grow with 100% CO as the sole energy source. For growth in CO, 0.2 g L⁻¹ yeast extract was added to the medium but did not support growth in the absence of CO (Table 2). Growth on CO under thermophilic conditions is shared between all isolated bacteria in the genus *Thermincola*. However, physiologically strain JR is more similar to *T. ferriacetica*, which can also reduce HFO and AQDS with acetate as electron, than it is to *T. carboxydophilia*, which is a reported obligate carboxydotrophic hydrogenogen.

Evidence for direct electron transfer

To elucidate the mechanism of electron transfer by *Thermincola potens* strain JR, we used a combination of physiological, electrochemical, and imaging analyses. To assess the contribution of biofilm-independent processes to current production in an operational MFC, we removed the spent culture broth surrounding the anode, rinsed the electrode biofilm, and replaced the removed fluids with fresh acetate amended medium lacking vitamins and minerals (Figure 3B). Replacement of spent culture fluids with fresh medium did not impact current production by the MFC, as current generation recovered to original levels (0.4 mA) immediately following medium replacement (29±15 minutes, n=3), indicating that the current-generating cells were primarily associated with the electrode biofilm. In contrast, when the anode in a working MFC was replaced with a fresh sterile anode, power generation was not observed until after an extended lag phase of 4 days indicating that the planktonic cells in the active culture broth had to colonize the anode surface before power recovery was measurable. These results signify that power production by strain JR in the MFC was dependent on attachment of the active cells to the electrode surface and was not mediated by soluble electron shuttles.

As an alternative method for detecting soluble electron mediators (37), we supplemented HFO-reducing cultures with cell-free spent anodic culture broth and demonstrated that the rate of HFO reduction with the spent broth was not stimulated relative to unamended controls (Figure 4). As expected, supplementation with AQDS (10µM), a soluble electron shuttle, produced a significant increase in HFO reduction rate.

Findings with strain JR clearly contrast with data from organisms known to utilize contact-independent strategies, where the addition of spent medium increased the rate of iron reduction relative to controls and anodic media replacement resulted in initially reduced current production and a significant delay in recovery of original current levels. Specifically, a reduction in current greater than 50% and a lag of 3-10 days was observed in media replacement studies with *Geothrix fermentans* and *Shewanella oneidensis* MR-1 (37, 38). Alternatively, results with strain JR are more similar to those previously obtained with *Geobacter sulfurreducens* and *Rhodospirillum rubrum*, two organisms known to use a direct mechanism of electron transfer to anodes and Fe(III)-oxides (21, 39), and signify that current production by strain JR was not dependent on planktonic (non-electrode bound) cells or biogenic soluble electron mediators excreted into the anode medium.

Previous studies have also used cyclic voltammetry (CV) to characterize bacterial anode electron transfer mechanisms from phylogenetically diverse bacteria (22, 40-45). We similarly applied CV over the course of three months to multiple MFCs inoculated with strain JR to rule out any possibility of electron transfer via soluble redox-active mediators. Only the results collected after three months of batch operation were shown for simplicity. No electrochemical differences were observed between the spent MFC culture broth and uninoculated control medium at any time point, indicating that redox-active components were either not present or were below detection (Figures 3C and 3D). Likewise, CV performed on spent culture broth from reduced ferric hydroxide cultures did not differ from sterile medium controls. To determine the detection limits of the biologically relevant redox-active mediators AQDS (data not shown) and riboflavin (Figure 2D) in our system, we added these compounds to spent MFC culture broth in titrations prior to CV analysis. Both compounds were detectable at the appropriate midpoint potential with detection limits (100 and 200 nM respectively) below those reported in studies with biologically produced mediators by *Shewanella* (22). In light of these CV results combined with the culture broth replacement studies outlined previously, it is unlikely that *Thermincola potens* strain JR produces soluble electron shuttling compounds for mediation of current production in the MFCs.

Confocal scanning laser microscopy (CSLM) with live-dead stain has previously been used to visualize anode biofilms (46-48). Here, CSLM was used to monitor temporal changes in strain JR biofilm thickness and activity on an anode surface. Once stable power production was achieved (7-10 days), electrode samples revealed a monolayer containing 92% active cells within a 2 μm distance from the electrode surface (Figure 5A). One month later, despite no change in current production, the biofilm on the electrode surface had grown tenfold in height ($21.7\mu\text{m} \pm 4.8\mu\text{m}$, $n=6$) (Figures 5B and 5C). However, this increased stacking of cells did not correspond to an increase in current production. Since the carbon electrode surface was non-uniform with a topographic variance of approximately 2 μm , a conservative distance of 5 μm was chosen as the electrode surface region in later time points to ensure the entire electrode surface was included in the analysis. Cell density analyses indicated a decrease with distance from the electrode surface from 2.2×10^3 cells per field of view at the electrode surface (0-5 μm) down to 1.4×10^3 cells per field of view at the middle (5-10 μm) and top portions (10-25 μm) of the biofilm respectively. Similarly, viability staining indicated that the primary region of live cells was localized to the electrode surface (Figure 5B) with the proportion of viable cells decreasing significantly from 73% at the electrode surface to 42% for the rest of the biofilm (Figure 5C, Table 3).

These biofilm results demonstrate that there was not a corresponding increase in current production with an increased stacking of strain JR cells on the anode surface. The cells in direct

contact with the anode appeared to contribute exclusively to current production, as they remained viable over time and the per-cell efficiency of current production decreased with an increase in the height of the biofilm. This finding is in direct contrast to confocal microscopy results obtained with *G. sulfurreducens*, which also developed multilayer biofilms on the anode surface, where cells up to 50 μm from the electrode surface remained equally viable and current production correlated to biofilm height (47). It was suggested that while *Geobacter* cells in intimate contact with the anode surface may rely on electron transfer via outer membrane *c*-type cytochromes, conductive pili were responsible for long-range transfer across the multilayer biofilm (47, 48).

Role for *c*-type cytochromes

Taken together, the results from physiological, electrochemical, and microscopic investigations suggest that *Thermincola potens* strain JR uses a direct mechanism of electron transfer to ferric iron oxides and anodes. In this context, it is currently recognized that *c*-type cytochromes play an important role in extracellular reduction of iron by Gram-negative bacteria (8, 9, 49); however, little is known about their role in metal-reducing bacteria outside the Proteobacteria. For strain JR, both genomic and physiological evidence support the role of *c*-type cytochromes in reduction of insoluble electron acceptors.

In direct electron transfer to external electron acceptors by *Shewanella oneidensis* and *Geobacter sulfurreducens*, *c*-type cytochromes located in the periplasm and outer membrane are required for transporting electrons generated on the inner membrane to the cell surface. The physiological importance of these proteins is reflected in the genome sequences of each of these organisms with 42 and 111 putative *c*-type cytochromes identified in *Shewanella* and *Geobacter* respectively (8, 9). Most of these cytochrome polypeptides possess more than one heme motif, with one *c*-type cytochrome in *Geobacter* containing 27 heme motifs (50, 51). In *S. oneidensis* MR-1, periplasmic multiheme *c*-type cytochromes CymA and MtrA are believed to transfer electrons from the quinone pool of the electron transport chain to a multiheme outer membrane cytochrome (Omc) protein complex containing MtrC and OmcA (52, 9). Likewise, outer membrane multiheme *c*-type cytochromes OmcB, OmcE and OmcS are implicated in the reduction of ferric oxides in *Geobacter sulfurreducens* (8, 51).

Spectral analysis was initially used to confirm the presence of cytochromes in strain JR and assess the physiological capacity of these proteins for reducing insoluble ferric iron. Dithionite-reduced minus HFO-oxidized spectra of whole cells showed absorption peaks at 420, 526, and 552 nm, corresponding to the gamma, beta, and alpha bands of *c*-type cytochromes (Figure 6A). The presence of *c*-type cytochromes that could be oxidized by HFO, implicates their functional role in the reduction of insoluble electron acceptors by this Gram-positive bacterium.

To identify these cytochromes, we searched the draft genome of *Thermincola potens* strain JR and found 35 ORFs containing doubled heme domains (PF09699), which are associated with *c*-type cytochromes (43). Of these putative *c*-type cytochromes, 74% (31) contain multiple doubled heme domains, with one ORF (ThermJRDraft 1055) containing 56 heme-binding motifs. Moreover, 97%, or 34, contain N-terminal signal peptides (SignalP 3.0) and an additional 37%, or 13, contain a single N-terminal transmembrane domain (TMHMM 2.0), predicting that many cytochromes are embedded in or external to the Gram-positive cell membrane (53). Initial analysis of these multiheme *c*-type cytochromes yielded gene models homologous to membrane

and periplasmic *c*-type cytochromes identified as being involved in extracellular electron transfer in both *Geobacter* and *Shewanella* species. Specifically, we identified ORFs related to MtrA (ThermJrDRAFT 350, 1060), as well as other proteins displaying partial sequence similarity to outer-membrane *c*-type cytochromes from these well-characterized DMRB (Table 4 and 5). However, this similarity is largely confined to the double heme motifs, suggesting that subfamilies of *c*-type cytochromes with novel domain structure may be associated with extracellular electron transfer by this Gram-positive bacteria.

The extensive *c*-type cytochrome complement of strain JR exhibits a complex and non-random genomic organization. The majority of these multiheme *c*-type cytochromes are predicted as being transcribed in clusters containing two or more cytochrome encoding genes, spanning five regions on the reverse strand, suggesting they encode co-expressed gene products. Unique to strain JR, these *c*-type cytochrome-rich gene clusters contain cytochrome assembly proteins, domains that target proteins to the cell surface (S-layer homology domain PF00395), and at least one gene encoding proteins containing NHL repeat domains (PF01436) and one with TPR repeat domains (PF00515, PF07719) per cluster. Information on predicted protein length, presence of signal peptide/transmembrane, best BLAST annotation and organisms, and E-value for each loci is included (Table 4 and 5). In the clusters, the NHL repeat-containing proteins are located immediately upstream of putative *c*-type cytochromes, and are often preceded by TPR domain-containing proteins (Figure 6B). The recent generation of a library of transposon mutants in *Geobacter sulfurreducens* identified TPR domains in proteins that impacted biofilm attachment, while insertions in NHL domain containing proteins located near *c*-type cytochromes significantly reduced the ability for electron transfers to anodes and iron minerals but not soluble iron (54). The recurrent linkage of these domains with *c*-type cytochromes in strain JR may denote a conserved functional role in Gram-positive extracellular electron transfer.

The prevalence and activity of *c*-type cytochromes in strain JR is reminiscent of the physiology of the metal-reducing Proteobacteria, rather than other characterized members of the Firmicutes or thermophilic bacteria. While genetic evidence and spectral analysis have identified a role for *c*-type cytochromes in Fe(III) reduction from Gram-negative species (8), until now, no correlation between presence of *c*-type cytochromes and iron reduction has been reported outside the Proteobacteria. Within the Firmicutes, *c*-type cytochromes were identified in one ferrihydrite-reducing Gram-positive bacteria, *Thermoterrabacterium ferrireducens* (55). However, not all DMRB contain detectable amounts of *c*-type cytochromes, as cytochrome spectra were reportedly not detected in whole cell extracts of *Desulfitobacterium metallireducens*, a mesophilic Firmicutes capable of metal reduction (56). No spectral or genomic evidence for *c*-type cytochromes was identified in the hyperthermophilic archaeon, *Pyrobaculum islandicum*, another known DMRB. This organism was reported to use a contact-independent mechanism of electron transfer, suggesting that mediators may be involved in extracellular electron transfer (57, 58). Consequently, the results presented in this manuscript are the first to demonstrate a role for cell envelope *c*-type cytochromes in a bacterium outside the Proteobacteria and the first to implicate these in direct extracellular electron transfer.

Direct extracellular electron transfer by Gram-positive bacteria

Compared to Gram-negative bacteria, Gram-positive bacteria lack an outer membrane, have a thicker cell wall (10-80nm), and may be encased in a glycoprotein S-layer. Due to these structural differences, it was generally thought that Gram-positive bacteria were incapable of

direct electron transfer to external electron acceptors (59). However, in addition to the work reported here, there are a handful of mechanistic studies demonstrating direct electron transfer to an insoluble electron acceptor by Gram-positive genera. One of the first studies investigated the mechanism of iron reduction and acquisition by *Listeria monocytogenes*, a Gram-positive food-borne pathogen, where iron is required for growth during experimental infections. Dialysis membrane separation was used to rule out secreted mediators and implicate cell surface ferric reductases in assimilatory iron reduction by *Listeria monocytogenes* (60). In addition to iron assimilation by Gram-positive pathogens, contact-dependent dissimilatory metal reduction mechanisms have also been identified in two Gram-positive DMRB. The inability of culture filtrate to augment reduction of HFO was evidence that *Thermoanaerobacter* sp. strain BSB-33 did not release extracellular redox mediators (61), while CV demonstrated that *Thermincola ferriacetica* also employed a direct mechanism of electron transfer to MFC anodes (62). Although not isolated from a MFC, *T. ferriacetica* has the capacity to reduce anodes (32), HFO, magnetite, and AQDS (31). Together with our findings these studies suggest that direct electron transfer to insoluble electron acceptors may be important to many Gram-positive bacteria. The shared mechanism of electron transfer to anodes within the genus *Thermincola* is significant, as differences in iron reduction strategies within a single genus were observed with both *Pyrobaculum* and *Shewanella* genera (58).

Research regarding extracellular reduction by Gram-positive bacteria is in its infancy. Based on our experimental results and observation of the cell envelope structure, we propose four models for direct electron transfer at a molecular level: fully contained redox mediator, ‘nanowires’, conductive cell walls, or a cytochrome conduit linking the inner membrane to the cell surface (Figure 7). We have considered the possibility that *Thermincola* spp. secrete a redox mediator that is fully contained within the electrode biofilm, which was not detectable in the surrounding medium (Figure 7A). We find this option unlikely as our results outlined above and reports by Marshall and coworkers (2009) (62) independently failed to detect secreted compounds. Moreover, this same methodology was sufficient to identify flavin as the redox mediator secreted and largely sequestered within biofilms of *Shewanella oneidensis* (22), as well as sufficient to demonstrate that *Geobacter sulfurreducens* uses a direct mechanism of electron transfer (21). We have also considered that similar to Gram-negative bacteria (8, 9), Gram-positive bacteria may use conductive pili to transport electrons from the cell membrane to the cell surface (Figure 7B). Nanowires in *Geobacter* act as electronic conductors extending the electron transfer capability to beyond the cell wall, and enabling long-range transfer through anode biofilms. To date this model is not supported by CSLM results of *Thermincola potens* biofilms, but three dimensional high resolution microscopic analysis of strain JR biofilms is in progress.

Unlike the fully-contained shuttle or nanowire models, the latter two models deserve further consideration, as they cannot be refuted by the experimental results to date. Ehrlich (2008) suggested a model of direct electron transfer by Gram-positive bacteria where the terminal reductase is located in the periplasm. The electrons are transferred from the reductase across the periplasmic space to non-peptide components of the cell wall, e.g. teichoic and teichuronic acids, which conduct the electrons to the cell surface (Figure 7C) (63). Conceptually, this model fails to consider that Gram-positive bacteria have extensive methods for covalently and non-covalently attaching proteins to the cell wall (64) and much of the circumstantial evidence for this model insufficiently distinguishes attached proteins from cell wall constituents. Furthermore, an initial structural examination of teichoic and teichuronic acids does not indicate features that could

confer redox activity onto these components. However, ongoing studies evaluating the intrinsic redox capacity of cell wall components in strain JR as well as other Gram-positive DMRB are underway.

In direct electron transfer by *Thermincola potens* strain JR, we propose that *c*-type cytochromes act as a conduit facilitating electron transfer across the 33 nm wide Gram-positive cell envelope (Figure 7D). This model is supported by spectral analysis that demonstrated *c*-type cytochromes with the capacity to be oxidized by insoluble ferric oxides. Additional support for the proposed role of *c*-type cytochromes can be found in the *Thermincola potens* strain JR draft genome, which represents the first Gram-positive DMRB to be fully sequenced (23). Despite the absence of an outer membrane in strain JR, we identified a large number of *c*-type cytochromes similar to those that function in extracellular electron transfer in model Gram-negative DMRB. Together our results represent the first time multi-heme *c*-type cytochromes have been functionally characterized and putatively associated with the periplasm and cell surface in a Gram-positive bacterium.

We used a combination of physiological, electrochemical, and genomic observations to expand the knowledge of Gram-positive extracellular respiration of metals and anodes using *Thermincola potens* strain JR as a suitable model organism. Our findings demonstrate that this organism is capable of direct electron transfer to insoluble electron acceptors like Fe(III) and anode surfaces, and while the molecular basis for this mechanism is presently not understood, we hypothesize *c*-type cytochromes play a role in facilitating electron transfer across the Gram-positive cell envelope. Implementation of additional technologies, including proteomics, transcriptomics, and genetics to the cell envelope fractions should reveal how *c*-type cytochromes are functionally integrated into the physiology of strain JR. Ultimately, this research will help elucidate the phylogenetic and physiological variation in bacterial electron transfer mechanisms and improve our understanding and modeling of Gram-positive metabolism with applications to pathogen physiology, bioremediation, and energy generation.

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Byrne-Bailey KG, Wrighton KC, Melnyk RA, Agbo P, Hazen TC, & JD Coates (2010) Draft genome sequence of *Therminicola potens* strain JR. *J Bacteriol* (in press).

Table 1. Electron acceptors utilized by *T. potens* strain JR. A positive result was denoted after three successive transfers.

Electron acceptors (with 10mM acetate)	Concentration	strain JR
Fe(III) HFO	100mM	+
Mn(IV) oxide	30mM	+
AQDS	10mM	+
Fe(III) pyrophosphate	5 and 10 mM	-
Fe(III) citrate	5 and 10 mM	-
Fe(III) NTA	2, 5, and 10 mM	-
Fumarate	10 mM	-
Sulfate	10 mM	-
Nitrate	10 mM	-
Nitrite	2 and 5 mM	-
Thiosulfate	5 mM	-
Selenate	10 mM	-
Oxygen	5 ml	-

Table 2. Electron donors utilized by *T. potens* strain JR. A positive result was denoted after three successive transfers.

Electron donors (with 10mM AQDS)	Concentration	strain JR
Acetate	100 mM	+
Hydrogen	10 ml	+
Carbon monoxide	10 ml	+
Benzoate	0.5 mM	-
Butyrate	5 mM	-
Formate	5 mM	-
Fumarate	0.5 mM	-
Glucose	10 mM	-
Glycerol	10 mM	-
Lactate	10 mM	-
Maltose	10 mM	-
Methanol	5 mM	-
Palmitate	1 mM	-
Phenol	1 mM	-
Pyruvate	5 mM	-
Succinate	1 mM	-
Toluene	0.03 mM	-
Trehalose	10mM	-
Yeast extract	1g/L	-

Table 3. Relative distribution of live cells throughout a cross section of the anode biofilm.

Location	Distance (μm)	Abundance Live (% of total cells)	Cell number (cells/field of view)
Electrode surface	0-5	73%	2.2×10^3
Middle of biofilm	5-10	41%	1.4×10^3
Top of biofilm	10-28	43%	1.4×10^3

Table 4. Information on the predicted protein length, presence of signal peptide/transmembrane, best BLAST annotation and organisms, and E-value for each *c*-type cytochrome loci located in cluster 0150-0166.

Locus	Protein Length	Signal Peptide/ Transmembrane	Best BLAST Annotation	Best BLAST Organism	E-value
0150	445	-/-	Radical SAM domain protein	<i>Geobacter sp.</i> M18	1.00E-142
0151	452	-/-	hypothetical protein GSU2893	<i>Geobacter sulfurreducens</i> PCA	1.00E-102
0152	490	-/-	Radical SAM domain protein	<i>Geobacter sp.</i> FRC-32	1.00E-144
0153	131	-/+	hypothetical protein GSU2889	<i>Geobacter sulfurreducens</i> PCA	9.00E-08
0154	4140	-/+	hypothetical protein	<i>Candidatus Kuenenia stuttgartiensis</i>	1.00E-76
0155	294	-/-	NHL repeat containing protein	<i>Dethiobacter alkaliphilus</i> AHT 1	1.00E-15
0156	293	-/+	cytochrome c assembly protein	<i>Geobacter sp.</i> M21	7.00E-43
0157	421	-/+	ResB-like	<i>Moorella thermoacetica</i> ATCC 39073	3.00E-14
0158	3091	-/+	fibronectin type III domain-containing protein	<i>Opitutus terrae</i> PB90-1	3.00E-30
0159	798	-/+	multiheme cytochrome	<i>Dethiobacter alkaliphilus</i> AHT 1	9.00E-22
0160	332	-/+	NHL repeat-containing protein	<i>Carboxydotherrmus hydrogenoformans</i> Z-2901	5.00E-45
0161	400	+/+	multiheme cytochrome	<i>Dethiobacter alkaliphilus</i> AHT 1	1.00E-18
0162	314	+/+	PKD domain containing protein	<i>Candidatus Methanosphaerula palustris</i>	5.00E-28
0163	211	-/+	Tetratricopeptide TPR_2 repeat protein	<i>Dethiobacter alkaliphilus</i> AHT 1	4.00E-22
0164	388	-/+	metallophosphoesterase	<i>Alkaliphilus metalliredigens</i> QYMF	5.00E-95
0165	183	+/+	PpiC-type peptidyl-prolyl cis-trans isomerase	<i>Anaerocellum thermophilum</i> DSM 6725	2.00E-13
0166	872	+/+	middle cell wall protein precursor	<i>Brevibacillus brevis</i> NBRC 100599	4.00E-47

Table 5. Information on the predicted protein length, presence of signal peptide/transmembrane, best BLAST annotation and organisms, and E-value for each c-type cytochrome loci located in cluster 1052-1066.

Locus	Protein Length	Signal Peptide/ Transmembrane	Best BLAST Annotation	Best BLAST Organism	E-value
1052	938	-/-	Radical SAM domain protein	<i>Geobacter</i> sp. FRC-32	1.00E-124
1053	283	-/+	cytochrome c assembly protein	<i>Geobacter metallireducens</i> GS-15	2.00E-58
1054	359	+/+	hypothetical protein Gura_0510	<i>Geobacter uraniireducens</i> RF4	2.00E-13
1055	2110	-/+	cytochrome c family protein	<i>Geobacter sulfurreducens</i> PCA	7.00E-70
1056	540	+/+	hypothetical protein DealDRAFT_1439	<i>Dethiobacter alkaliphilus</i> AHT 1	1.00E-30
1057	363	+/+	multiheme cytochrome	<i>Dethiobacter alkaliphilus</i> AHT 1	3.00E-10
1058	340	-/+	NHL repeat-containing protein	<i>Carboxydotherrmus hydrogenoformans</i> Z-2901	5.00E-43
1059	217	-/+	TPR domain-containing protein	<i>Carboxydotherrmus hydrogenoformans</i> Z-2901	2.00E-24
1060	507	-/+	cytochrome C family protein	<i>Dethiobacter alkaliphilus</i> AHT 1	4.00E-58
1061	407	+/+	putative cytochrome c-type protein	<i>Carboxydotherrmus hydrogenoformans</i> Z-2901	2.00E-81
1062	347	-/+	putative cytochrome c-type protein	<i>Carboxydotherrmus hydrogenoformans</i> Z-2901	5.00E-76
1063	323	-/-	Trans-hexaprenyltranstransferase	<i>Desulfotomaculum acetoxidans</i> DSM 771	1.00E-105
1064	66	-/+	Sec-independent protein translocase TatA	<i>Moorella thermoacetica</i> ATCC 39073	2.00E-14
1065	556	-/-	alpha-glucan phosphorylase	<i>Hellobacterium modesticaldum</i> ICE1	0.00E+00
1066	270	-/-	glutamate racemase	<i>Alkaliphilus metalliredigens</i> QYMF	4.00E-67
1052	938	-/-	Radical SAM domain protein	<i>Geobacter</i> sp. FRC-32	1.00E-124
1053	283	-/+	cytochrome c assembly protein	<i>Geobacter metallireducens</i> GS-15	2.00E-58

Figure 1. *Thermincola potens* strain JR is phylogenetically (A) and morphologically (B) Gram-positive. **A)** 16S rRNA gene tree constructed using Bayesian analysis reveals *T. potens* is a member of the phylum Firmicutes within the family Peptococcaceae. Closed circles at nodes indicate posterior probabilities ≥ 0.97 ; open circles indicate a posterior probability < 0.97 . **B)** Cryo-EM projection views of vitrified whole mounts fail to detect an outer membrane but show the plasma membrane (PM) is enclosed by a 16 nm thick low-density periplasmic space (PS) which is bound by a 17nm thick high-density cell wall (CW).

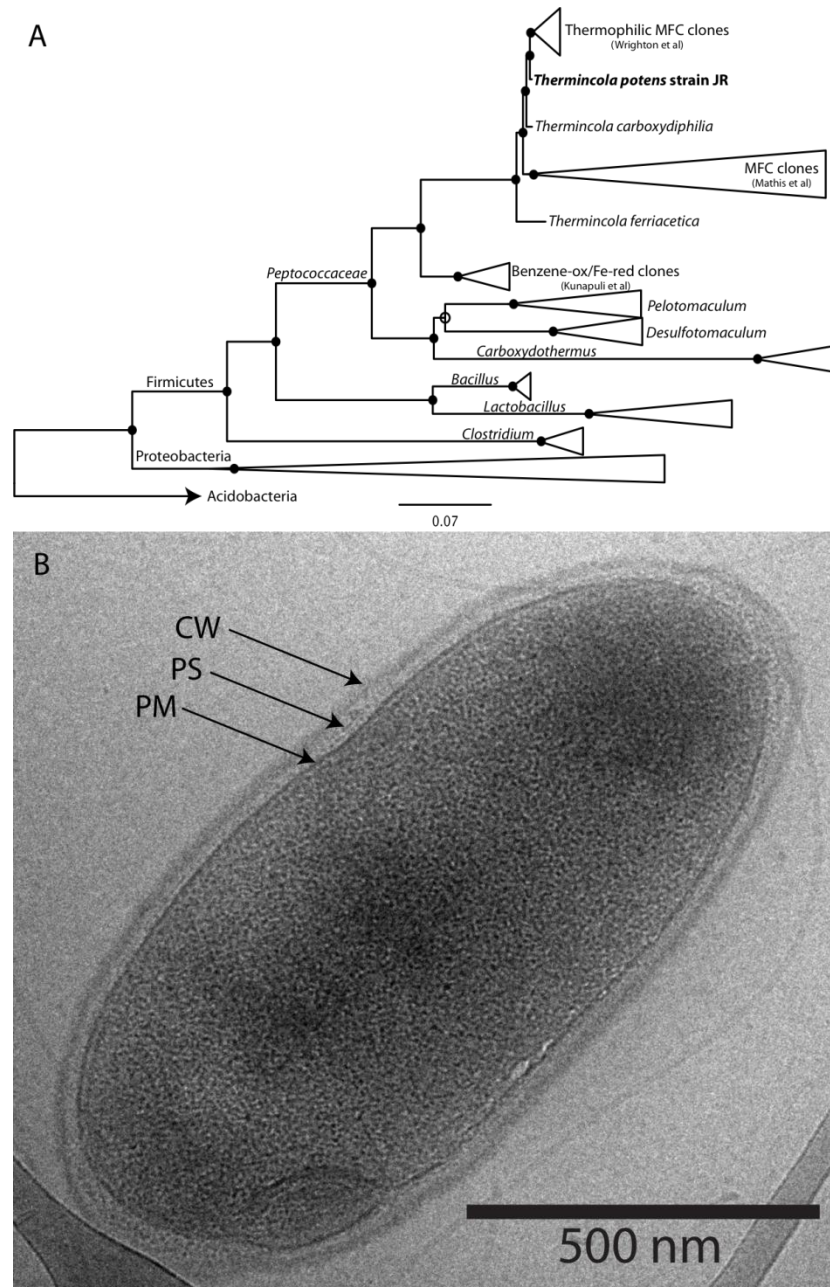


Figure 2. A) Effect of temperature on current production in a microbial fuel cell by strain JR. The optimum temperature for current production is between 60° and 70°C with a maximum sustainable temperature of 78°C and minimum sustainable of 45°C. **B)** SEM of a graphite fiber anode after growth of strain JR in a microbial fuel cell.

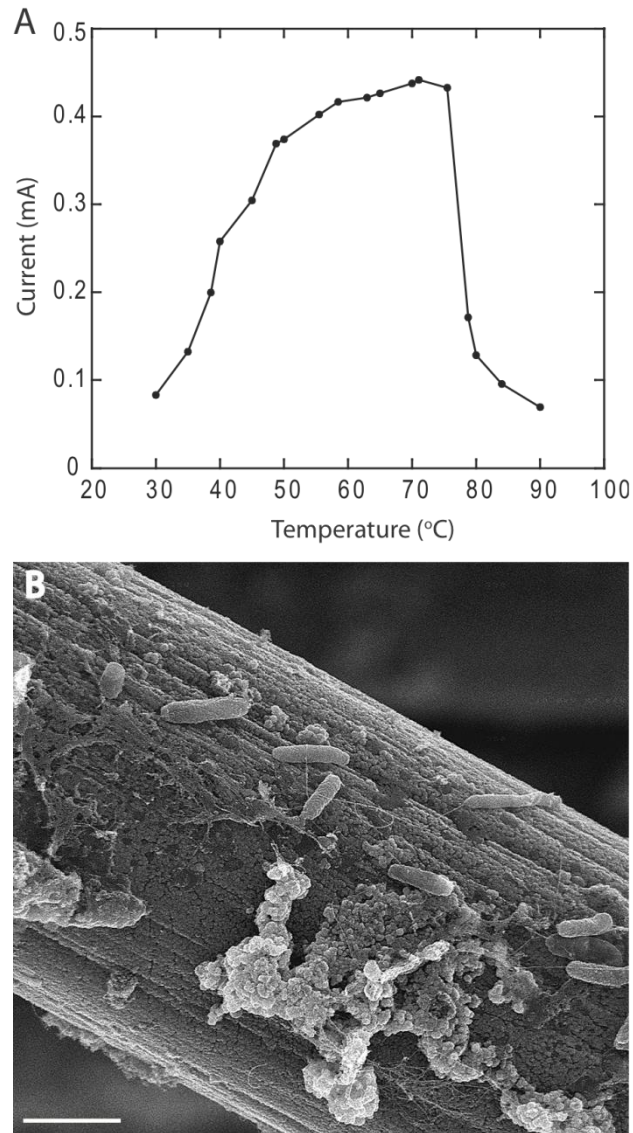


Figure 3. **A)** Growth (\blacktriangle , \triangle) of strain JR and Fe(III) reduction (\blacksquare , \square) with hydrous ferric oxide as the electron acceptor. Ferric iron basal media was amended with cells and acetate (closed symbols) or a control containing cells but no acetate (open symbols). Mean results of triplicate cultures are reported. **B)** Current production by strain JR returned to original levels after replacement of anode media ($n=3$). **C)** CV of cell-free supernatant from a microbial fuel cell that was batch-operated for 12 weeks (red line) and a control CV of media baseline (black line). **D)** CV of cell-free supernatant from a microbial fuel cell in (A) spiked with riboflavin (black line) and the baseline subtracted difference between MFC and control voltammograms from Figure 2C (blue line).

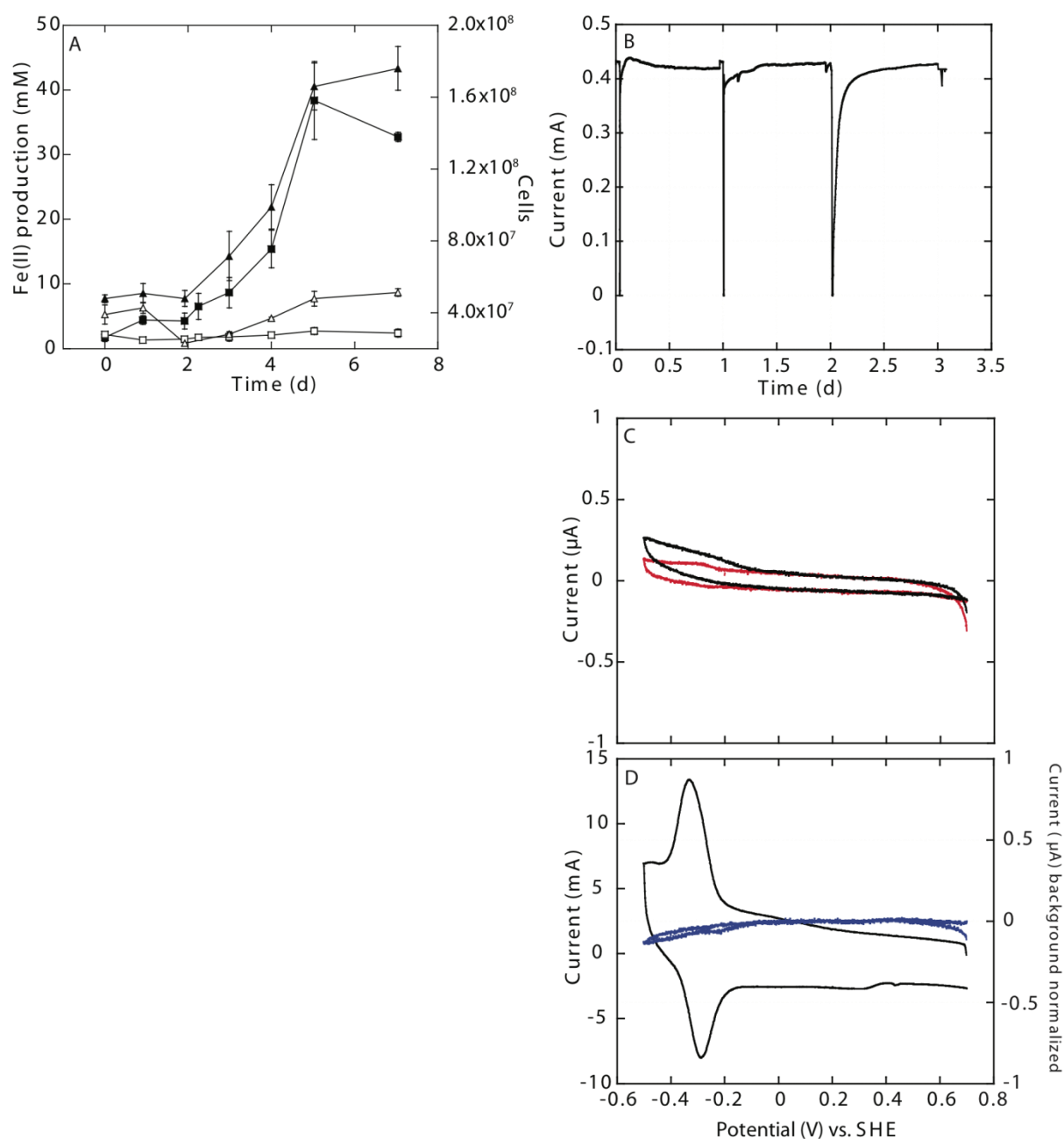


Figure 4. Fe(III)-oxide reduction by *Thermincola potens* strain JR incubated with 12 week old batch-operated MFC supernatants (80% v/v). Effects on the rate of iron reduction over time with the addition of 8mL of either supernatant (dashed line, o), fresh medium (■), 50 μ M AQDS and fresh medium (x), or fresh medium without acetate (\square) are shown.

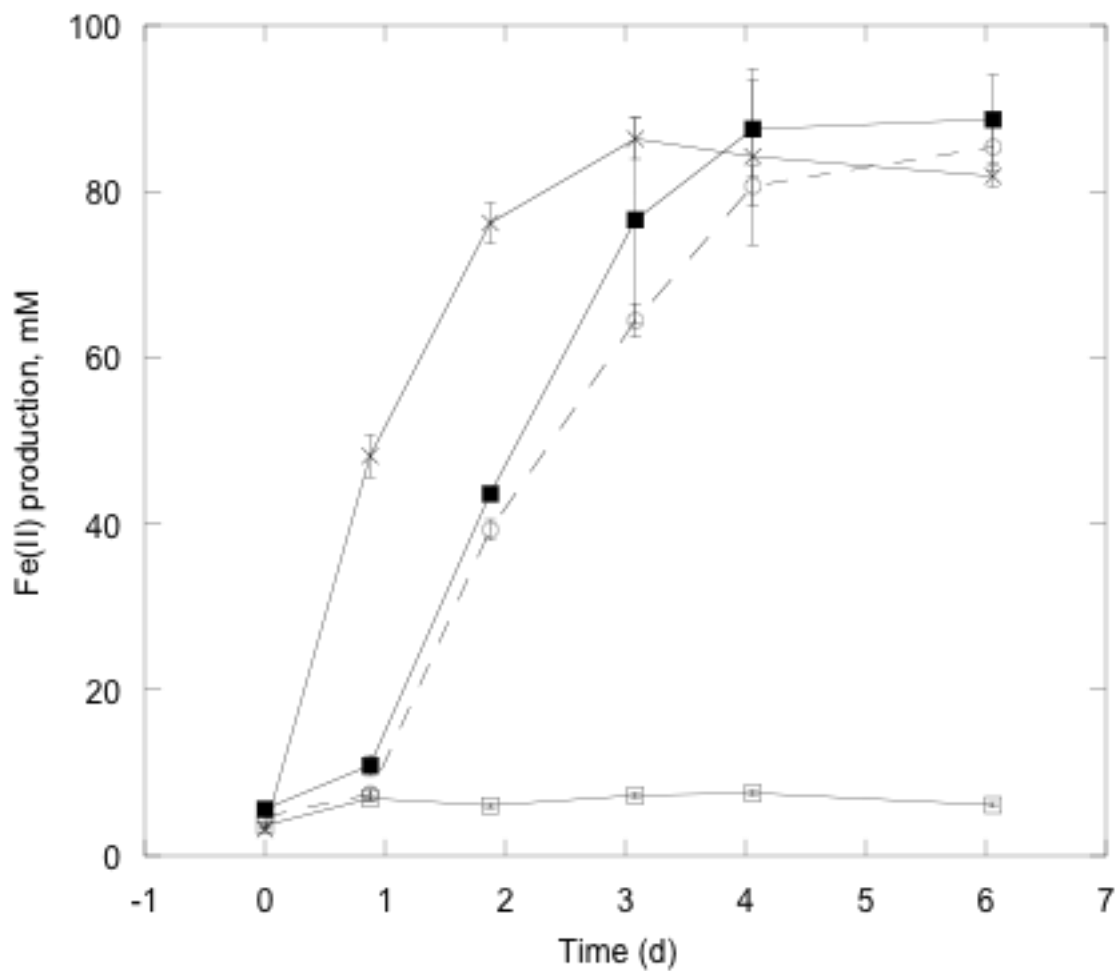


Figure 5. Confocal laser scanning microscopy of *Thermincola potens* strain JR on a graphite fiber anode surface. **A)** Electrode surface colonization after 4 days after the current-production stabilized with 1000 \times magnification. **B)** The same electrode surface is viewed 6 weeks later with 630 \times magnification. **C)** A cross section of Figure 3B revealed 20 μ m thick biofilm with a primary layer of live cells along the electrode surface. Scale bar represents 10 μ m.

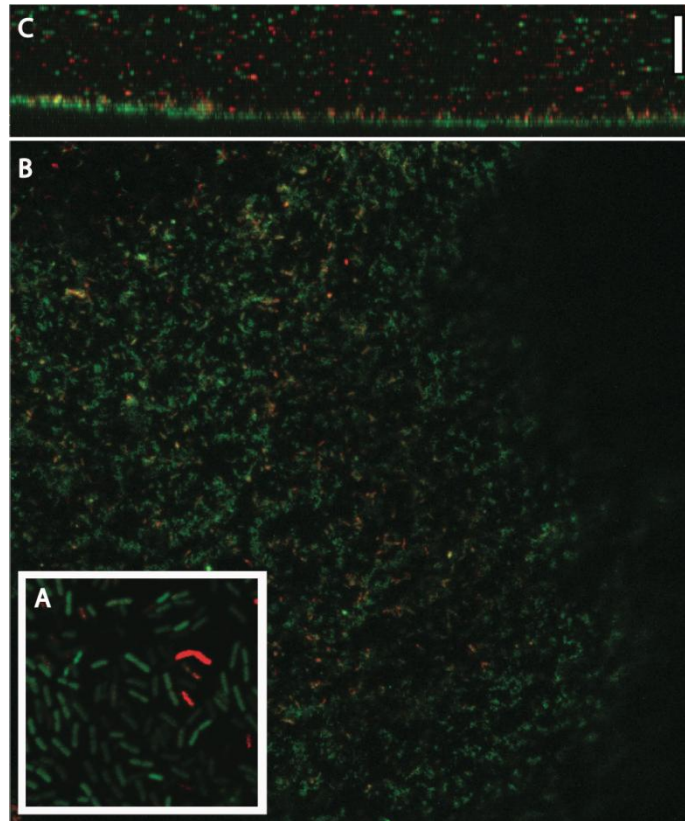


Figure 6. A) Difference spectrum of strain JR whole cells of dithionite reduced represented by solid line and HFO oxidized represented by dashed line. Absorbance maxima at 426, 522, and 553 nm are consistent with the involvement of *c*-type cytochromes in ferric iron reduction. Tick marks on the y-axis represents a 0.2 change in absorbance. **B)** The diagrammatic representation of 2 of 5 predicted *c*-type cytochrome-rich gene clusters located on the reverse strand. In addition to genes that encode multiheme *c*-type cytochromes (red), the cytochrome-rich clusters contain genes that encode cytochrome assembly proteins (pink) and genes that encode proteins with domains that target proteins to the cell surface (grey), NHL repeat domains (yellow), TPR domains (green), as well as domains for biosynthesis of both *c*-type cytochromes (blue) and heme (orange).

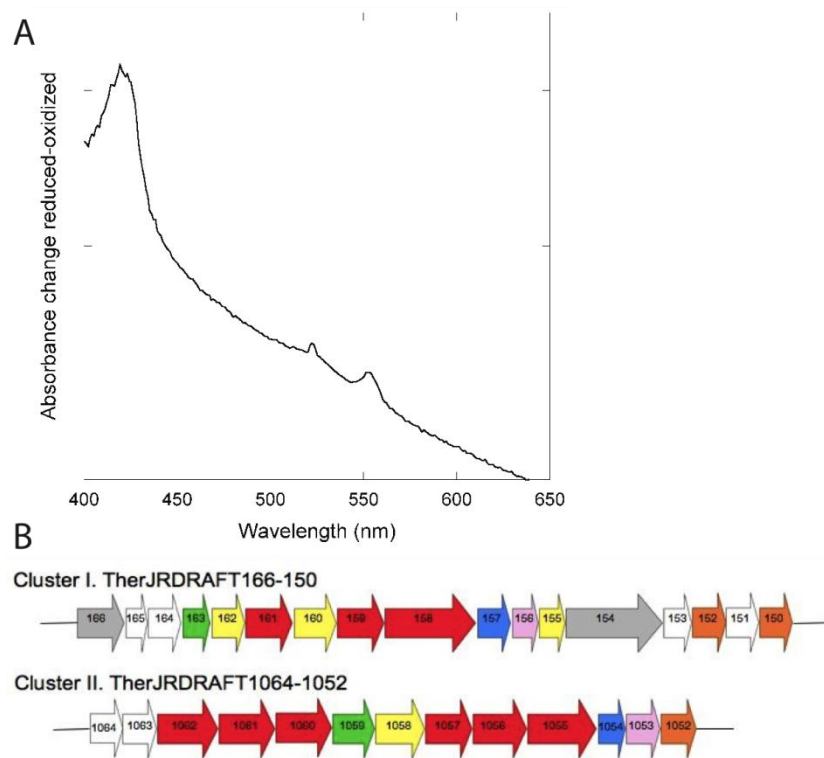
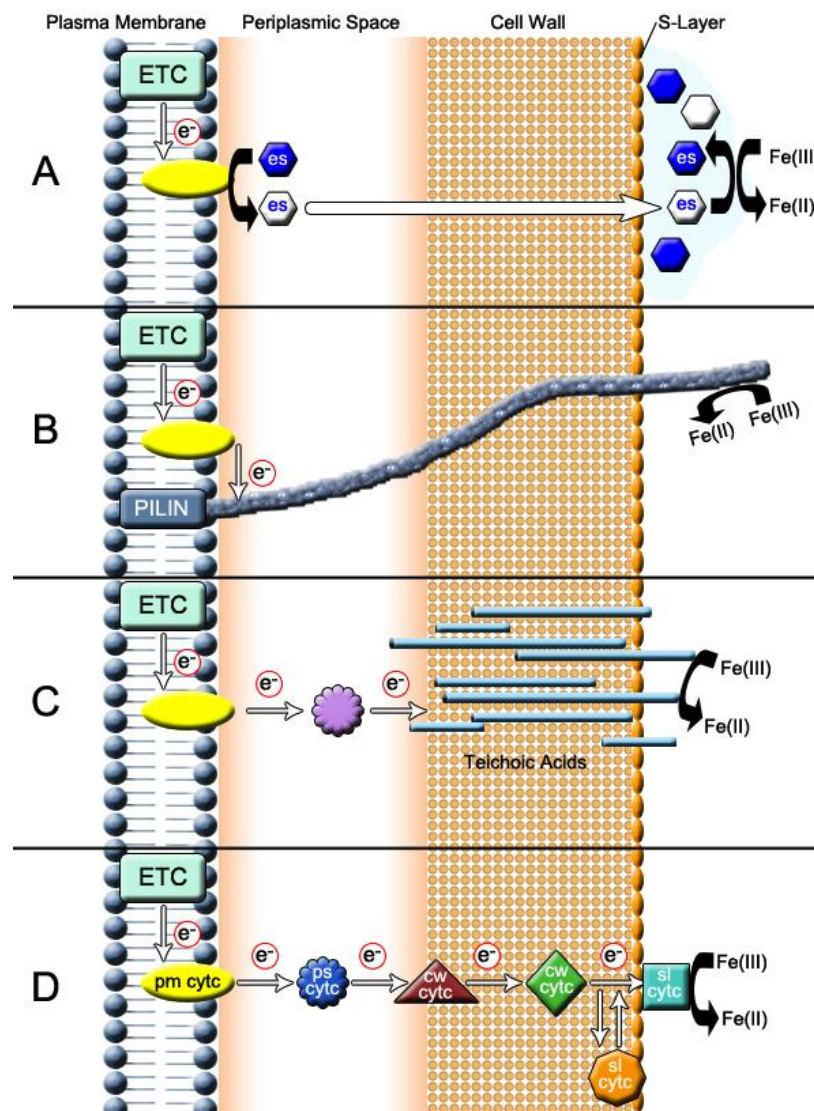


Figure 7. Illustration of the four models for direct electron transfer by a Gram-positive bacterium. **A)** Biofilm contained redox mediator, **B)** ‘nanowires’, **C)** conductive cell walls, or **D)** cytochrome chain linking the inner membrane to cell surface. Ferric iron is shown as a representative exogenous electron acceptor. ES (electron shuttle), Cyt_c (multiheme *c*-type cytochrome), PM (plasma membrane), PS (periplasmic space), CW (cell-wall), SL (s-layer).



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Chapter 5

Summary and Future Directions

Given the rising cost of energy and the abundance of affordable and sustainable fuel sources, it is plausible that in some form MFCs will may play a role in the future energy picture (Chapter 1). In addition to electricity generation, knowledge gleaned from this dissertation has broad applications to bioremediation, pathogen physiology, and to understanding carbon and iron biogeochemical cycles in anoxic soils and sediments. To accomplish the primary goal of understanding the microbial ecology and physiology of thermophilic electricity generation, this dissertation employed a cross-disciplinary approach relying on principles and methods from microbial physiology, microbial ecology, biogeochemistry, and electrochemistry.

In chapter 2, electricity production was demonstrated from MFCs operated at 55°C, with the system functioning effectively and reproducibly for over 100 days. Meta-analysis suggested that thermophilic operation could be competitive with mesophilic operation in terms of current generation and coulombic efficiency (1-4), but direct comparisons with mixed communities in highly functional MFCs were not performed. Accurate companion studies will require MFCs optimized for current production, as the H-cell configuration used in this dissertation is plagued by a high internal resistance. As a consequence of the high internal resistance, current and power densities are severely restricted, thus negatively impacting comparative analyses. Future comparisons should also include a cost-benefit analysis normalizing current for operation costs. It is not expected that implementation and operation costs will scale linearly, however, these types of comparisons may be a first step in implementing MFC technology to the treatment of thermophilic waste streams.

In addition to demonstrating themophilic MFC operation, the objectives of this dissertation were to expand the known diversity of organisms capable of anodic respiration as well as explore the mechanisms of electron transfer by these organisms. The composition and activity of the anode bacterial community was examined in Chapter 3. Here molecular approaches revealed that Gram-positive bacteria belonging to the Firmicutes were dominant and metabolically active in current producing anodes, while culture-based studies resulted in the isolation of three of the most dominant genera in the anode biofilm. Two isolates, *Geobacillus* strain S2E and *Thermincola* strain JR, could reduce solid phase iron coupled to acetate oxidation, yet only strain JR could generate current independently, as strain S2E required the addition of a shuttling compound to generate low levels of current.

Current-production by *Geobacillus* strain S2E shows that the utilization of an insoluble electron acceptor does not translate to the utilization of anodes as an electron acceptor. Reasons for the independent utilization of iron but not anodes were not explored, but demand further attention. These results could be explained by unfavorable redox potential of the anode, with anodes typically having a lower redox potential than ferric iron (5). Alternatively, ligand production by S2E is another reasonable explanation for this discrepancy, as the bacterium could solubilize solid-phase iron and subsequently utilize soluble ferric iron as electron acceptor but the bacterium lacks a mechanism for transferring electrons directly onto solid-phase electron acceptors. The apparent difference in anode respiration strategies between Gram-positive members of the anode biofilm lends itself to comparative studies of these organisms exploring the similarities and differences in solid-phase respiration between them. Future studies dedicated to elucidating the mechanism of electron transfer to iron and anodes by strain S2E are warranted and will help unravel the complexity of interactions in the anode biofilm.(6)

The reduction of solid phase iron by *Geobacillus* strain S2E was a novel finding, as dissimilatory metal reduction had not been identified in this genus (7). Further physiological characterization of this bacterium is justified; members of this genus are a source of thermostable

enzymes with broad biotechnological, remediation, and biofuel applications (9). Accounting for the fact that *Geobacillus spp.* are commonly isolated from high temperature and pressure petroleum reservoirs, can ferment the major sugars in cellulosic biomass, and tolerate high (10%) concentrations of ethanol makes them ideally suited for biofuel production (7-9). It is also possible that *Geobacillus* strain S2E and *Thermincola* strain JR could ecologically complement one another, resulting in electricity generation from waste streams containing complex organic matter. To this end, qPCR primers and FISH probes have been designed to monitor the presence and relative abundance of *Thermincola* and *Geobacillus* populations in future environmental samples (Table 1). Furthermore, these molecular tools could be used to in temporal sampling of coculture experiments with both organisms in MFCs to provide valuable information on the competition, cooperation, and succession of mixed population anode biofilms, which represent areas of anode research that are poorly understood at this time.

Since *Thermincola sp.* strain JR was capable of transferring electrons to anodes, the ability to utilize other electron acceptors was investigated in Chapter 4. In addition to anodes, strain JR can reduce solid phase Fe(III) and Mn(IV) oxides, but failed to reduce soluble forms of ferric iron. Strain JR is capable of chemolithoautotrophy with hydrogen as an electron donor and amorphous hydrous ferric iron (HFO) as an electron acceptor, while strain JR is also capable of anaerobic carboxydutrophy utilizing CO as the sole carbon and energy source. However it is undetermined whether strain JR is capable of growing by these metabolisms or whether the organism can couple CO/CO₂ fixation to electron transfer to anodes.

The biochemically constrained nature of strain JR physiology demonstrated in Chapter 4 (Table 1 and 2) was supported by genomics, as key enzymes were missing from energy and carbohydrate pathways: Calvin cycle, Embden-Meyerhof pathway, Enter-Doudoroff pathway, glycolysis and the pentose phosphate pathways (10). The genome revealed strain JR has the capacity for CO/CO₂ fixation using the Wood-Ljungdahl pathway (reverse acetyl-coA pathway), as the organism maintains 4 copies of the carbon monoxide dehydrogenase (CODH) catalytic subunit. Furthermore, strain JR lacks key genes for alternative carbon fixation pathways including the reverse TCA cycle and the 3-hydroxypropionate pathway, signifying that reverse acetyl-coA is the exclusive pathway for CO/CO₂ fixation. It is possible that energy conservation could be achieved using a carbon monoxide dehydrogenase/hydrogenase complex; however further transcriptional analyses are required to support this notion.

Using genomics and phylogeny to formulate hypotheses, strain JR may be capable of homoacetogenesis using hydrogen and carbon dioxide (11), and as such studies investigating this physiology in strain JR are underway. The ability to use acetate to produce H₂/CO₂ and vice versa by the same organism and pathway implies that this bacterium can change substrate and product reversibility depending on the environmental conditions (12). If homoacetogenesis is confirmed in strain JR, it would also be prudent to evaluate this organism's capacity to use cathodic reducing equivalents, rather than hydrogen, to sequester inorganic carbon and produce acetate and biomass in the cathode chamber. This research not only has applications to biofuel technology, but would also support basic scientific research regarding cathodic bacterial physiology. To date the growth of organisms using cathodic reducing equivalents has not been documented nor have the mechanisms of electron acceptance from cathode surfaces been elucidated (13).

In chapter 4, in addition to characterizing the physiology of strain JR, a combination of electrochemical and microscopic methods demonstrated that *Thermincola sp.* strain JR does not produce an electron shuttling compound but requires direct contact for current production to

microbial fuel cell anodes. Compared to Gram-negative bacteria, strain JR lacks an outer membrane, has a thin periplasmic space (16nm), a thicker cell wall (17nm), and is encased in a glycoprotein S-layer. Accounting for these structural differences direct electron transfer by strain JR can be rationalized using nanowire, cytochrome chain, or conductive cell wall models. Physiological and genomic evidence implicated a role for *c*-type cytochromes in direct extracellular electron transfer by strain JR.

Future research confirming the molecular mechanism of electron transfer by a Gram-positive bacterium is necessary. To corroborate the hypothesis that a chain of *c*-type cytochromes is responsible for electron transfer across the cell envelope (Chapter 4), cells grown on HFO, AQDS, and CO can be fractionated to identify the proteome of the cell wall, cell membrane, and soluble fractions. In addition to whole-fraction proteomics, fractionated regions can also be treated with SDS-PAGE and heme stained to confirm *c*-type cytochrome mass spectrometry signatures in each fraction. Proteomics data will be cross-referenced to the genome to identify *c*-type cytochromes located in each fraction. Transcriptomics, or primers designed for predicted cytochromes from the genome and proteome, will confirm the mRNA expression of the cytochromes under growth on insoluble and soluble electron acceptors. If *c*-type cytochromes are not found localized to the cell wall or envelope, whole fraction proteomics will be used to formulate hypotheses on other likely candidate redox active proteins. Additionally, cyclic voltammetry will be performed on native and proteinase-treated cell walls to confirm that cell wall structures independent of proteins are not redox active. Ultimately the creation of a genetic system in strain JR will be necessary to prove a function for, as well as enable visualization and localization of redox-active proteins along the cell envelope during metal and anode reduction. Beyond a molecular understanding of electron transfer, the sequenced genome in strain JR facilitates comparative genomics between sequenced dissimilatory metal-reducing bacteria (DMRB), resulting in hypotheses for the evolution of *c*-type cytochromes in metal-reducing bacteria.

Overall, the research described in this dissertation was highlighted by several key accomplishments. The community characterization represented the first successful purification of RNA from electrode communities. Also as part of this research two novel Gram-positive DMRB were isolated. One of them, *T. potens* strain JR, was the first demonstration of independent current production by a Gram-positive bacterium and also represents the first MFC isolate to be genome sequenced. Research from genomics and physiological studies from strain JR provided the first evidence for *c*-type cytochromes in direct extracellular electron transfer by Gram-positive bacteria. As described in this chapter, however, this research is only a springboard for future research trajectories dedicated to understanding Gram-positive ecology, physiology, biotechnology applications, and biochemistry.

Table 1. FISH and/or qPCR probes designed as part of this dissertation.

Specificity	Probe	Probe Sequence (5' to 3')	16S rRNA target site
<i>Thermincola spp.</i>	JR509F	AGACTGGGCGTCTTGAGG	509
<i>Thermincola spp.</i>	JR906R	TCTTCGGGTCACAAGCAA	906
<i>Geobacillus spp.</i>	S2E1156F	GTGACTGCCGGCTAAAAGTC	1156
<i>Geobacillus spp.</i>	S2E1313R	TGCAATCCGAACTGAGAGTG	1313

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