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Electricity generation by thermophilic microorganisms from marine sediment

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Abstract The search for microorganisms that are capable of catalyzing the reduction of an electrode within a fuel cell has primarily been focused on bacteria that operate mesobiotically. Bacteria that function optimally under extreme conditions are beginning to be examined because they may serve as more effective catalysts (higher activity, greater stability, longer life, capable of utilizing a broader range of fuels) in microbial fuel cells. An examination of marine sediment from temperate waters (Charleston, SC) proved to be a good source of thermophilic electrode-reducing bacteria. Electric current normalized to the surface area of graphite electrodes was approximately ten times greater when sediment fuel cells were incubated at 60°C (209 to 254 mA/m^2) vs 22°C (10 to 22 mA/m^2). Electricity-generating communities were selected in sediment fuel cells and then maintained without sediment or synthetic electron-carrying mediators in single-chambered fuel cells. Current was generated when cellulose or acetate was added as a substrate to the cells. The 16S ribosomal ribonucleic acid genes from the heavy biofilms that formed on the graphite anodes of acetate-fed fuel cells were cloned and sequenced. The preponderance of the clones (54 of 80) was most related to a Gram-positive thermophile, *Thermincola carboxydophila* (99% similarity). The remainder of clones from the community was most related to *T. carboxydophila*, or uncultured Firmicutes and Deferribacteres. Overall, the data indicate that temperate aquatic sediments are a good source of thermophilic electrode-reducing bacteria.

Keywords Thermophiles · Microbial fuel cells · Electricity · *Thermincola* · Deferribacteres

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Introduction

The microbial oxidation of organic and inorganic compounds can be coupled to electrode reduction in a microbial fuel cell (MFC) to generate a direct electric current (Logan et al. 2006; Lovley 2006; Rabaey et al. 2007). This phenomenon is receiving renewed attention because of the intriguing possibility of developing sustainable energy processes that do not require the consumption of fossil fuels. Thus far, the current and power densities achieved with MFCs are relatively low but still could be used to power sensors and other electronic devices, generate power for remote applications, and treat waste while generating electricity. Multiple factors limit the performance of a fuel cell, and presently, much of the research on MFCs is focused on improving the hardware of the cells. The performance and capabilities of the biological catalysts will also be critical as MFC technology improves. A group of microorganisms that has been examined only to a limited extent in MFCs is the extremophiles. Extremes in pH, salinity, and temperature when combined with materials that operate best under such conditions would potentially result in more powerful MFCs.

The search for bacteria that function optimally at higher temperatures and thereby would have higher catalytic rates was the aim of the present study. MFCs operated at

mesophilic temperatures (below 50°C) have produced power from the oxidation of fuels in ocean sediments (Holmes et al. 2004; Reimers et al. 2001; Tender et al. 2002), wastewater (Angenent et al. 2004; Logan 2005; Min et al. 2005), and biomass (Wilkinson 2000). Temperatures above 50°C produced by direct sunlight, volcanic hot springs, hydrothermal vents, composting of municipal and agricultural waste, steam lines and hot water pipes, and the waste heat from a variety of industrial processes are supportive of the growth of thermophilic bacteria (Madigan and Oren 1999; Madigan and Martinko 2005). Soil and aquatic sediments from temperate environments are known to possess thermophilic bacteria with optimal growth temperatures above 50°C (Madigan and Martinko 2005). Sediments are also rich with many of the recently discovered mesophilic, direct electrode-reducing bacteria (Bond et al. 2002; Holmes et al. 2004). For these reasons, this study was set to determine if common aquatic sediments are also a good source of thermophilic electrode-reducing bacteria.

In a MFC, bacteria use an anode as a terminal electron acceptor. The bacteria may use a soluble factor from the environment as an electron carrier to mediate transfer of electrons to the electrode, require the addition of a synthetic mediator (Park and Zeikus 2000), generate a soluble mediator (Rabaey et al. 2004, 2005), or, through direct bacterium-to-electrode contact, deliver electrons to the surface of an electrode. The mechanism of the latter is still not fully understood but has been defined as a property of electricigenic bacteria (Lovley 2006). Electricity generation with synthetic mediators (Azure A) and thermophilic *Bacillus* spp. has been documented (Choi et al. 2004), and wastewater without added mediators has recently been shown to serve as a source of thermophilic electrode reducers (Jong et al. 2006). The present study is concerned with generating electricity under thermophilic conditions with bacteria from marine sediment without the addition of an electron-carrying shuttle and determining if the thermophilic bacterial community is capable of generating electric currents higher than its mesophilic counterpart.

Materials and methods

Sediment fuel cells Anoxic marine marsh sediment 2 to 30 cm below the sediment surface was collected along the banks of the mouth of the Ashley River within Charleston Harbor (Charleston, SC). Sediment fuel cells similar to those described by Holmes et al. (2004) were constructed as follows: sediment free of shells and plant detritus was made homogenous by stirring and was added to the 250 ml mark of 600 ml beakers, which were then filled to the 500 ml mark with harbor water. Approximately 50 ml of double-distilled H_2O was added daily to replace water lost to evaporation. Placing a flask of water in the oven helped to minimize evaporation in the sediment fuel cells. Graphite electrodes with a surface area of 6.7 cm^2 were prepared with marine-grade wire as previously described (Milliken and May 2007). Those serving as anodes were placed 5 cm below the surface of the sediment, 4 cm away from the sides of the beakers, and 2 cm away from the bottom of the beakers. Cathodes of the same size were suspended in the overlying water 2 cm above the sediment surface and 7 cm from the buried anodes. The electrodes were connected through a 1,000-Ω resistor, which was maintained at the temperature applied to the fuel cells. To determine the effect of temperature on the load, a 1,000-Ω resistor was incubated at 60°C, which resulted in a 0.5% decrease in resistance vs when the resistor was maintained at 22°C. The sediment fuel cells were incubated in an incubator-oven that was preset at the designated thermophilic temperature. Air was delivered to the overlying water continuously at 140 ml/min though surgical tubing by an aquarium pump. A set of three killed-cell control sediment fuel cells were prepared similarly but were treated with 1% formaldehyde before the study.

Single-chamber fuel cells Single-chamber fuel cells (25 ml total volume) made of glass were prepared as described previously (Milliken and May 2007). The anodes were identical to those used in the sediment fuel cells, and the cathodes were made of platinum–carbon cloth with 0.5 mg Pt/cm^2 using 10% Pt on Vulcan XC-72 (E-Tek, Somerset, NJ) and had a surface area of 1.7 cm^2. Nafion®117 (The Fuel Cell Store, Boulder, CO) was clamped to the inner surface of the cathode. A minimal anaerobic medium (ECl, pH 6.8; Berkaw et al. 1996) without any soluble synthetic mediators, resazurin, sulfide, or cysteine was prepared under strict anoxic conditions under N_2/CO_2 (80:20). Before transfer of the sediment fuel cell anodes, the single-chamber assembly was wrapped in foil and autoclaved for 45 min and then placed for at least 12 h in an anaerobic Coy chamber (Grass Lake, MI). The anodes were taken directly out of the sediment, gently shaken to remove excess sediment, and placed into the single-chamber fuel cell under positive-pressure N_2/CO_2 (80:20) supplied by canula. The system was filled with 20 ml of the medium, sealed with a black butyl stopper, and placed in a 60°C incubator. The medium within the anode chamber was exchanged every 2 to 3 days by syringe under an atmosphere of N_2/CO_2 (80:20). At the time of exchange, the spent medium had a pH of 6.3 to 6.5 and had lost 8 to 10 ml of volume. Replacement of the medium restored the pH to 6.8 and the volume to 20 ml. To prepare a sterile, killed-cell control, an anode from an electricity-producing sediment fuel cell was sealed in an anaerobe tube with

10 ml of medium and autoclaved for 45 min. This anode was then transferred to a fuel cell assembly as described for the live systems.

Monitoring electricity Voltage measurements on sediment fuel cells and single chamber cells were made as described previously (Milliken and May 2007). Continuous 60-min interval voltage measurements across a 1,000-Ω load resistor were taken throughout the experiments. Current (I) was calculated as I (mA)=V (mV)/R (Ω) where V is the voltage and R is the external resistance. Power (P) in milliwatts was calculated as P (mW)=I^2 (mA) R (Ω). Current and power densities were normalized to the surface area of the electrodes.

Monitoring acetate and electron recovery Acetate measurements were made by application of fuel-cell medium to an ion chromatograph using methods previously described (Milliken and May 2007). An eight-electron oxidation of the acetate to CO_2 was used in the calculations. The electron recovery (Coulombic efficiency, Ec) was based on changes in acetate consumption and current across 1,000 Ω over time, where Ec=Coulombs of current divided by Coulombs available based on measured acetate consumption. Conversions to Coulombs were based on 1 C= 1 A×1 s, 1 C=6.24×10^{18} electrons, 1 mol=6.02×10^{23} electrons and therefore 96,500 C/mol. Methane analysis was done by application of 50 μl of headspace gases from the fuel cells to a gas chromatograph (Hewlett-Packard 6890) equipped with a flame ionization detector (Cutter et al. 2001).

Scanning electron microscopy An anode from an acetate-fed cell, after ten exchanges with sediment-free media, was immersed in 2% glutaraldehyde in sodium cacodylate buffer overnight, then chemically dehydrated with hexamethyldisilazane overnight. An SC7640 desktop sputter coater (Polaron, Hertfordshire, UK) was used to coat the samples with approximately 100 Å of gold and palladium mix. The sample was then analyzed in a JEM-5410LV scanning electron microscope (JEOL, Tokyo, Japan) at 15 kV accelerating voltage.

Amplified ribosomal DNA restriction analysis The anode of an electricity-generating single-chamber fuel cell, fueled with acetate (25 mM) and receiving ten exchanges of sediment-free media, was aseptically scraped with a sterile scalpel to collect the community that had formed a biofilm on the electrode. Whole genomic deoxyribonucleic acid (DNA) extraction from the microbial community was performed according to the manufacturer's instructions with a PowerSoil DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA). Polymerase chain reaction (PCR) amplification of the 16S ribosomal ribonucleic acid (rRNA) gene used the universal primers 27F (5′-AGAGTTTGATCM TGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACG ACTT-3′) and the Choice *Taq* Blue Mastermix (Denville Scientific, Metuchen, NJ). The PCR method performed on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) had an initial denaturation step of 1:30 at 94°C, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by the final extension step of 72°C for 7 min. The PCR product was ligated and cloned using the pGEM-T Easy Vector System II according to the manufacturer's protocol (Promega, Madison, WI). Positive clones were screened on Luria–Bertani (LB)/ampicillin/isopropyl-β-D-thiogalactopyranoside/X-gal plates, and 80 clones were grown overnight in LB/ampicillin 100 media. A culture PCR was performed to amplify the 16S rRNA gene needed for restriction analysis and sequencing. The same PCR conditions were used as described above with the slight modification of the PCR method as follows: 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and the final extension step of 72°C for 5 min. The PCR product in this step was subjected to two separate restriction digests of the *Hha*I and *Hae*III restriction enzymes. The restriction digest was performed at 37°C for 2 h in 1.5 μl of supplied buffer C, 1.5 μl 10× bovine serum albumin, 0.1 μl restriction enzyme, and 11.9 μl PCR product. Each restriction digest was visualized on a 2% Trevigel (Trevigen, Gaithersburg, MD) in 1× Tris–acetate–ethylenediamine tetraacetic acid buffer, and the isolates with distinct patterns in each digest were selected for sequencing.

Sequencing and analysis of the 16S rRNA genes One to four clones of each of the 11 different representative restriction fragment length polymorphisms (RFLP) patterns were selected for sequence analysis. Plasmid DNA was isolated using the Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA) and sent to the BioAnalytical Services Laboratory at the University of Maryland Biotechnology Institute. The samples were sequenced on an ABI 3130 XL Genetic Analyzer using the sequencing primers M13F and M13R.

The consensus sequences for each of the 11 different RFLP patterns were assembled using the SeqMan program in the DNASTAR software package (DNASTAR, Madison, WI). Each consensus sequence contained at least 1,460 bp and was subjected to Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project analysis. Phylogeny was determined with the Ribosomal Database Project's Classifier (Wang et al. 2007) and Seqmatch (Cole et al. 2007). The 16S rRNA gene sequences were compared to the GenBank database, and similarity scores were calculated using BLAST analysis (Atschul et al. 1990). The DNASTAR software package previously mentioned

was used for alignment of the 16S rRNA genes using the MegAlign program and the CLUSTALW algorithm. The nucleotide sequences generated in this study were submitted to GenBank under the accession numbers EU194827 through EU194837.

Results

Electricity generation under thermophilic conditions Sediment fuel cells, constructed with marine sediment and operated at 60°C without added energy sources or synthetic electron-carrying mediators, generated direct electric current well above that produced by counterparts incubated at 22°C (Fig. 1a and b). Maximum currents per m^2 of anode surface were established between 2 and 5 days and ranged from 209 to 254 mA/m^2 (29 to 43 mW/m^2) for the triplicate live cells incubated at 60°C. Background currents for the killed-cell control MFCs leveled off between 3 and 8 mA/m^2. Similarly prepared sediment fuel cells (again in triplicate) incubated at 22°C generated 10 to 22 mA/m^2 within 5 days (Fig. 1a), an order of magnitude less than that produced by the thermophilic cells. Electricity generation peaked at 60°C in relation to other temperatures (Fig. 2) but was sustained at 75°C. Current ceased when active cells were exposed to 90°C. Summation of the data from Figs. 1 and 2 shows that when all parameters but temperature were held constant, the thermophilic sediment fuel cells generated nearly tenfold higher current than the mesophilic counterparts.

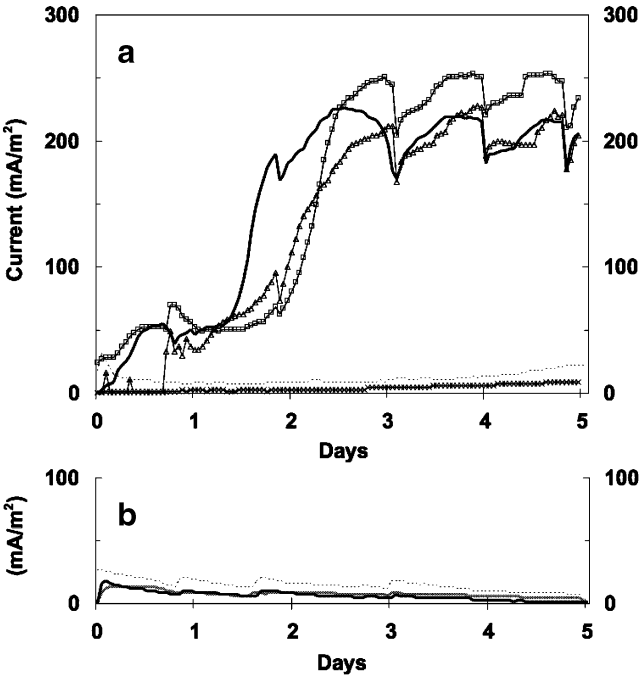

Fig. 1 Generation of electric current by thermophilic sediment fuel cells prepared with marine sediment. **a** Current generated by three cells incubated at 60°C (*top curves*: *thick solid line*, *squares*, and *triangles*) and three cells incubated at 22°C (*lower curves*: *dotted*, *thin solid*, and *x lines*). **b** Current generated by three formaldehyde-killed cells incubated at 60°C. All fuel cells were operated with 1,000-Ω load of resistance. Temporary decreases in current correspond with the replacement of evaporated water

Electricity generation without sediment in single-chamber cells Anodes from the sediment fuel cells described in Fig. 1 were transferred into single-chamber fuel cells equipped with air-bathed, Pt–carbon cloth cathodes. This increased the availability of oxygen to the cathode and enabled the examination of the thermophilic microbial electrode reduction in the absence of sediment and externally supplied mediators. The anodes were gently shaken to minimize transfer of sediment to the single chamber cells, and anaerobic minimal medium plus 25 mM sodium acetate was added to each of the cells, which were then incubated at 60°C. In less than 2 days, the current produced by these cells had stabilized at 478 to 537 mA/m^2 of anode surface (Fig. 3). A polarization and power curve analysis normalized to the surface area of the anode (Fig. 4) revealed an open-circuit voltage of approximately 0.5 V and a maximum power density of 207 mW/m^2 of anode surface. The surface area of the cloth cathode was approximately fourfold less than that of the anode; therefore, the power density per cathode surface area was 815 mW/m^2.

Acetate as a fuel Current was immediately restored in acetate-fed, single-chamber fuel cells after successive exchanges of the medium, and this resulted in the elimination of visible sediment (Fig. 3). This also resulted in a very heavy biofilm of rod-shaped bacteria on the

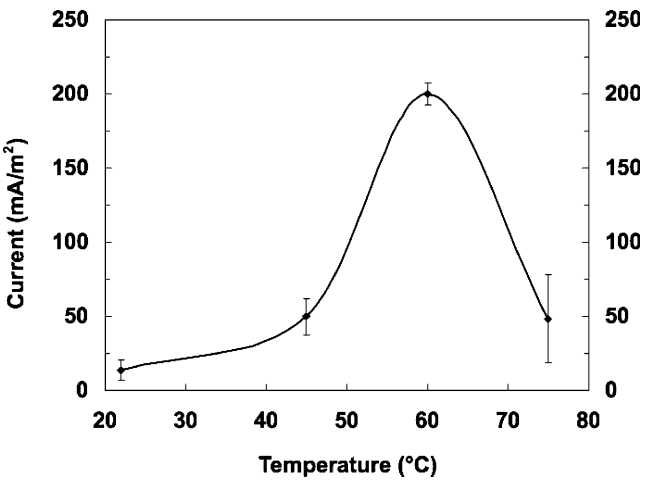

Fig. 2 Maximum sustained current density generated by sediment fuel cells incubated at 22, 45, 60, and 75°C for 5 days with a 1,000-Ω load resistance. Three cells at each temperature were examined, and the *error bars* represent the standard deviation from the sustained (more than a day) maxima

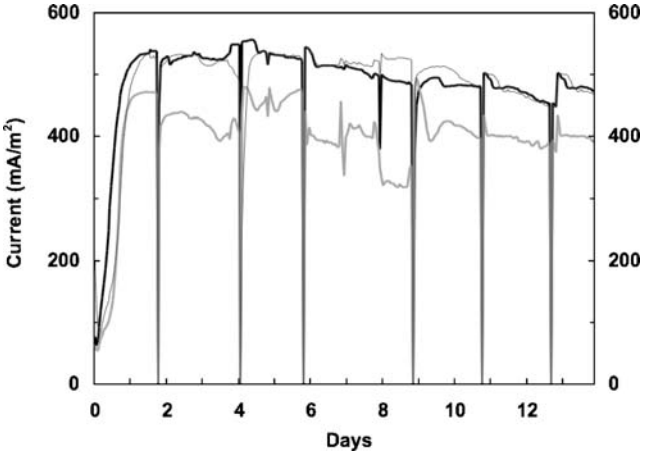

Fig. 3 Generation of electricity by thermophilically enriched microbial communities in single chamber fuel cells with Pt–C cloth, air-bathed cathodes, and a 1,000-Ω resistance. Three anodes from sediment fuel cells were moved to three single chamber cells (*dark*, *gray*, and *thin solid lines*) at time zero, were supplied with 25 mM sodium acetate, and were incubated at 60°C. The sediment-free medium and acetate were replaced at each *vertical line* that meets the *x*-axis within the plot

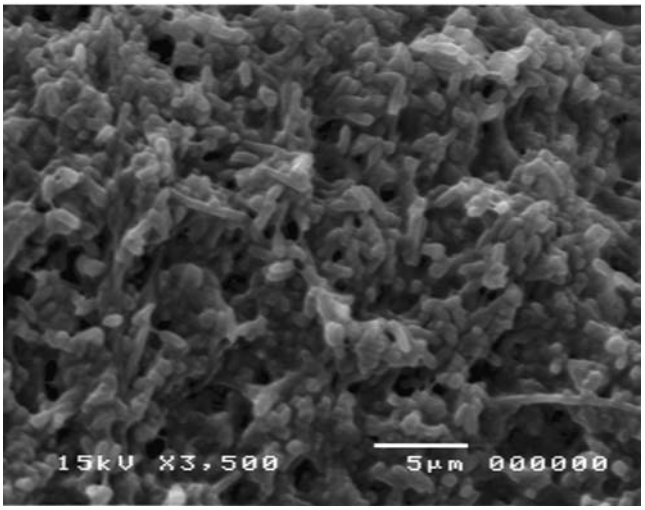

Fig. 5 Scanning electron micrographs of bacteria on the anode surface of a MFC incubated at 60°C with acetate as fuel. No biofilm was observed when a MFC was incubated with an open (unconnected) circuit

surface of the anode (Fig. 5). A 10–15% decline in current was observed over a 2-week period, but the current could be restored if the Nafion membrane was replaced after 2 weeks. This bacterial community could then be transferred from cell to cell in the ECl medium or to a serum bottle containing the ECl medium with 15 mM sodium acetate and 10 mM sodium fumarate and then back to a fuel cell, and electricity was again generated. The community has been thus transferred and maintained without sediment for 1 year and more than ten transfers and has continued to produce electricity as demonstrated in Fig. 3. In one of the single-chamber fuel cells, the microbial community was starved for fuel. The addition of sodium acetate after the current had declined caused a rapid restoration of electricity generation, indicating that acetate was serving as the fuel for electricity production by the thermophilic bacterial community (Fig. 6). The Coulombic efficiency (electron recovery) from acetate was 35.5±9.6% (n=6) and was determined from several different MFCs by measuring the acetate consumption over time while the current remained above 450 mA/m^2. Methane was not detected in the headspace of the cells (detection limit of 0.5 μmol). The most likely explanation for the low recovery of electrons is that the mixed microbial community includes aerobic or

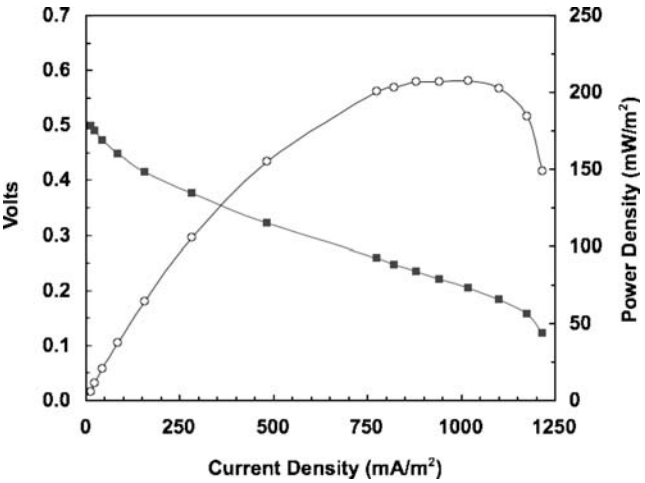

Fig. 4 Polarization (*solid squares*) and power curve (*open circles*) analysis from a single-chamber fuel cell incubated at 60°C. A variable resistor box was used to set the resistance for each resistive load (150 to 64,000 Ω) to measure the polarization curve at pseudo-steady state as defined by Logan et al. (2006)

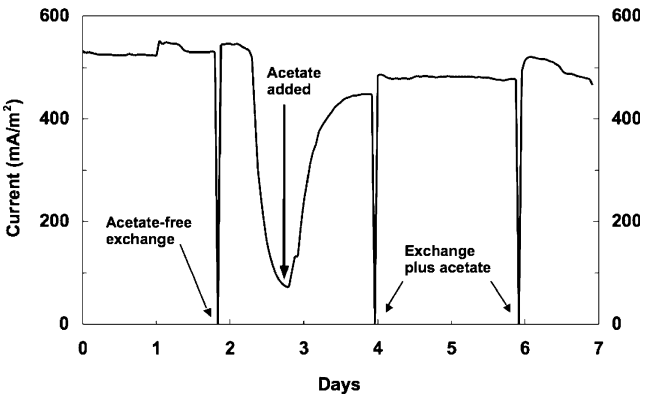

Fig. 6 Acetate as a fuel for the thermophilically enriched microbial community in a single-chamber fuel cell. After transfer of an anode from a sediment fuel cell to a single chamber cell without sediment and six exchanges of media without sediment, the medium was replaced without acetate. As designated on the plot, acetate was added after the current had dropped by more than 80%, and the electric current was re-established. The cell was operated with a 1,000-Ω resistance

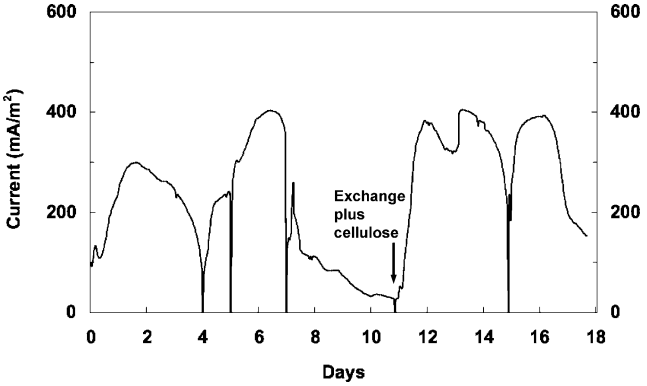

Fig. 7 Cellulose as a fuel for the thermophilically enriched microbial community in a single-chamber fuel cell. An anode from a sediment fuel cell was transferred to a single chamber cell without sediment with 0.5% w/v cellulose and 0.1% w/v yeast extract. Sediment-free medium without cellulose or yeast extract was exchanged at each *vertical line* that meets the *x*-axis within the plot. Near day 11, an exchange was made with cellulose added. Similar data were obtained with replicate fuel cells. The cell was operated with 1,000 Ω of resistance

microaerophilic acetate-consuming bacteria and that oxygen enters during the manipulation (medium exchange) and operation of the cell.

Electricity generation with cellulose Cellulose can also serve as a fuel source for a thermophilic electrode-reducing community. As Fig. 7 shows, current could be sustained with cellulose added as a sole carbon and energy source to a single-chamber cell containing an anode that had been started in a sediment fuel cell at 60°C. The cell was initiated with 0.5% w/v cellulose powder (~20 μm-sized particles), and then the medium was exchanged several times to eliminate residual sediment and fuel. Eventually, the current began to fade, at which time an exchange of medium with 0.5% w/v cellulose was made, recovering the current. In contrast with the acetate-fed single-chamber MFC experiment described above, yeast extract (0.1% w/v) needed to be added at time zero. The fuel cells were operated in batch (semibatch) mode; therefore, cellulose fermentation in relation to electrode reduction was not optimized, and the overall efficiency of the system may have been lowered because of the buildup of end-products that could inhibit or modify cellulose fermentation and electrode reduction. Once electricity generation was established, the yeast extract was no longer required and was not added with the exchanges of media.

Community analysis of an acetate-fueled cell Cloning of an acetate-fed community from the surface of a graphite anode resulted in 80 clones with 1,460 bp of 16S rRNA gene sequence (Table 1). All possessed sequence belonging to the Firmicutes or Deferribacteres, with a clear majority of Firmicutes (64 clones). Of the Firmicutes, 48 had identical RFLP patterns (B) and sequence most similar (99%) to that of *Thermincola carboxydophila* strain 2204. The 16S rRNA genes from six other clones produced a different RFLP pattern (E), yet the sequence was also 99% similar to that of *T. carboxydophila* strain 2204. Five more clones (RFLP patterns F and H) held sequence most similar to *T. carboxydophila* but more distantly (88 to 90% similarity). The remainder of the Firmicutes (RFLPs G, I, and J) was most related to a series of uncultured bacteria. All of the remaining 12 clones (RFLPs A, C, D, and K) held 16S rRNA gene sequences most related to uncultured Deferribacteres (87 to 96% similarity).

Discussion

This study has shown the feasibility of using thermophilic electrode reducing bacteria as catalysts in MFCs. Marine

Table 1 Analysis of 16S rRNA genes from acetate fueled thermophilic MFC

RFLP	Accession number	Phylum (>90%)	Closest match (accession number)	Percent similarity
A (4)[a]	EU194828	Deferribacteres	Uncultured bacterium clone 165B42 (DQ925879.1)	87
B (48)	EU194830	Firmicutes	*Thermincola carboxydiphila* strain 2204 (AY603000.2)	99
C (1)	EU194829	Deferribacteres	Uncultured bacterium clone C74 (DQ424926.1)	96
D (6)	EU194834	Deferribacteres	Uncultured bacterium clone 1A162 (DQ424915.1)	89
E (6)	EU194831	Firmicutes	*Thermincola carboxydiphila* strain 2204 (AY603000.2)	99
F (4)	EU194832	Firmicutes	*Thermincola carboxydiphila* strain 2204 (AY603000.2)	90
G (3)	EU194835	Firmicutes	Uncultured bacterium clone TTA_B61 (AY297976.1)	98
H (1)	EU194833	Firmicutes	*Thermincola carboxydiphila* strain 2204 (AY603000.2)	88
I (1)	EU194837	Firmicutes	Uncultured low G+C Gram-positive bacterium clone DR546BH1103001SAD28 (DQ234647.1)	92
J (5)	EU194836	Firmicutes	Uncultured soil bacterium clone UE5 (DQ248237.1)	89
K (1)	EU194827	Deferribacteres	Uncultured bacterium clone 165B42 (DQ925879.1)	87

[a] RFLP pattern (number of clones)

sediment from a temperate environment (Charleston, SC) proved to be a good source of thermophilic electrode-reducing bacteria. Such an environment is not commonly thought of as a habitat for thermophiles, but mesophilic environments do possess thermophiles (Madigan and Martinko 2005), and it is conceivable that thermophilic electricity-generating bacterial communities could be ubiquitous. Sediment fuel cells produced much higher electric currents at 60 vs 22°C, indicating that the thermophilic microbial population possesses a superior capability to generate electricity. The use of extremophilic bacteria as catalysts in fuel cells holds much promise because they will generate higher rates of metabolic activity (in this case resulting in more electricity) and will be more stable under severe conditions common to industry. Indeed, conventional hydrogen or methanol fuel cells are routinely operated at high temperatures and at low or high pH, wastewaters are frequently treated at thermophilic temperatures and have been with MFCs (Jong et al. 2006), and biofuels or bioenergy may be more effectively and inexpensively produced at thermophilic temperatures (Lynd et al. 2005).

The energy to be harvested with a MFC is as dependent upon the fuel as it is the fuel cell and the microorganisms. Biomass or organic waste will commonly include cellulose, the most abundant carbon source on the planet and an excellent potential renewable energy source. For a review of cellulose and its fermentation, see Demain et al. (2005). Electricity generation by MFCs supplied with cellulose has been reported but only with mesophilic bacterial catalysts (Rismani-Yazdi et al. 2007; Ren et al. 2007). Niessen et al. (2005) demonstrated that hydrogen generated by mesophilic cellulolytic bacteria can be collected and then abiotically transformed into electricity by a fuel cell. However, in the present study, electricity was generated for the first time with cellulose in a MFC operated under thermophilic conditions. Whether the cellulose directly served as a fuel or a product of cellulose fermentation fueled electricity generation was not determined. The latter is probable because acetate is a well-known substrate of mesophilic electricity-generating bacteria (Lovley 2006), and acetate is a major end-product of anaerobic cellulose fermentation (Demain et al. 2005).

It is clear from the results presented herein that acetate can serve as a fuel for electricity generation by thermophilic bacterial communities enriched from marine sediment. The examination of the 16S rRNA genes from an acetate-consuming community on the anode of a fuel cell revealed a community dominated by Gram-positive bacteria. Most of the clones (61 of 80) held DNA most similar to that of *T. carboxydophila* (99% similarity). Two *Thermincola* spp., *T. carboxydophila* and *T. ferriacetica*, are described in the literature (Sokolova et al. 2005; Zavarzina et al. 2007). Both are Gram-positive spore-forming moderate thermophiles that have been isolated from terrestrial hot springs. Three more clones (RFLP G) were also most related to bacteria from a thermophilic environment, in this case an uncultured Firmicute from a terephthalate-degrading thermophilic community grown in an anaerobic reactor (Chen et al. 2004). The five remaining Firmicute-related clones could not be identified with thermophiles or mesophiles based on their most related sequences in Genbank. Twelve clones (RFLPs A, C, D, and K) did not contain DNA of Gram-positive bacteria. Instead, these were most related to uncultured Deferribacteres (87 to 96% similarity). *Deferribacter* spp. are Gram-negative moderate thermophiles isolated from deep subsurface waters and other thermal environments (Greene et al. 1997; Miroshnichenko et al. 2003; Takai et al. 2003). Six of the clones (RFLPs C and D) were most closely related to two uncultured bacteria discovered in a thermophilic MFC inoculated with brewery waste (Jong et al. 2006).

It is apparent that the community described consists of generally two types of bacteria: Gram-positive bacteria most related to *Thermincola* spp., which were dominant in the clonal analysis, and Gram-negative bacteria related to *Deferribacter* spp. All cultured strains of these genera are known to be thermophilic. Marine sediments have been used to enrich electricity-generating communities under mesophilic conditions (Bond et al. 2002; Holmes et al. 2004), but *Thermincola* and *Deferribacter* are not part of these communities. *Deferribacter thermophilus* (Greene et al. 1997), *D. abyssi* (Miroshnichenko et al. 2003), and *Thermincola ferriacetica* (Zavarzina et al. 2007) are capable of using acetate as a carbon and energy source and insoluble iron external to the cell as an electron acceptor. Although it is not always the case, reduction of iron external to a bacterial cell is a common property of electricigenic bacteria (Lovley 2006; Yan et al. 2007).

Studies with Gram-positive bacteria in fuel cells have been limited (Aelterman et al. 2006; Choi et al. 2004; Milliken and May 2007; Park et al. 2001), and their role in fuel cells is still relatively unknown (reviewed in Rabaey et al. 2007). The thermophilic Gram-positive *Thermincola* spp. in the acetate-fed fuel cell may serve as electrode reducers, produce electron-carrying shuttles for use by themselves or other bacteria, or possibly are required to consume the acetate and donate hydrogen to electrode-reducing bacteria, e.g., Gram-negative Deferribacteres, through a syntrophic interspecies hydrogen transfer. The production of electron-carrying shuttles has been demonstrated with mesophilic electrode-reducing bacteria (Bond and Lovley 2005; Rabaey et al. 2004, 2005), and many of the mesophilic electrode-reducing bacteria are members of the Gram-negative δ-proteobacteria. Regardless of the mechanism of electron transfer to the electrode or the type of microorganism, the thermophilic communities selected

first in sediment fuel cells and then established after multiple transfers and exchanges of the medium within the fuel cells continue to generate electricity without added electron-carrying mediators.

In conclusion, this is the first time that marine sediment has been shown to possess thermophilic electricity-generating bacteria as well as the first time that a thermophilic MFC has generated electricity with cellulose. A unique and narrowly defined group of Firmicutes and Deferribacteres were found to colonize the surface of an acetate-fed cell. This and previous work (Jong et al. 2006) indicate that thermophilic electricity-generating communities are ubiquitous and not restricted to thermophilic environments. The higher electrical currents produced under thermophilic conditions vs mesophilic, plus the omnipresence of these thermophiles, demonstrates a promising opportunity for the application of MFCs at high temperatures. This will require a broader and deeper understanding of the thermophilic electricity-generating bacterial communities and further engineering of the fuel cells in relation to higher temperatures.

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