Microbial Ecology

Microbial Communities Associated with Electrodes Harvesting Electricity from a Variety of Aquatic Sediments

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Abstract

The microbial communities associated with electrodes from underwater fuel cells harvesting electricity from five different aquatic sediments were investigated. Three fuel cells were constructed with marine, salt-marsh, or freshwater sediments incubated in the laboratory. Fuel cells were also deployed in the field in salt marsh sediments in New Jersey and estuarine sediments in Oregon, USA. All of the sediments produced comparable amounts of power. Analysis of 16S rRNA gene sequences after 3–7 months of incubation demonstrated that all of the energy-harvesting anodes were highly enriched in microorganisms in the δ-Proteobacteria when compared with control electrodes not connected to a cathode. *Geobacteraceae* accounted for the majority of δ-Proteobacterial sequences or all of the energy-harvesting anodes, except the one deployed at the Oregon estuarine site. Quantitative PCR analysis of 16S rRNA genes and culturing studies indicated that *Geobacteraceae* were 100-fold more abundant on the marine-deployed anodes versus controls. Sequences most similar to microorganisms in the family *Desulfobulbaceae* predominated on the anode deployed in the estuarine sediments, and a significant proportion of the sequences recovered from the freshwater anodes were closely related to the Fe(III)-reducing isolate, *Geothrix fermentans*. There was also a specific enrichment of microorganisms on energy harvesting cathodes, but the enriched populations varied with the sediment/water source. Thus, future studies designed to help optimize the harvesting of electricity from aquatic sediments or waste organic matter should focus on the electrode interactions of these microorganisms which are most competitive in colonizing anodes and cathodes.

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Introduction

Energy can be harvested from anoxic marine sediment by placing a graphite electrode in the sediment (anode) and connecting it in an electrical circuit to another electrode (cathode) in the overlying aerobic seawater [8, 52, 61]. It was initially thought that the current from sediment batteries resulted only from oxidation of reduced products of anaerobic respiration, such as sulfide, Fe(II), and reduced humic substances [52]. However, the observation that specific groups of organisms were enriched at the surface of energy-harvesting anodes [8, 61], suggested a more direct role for microbial activity in the process.

Initial analysis of 16S rRNA gene sequences from microorganisms attached to anodes from laboratory and field operated marine sediment fuel cells revealed a significant enrichment of bacteria related to *Desulfuromonas acetoxidans*, a member of the *Geobacteraceae* family within the δ-subdivision of Proteobacteria [8, 61]. Pure culture studies of *D. acetoxidans* and two other members of the *Geobacteraceae*, *Geobacter metallireducens* and *G. sulfurreducens*, demonstrated that microorganisms within this family can completely oxidize organic compounds, such as acetate, to carbon dioxide with an electrode serving as the sole electron acceptor [8, 9]. These results suggest that a significant portion of current generation from sediment fuel cells may be the result of microorganisms directly transferring electrons to the anode.

The direct electron transfer between *Geobacteraceae* and electrodes differs from previously described microbial fuel cells which required the addition of electron-shuttling compounds such as neutral red, thionin, methyl viologen, and phenazine ethosulfate [15, 16, 46, 47, 54] for effective transfer of electrons between cells and the

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electrode. These electron shuttles accept electrons from intracellular and membrane-bound redox proteins and transfer the electrons to the electrode surface, with the regeneration of the oxidized form of the shuttle. Although exogenous electron shuttles are not required for energy production in sediments, it was hypothesized that addition of these compounds on or near deployed electrodes may increase energy production. Of particular interest were extracellular quinones such as humic substances and the humics analogue anthraquinone 2,6-disulfonate (AQDS), which are known to enhance electron transfer between *Geobacteraceae* and insoluble Fe(III) oxides [26, 27]. AQDS stimulated the rate of current production by a pure culture of *D. acetoxidans* [8], and thus it seemed possible that addition of AQDS to sediments would enhance current production.

In order to learn more about the role of microorganisms involved in electricity harvesting from sediments, we surveyed the microbial communities associated with laboratory fuel cells constructed with marine, salt-marsh, and freshwater sediments, as well as sediment fuel cells deployed in the field. In addition to studying the anodes of these fuel cells, the microorganisms attached to the cathode were analyzed for the first time. The potential for the electron shuttle, AQDS, to stimulate energy harvesting or alter the microbial community was also investigated. The results demonstrate that (1) electricity can be harvested from freshwater, estuarine, and salt-marsh sediments as well as marine sediments; (2) *Geobacteraceae* are usually the predominant microorganisms colonizing the anode, however, other electrode-reducing microorganisms are also enriched and may predominate in some instances; (3) the addition of AQDS has little effect on energy harvesting but causes subtle changes in the composition of the microbial communities associated with anodes; and (4) there are specific enrichments of microorganisms on the cathode of sediment batteries that vary with the sediment source.

Materials and Methods

Sediment Sources. To construct sediment fuel cells in the laboratory, anoxic marine sediments were collected from Boston Harbor, MA, near the World's End peninsula, at a water depth of 5 m. Sediments were collected in 5-gal plastic buckets and sealed. Salt-marsh sediments and water were collected from a saline pond in the Great Sippewisset Marsh (West Falmouth, MA) which is primarily vegetated with *Spartina* grasses. Salt-marsh samples were collected at a mean water depth of 0.5 m in canning jars that were filled to the top and then sealed. Freshwater sediments were collected from Gunston Cove, VA on the Potomac River [32], at a mean water depth of 6 m, in canning jars. Water from each sampling site was also collected in plastic containers. All sediment and water samples were stored at 15°C.

The fuel cells operated in the field were subject to seasonally changing environmental conditions and utilized local sediments at coastal sites near Tuckerton, New Jersey, and the Yaquina Bay Estuary near Newport, Oregon [61].

Assembly of Sediment Batteries. For laboratory incubations, electrodes were unpolished 0.5-inch-thick, 2.5- or 3.5-inch-diameter graphite disks (grade G10, Graphite Engineering and Sales, Greenville, MI). All current production values were normalized to electrode surface area. Connections were made with threaded watertight connectors with #20 AWG marine-grade wire (Impulse, San Diego, CA) screwed into holes drilled directly in the graphite electrodes. Holes were filled with silver epoxy (Epoxy Technology, Billerica MA) and sealed with marine epoxy (Hysol adhesives, Seabrook NH). As new electrodes contained contaminants (such as SO_4^{2-} and Fe^{2+}) from manufacture, electrodes were soaked in successive changes of 1 N HCl until extractable Fe(II) was undetectable. For routine cleaning and removal of adherent biomass, electrodes were exposed to 1 N HCl and 1 N NaOH each for at least 24 h and were stored in deionized water.

Laboratory sediment fuel cells were prepared by filling 1-L beakers, 2-L jars, or 20-L quaria 1/4 full with anoxic sediment, and filling the remainder with water collected from the same site. Anodes were placed 2–5 cm below the sediment surface, and cathodes were suspended in the overlying seawater within 15 cm of the sediment. The overlying water was continuously bubbled with air, which was first sparged though water to minimize evaporation. Water lost by evaporation was replaced with deionized water. Sediments were equilibrated for 24 h, then current harvesting was initiated (where indicated) by connecting the anode to the cathode via a fixed resistor of 1000 ohms. Fuel cells were operated at 15°C in the dark. Triplicate or duplicate fuel cells and their appropriate non-current controls were operated for each sediment type, and a single anode/cathode set was analyzed from each fuel cell and non-current control.

For each sediment type or treatment, at least two independent incubations were conducted.

Field-deployed sediment fuel cells were constructed with 19-inch diameter graphite disk electrodes and operated as previously described [52, 61]. These experiments were each approximately 7 months in duration and involved energy harvesting either at fixed current or fixed voltage.

When noted, laboratory sediment fuel cells were sterilized either by adding formaldehyde directly to the sediment at a final concentration of 0.5%, or by autoclaving a complete sediment fuel cell (including emplaced

electrodes) on three consecutive days for 1 h each. In order to determine the effect of an electron shuttling compound on current production, anthraquinone 2,6-disulfonate (AQDS) dissolved in deionized water was added to sediments at a final sediment concentration of 100 μM, and an equivalent amount of water was added to controls. Current and voltage measurements for long-term sediment experiments were collected with a Keithley model 2000 multimeter (Keithley Instruments, Cleveland, OH).

Enrichment Cultures and MPN Analysis. Microorganisms colonizing the surface of anaerobic electrodes (anodes) from a laboratory marine sediment fuel cell (Boston Harbor sediment) and the fuel cell deployed near Tuckerton, New Jersey, served as inocula for culture-based analyses. Immediately following removal from sediments, a small area of the anode surface was washed with a sterile medium (see below), lacking any electron donors or acceptors and containing 1 mM Na_2S as a reductant, and vigorously scraped with a sterile razor blade and resuspended in sterile medium (3 mL) forming a suspension consisting of graphite and electrode-associated microbes. Anoxic pressure tubes (Bellco Glass, Inc., Vineland, NJ) containing 10 mL of the appropriate medium were immediately inoculated with 0.25 mL of this graphite/cell suspension and serially diluted to 10^{-6}. Tubes from the highest dilutions showing growth after ~6 months were used to inoculate new dilutions with the appropriate electron acceptor. Cultures were diluted to extinction in this manner three times.

The medium contained (per liter): 20 g NaCl, 0.77 g KCl, 0.25 g NH_4Cl, 0.1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 mL DL vitamins [28], 10 ml DL minerals [28], and 2.0 g $NaHCO_3$. Before $NaHCO_3$ was added, pH was adjusted to 6.8 with 5 N NaOH. The culture medium was dispensed into anaerobic pressure tubes and sparged with oxygen-free N_2:CO_2 (80:20, vol/vol) for 15 min to remove dissolved oxygen. The tubes were then sealed with thick butyl rubber stoppers and autoclaved. After autoclaving, the medium was supplemented with 1 mM $FeCl_2 \cdot 2H_2O$, 20 mM $MgCl_2 \cdot 6H_2O$, and 4 mM $CaCl_2 \cdot 2H_2O$ from sterile anoxic stock solutions. Poorly crystalline Fe(III) oxide (100 mmol/L) [35, 36] or S° powder (10 mmol/L) was provided as electron acceptor, and acetate (5 mM) was provided as electron donor. All incubations were at 15°C in the dark.

Most-probable number (MPN) analysis was also performed with microorganisms associated with the New Jersey anode, and a control electrode incubated in the same sediments but not used to harvest current. For each electrode, an area equivalent to 5 cm^2 was rinsed and scraped into a total volume of 2 mL, using the Na_2S-containing medium described above. Anoxic pressure tubes containing Fe(III) oxide and acetate were inoculated with 0.25 mL of this graphite/cell suspension. Each of these cultures was serially diluted to 10^{-8} in triplicate. After ~6 months of growth at 15°C, the highest dilution with growth was noted, and a standard three-tube MPN chart was consulted. Following the enumeration procedure, enrichment cultures were obtained by further dilutions from the highest dilution showing growth, using the Fe(III) oxide medium.

DNA Extraction, PCR, and Cloning of the 16S rRNA Gene. After at least 3 months of current harvesting, electrodes were removed from the water or sediment. The surface of electrodes was rinsed free of visible debris with a stream of sterile artificial seawater or freshwater medium. The first millimeter of the graphite electrode was scraped vigorously with a sterile razor blade into 1.5 mL TE buffer (pH 8), producing a suspension consisting of graphite and electrode-associated microbes.

DNA was extracted from the graphite/cell suspension with a modified version of the Miniprep of Bacterial Genomic DNA protocol [7]. The suspension was resuspended in TE/sucrose buffer (pH 8, 6.7% sucrose), SDS (0.5% final concentration), and lysozyme solution (1 mg/mL final concentration). The tubes were incubated at 37°C for 30 min and vortexed every 5 min. Following proteinase K treatment (0.1 mg/mL) at 37°C for 1 h, 0.5 g of MULTIMIX 2 Tissue Matrix beads (Bio101 Systems, Carlsbad, CA) was added to the suspension. Samples were then placed in a mini-beadbeater (BioSpec Products, Bartlesville, OK) for 30 s at 550 rpm, and DNA was extracted once with chloroform:isoamyl alcohol (24:1), and once with phenol:chloroform:isoamyl alcohol (25:24:1). This extracted DNA was further purified with the Wizard DNA Clean-Up System (Promega, Madison, WI). Extractions typically yielded 20–100 μg of DNA per 10 cm^2 of electrode. 16S rRNA gene fragments were amplified with the primer 8 forward [20, 21] or 63 forward [40] with 519 reverse [21] and 338 forward [2] and 907 reverse [20]. The total volume of each PCR mixture was 100 μL and contained ~6 ng DNA template; 10 μL Qiagen 10× buffer (15 mM $MgCl_2$); 5 μL of buffer Q (Qiagen, Valencia, CA); 8 μL of a 0.25 μM dNTP solution (Sigma Chemical Company, St. Louis, MO); 60 pmol each forward and reverse primers; 5 μL dimethyl sulfoxide (DMSO); and 3 units *Taq* polymerase (Qiagen). To eliminate contaminating DNA template, the PCR mixtures were exposed to UV radiation for 8 min prior to the addition of template and *Taq* polymerase. PCR amplification was performed in a DNA Engine thermal cycler (MJ Research, Inc., Waltham, MA) with an initial denaturation step at 94°C for 5 min, followed by 20 cycles of 94°C (30 s), 50°C (30 s), and 72°C (45 s) with a final extension at 72°C for 7 min. PCR products amplified with the three primer sets were pooled prior to the construction of clone libraries. Clone libraries were

constructed from the pooled 16S rRNA gene fragments using the TOPO TA cloning kit, version K2 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Replicate libraries discussed in the Results were generated from electrodes incubated in separate containers and subjected to separate DNA extractions and library construction.

Restriction Enzyme Analysis and Sequencing of the 16S rRNA Gene. In order to ensure that the sample size was large enough for statistical comparisons within and between the different conditions, 60 clones from each clone library were selected for restriction enzyme analysis. 16S rRNA gene fragments were amplified from various clones using M13 forward and reverse primers (Invitrogen). The amplified inserts (~500 ng) were digested for 16 h at 37°C with *Hha*I and *Msp*I (New England Biolabs, Beverly, MA) according to the manufacturers instructions. Restriction fragments were visualized on a 3% Metaphor agarose gel (BioWhittaker Molecular Applications, Rockland, ME), and a single clone from groups of identical restriction patterns was sequenced. Plasmid inserts were sequenced at the UMass Amherst automated sequencing facility with the M13 forward primer. Although unidirectional sequencing can result in higher sequence error rates, a few base-pair ambiguities would not affect the results of this study.

Phylogenetic Analysis. Sequences were compared to the GenBank database with the BLAST algorithm [1]. Representative sequences were manually aligned in the Wisconsin Package, version 10.2 (Genetics Computer Group, Madison, WI). Ambiguous characters in the alignment were ignored when these aligned sequences were imported into PAUP 4.0b 4a [60] where phylogenetic trees were inferred. Branching order was determined and compared using character-based (maximum parsimony) and the distance-based algorithm, HKY85 4-parameter model.

The GenBank accession numbers of the referenced sequences are as follows: *Desulfuromonas acetoxidans*, M26634; *Desulfuromonas thiophila*, Y11560; *Desulfuromusa kysingii*, X79414; *Desulfuromusa bakii*, X79412; *Geobacter chapelleii*, U41561; *Desulfosarcina variabilis*, M34407; *Desulfobacter curvatus*, M34413; *Desulfocapsa sulfoexigens*, Y13672; *Desulfobulbus propionicus*, M34410; *Paracoccus alkaliphilus*, AY014177; *Roseobacter denitrificans*, M96746; *Caulobacter fusiformis*, AJ007803; *Acetobacter peroxydans*, AB032352; *Methylobacter luteus*, M95657; *Cycloclasticus pugetii*, L34955; *Geovibrio ferrireducens*, X95744, *Geothrix fermentens*, GFU41563; *Thermus* sp. SA-01, AF020205. The accession numbers for clones and isolates from this study that were submitted to GenBank were AF534230–AF534263, and AY193732–AY193767.

Assessment of Microbial Diversity. A similarity matrix with 450 base pairs considered was constructed with the similarity matrix program [39], available on the Ribosomal Database Project II website, and ALIGN version 2.0 [41]. In order to determine microbial diversity represented by each clone library, clones were grouped into phylotypes, based on 16S rRNA gene similarity of >97%. The Shannon–Weaver index of diversity [$H = C/N\,(N \log N - \sum n_i \log n_i)$, where $C = 2.3$, N = number of phylotypes, and n_i = number of individuals in the ith phylotype] was calculated [4–6]. Because the sample size for each library was relatively small (60 clones), equitability (J), which is independent of sample size, was calculated from the Shannon–Weaver indices ($J = H/H_{max}$), where H_{max} is the theoretical maximal Shannon–Weaver diversity index for the population examined [4–6].

Geobacteraceae-Specific Primer Design and Testing. A manual alignment of 16S rRNA gene sequences from various *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa* isolates and clones was done in the Wisconsin Package, version 10.2 (Genetics Computer Group, Madison, WI). This alignment was then imported into Primer Express, version 1.0 (PE Applied Biosystems, Foster City, CA), and primers *Geobacteraceae* 494 F (5′-AGG AAG CAC CGG CTA ACT CC-3′) and *Geobacteraceae* 1050R (5′-CGA TCC AGC CGA ACT GAC C-3′) were designed from consensus regions indicated by the program [8]. Optimum temperature and cycle parameters were determined in a gradient thermal cycler (MJ Research, Inc., Waltham, MA). The primers amplified a 556-bp gene fragment from the 16S rRNA gene in the organisms of interest best with the following parameters: an initial step of 94°C for 5 min, 35 cycles of 94°C for 20 s, 51°C for 20 s, and 72°C for 30 s, followed by a final elongation step at 72°C for 7 min. Primers were tested on DNA extracted from pure cultures of *Desulfuromonas acetoxidans*, *Pelobacter carbinolicus*, *Geobacter sulfurreducens*, *Escherichia coli*, and *Desulfuromusa succinoxidans*. When these primers were tested on DNA extracted from the cathode surface, PCR amplification of the appropriate gene fragment was not observed. In order to further evaluate the specificity of the primers, a clone library was constructed from 16S rRNA gene fragments amplified from an anode with 494F and 1050R using the TOPO TA cloning Kit, version K2. Thirty-eight clones were selected and analyzed using the same restriction digestion and sequencing protocol outlined above, and all clone inserts were most similar to 16S rRNA gene sequences from *Desulfuromonas*, *Desulfuromusa*, or *Pelobacter* species.

Most Probable Number PCR Analysis. Five-tube MPN-PCR analyses were performed, as previously described [3, 8, 19] with DNA extracted from various electrode surfaces from current and non-current har-

vesting aquaria. Serial 10-fold dilutions were made, and 5 μl aliquots of the diluted DNA were the template in the PCR. *Geobacteraceae* 16S rRNA gene fragments were amplified with *Geobacteraceae* 494F and *Geobacteraceae* 1050R. PCR products were visualized on an ethidium bromide stained agarose gel. The highest dilution that yielded product was noted, and a standard five-tube MPN chart was consulted to estimate the number of *Geobacteraceae* 16S rRNA gene fragments in each sample.

Results

Current Production in a Diversity of Sediments. Current production from three different sediment types, marine (Boston Harbor, MA), salt marsh (Great Sippewisset, Falmouth MA), and freshwater (Gunston Cove, VA), was monitored in laboratory incubations under identical loads (1000 ohms) (Fig. 1A). Current production in field-deployed sediment fuel cells in salt marsh and estuarine sediments in Tuckerton, New Jersey, and Yaquina Bay Estuary, Oregon, is described elsewhere [61]. As previously observed [8, 52, 61], when graphite electrodes placed in marine sediments were connected via a fixed resistor to cathodes in overlying seawater, current was produced and peaked within 20 days. In a typical marine sediment incubation (Fig. 1A), a maximum current production of ~30 mA per square meter of electrode surface area (mA/m^2) was reached within 10–20 days of incubation, followed by a decrease to ~20 mA/m^2 after 90 days of operation. These trends in current production were the same whether cells were constructed in 1-L beakers, 2-L jars, or 20-L laboratory aquaria, but absolute values varied as much as 35% between incubations because of unknown factors. Inhibiting microbial activity in the sediments by autoclaving or formaldehyde stopped current flow (Fig. 1B).

When graphite electrodes were incubated in salt-marsh sediments, initial current production peaked within 10 days at current densities averaging 29 mA/m^2, and decayed to an average of 7 mA/m^2 for the remainder of the incubations (Fig. 1A). Sediment fuel cells constructed with freshwater sediment had a similar pattern, but reached maximum levels of current production (21 mA/m^2) within 5 days, and maintained current densities averaging 9 mA/m^2 afterwards (Fig. 1A). Although current production from coastal marine and salt-marsh sediments has been reported previously [8, 52, 61], this is the first report of current production from freshwater sediments.

To investigate the effect of an electron shuttling compound on current production, anthraquinone 2,6-disulfonate (AQDS) was included in marine sediments at a final concentration of 100 μM. Initial current production in AQDS-amended sediments peaked at levels 15–20% higher than in nonamended sediments, but always

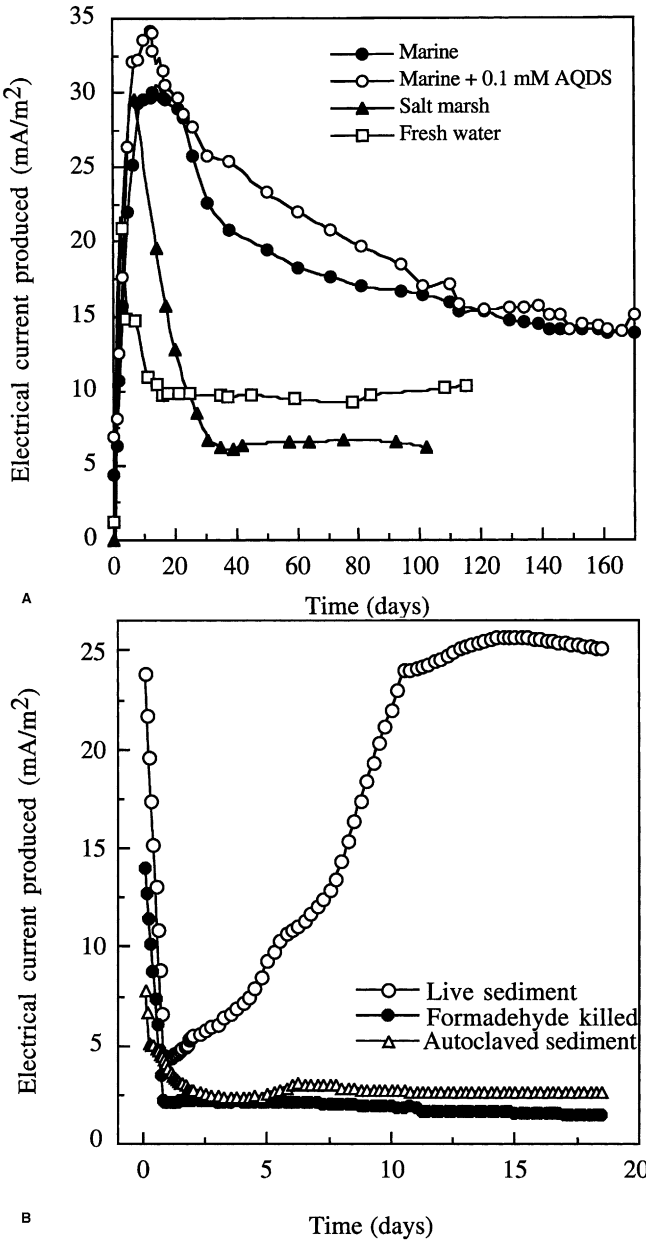

Figure 1. (A) Examples of current production by laboratory fuel cells constructed using sediments from three different sources under identical loads (1000 ohms), and effect of amending the marine sediments with 100 μM AQDS. (B) Effect of sterilization on current production in laboratory marine sediment fuel cells.

decayed to levels observed in nonamended sediments within 100 days (Fig. 1A). Introduction of additional AQDS to the surface of 150-day-old buried anodes via syringe did not significantly stimulate current production, indicating that some factor(s) other than AQDS availability were responsible for the decline in current.

Microbial Community Associated with Anodes. In order to gain further insight into the role of microorganisms in current production, the microbial commu-

Table 1. Comparison of equitability values (*J*) from non-current control and current-harvesting clone libraries obtained from electrode surfaces

Clone library	*Non-current control electrode*[a]	*Current-harvesting electrode*[a]
Marine anode (lab)	0.79 (±0.06)	0.37 (±0.04)
Salt-marsh anode (lab)	0.82 (±0.01)	0.33 (±0.01)
Freshwater anode (lab)	0.87 (±0.01)	0.47 (±0.01)
Salt-marsh anode (field)	0.75	0.45
Estuarine anode (field)	0.79	0.25
Marine cathode (lab)	0.85 (±0.03)	0.34 (±0.04)
Salt-marsh cathode (lab)	0.80 (±0.02)	0.35 (±0.01)
Salt-marsh cathode (field)	0.75	0.48

[a]Mean and standard deviation calculated from equitability values obtained from three separate clone libraries for all conditions except the New Jersey salt marsh [61] and estuarine field experiments because only one active fuel cell was operated at each field site.
[b]A value of $J = 0$ represents a pure culture, whereas $J = 1$ if each clone is a unique phylotype.

nities on the anodes (electron-harvesting electrodes in the sediments) were examined using a 16S rRNA gene clone library approach. These analyses included fuel cells constructed in the laboratory with the three different sediments described above, as well as two field-deployed sediment fuel cells. In each instance, the microbial diversity on the surface of the anode was dramatically lower than on control electrodes incubated in the sediment for the same amount of time, but not connected to a cathode in the overlying water (Table 1). Far fewer unique 16S rRNA gene sequences were recovered from energy-harvesting electrodes than from the controls. Equitability values calculated from Shannon–Weaver indices were about twofold greater in the control libraries than in the libraries from current-harvesting electrodes (Table 1).

In all six cases, the decrease in diversity on anodes was due to a significant enrichment of δ-Proteobacteria (Table 2). Depending upon the sediment examined, 54–76% of the 16S rRNA gene sequences recovered from the energy-harvesting anodes were δ-Proteobacteria. In contrast, only ~15% of the sequences on control electrodes not connected to a cathode could be assigned to the δ-Proteobacteria.

Enrichment of Geobacteraceae on Anodes. On five of the six anodes examined, *Geobacteraceae* accounted for the majority of δ-Proteobacterial sequences recovered from the anodes (45–89%) (Fig. 2). MPN-PCR analyses of anodes using *Geobacteraceae*-specific primers indicated that there were 100-fold more *Geobacteraceae* sequences/μg DNA on the current-harvesting anodes than on their corresponding control electrodes (Fig. 3).

Of the *Geobacteraceae* sequences recovered from different anodes, trends were observed that correlated with sediment type. For example, in marine and salt-marsh sediments, between 65 and 68% of the bacterial sequences were *Geobacteraceae*, and nearly all of these *Geobacteraceae* were most similar to *Desulfuromonas* species (83–89%) (Fig. 4). However, in freshwater sediments (Fig. 4), all of the *Geobacteraceae* sequences were most closely related to *Geobacter* species or to *Pelobacter propionicus*, which falls within the *Geobacter* cluster of the *Geobacteraceae* [23].

The overall abundance of *Geobacteraceae* sequences associated with the anodes from the AQDS-amended sediments was similar to anodes from sediments not amended with AQDS (Fig. 2). However, there was a shift in species composition within the *Geobacteraceae* when

Table 2. Percentage of 16S rRNA gene sequences in clone libraries from anodes placed in anoxic sediment and used to harvest current, or placed in similar sediment but not connected to cathodes

Fuel cell conditions	*α-Proteobacteria*	*β-Proteobacteria*	*γ-Proteobacteria*	*δ-Proteobacteria*	*Cytophagales*	*Firmicutes*	*Other bacteria*
Marine no current (lab)	5.80 (±0.80)	0	7.50 (±2.50)	12.50 (±3.50)	19.0 (±13.0)	8.0 (±2.0)	19.40 (±3.90)
Marine current (lab)	7.50 (±2.50)	0	2.35 (±0.65)	70.0 (±5.0)	9.15 (±5.8)	11.60 (±6.6)	5.65 (±0.60)
Marine (AQDS) current (lab)	4.15 (±0.85)	0	3.50 (±1.50)	66.7 (±1.70)	6.0 (±1.0)	8.30 (±5.0)	9.80 (±0.15)
Salt marsh no current (lab)	16.65 (±3.35)	0	24.50 (±0.50)	15.0 (±5.0)	10.30 (±0.3)	12.0 (±2.80)	20.90 (±3.80)
Salt marsh current (lab)	7.05 (±2.95)	0	8.75 (±2.10)	65.20 (±10.1)	7.05 (±2.90)	3.0 (±0.30)	8.85 (±6.10)
Freshwater no current (lab)	2.0 (±2.0)	18.50 (±8.50)	15.0 (±15.0)	16.20 (±3.70)	9.20 (±9.20)	10.0 (±10.0)	29.0 (±9.0)
Freshwater current (lab)	0	7.0 (±3.0)	9.70 (±0.3)	53.50 (±3.0)	3.15 (±3.10)	3.0 (±3.0)	23.60 (±3.60)
Salt marsh no current (field)	17.0	0	21.0	23.0	6.0	5.0	28.0
Salt marsh current (field)	3.0	0	9.0	76.0	5.0	3.0	4.0
Estuarine no current (field)	7.10	0	16.70	35.70	9.5	4.80	23.80
Estuarine current (field)	0	0	0	62.50	32.5	2.50	2.50

[a]Mean and standard deviation calculated from values obtained from three separate libraries excluding the salt-marsh [61] and estuarine [61] field experiments.

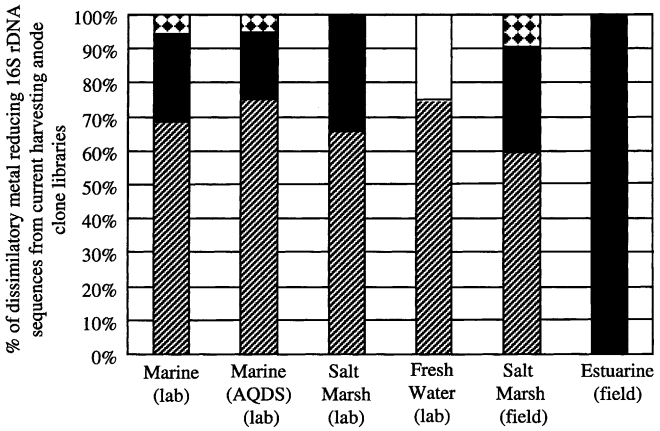

Figure 2. Relative proportions of dissimilatory metal reducing 16S rRNA gene sequences associated with the current harvesting anode from five separate fuel cells. Hashed lines: *Geobacteraceae*; black: *Desulfobulbus/Desulfocapsa* species; diamond pattern: other sulfate reducing δ-Proteobacteria; white: *Geothrix* species.

AQDS was added (Fig. 4). In marine sediments not amended with AQDS, *Desulfuromonas* sequences accounted for 88.9% ± 2.7% (mean ± standard deviation; $n = 3$) of the *Geobacteraceae* sequences with 8.3% ± 2.7% being most closely related to the sequences of *Desulfuromusa* species. In contrast, in the sediments amended with AQDS, *Desulfuromusa* accounted for 44.6% ± 3.0% of the sequences with 46.5% ± 3.6% of the sequences most closely related to *Desulfuromonas* species (Fig. 4).

Enrichment of Desulfobulbus/Desulfocapsa, Cytophaga, and Geothrix Species on Anodes. The study of multiple anodes incubated in different sediments allowed

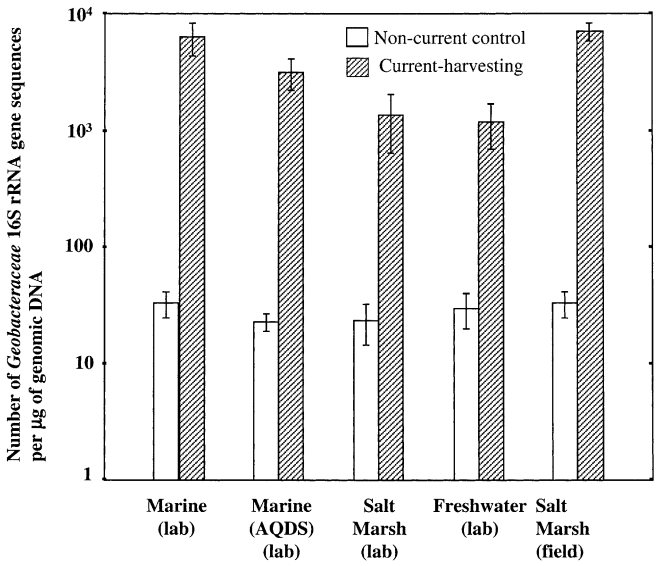

Figure 3. Number of *Geobacteraceae* 16S rRNA gene sequences as quantified by MPN-PCR. Each point is the average of five replicates. The error bars represent 95% confidence intervals.

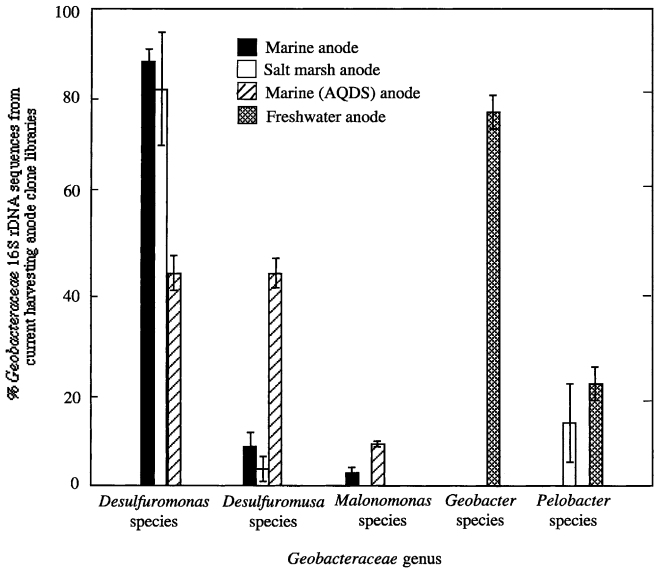

Figure 4. Relative proportions of *Geobacteraceae* 16S rRNA gene sequences associated with the current harvesting anode from four separate laboratory fuel cells.

the identification of groups of organisms not previously known to be enriched at the surface of anodes. For instance, although the anode from the fuel cell deployed in Yaquina Bay, Oregon, was highly enriched in δ-Proteobacterial sequences, all of these δ-Proteobacterial sequences were most closely related to *Desulfobulbus/Desulfocapsa* species, rather than *Geobacteraceae* (Fig. 2). Furthermore, *Desulfobulbus/Desulfocapsa* sequences accounted for 26–34% of the δ-Proteobacterial sequences on anodes incubated in other marine sediments, or ~20% of the overall microbial community (Fig. 2). In contrast, no *Desulfobulbus/Desulfocapsa* sequences were recovered from control electrodes, or from anodes emplaced in freshwater sediments (where all δ-Proteobacterial sequences were in the *Geobacteraceae*).

Sequences related to previously described microorganisms in the *Cytophagales* were also enriched on the estuarine sediment anode deployed in Yaquina Bay (Table 2). This was not observed in any of the other sediments, which had a lower proportion of *Cytophagales* sequences on energy-harvesting anodes than on control electrodes (Table 2).

Although the most notable effect of energy-harvesting on the composition of the freshwater anodes was the enrichment of *Geobacteraceae*, there was also an enrichment of sequences most closely related to *Geothrix fermentans* (Fig. 2). *Geothrix* sequences accounted for up to 19% of the sequences on the current-harvesting anodes, but none were detected on the non-current controls.

Enrichment Cultures. Most-probable number analysis (MPN) of Fe(III)-reducing microorganisms was conducted with material scraped from anodes at the

Table 3. Enrichment cultures of current-harvesting microbes associated with the anode surface

Inoculum	*Culture name*	*Electron acceptor*	*Most similar organism(s)*	*Sequence similarity*
Marine anode (lab)	Isolate A1	Fe(III) oxide	*Malonomonas rubra*	92.0%
Marine anode (lab)	Isolate A2	Fe(III) oxide	*Desulfuromusa succinoxidans*	94.0%
Marine anode (lab)	Isolate S1	Colloidal sulfur	*Desulfuromusa succinoxidans*	97.0%
Marine anode (lab)	Isolate S2	Colloidal sulfur	*Desulfuromusa succinoxidans*	97.0%
Salt-marsh anode (field)	Enrichment culture T1	Fe(III) oxide	*Desulfuromusa bakii*	99.0%
			Desulfoarculus baarsii	90.0%
Salt-marsh anode (field)	Enrichment culture T2	Fe(III) oxide	*Desulfuromusa bakii*	99.0%
			Desulfuromusa bakii	94.0%
Salt-marsh anode (field)	Enrichment culture T3	Fe(III) oxide	*Desulfuromusa bakii*	99.0%
			Malonomonas rubra	93.0%

[a]Acetate (5 mM) was used as electron donor for all enrichments on Fe(III) oxide (100 mM) and colloidal S^0 (10 mM).

field-deployed salt-marsh site. After 6 months of incubation in medium containing Fe(III) oxide and acetate, microorganisms from the active anode grew in dilutions 100-fold higher than the non-current control; 1.9×10^3 to 2.1×10^4 cells/cm^2 (95% confidence interval) were detected on the active anode compared to only 130 to 930 cells/cm^2 (95% confidence interval) on the control.

Further serial transfers from the current harvesting samples into new media yielded enrichment cultures with no more than two organisms. Several isolates were also obtained from current-harvesting anodes from a marine sediment fuel cell using medium containing Fe(III) oxide or colloidal S^0 as an electron acceptor. Phylogenetic analysis of these organisms indicated that all of the enriched microorganisms were δ-Proteobacteria, mainly species from the family *Geobacteraceae* (Table 3). One organism detected in an enrichment culture was most similar to the sulfate-reducing δ-Proteobacterium *Desulfoarculus baarsii*. The majority of 16S rRNA gene sequences associated with the marine fuel cell clone libraries were between 76% and 95% similar (~500 base pairs considered) to sequences of organisms found in the marine anode enrichment cultures (Fig. 5). The field deployed salt-marsh anode showed similar trends; the majority of enrichment culture and clone library sequences were 75–89% similar (Fig. 5).

Microbial Community Associated with Cathodes. In order to determine whether specific microorganisms might be associated with reactions at the cathode surface, microbial communities associated with cathodes from two laboratory sediment fuel cells (marine and salt marsh) and one deployed in the field (salt marsh in Tuckerton, New Jersey) were also examined. As observed with current-harvesting anodes, the community diversity on all three cathodes was significantly lower than on electrodes suspended in the water for the same amount of time, but not connected to an anode in the sediment (Table 1). However, the nature of the enrichment on the cathodes appeared to be dependent on the environment from which the sediment fuel cells were constructed.

For example, cathodes from the laboratory marine sediment fuel cells were enriched in 16S rRNA gene sequences from the γ-subdivision of Proteobacteria (Table 4). The majority (84.4%) of the γ-Proteobacterial sequences on the cathodes grouped in the *Cycloclasticus*/Type I Methanotroph cluster, while none of the γ-Proteobacterial sequences detected on the non-current-generating cathodes belonged to this group (Fig. 6).

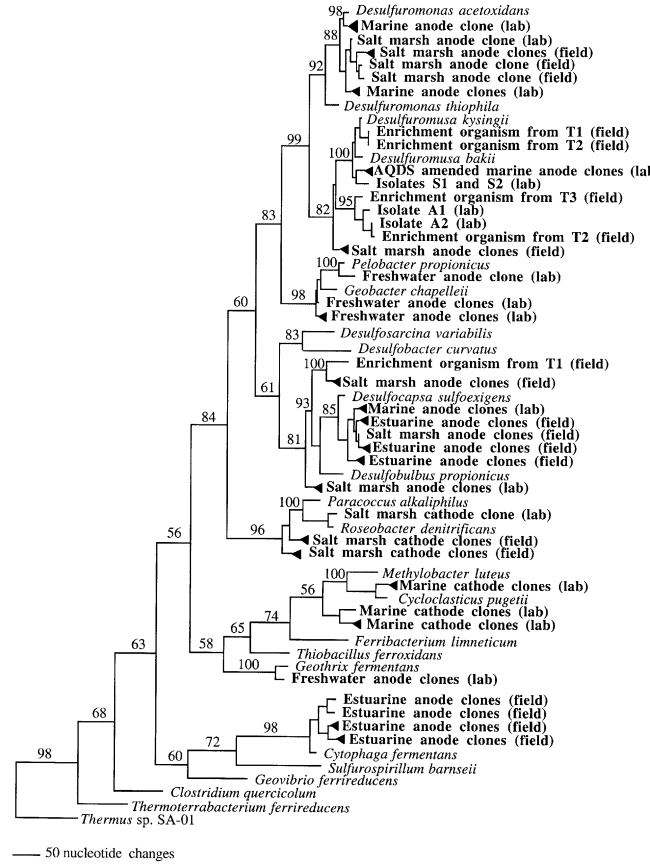

Figure 5. Phylogenetic tree constructed by maximum parsimony analysis showing the relationship of representative clones associated with various current-harvesting anodes and cathodes to 16S rRNA gene sequences of previously described bacteria. *Thermus* sp. SA-01 was used as the outgroup, and bootstrap analysis was conducted with 100 replicates.

Table 4. Percentage of 16S rRNA gene sequences associated with current and non-current harvesting cathodes from three separate fuel cells

Fuel cell condition	*α-Proteobacteria*	*γ-Proteobacteria*	*Cytophagales*	*Other bacteria*
Marine no current (lab)	30.0 (±5.0)	11.10 (±2.50)	26.65 (±2.35)	33.35 (±3.65)
Marine current (lab)	18.85 (±2.15)	63.30 (±1.70)	11.50 (±8.50)	5.50 (±3.80)
Salt marsh no current (lab)	25.05 (±11.75)	13.65 (±0.35)	10.35 (±3.65)	50.95 (±23.45)
Salt marsh current (lab)	69.70 (±3.0)	9.35 (±7.35)	6.75 (±2.25)	15.15 (±1.55)
Salt marsh no current (field)	20.0	35.0	0	45.0
Salt marsh current (field)	75.50	9.40	0	15.1

[a]Mean and standard deviation calculated from triplicate clone libraries for all conditions excluding the New Jersey salt marsh cathode (only one clone library assembled from New Jersey salt marsh current and non-current cathodes).

In contrast to the laboratory marine sediment fuel cell, the microbial communities recovered from cathodes in the laboratory and New Jersey field deployed salt marsh sediment fuel cells had fewer γ-Proteobacteria sequences on cathodes than on controls. Instead, these cathodes were enriched with α-Proteobacterial sequences (Table 4). The majority of α-Proteobacterial sequences fell within the *Rhodobacter* family (primarily *Paracoccus* and *Roseobacter* species) (Fig. 5): 68–78% of the α-Proteobacterial sequences on the current-generating cathode, compared to only 13–20% of the α-Proteobacteria on the non-current control. None of the γ-Protobacterial sequences detected on cathodes from laboratory salt-marsh fuel cells or the New Jersey field deployment were from the *Cycloclasticus*/Type I Methanotroph cluster.

Discussion

These results demonstrate that specific microorganisms colonize both the anodes and cathodes of sediment fuel cells. As detailed below, this information, coupled with the known physiology of microorganisms closely related to those enriched on the anodes, suggests that microorganisms play an important role in energy harvesting with sediment fuel cells. The detailed studies presented here expand on previous preliminary studies on the microbial communities associated with the anodes of sediment fuel cells [8, 61] and suggest that the microorganisms involved in electron transfer may vary in different sedimentary environments. Furthermore, these studies show that the compositions of microbial communities on the cathodes of sediment fuel cells are unique, and the studies demonstrate the need for further investigation of microbe–electrode interactions on the cathode.

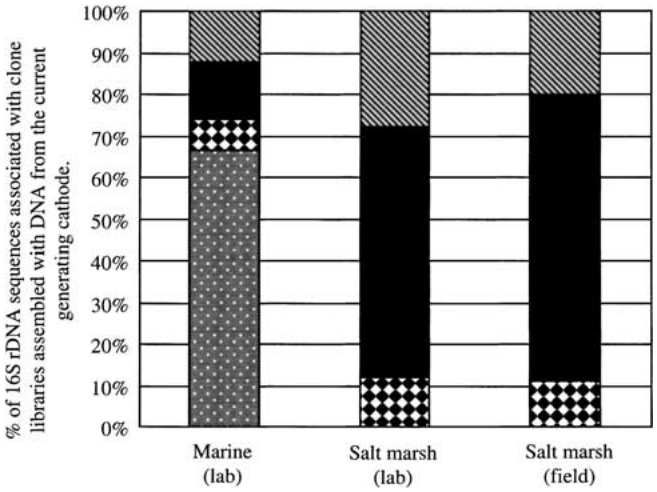

Figure 6. Relative proportions of α- and γ-Proteobacterial 16S rRNA gene sequences associated with the current-harvesting cathode from three separate fuel cells. Dotted gray: *Cycloclasticus*/Methylotroph I; diamond pattern: other γ-Proteobacteria; black: *Rhodobacter*; hashed lines: other α-Proteobacteria.

Evaluation of Methods. Each of the three methods that were used to evaluate the microbial communities on the electrodes has advantages and disadvantages. Although clone library sequence analysis of environmental 16S rRNA genes eliminates a number of PCR artifacts that can occur with other PCR-based approaches, this technique still has potential biases [11, 17, 51, 57, 59, 63]. In order to minimize bias in the 16S rRNA gene analysis, genomic DNA was carefully extracted and further purified with a cleanup system, the 16S rRNA gene was amplified with three different nonspecific primer sets, and PCR protocols that were previously shown to reduce PCR-generated chimeras, mutations, and heteroduplexes were utilized [51]. In most instances, the results from the clone libraries were further verified using quantitative MPN-PCR with specific primers. Furthermore, culturing studies helped support results obtained from molecular analyses. Although culturing also has a strong potential for bias [18], these results provided independent verification of the enrichment of Fe(III)- and S^0-reducing microorganisms on anode surfaces. In addition, this approach allowed the recovery of microorganisms that can be used for future physiological studies.

Although it may be difficult to infer the absolute number of *Geobacteraceae* species present in the environment from MPN-PCR and clone library analyses, these techniques do provide an indication of the relative proportion of *Geobacteraceae* sequences in comparable samples. Clone library analysis indicated that 2.5 to 6

times more *Geobacteraceae* species were associated with the current-harvesting anode than the non-current control, whereas a 100-fold increase in *Geobacteraceae* sequences was estimated with MPN-PCR. A possible explanation for the lower values detected with clone library analysis is the fact that clone libraries tend toward a 1:1 ratio, despite the initial template concentration [49]. Studies have also shown that a 10-fold discrepancy is frequently associated with quantitative PCR approaches, such as MPN-PCR [59]. In addition, only a subsection of the *Geobacteraceae* population would be detected from analysis of a relatively small clone library (60 clones) constructed with nonspecific bacterial primers, whereas all of the *Geobacteraceae* 16S rRNA gene sequences should be amplified with *Geobacteraceae*-specific primers.

Enrichment of Microorganisms on Anodes. The results suggest that there is a specific enrichment of microorganisms capable of Fe(III) reduction on the anodes of sediment fuel cells. However, sediment type appears to affect which Fe(III)-reducing microorganisms predominate. In all sediment fuel cells, except the one deployed in Yaquina Bay, anodes were specifically enriched with sequences from the family *Geobacteraceae*. In the freshwater sediments, these *Geobacteraceae* sequences were exclusively from the *Geobacter* cluster of the *Geobacteraceae*, which contains primarily freshwater microorganisms [23, 24]. *Geobacteraceae* sequences that were amplified from anodes in marine sediments, on the other hand, fell within the *Desulfuromonas* cluster, which contains primarily marine organisms [23, 24].

The specific enrichment of *Geobacteraceae* sequences on electrodes emplaced in a diversity of sediments is not surprising because *Geobacteraceae* are the predominant microorganisms in a variety of sedimentary environments in which Fe(III) oxide reduction is the main terminal electron-accepting process [19, 53, 55, 56, 58]. There are significant similarities between insoluble Fe(III) oxides and graphite electrodes. For example, both electrodes and Fe(III) oxides represent a potential electron sink that, unlike other common electron acceptors, such as oxygen, nitrate, or sulfate, is insoluble. Studies on the mechanism of electron transfer to Fe(III) oxide by microorganisms in the *Geobacteraceae* have suggested that they have a membrane-bound Fe(III) reductase [38] and that direct contact between the organisms and Fe(III) oxide is required for electron transfer [12, 42]. In contrast, other well-studied Fe(III)-reducing bacteria, such as *Shewanella* species [43, 45] and *Geothrix fermentans* [44], release electron-shuttling compounds which transfer electrons from the cell surface to the surface of the Fe(III) oxide. This alleviates the need for direct contact between cells and the Fe(III) oxide surface, but at a potentially high energetic cost that may limit the ability of these organisms to compete with the *Geobacteraceae*. Given the ability of *Geobacteraceae* to directly transfer electrons to the surface of Fe(III) oxide, it may not be surprising that members of this family, such as *Geobacter metallireducens*, *Geobacter sulfurreducens*, and *Desulfuromonas acetoxidans*, can conserve energy to support growth by completely oxidizing organic compounds, such as acetate, and quantitatively transferring these electrons to electrodes [8, 9].

The enrichment of *Geothrix fermentans* on energy-harvesting anodes in freshwater sediments is consistent with the finding that *Geothrix* species may be the second most abundant type of Fe(III)-reducing microorganism in some subsurface environments in which Fe(III) reduction is important. Molecular analysis of aquifer sediments indicated that *Geothrix* sequences increased when Fe(III)-reducing conditions were induced, albeit at levels much lower than for the *Geobacteraceae* [56]. A sequence closely related to *G. fermentans* was also detected in benzene oxidizing, Fe(III)-reducing enrichment cultures from aquifer sediments [55]. In addition, recent studies have shown that *G. fermentans* can transfer electrons to electrodes (Bond DR, Holmes DE, Lovley DR, Abstracts, ASM General Meeting, 2003).

In all sediment fuel cells constructed with marine and salt-marsh sediments, there was also an increase in sequences from the *Desulfobulbus/Desulfocapsa* phylogenetic cluster on the anodes. These organisms accounted for >60% of the clone library from the anode deployed in Yaquina Bay, and for ~20% of the sequences amplified from the other four current harvesting anodes. *Desulfobulbus propionicus*, a pure culture isolate from this group, is capable of Fe(III) reduction [37], and more recent studies have demonstrated that it can also use propionate, lactate, and pyruvate as electron donors for electron transfer to electrodes (Holmes DE, Bond DR, Lovley DR, unpublished results). However, *D. propionicus* does not use acetate, which can be one of the most abundant organic acid intermediates in anaerobic metabolism in sediments [24, 64].

The importance of organisms closely related to *D. propionicus* in current production may lie in their novel ability to anaerobically oxidize S^0 to sulfate with a suitable electron acceptor. While this was initially shown to occur with Mn(IV) serving as an electron acceptor [31], a similar reaction is also possible using an electrode (Holmes et al., unpublished results). S^0 is known to precipitate on the anodes of marine sediment fuel cells as the result of abiotic sulfide oxidation at the anode surface [61]. This may favor organisms such as *D. propionicus* and explain the specific enrichment of *Desulfobulbus/Desulfocapsa* sequences on anodes from marine sediment fuel cells. If so, this would suggest that sulfur sources rather than organic electron donors might have served as the critical fuels for current production in the field deployment in Yaquina Bay. However, this requires further investigation.

The fact that the addition of the electron-shuttling compound AQDS did not have a long-term effect on current generation in the case of the laboratory fuel cell drawing power from marine sediment suggests that the ability of the microorganisms to transfer electrons to the electrode surface is not the ultimate limiting factor in current production. Although long-term current production was not affected by the presence of AQDS, the addition of AQDS did result in subtle shifts in the microbial community, most notably within the *Geobacteraceae*. In contrast to the predominance of *Desulfuromonas* species on anodes in marine sediments not amended with AQDS, *Desulfuromusa* species accounted for nearly half of the *Geobacteraceae* detected on anodes from AQDS-amended sediments. *Desulfuromusa* species are also capable of Fe(III) reduction [22, 23], and *Desulfuromusa*-like species recently isolated from the surface of energy-harvesting anodes recovered from marine sediments are capable of donating electrons to graphite anodes (Holmes DE, Nicoll J, Bond DR, Lovley DR, manuscript in preparation). The reasons why the provision of an electron-shuttling compound might favor the growth of *Desulfuromusa* species on the anodes have yet to be determined.

In anaerobic sediments, fermentative microorganisms metabolize fermentable compounds primarily to acetate and hydrogen, which are then oxidized by Fe(III) or SO_4^{2-}-reducing microorganisms, or by methanogens [25, 29, 30, 34, 62]. Fe(III)-reducing microorganisms, rather than fermentative microorganisms, were enriched on the anode, even when AQDS was present as an electron shuttle. This suggests that, just as fermentative microorganisms are not able to effectively oxidize organic compounds with the reduction of Fe(III) oxide [33], they also do not effectively use the anode as an electron acceptor. For example, a *Clostridium* species recently isolated from the surface of an energy-harvesting anode of a fermentative fuel cell was only capable of transferring approximately 0.04% of electrons released from glucose fermentation to an electrode [48].

Enrichment of Microorganisms on Cathodes. Energy harvesting also resulted in a shift in the microbial community on cathodes used to generate electricity. However, the specific organisms enriched on the cathode were dependent on the sediment/water source. Cathodes involved in energy harvesting in marine sediments and seawater were heavily enriched with microorganisms in the γ-Proteobacteria, whereas 16S rRNA gene sequences most similar to species within the α-subdivision of Proteobacteria increased on anodes from laboratory and field deployed sediment fuel cells constructed with salt marsh sediment.

Although Proteobacteria, particularly those from the α-subdivision, commonly colonize surfaces in marine environments [13, 14], the organisms enriched on energy-generating cathodes differed from those usually found in marine biofilms. Both groups of enriched organisms are often involved in nitrogen transformations such as ammonia oxidation and denitrification [10, 50], which suggests the possibility of nitrogenous compound cycling at or near the cathode surface. Alternatively, it is possible that current harvesting significantly alters the charge, pH, or oxygen level near the cathode surface, leading to changes in microbial colonization. However, microbiological or geochemical evidence supporting these speculations is not yet available, and how this altered community affects current production remains a focus of future investigation.

In summary, these studies suggest that further optimization of electricity harvesting from sediment organic matter should consider factors which would enhance the ability of Fe(III)-reducing microorganisms to contact and transfer electrons to the anode. The role of the microorganisms that are enriched on the cathode is less clear. However, now that it is known that there are specific enrichments of microorganisms with unique metabolic capabilities on both electrodes, further investigation of the interaction of these microorganisms with electrodes is warranted.

Acknowledgments

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