

Electricity generation from cysteine in a microbial fuel cell

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Abstract

In a microbial fuel cell (MFC), power can be generated from the oxidation of organic matter by bacteria at the anode, with reduction of oxygen at the cathode. Proton exchange membranes used in MFCs are permeable to oxygen, resulting in the diffusion of oxygen into the anode chamber. This could either lower power generation by obligate anaerobes or result in the loss in electron donor from aerobic respiration by facultative or other aerobic bacteria. In order to maintain anaerobic conditions in conventional anaerobic laboratory cultures, chemical oxygen scavengers such as cysteine are commonly used. It is shown here that cysteine can serve as a substrate for electricity generation by bacteria in a MFC. A two-chamber MFC containing a proton exchange membrane was inoculated with an anaerobic marine sediment. Over a period of a few weeks, electricity generation gradually increased to a maximum power density of 19 mW/m² (700 or 1000 Ω resistor; 385 mg/L of cysteine). Power output increased to 39 mW/m² when cysteine concentrations were increased up to 770 mg/L (493 Ω resistor). The use of a more active cathode with Pt- or Pt–Ru, increased the maximum power from 19 to 33 mW/m² demonstrating that cathode efficiency limited power generation. Power was always immediately generated upon addition of fresh medium, but initial power levels consistently increased by ca. 30% during the first 24 h. Electron recovery as electricity was 14% based on complete cysteine oxidation, with an additional 14% (28% total) potentially lost to oxygen diffusion through the proton exchange membrane. 16S rRNA-based analysis of the biofilm on the anode of the MFC indicated that the predominant organisms were *Shewanella* spp. closely related to *Shewanella affinis* (37% of 16S rRNA gene sequences recovered in clone libraries).

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1. Introduction

Electricity can be produced by bacteria growing on carbon electrodes (anodes) in anaerobic sediments when

connected to electrodes in the oxic overlying water (cathodes) (Reimers et al., 2001; Tender et al., 2002). These sediment fuel cells represent a new type of microbial fuel cell (MFC) that does not require a proton exchange membrane. In MFCs, electrons obtained from the oxidation of organic matter can be passed by respiratory enzymes to the electrode, creating current flow. Protons must then diffuse to the cathode to combine with the electron and oxygen to form water.

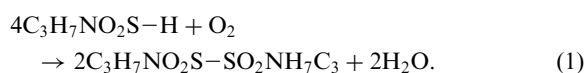
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The power generated is in proportion to the organic content of the sediment.

The microbial ecology of anaerobic sediments is complex, and primarily iron reducing bacteria in the family Geobacteraceae have been identified as associated with power generation in sediment fuel cells (Bond et al., 2002). Geobacteraceae are obligate anaerobes, while other iron reducing bacteria such as *Shewanella*, are facultative anaerobes. While sulfate reduction predominates in marine sediments, methanogenesis can occur in highly organic rich sediments in marine systems as well (Capone and Kiene, 1988; Mountfort et al., 1999) and in many places methane derived from subsurface coal measures can result in large fluxes of methane in marine sediments even in the absence of significant levels of methanogenesis (Judd et al., 1997). Gas seeps in Torry Bay, Firth of Forth, Scotland, exhibit methane fluxes in excess of 2000 m³ per year (Judd et al., 1997, 2002). We therefore wondered if these anoxic sediments rich in methane could also be used to produce electricity.

The use of membrane MFCs have several advantages compared with sediment fuel cells. Higher current densities have been achieved in MFCs and they can easily be used with pure cultures and bacteria enriched on electrodes (Reimers et al., 2001; Tender et al., 2002; Bond and Lovley, 2003; Park and Zeikus, 2003; Rabaey et al., 2003). Proton exchange membranes such as NafionTM have been developed to have the high proton conductivities needed in hydrogen fuel cells, but unfortunately these membranes are permeable to oxygen (Basura et al., 1998). It was estimated that up to 28% of the glucose added to a MFC could be lost through aerobic respiration by bacteria sustained by oxygen fluxes through the membrane (Liu and Logan, 2004). Oxygen diffusion into a reactor can create a problem as many bacteria, such as *Geobacter* sp., are obligate anaerobes. Diffusion of oxygen from the aerobic cathode chamber into the anoxic anode chamber can raise the redox potential and halt cell respiration. One approach routinely used for culturing obligate anaerobes such as methanogens, is to maintain a low redox potential using oxygen scavengers, such as cysteine (Hungate, 1969). When dissolved in water, cysteine reacts with oxygen to form a disulfide dimer (cystine), according to



Some bacteria, such as *Escherichia coli*, partially degrade cysteine to pyruvate in conjunction with the production of ammonia and hydrogen sulfide. *Shewanella putrefaciens* NCMB 1735 (formerly *Alteromonas putrefaciens*) can grow anaerobically using cysteine with trimethyla-

mine oxide as a terminal electron acceptor (Stenberg et al., 1984).

Cysteine has been used in MFCs to scavenge dissolved oxygen (Min et al., 2004) and it has also been claimed to function as a mediator in systems containing iron and acetate (Doong and Schink, 2002). The introduction of another mediator (anthraquinone-2,6-disulfonate, or AQDS) into a MFC was shown to increase power by 24% when power was already generated by bacteria through direct contact with the electrode (Bond et al., 2002). Because cysteine can be degraded by some bacteria, we wondered if this compound alone would sustain electricity generation in a MFC. To date there has been no record of electricity generation supported solely by a protein or amino acid in a MFC.

2. Materials and methods

2.1. Medium and cell culture

Sediment samples were obtained from Torry Bay, Firth of Forth, Scotland (56°03'08"N, 3°34'59"W). The sediment was obtained using a sterilized stainless steel core tube. The core was returned intact to the laboratory and placed in an anaerobic glove box. The core was sectioned at 2 cm intervals under anoxic conditions. A sample from 30 cm depth was transferred aseptically to a sterile plastic bottle and stored for 24 h at 4 °C. A sample (6 g) was transferred into a mineral salts medium (MSM) containing (mg/L in distilled water): NaCl, 8800; NaHCO₃, 3000; MgCl₂·7H₂O, 330; CaCl₂, 275; KH₂PO₄, 14; K₂HPO₄, 21; Na₂HPO₄·7H₂O, 56; FeSO₄·7H₂O, 10; MnSO₄·H₂O, 5; NH₄Cl, 3.1; KCl, 2; CoCl₂·6H₂O, 1; ZnCl₂, 1; CuSO₄·5H₂O, 0.1; H₃BO₃, 0.1; Na₂MoO₄, 0.25; NiCl₂·6H₂O, 0.24; EDTA, 1. The solution pH was 7. Cysteine (L-cysteine HCl, Sigma Chemicals) was added to a final concentration of 385 mg/L, except when noted.

2.2. MFC construction and operation

The MFC consisted of two bottles (250-mL bottles, Corning Inc.; 300 mL capacity) with carbon paper electrodes (2.5 × 4.5 cm). The bottles were joined by a glass bridge containing a proton exchange membrane (NafionTM 117, Dupont Co.) held by a clamp between the flattened ends of the two glass tubes (inner diameter = 1.3 cm) fitted with rubber gaskets (Fig. 1). Anodes (two) were made of plain carbon paper, while the cathode, except as noted below, contained catalyst (0.35 mg/cm², 10% Pt; De Nora North America, Inc.) on one side. In order to determine if the cathode used here limited maximum power output, home made cathodes containing either 0.35 mg/cm² of Pt, or

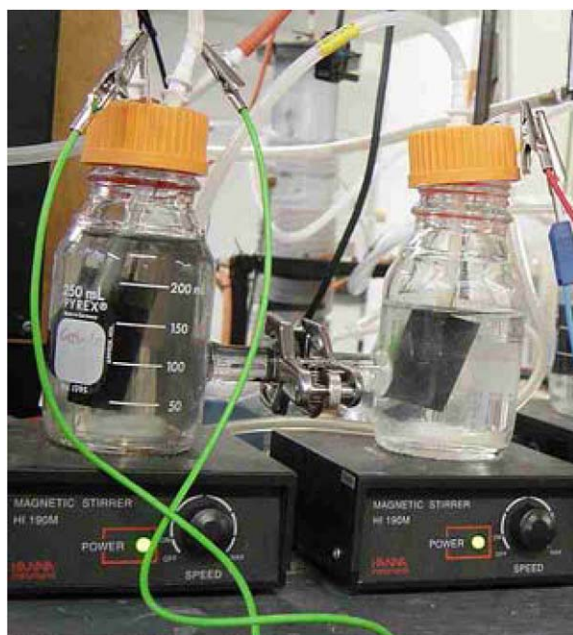


Fig. 1. MFC setup (anode chamber containing two electrodes on the left; cathode on right).

0.50 mg/cm² Pt/Ru (1:1 molar ratio) on both sides of the carbon paper were prepared using the method of Hogarth (1995). The electrodes were attached using copper wire with all exposed metal surfaces sealed with a nonconductive epoxy (Dexter Corp., NJ, USA). The anode and cathode were filled (250 mL) with MSM, mixed with a magnetic stir bar, and operated at room temperature (22 °C). The anode was continuously purged with methane (20 mL/min) to simulate the gas environment of the anaerobic sediment, while the cathode was sparged with air (110 mL/min) using an aquarium membrane pump.

2.3. Analysis

The fuel cell was continuously monitored using a data acquisition system for voltage (ADC22, v. 5.07.4, Pico Technology, Ltd.) and checked with a multimeter (Black Star, model 3225). The circuit was usually operated under a fixed load of 493 Ω (first anode) and 468 Ω (second anode), except during power measurements when resistance was set using a variable resistor box (model 1501, Time Electronics, Ltd, Tonbridge, Kent, UK). Current (i) was calculated at a resistance (R) from the voltage (V) as $i = V/R$. Power (P) was calculated as $P = iV$, normalized by the surface area of the anode. Power was modeled as a function of cysteine concentration using a nonlinear regression analysis (Sigmaplot, 2000, Jandel Scientific). Total electron flow was calcu-

lated by summing the product of the time interval and the current passed through the circuit. Coulombic efficiency was calculated as the total coulombs measured divided by the moles of electrons available from the added cysteine. We assumed either one mole of electrons per mole of cysteine on the basis of cysteine conversion to cystine, or 10 moles of electrons for cysteine oxidized to carbon dioxide.

Cysteine concentrations were measured using Ellman's reagent (Ellman, 1959) prepared by dissolving 40 mg of 5,5'-dithiobis(2-nitrobenzoic acid) in 10 mL of phosphate buffer, adjusted to a pH = 7 using NaOH. Reactor samples (50 μL) were combined with buffer (2 mL, 0.1 mM, NaH₂PO₄, pH = 8) and distilled water (5 mL); 3 mL of this sample was then poured into a cuvette containing 20 μL of Ellman's reagent, and the absorbance measured at 412 nm after 2 min.

To determine if cysteine could function as a mediator, we used cyclic voltammetry (PC 4/750TM Potentiostat, Gamry, USA) to characterize the oxidation–reduction reactions on the electrode surface when cysteine was added into an uninoculated reactor. The current response at an electrode surface was measured over a range of potentials in an unstirred solution at a scan rate of 25 mV/s (minimum of 5 scans). The anode was the working electrode and the counter electrode was the MFC cathode with a Ag/AgCl reference electrode. The potentials examined were in the range of –600 to 200 mV.

2.4. DNA extraction from bacterial cells present in the MFC

An aliquot of MSM (2 mL) containing both suspended cells and biofilm material scraped from the anode with a sterile pipette tip was removed from the anodic chamber of the MFC. The suspension was centrifuged (10,000g, 5 min) and the supernatant removed. The cells were resuspended in sterile molecular biology grade water (100 μL, Sigma, UK) and DNA was extracted using a FastDNA Spin Kit for soil (BIO 101, Q-BioGene, UK). Cell morphology was examined using fluorescence microscopy after staining the cells with 4', 6 diamidino-2-phenylindole (DAPI; final concentration 3.3 μg/mL) for 12 min and filtering them onto 0.2 μm pore size black polycarbonate filters (Costar Scientific Corporation, Bucks., UK).

2.5. PCR amplification and cloning of bacterial 16S rRNA genes

Eubacterial 16S rRNA gene fragments were amplified using primers pA (5'-AGAGTTTGATCCTGGCT-CAG-3') and pHr (5'-AAGGAGGTGATCCAGCCG-CA-3') (Edwards et al., 1989) according to a previously

described method (Rowan et al., 2003). PCR products were purified using a Qiagen PCR clean up kit (Qiagen, Crawley, UK). Cloning was carried out with a Qiagen PCR cloning kit (Qiagen, Crawley, UK) using the pDrive cloning vector. Competent *E. coli* cells (Qiagen EZ) were transformed with the ligated DNA. All procedures were carried out according to the supplier's instructions. White colonies were selected at random and nucleic acids were extracted from a sector of *E. coli* colonies by boiling in TE for 3 min. Amplification of DNA inserts was performed using primers pUCr (5'-CAGGAAACAGCTATGAC-3') and pUCf (5'-GTTTTCCCAGTCACGAC-3') as described previously (Rowan et al., 2003). PCR products of the correct size from 35 randomly selected colonies were screened by DGGE to identify different clone types.

2.6. DGGE screening of cloned 16S rRNA gene fragments

Cloned 16S rRNA gene fragments were analyzed by denaturing gradient gel electrophoresis (DGGE) to screen clones prior to sequencing. DGGE analysis was conducted using the D-Gene DGGE system (Bio-Rad, Hercules, CA, USA). Polyacrylamide gels (10% polyacrylamide, acrylamide:*N,N'*-methylenebisacrylamide, 37.5:1; 0.75 mm thick; 16 × 16 cm) were run in 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.3). A gradient ranging from 30 to 60% denaturant (100% denaturant is 7 M urea plus 40% vol/vol formamide in 1 X TAE) was used. Gels were run at 60 °C for 4 h at a constant 200 V and stained for 30 min in SYBR green I (Sigma, Poole, UK; diluted 1/10,000 in 1 X TAE). Stained gels were viewed and documented using a Fluor-S Multimager (Bio-Rad, Hercules, CA, USA). Clones with different migration characteristics in DGGE analysis were selected for sequence determination.

2.7. Sequencing and phylogenetic analysis of cloned rRNA gene fragments

Representatives from the different clone types identified by DGGE analysis were sequenced. Near-complete 16S rRNA sequences were obtained from selected clones using the primers of Edwards et al. (1989). All sequencing was conducted using the DyeDeoxy chain termination method and an ABI prism automated DNA sequencer (PE Applied Biosystems, Warrington, UK). The 13 partial 16S rRNA sequences obtained have been deposited in the GenBank database with accession numbers AY785248–AY785260. Phylogenetic distance analyses were conducted using the Jukes and Cantor (Jukes and Cantor, 1969) correction for multiple substitutions at a single site and the neighbor joining

method (Saitou and Nei, 1987) as implemented in the TREECON package (Van de Peer and de Wachter, 1994). Bootstrap re-sampling was conducted with 100 replicates.

3. Results

3.1. Power generation

Power generation by the MFC increased during the first few weeks, eventually reaching an average of 18 mW/m² with fresh medium (493 Ω resistor, 385 mg/L of cysteine). Electricity was generated in proportion to cysteine concentration (Fig. 2). Power increased directly in proportion to cysteine concentrations up to ~200 mg/L, and then power increased more slowly with cysteine concentration reaching a maximum of 38 mW/m² at 770 mg/L of cysteine. In the absence of cysteine in the medium, no power was generated. Changing the gas used for sparging the anode chamber from methane to nitrogen did not affect power generation (data not shown). The shape of the curve in Fig. 2 is typical of substrate-limited kinetics, so we therefore fit the data to a Michaelis–Menten type of equation producing a half-saturation constant of $K_s = 460$ mg/L and $V_{max} = 56$ mW/m². Extensive tests in our laboratory with other substrates using a MFC of the same type used here indicated that the maximum power achievable in this system is ca. 44 mW/m² (Oh and Logan, 2004). Thus, while the fit of the data to this type of equation was good over the region of cysteine concentrations examined, it is unlikely the maximum power density of 56 mW/m² could be achieved in this system due to limitations of maximum power density that can be achieved with this MFC.

A second carbon electrode was added into the anode chamber in order to determine if power output could be enhanced by colonization of bacteria grown in the fuel cell as opposed to all bacteria present in the original sediment inoculum. Each electrode was wired with a separate resistor to a common cathode. The reactor was run for several weeks to allow time for colonization of the second electrode. During this period the anode and cathode chambers were replaced with fresh medium each time the power density decreased to less than ~0.010 V (approximately 100–120 h). When only the original electrode was connected during an experiment, the voltage produced was 0.115 V (493 Ω; 12 mW/m²). Slightly less power was generated using only the new electrode (468 Ω; 0.088 V, 7.4 mW/m²). When both electrodes were connected, the voltage for each line was 0.086 V, respectively, or a total power density of 13.4 mW/m². The fact that the power was not doubled when the second electrode was added indicates that

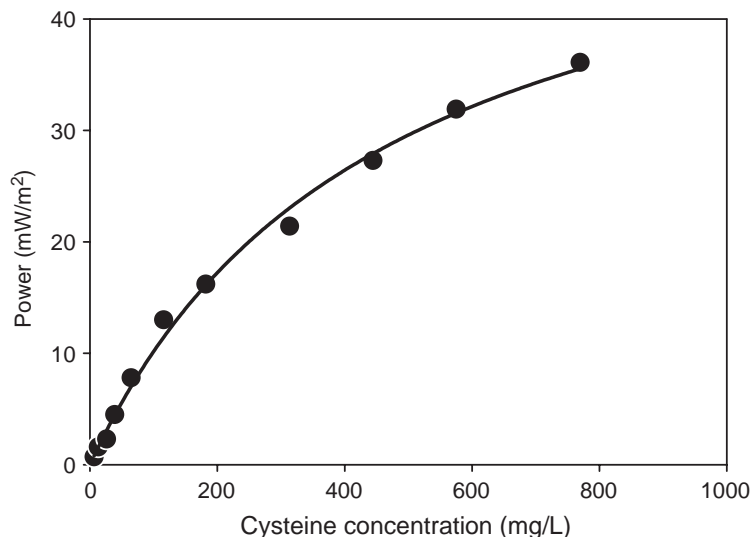


Fig. 2. Power output by MFC as a function of cysteine concentration. The regression line was fit according to Michaelis–Menton kinetics, with $V_{\max} = 56 \text{ mW/m}^2$ and $K_s = 460 \text{ mg/L}$.

power generation was limited by some factor other than anode surface area.

Power output as a function of load was measured by varying the resistance across a single electrode over a range of 1Ω – $10 \text{ k}\Omega$ in fresh medium (Fig. 3A). The maximum power of 19 mW/m^2 was obtained when the resistance was 1000Ω , with power at 18 mW/m^2 under normal conditions (493Ω).

Power output was affected by the cathode material. When the cathode was replaced with a Pt-free carbon electrode, power output decreased from a maximum of 19 to 3.4 mW/m^2 (Fig. 3A). Using another identically prepared carbon electrode produced even less power (0.9 mW/m^2). Replacing the cathode with a Pt cathode prepared in our laboratory increased power to 33 mW/m^2 (Fig. 3B). The use of a Pt–Ru catalyst provided the same power output as the new Pt cathode (Fig. 3B). Ru has been used to prevent poisoning of Pt electrodes (Gojkovic et al., 2003).

The total current recovery by a MFC containing one or two anode electrodes was measured over time (Fig. 4). Based on current recovery, cysteine was not just converted to cystine. Assuming one mole of electrons produced per mole of cysteine (Eq. (1)), the total current produced in the two anode experimental setup over 109 h (Fig. 4A) would account for more cysteine than was added (141%). Assuming complete bacterial oxidation of cysteine to CO_2 resulted in a more realistic recovery of 10% of the electrons as electricity. The fate of the cysteine not used for electricity generation could not be identified based on analysis of organic compounds in solution. The concentration of dissolved

organic matter remaining in solution after depletion of cysteine was 20 – 30 mg C/L but an analysis of volatile acids and alcohols did not identify any acetate, butyrate, or propionic acids, acetate, methanol, ethanol, *N*-propanol or *N*-butanol. Cysteine was completely consumed following the cessation of power generation (Fig. 4B). The addition of simple organic acids (acetic, butyric and butyric) failed to generate electricity within 4 h (data not shown).

In order to examine if cysteine could function as an electron shuttle, we used cyclic voltammetry (Rabaey et al., 2004) with cysteine but no biofilm present in the system. We found no evidence of oxidation or reduction peaks in the anode chamber with cysteine indicating that it did not function as a mediator in this system.

3.1.1. Microbial community analysis

Bacteria obtained from the MFC appeared to be predominantly rod shaped (Fig. 5). Analysis of the 35 clones derived from the MFC identified nine separate groups on the basis of migration in DGGE gels. For each of these nine groups one or more representative clone was chosen and sequenced (11 nearly full length and two partial 16S rRNA gene sequences were obtained; Table 1 and Fig. 6). The clone library was dominated by *Gammaproteobacteria* (34 of 35 clones). The most abundant sequence type in the clone library (14 out of 35 clones) was closely related (97.3–99.8% identity) to *Shewanella affinis* KMM 3586 with sequences most similar to

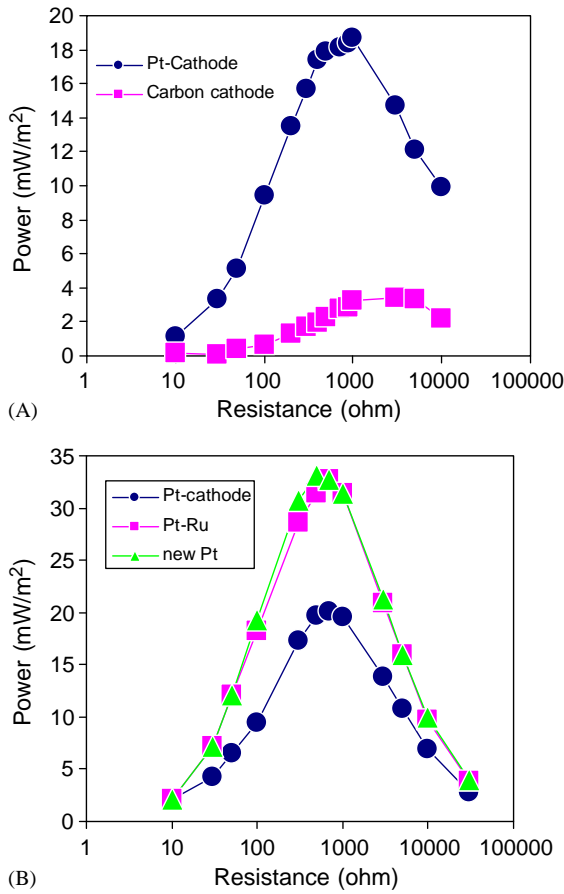


Fig. 3. Power output by MFC with different cathodes: (A) power output with a commercially available Pt-coated carbon cathode versus that of a plain carbon cathode; and (B) Pt-coated cathode versus carbon cathodes prepared in our laboratory containing Pt–Ru or Pt catalysts.

Vibrio spp. and *Pseudoalteromonas* spp. comprising the next most frequently detected.

4. Discussion

Electricity generation can be sustained solely on cysteine oxidation in a MFC. The amount of power generated through cysteine oxidation (maximum of 19 mW/m² for typical operating conditions) is comparable to power reported in many other MFC studies using lactate (0.6–15 mW/m²; Kim et al., 1999, 2002; Park and Zeikus, 2003), acetate (0.3–49 mW/m²; Bond et al., 2002; Bond and Lovley, 2003; Min et al., 2004) or glucose (33 mW/m²; Chaudhuri and Lovley, 2003) in similar types of two-chambered MFCs. Substantially higher power has only been achieved using specially designed electrodes, direct-air cathodes, or uniquely

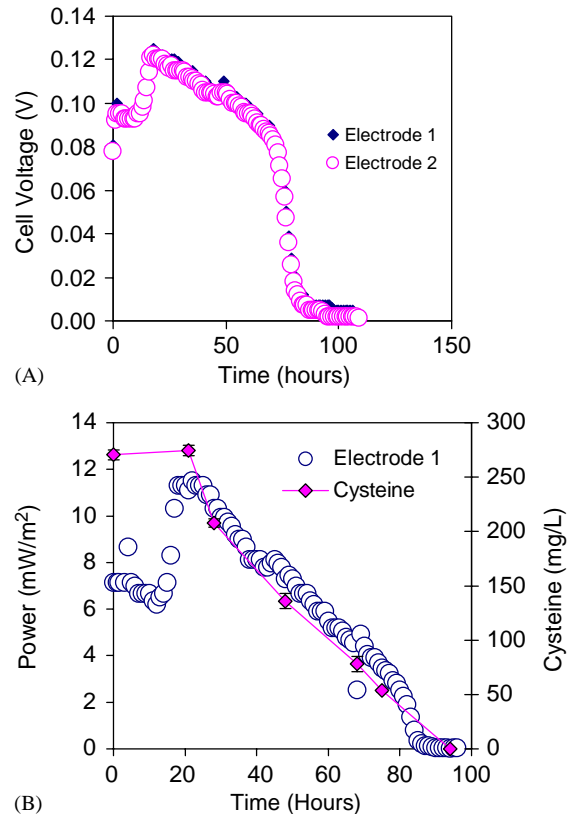


Fig. 4. (A) Voltage measured in the MFC cell containing two anodes, and (B) power generated in the reactor with a single anode with simultaneous monitoring of the concentration of cysteine in the reactor.

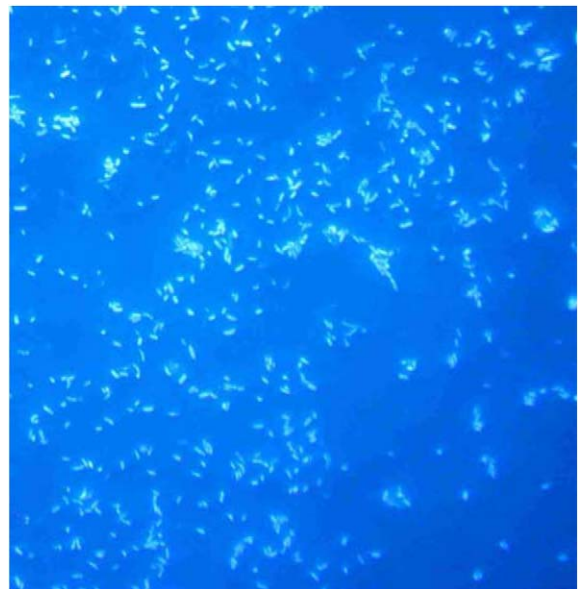


Fig. 5. DAPI stained preparation of bacteria from the MFC reactor.

Table 1
Closest matches with GenBank sequences of cloned 16S rRNA gene fragments derived from the MFC

| DGGE group | Sequence designation ^a | Closest FASTA match | % Identity | Reference | % Representation of sequences in clone library |
|------------|-----------------------------------|--|-------------------|------------------------|--|
| A | MFC 26 | AY174869 <i>Vibrio</i> sp. QY101 | 99.5 ^b | Han et al. (2002) | 22.9 (8 clones) |
| B | MFC 2 | AP005073 <i>Vibrio parahaemolyticus</i> RIMD 2210633 | 97.4 ^b | Makino et al. (2003) | 2.8 (1 clone) |
| C | MFC 15 | AY339889 <i>Pseudomonas</i> sp. NJU001 | 99.9 ^b | Gao and Shao (2003) | 5.7 (2 clones) |
| D | MFC 7 | AF500080 <i>S. affinis</i> KMM 3586 | 99.8 ^b | Ivanova et al. (2003) | 37.1 (14 clones) |
| | MFC 10 | AF500080 <i>S. affinis</i> KMM 3586 | 98.2 ^b | | |
| | MFC 35 | AF500080 <i>S. affinis</i> KMM 3586 | 97.3 ^c | | |
| E | MFC 8 | AY305857 <i>Pseudoalteromonas</i> sp. SM991 | 99.9 ^b | Chen et al. (2003) | 14.3 (5 clones) |
| | MFC 28 | AY305857 <i>Pseudoalteromonas</i> sp. SM991 | 99.7 ^b | | |
| F | MFC 12 | AP005083 <i>Vibrio parahaemolyticus</i> | 93.5 ^b | Makino et al. (2003) | 2.8 (1 clone) |
| G | MFC 17 | AJ316179 <i>Vibrio</i> sp. LMG 20023 | 97.3 ^b | Thompson et al. (2001) | 5.7 (2 clones) |
| | MFC 19 | AJ316194 <i>Vibrio</i> sp. LMG 19999 | 96.3 ^b | Thompson et al. (2001) | |
| H | MFC 27 | AF290495 Uncultured sheep mite bacterium | 99.4 ^b | Hogg and Lehane (2001) | 2.8 (1 clone) |
| I | MFC 32 | AJ227787 <i>Brevundimonas aurantiaca</i> | 98.6 ^d | Abraham et al. (1999) | 2.8 (1 clone) |

^aThirteen nearly full or partial 16S rRNA gene sequences obtained from a total of 35 clones.

^bBased on nearly full length 16S rRNA sequence.

^cBased on 977 bp of 16S rRNA sequence.

^dBased on 357 bp of 16S rRNA sequence.

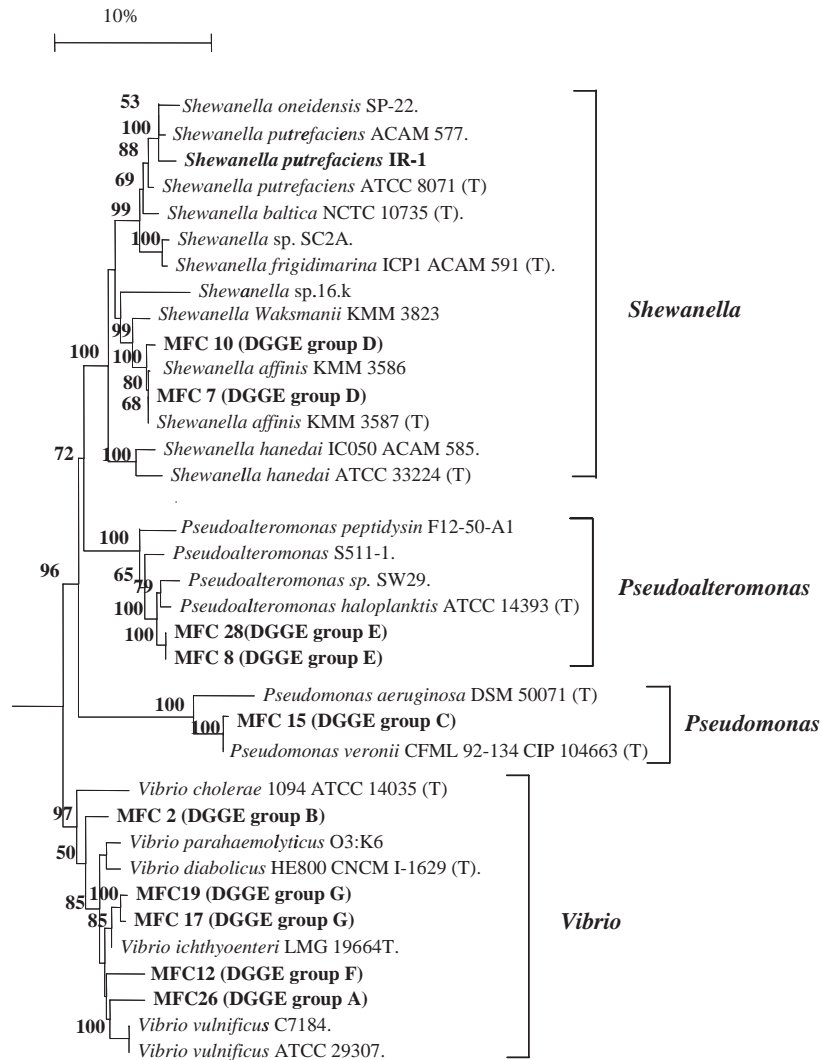


Fig. 6. Phylogenetic distance tree based on the comparative analysis (1201 positions) of 16S rRNA sequences recovered from the MFC. The scale bar denotes 10% sequence divergence and the values at nodes indicate the percentage of bootstrap trees that contained the cluster to the right of the node. The tree was rooted with reference to *E. coli*. Bootstrap values less than 50 are not shown. Sequences recovered from the MFC in this study and the 16S rRNA sequence from *Shewanella putrefaciens* IR-1 previously shown to be active in a microbial fuel cell (Kim et al., 2002) are shown in bold typeface.

acclimated cultures (Park and Zeikus, 2003; Rabaey et al., 2003; Liu and Logan, 2004).

The use of an oxygen scavenger such as cysteine in MFCs is motivated by identification of obligate anaerobes in sediment fuel cells, such as bacteria in the family *Geobacteraceae* (Bond et al., 2002). However, *Geobacter* spp. have been reported to be able to scavenge small amounts of dissolved oxygen, presumably as a method of maintaining low redox conditions needed for their growth (Jara et al., 2003). The maximum rate that oxygen would need to be removed by these or other bacteria in the fuel cell can be estimated based on previous measurements of oxygen flux through Na-

tionTM membranes in hydrogen fuel cells (Basura et al., 1998; Liu and Logan, 2004). The maximum rate (W) of oxygen diffusion through a membrane of cross section A can be approximated as $W \cong -DAC_{eq}/\delta_m$ (Liu and Logan, 2004). For the membrane used here, and assuming an oxygen solubility in the membrane equal to oxygen saturation in water at equilibrium of $C_{eq} = 2.6 \times 10^{-7}$ mol/cm³, a membrane thickness of $\delta_m = 190 \mu\text{m}$, and a diffusion constant of oxygen in the membrane of 4.4×10^{-6} cm²/s (Liu and Logan, 2004), oxygen could diffuse into the anode chamber at a rate of 0.009 mg/h. This could result in a daily net oxidation of cysteine to cystine of 13 mg/L.

This finding that cysteine alone can support electricity generation has important implications for electron balances using pure and mixed cultures in MFCs. When facultative bacteria are used in pure culture experiments, it is likely that oxygen diffusion into the anode will be scavenged by the bacteria. The major effect on the fuel cell operation in this case should be a loss of substrate to microorganisms through aerobic respiration. The bacteria identified in the present study were primarily species of *Shewanella* which are known to be facultative anaerobes. An electron balance showed that 14% of the cysteine could be accounted for through electricity generation. If the above oxygen fluxes are included into the electron balance for cysteine, an additional 14% (28% total) of the cysteine could be lost to a combination of electricity generation and cysteine oxidation to cystine. Coulombic efficiencies reported by others vary widely from 0.04% to 97% (Park et al., 2001; Bond et al., 2002; Bond and Lovley, 2003; Chaudhuri and Lovley, 2003).

Cysteine has been reported to function as an electron shuttle, or mediator, in experiments with *G. sulfurreducens* grown on acetate and iron (Doong and Schink, 2002). However, cysteine could not have functioned solely as a mediator here as cysteine was the only available oxidizable substrate (Fig. 4). Cyclic voltammetry was also used to show that it did not function as a mediator here because cysteine did not show any evidence of reversible oxidation and reduction. However, there was a consistent increase in power generation of ca. 30% over the first 24 h after addition of fresh medium. This increase may be indirect evidence of a mediator being produced by the bacteria that functioned as a shuttle between bacteria in the biofilm and the electrode surface. The increase in the voltage observed here, within the first 24 h, is an amount that is consistent with previous reports on the effects of a mediator added into a mediatorless MFC. Bond et al. (2002) found using cultures of *G. acetoxidans* that addition of the mediator anthraquinone-2,6-disulfonate (AQDS) increased electron transfer by 24%. It has been shown that some strains of *Shewanella* sp. are capable of producing mediators (Newman and Kolter, 2000), and *Shewanella* were identified as being present in the microbial community in this MFC.

Studies of MFCs have either focused on the ability of pure cultures of electrochemically active bacteria (Kim et al., 1999; Bond et al., 2002; Pham et al., 2003; Chaudhuri and Lovley, 2003) or the colonization of the anode by bacteria derived from a range of inocula (Bond et al., 2002; Lee et al., 2003; Kim et al., 2004; Phung et al., 2004). Bacterial communities of naturally colonized anodes from MFCs have been analyzed on a number of occasions (Bond et al., 2002; Lee et al., 2003; Kim et al., 2004; Phung et al., 2004). The composition of these bacterial communities is somewhat varied, and although

the observed variation has been interpreted as resulting from either the inoculum source or electron donor, definitive evidence that this is genuinely the case is lacking. It is clear from studies using both pure cultures and anode-enriched consortia from an environmental inoculum that many bacterial taxa are electrochemically active and can use electrodes as an electron acceptor. *Deltaproteobacteria* from the *Geobacteraceae* have been identified as abundant members of the anode community in fuel cells derived from marine sediments (Bond et al., 2002; over 70% of 16S rRNA genes in clone libraries) and activated sludge (Lee et al., 2003; ca. 13% of 16S rRNA genes in clone libraries). *Betaproteobacteria* related to *Leptothrix* spp. and *Acidovorax* spp. were found to be significant members of the anodic community of fuel cells inoculated with river sediment and fed with either river water or artificial wastewater, respectively. In the case of the artificial wastewater fed fuel cell the most abundant sequences were related to *Alphaproteobacteria*.

In this study over 97% of the sequences detected belonged to the *Gammaproteobacteria*. Only one other study has reported predominance of *Gammaproteobacteria* associated with a MFC where ca. 33% of the sequences recovered from a glucose-fed reactor were from *Gammaproteobacteria* (Lee and Kim, 2004). The *gammaproteobacterium* *S. putrefaciens* and presumably other metal-reducing *Shewanella* sp. have the majority of their cytochromes located in their outer membrane (Myers and Myers, 1992). Consequently *S. putrefaciens* IR-1 has been successfully used in a MFC (Kim et al., 2002). However, prior to the study reported here, members of this well-known metal-reducing genus have not been identified as important members of the anodic community of MFCs enriched from environmental inocula. The *Shewanella* sp. identified was most closely related to *S. affinis*, this bacterium and its closest relatives (*S. waksmanii* and *S. cowelliana*) have all been isolated from marine mud-dwelling invertebrates (Ivanova et al., 2003a, b, 2004), consistent with the intertidal marine sediment used to inoculate the MFC. The predominance of a *Shewanella* sp. in our MFC fed cysteine is not unexpected since *Shewanella* are known to grow using cysteine (Stenberg et al., 1984), but it was not previously known that growth on cysteine alone could be sustained in a MFC.

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