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ORIGINAL PAPER

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Enrichment of microbial community generating electricity using a fuel-cell-type electrochemical cell

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Abstract A fuel cell was used to enrich a microbial consortium generating electricity, using organic wastewater as the fuel. Within 30 days of enrichment the maximum current of 0.2 mA was generated with a resistance of 1 kΩ. Current generation was coupled to a fall in chemical oxygen demand from over 1,700 mg l^{-1} down to 50 mg l^{-1}. Denaturing gradient gel electrophoresis showed a different microbial population in the enriched electrode from that in the sludge used as the inoculum. Electron microscopic observation showed a biofilm on the electrode surface and microbial clumps. Nanobacteria-like particles were present on the biofilm surface. Metabolic inhibitors and electron acceptors inhibited the current generation. 16S ribosomal RNA gene analysis showed a diverse bacterial population in the enrichment culture. These findings demonstrate that an electricity-generating microbial consortium can be enriched using a fuel cell and that the electrochemical activity is a form of anaerobic electron transfer.

Introduction

Electrochemical techniques are used in various fields of biology, for example in biochemistry to characterize redox proteins and in biotechnology to develop biosensors, bioelectrochemical synthetic processes, and biofuel cells (Higgins and Hill 1985). Although a large number of redox proteins are electrochemically active, the peptide chain adjoining the redox center of the protein hinders direct electron transfer between the redox proteins and an electrode. Modifications of the protein or the electrode surface can increase the rate of the electrochemical reactions (Uosaki and Hill 1981). In most cases, intact microbial cells that contain active redox proteins are electrochemically inactive, as their cell walls and other surface structures are electrically non-conductive. Mediators can be used to facilitate the transfer of electrons between microbial cells and an electrode (Allen and Bennetto 1993; Kim and Kim 1988). Alternatively, the bacterial cells can be modified with hydrophobic conducting compounds to increase electrochemical activity (Park et al. 1997, 1999).

A microbial fuel cell (MFC) is a two-compartment structure divided by a cation-specific membrane. The electrons available through the metabolism of the electron donors by microorganisms are transferred to the anode of the fuel cell and then to the cathode through the circuit, where they reduce the oxidant (Allen and Bennetto 1993), consuming protons available through the membrane from the anode. There are at least two types of MFC. One involves the utilization of electrochemically active metabolites such as hydrogen sulfide produced by microbial metabolism (Haberman and Pommer 1991) and another involves the use of mediators (Allen and Bennetto 1993; Park and Zeikus 2000).

A number of bacteria have been isolated with the ability to use Fe(III) as a terminal electron acceptor (Lovley 1991; Nealson and Saffarini 1994). Although there is some evidence that a soluble electron carrier is involved in the electron transfer to the water-insoluble electron acceptor (Newman and Kolter 2000; Turick et al. 2002), direct contact between the bacterial cells and the electron acceptor is required for the dissimilatory Fe(III) reduction (Lovley 1991; Nevin and Lovley 2000). Among the Fe(III)-reducers, *Shewanella putrefaciens* (Myers and Myers 1992) and *Geobacter sulfurreducens* (Lloyd et al. 2000) are known to localize the majority of their

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membrane-bound cytochromes on the outer membrane; and the former is electrochemically active (Kim et al. 1999a, 1999b). *S. putrefaciens* grew on lactate in the absence of electron acceptors in an electrochemical fuel cell, but did not metabolize lactate when the anode was disconnected from the cathode (Kim et al. 1999b). A mediator-less MFC was successfully operated using *S. putrefaciens* (Kim et al. 2002). An electrochemically active strain of *Clostridium butyricum* was isolated from the mediator-less MFC described in this communication (Park et al. 2001).

Recently, it was reported that a two-electrode system can harvest energy from marine sediments (Bond et al. 2002; Reimers et al. 2001; Tender et al. 2002). Natural redox compounds such as sulfur/sulfide, Fe(II)/Fe(III) and humic acid were mentioned as possible mediators facilitating electron transfer from the microbial cells to the electrode (Reimers et al. 2001), while the addition of a humic acid analogue, anthraquinone-2,6-disulfonate, increased current production slightly (Bond et al. 2002). In this communication, a fuel cell-type electrochemical cell was used to enrich a microbial consortium generating electricity from wastewater as fuel.

Materials and methods

Wastewater and sludge

Wastewater was collected from a starch-processing plant (Samyang Genex Co., Inchon, Korea). The chemical oxygen demand (COD) value of the wastewater was around 1700 mg l^{-1}. The wastewater contained 25±7.7 mg l^{-1} total nitrogen and 10.7±1.7 mg l^{-1} total phosphorus, respectively. Inorganic nitrogen was less than 5 mg l^{-1}. Anaerobic digester sludge and activated sludge were collected from the wastewater treatment streams of the same plant.

Microbial fuel cell

Transparent polyacrylic plastic was used to construct the MFC according to Allen and Bennetto (1993) with an anode compartment capacity of 25 ml. Each cell compartment had three ports at the top, for the electrode wire, the addition and sampling of solutions, and gassing. The anode compartment was kept anoxic by gassing with oxygen-free nitrogen (15 ml min^{-1}) and the cathode compartment oxic with air (15 ml min^{-1}). Both the anode and cathode of the MFCs were of graphite felt (50×50×3 mm, 0.63 g, GF series; GEE Graphite, Dewsbury, UK). The surface area of this graphite felt was 332 mm^2 g^{-1} electrode. The change in potential was recorded using a digital voltammeter (model 2000; Keithley Instruments, Cleveland, Ohio) linked to a multichannel scanner (model 2000-SCAN; Keithley Instruments). Data were recorded every 2 min on an IBM compatible personal computer through an IEEE488 input/output system (model KPC-488.2AT; Keithley Instruments) and a cable (model CTMGPIB-1; Keithley Instruments). The measured potential was used to calculate the current according to the relationship between current and potential at a given resistance. Coulomb yields were obtained by integrating current over time (Kim et al. 2002).

Enrichment

The fuel cell was used to enrich electrochemically active bacteria, using wastewater as the electron donor and sludge as the bacterial source. Wastewater was used to fill a fuel cell containing 5–20 ml sludge before the potential was recorded. When the potential reached a steady state, the fuel cell was connected to 1 kΩ resistor to measure the current. For the batch-mode enrichment culture, five fuel cells were used for each set of experiments, including a control with wastewater alone and cells using activated sludge or anaerobic digester sludge as their bacterial source. Another five fuel cells were prepared in a similar way using activated sludge; and wastewater fuel was fed continuously at the rate of 0.3 ml min^{-1} for continuous enrichment culture. An additional three fuel cells were run in batch mode as a control. The anode was not connected to the cathode in the control fuel cells.

Inhibitors and electron acceptors

In some experiments, metabolic inhibitors were added to the fuel cells. These included rotenone (in acetone), 2-heptyl-4-hydroxyquinolone-*N*-oxide (HQNO, in ethanol), antimycin A (in ethanol), potassium cyanide (in water), sodium azide (in water), *p*-chloromercuriphenylsulfonate (*p*-CMPS, in water), 2,4-dinitrophenol (in acetone), and dicyclohexylcarbodiimide (DCCD, in acetone), and electron acceptors such as oxygen, nitrate, nitrite, and sulfate. They were purchased from Sigma (St Louis, Mo.) and Aldrich (Milwaukee, Wis.). Blank runs with solvent only showed no significant changes in current generation.

Phosphate-buffered media and viability counts

Phosphate-buffered basal medium (PBBM; Chang et al. 1999) was used to suspend bacterial cells from the anode. A piece of the electrode taken from a fuel cell enriched for over a year was placed in a pressure tube (Bellco Glass, Vineland, N.J.) containing anaerobically prepared PBBM and shaken vigorously to obtain a suspension of cells associated with the electrode. Strictly anaerobic conditions were maintained throughout the procedure. The suspensions were plated on starch wastewater/PBBM with or without acetate (20 mM) or lactate (30 mM) and ferric pyrophosphate (20 mM) and then incubated in an anaerobic glove box (Coy Lab, Ann Arbor, Mich.) at 30 °C for anaerobes and in a 30 °C incubator for aerobes. Nitrate or nitrite was used as the electron acceptor in some experiments. Starch wastewater/PBBM was prepared by dissolving PBBM ingredients in 1:1 diluted wastewater.

Microscopy

Low-vacuum electron micrographs (LVEM) were taken, using a JSM 5410 (Jeol, Tokyo, Japan). Scanning electron micrographs (SEMs) were taken after the enriched electrode samples were sputter-coated with gold. Bacterial suspensions were prepared by vigorous shaking before they were fixed, embedded, and stained according to the standard method for transmission electron microscopy (TEM). Confocal scanning laser micrographs (CSLM; Caldwell et al. 1992; Lawrence et al. 1991) were taken using a confocal scanning laser microscope (LSM-410; Carl Zeiss, Germany). The samples were stained with LIVE BacLight bacterial gram stain kit (L-7005; Molecular Probes).

Analyses

Standard methods were employed to analyze protein and DNA (Gerhardt 1993) and soluble COD (Eaton et al. 1995). The COD value was expressed as the concentration of organic contaminants. Methane was analyzed by gas chromatography as described by Chang et al. (1999). Gas samples for methane measurement were collected from the fuel cell under normal operational conditions, with or without nitrogen-gassing.

Denaturing-gradient gel electrophoresis

Anaerobic digester sludge (5 ml) and a piece of anode (0.3 cm^3) from the MFC enriched for over a year were used to extract whole genomic DNA, using Genomic DNA kits (Q-Biogene, Carlsbad, Calif.). The extracted DNA was amplified by polymerase chain reaction (PCR) in a GeneAmp 9600 PCR system (Applied Biosystems, Foster City, Calif.), using a forward primer (341f: 5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CGA CAG-3′) and a reverse primer (534r: 5′-ATT ACC GCG GCT GCT GG-3′) for denaturing-gradient gel electrophoresis (DGGE). The DCode universal mutation detection system (Bio-Rad, Hercules, Calif.) was used for DGGE according to the manufacturer′s procedure, using a denaturant gradient from 30% to 60% (Muyzer et al. 1993). Numbers given for the primers are those of nucleotide sequence of *Escherichia coli* 16S rDNA.

16S-rDNA sequencing and phylogenetic analysis

The extracted DNA was amplified for 16S rDNA sequencing, using universal primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-TAC GGY TAC CTT GTT ACG ACT T-3′). The amplicon was cloned into a vector (pGEM-T easy vector system I; Promega Co., Madison, Wis.) and transformed into *E. coli* DH5 α (Takara BIO, Shiga, Japan). Plasmid inserts were isolated using the QIAprep spin miniprep kit (Qiagen, Valencia, Calif.) and digested with *Rsa* I (Promega) for restriction fragment length polymorphism analysis to select different clones for sequencing. Different clones were sequenced using an ABI 310 automatic sequencer system (Applied Biosystems) at Macrogene Co. (Seoul, Korea). The sequences were compared with those in the GenBank nucleotide sequence database. Sequences were aligned and analyzed by CLUSTAL W (enclosed within the Lasergene software package; DNASTAR, Madison, Wis.). A phylogenetic tree was drawn using the CLUSTAL method (Megalign program in the DNASTAR package). The sequences were deposited in GenBank (accession numbers AF438166–AF438175).

Results

Enrichment

An open-circuit potential of around 0.6 V was developed immediately after the addition of the sludge. Similar results were obtained from all fuel cells to which activated sludge or anaerobic digester sludge had been added. The fuel cell containing only wastewater did not develop a significant potential. The magnitude of the open-circuit potential developed was dependent on the amount of sludge added (data not shown). These results show that the sludge contains electrochemically active material(s).

When the fuel cell was connected through a resistance of 1 kΩ, the potential dropped to 20 mV, which corresponds to a current of 20 μA. When a portion of the anode chamber content (5 ml) was replaced with fresh wastewater, the current increased to 0.2 mA before falling to a background level of 20 μA with a decrease in COD (Fig. 1). Repeated wastewater replacements were coupled to current generation together with a stepwise fall in soluble COD from 1,700 mg l^{-1} to 50 mg l^{-1} in 30 days. Similar trends were observed in all fuel cells which had received activated sludge or anaerobic digester sludge.

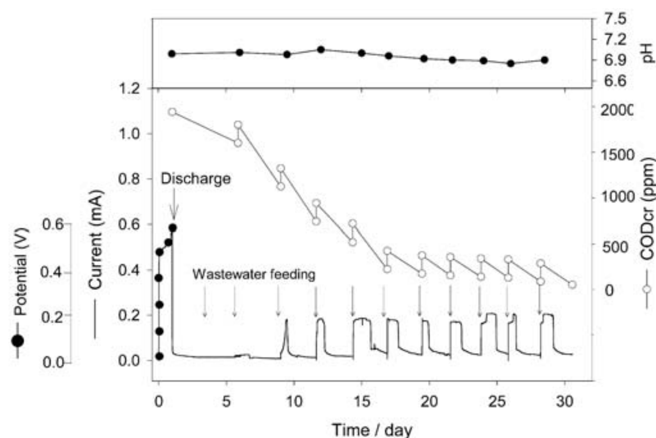

Fig. 1 Enrichment of electrochemically active microorganisms using a fuel cell inoculated with activated sludge and fed with starch-processing wastewater. The cathode compartment was filled with 50 mM phosphate buffer (pH 7.0) and aerated at the rate of 15 ml min^{-1}, whilst the anode compartment was gassed with oxygen-free nitrogen at the same rate. The fuel cell was kept at 30 °C. When the current dropped to the background level, 5 ml anode content was replaced by fresh wastewater. *COD* Chemical oxygen demand

The fuel cells without the bacterial source but containing wastewater showed similar results at a slower rate. The soluble COD value of the anode content of the control fuel cell was reduced to around 50 mg l^{-1} in 6 weeks. The off-gas from the anode compartments was analyzed periodically, but methane was less than the detection limit (0.001%) throughout the study, regardless of nitrogen-gassing through the anode compartment.

The open-circuit control fuel cells were operated in a similar way to the others for 8 weeks before the anode was connected to the cathode through 1 kΩ resistance. Wastewater (400 mg l^{-1}) was replenished daily. After 8 weeks, the control fuel cells were replenished by fresh wastewater and the current was measured for 4 h. These control fuel cells generated a steady current lower than 0.02 mA. This figure is much lower than the current generated from the closed-circuit MFCs. The initial COD (400 mg l^{-1}) decreased to around 100 mg l^{-1}. The change in COD was much greater than expected from the current generated, but smaller than that of the functional MFC. This might be due to aerobic oxidation of electron donors in the anode compartment, using oxygen diffused from the cathode compartment (Pham et al. 2003).

These results suggested that the electrochemically active particles (probably bacteria in the sludge) propagated in the closed-circuit fuel cells but not in the open-circuit control and that the wastewater contained electrochemically active particles at a lower concentration than the sludge. The current generation might be the result of electrons transferred to the electrode by the electrochemically active bacteria after they metabolized electron donor(s) in the wastewater in the absence of electron acceptors other than carbonate.

The stepwise decrease in soluble COD showed that certain electron donor(s) in the wastewater were metabolized faster than others by the microorganisms present in the sludge at the given conditions and that those electron donors consumed at a later stage were metabolized by the microorganisms enriched during operation of the fuel cell. The fuel cells generated current stably for over 5 years. Similar results were obtained from fuel cells fed by other wastewater, including artificial wastewater and effluent from septic tanks (unpublished data). In a separate set of experiments, the fuel cells were fed with diluted wastewater continuously. The current reached a steady-state value of 0.2 mA within 3 weeks (data not shown).

DNA and protein content of the enriched electrode

Chemical analyses of the microbial community associated with the electrode enriched for 6 months showed that 1 g of the electrode generated biomass containing 130.0±9.8 mg protein and 4.33±0.24 mg DNA. These figures were used to calculate the number of bacteria present as $6.34 \pm 0.6 \times 10^{11}$ bacteria g^{-1} electrode, assuming that the bacterial cells contain 55% protein and 1.8% DNA and that a bacterial cell has a weight of 3.75×10^{-13} g (Whitman et al. 1998).

Colony-forming units on the enriched electrode

A piece of electrode was vortexed and diluted serially to count the colony-forming units (CFUs) on starch wastewater/PBBM plates with or without ferric pyrophosphate as an electron acceptor. Colonies formed on the solid media ranged from $6.5 \pm 3.0 \times 10^{6}$ CFUs to $3.5 \pm 0.5 \times 10^{7}$ CFUs g^{-1} electrode. Clear halos of Fe(III) reduction were formed around some colonies on the plates containing Fe(III). They were isolated and purified to determine their Fe(III)-reducing and electrochemical activities.

The number of colonies did not increase with the addition of acetate and lactate or Fe(III) to the solid medium. Similar CFUs were obtained on the solid media when other electron acceptors replaced Fe(III) in the medium, including oxygen, nitrate, and sulfate. These results show that most of the colonies formed were fermentative bacteria. The total CFU count was four orders of magnitude smaller than would be expected from the chemical analyses. CSLM showed that the enriched biomass in the fuel cell consisted of live bacterial cells (Fig. 2). These results suggest that the microbes on the electrode are alive, but do not grow under the conditions employed for the determination of CFUs. All the colonies appearing on the plates were examined microscopically. All consisted of normal-sized rod- or coccus-shaped bacteria.

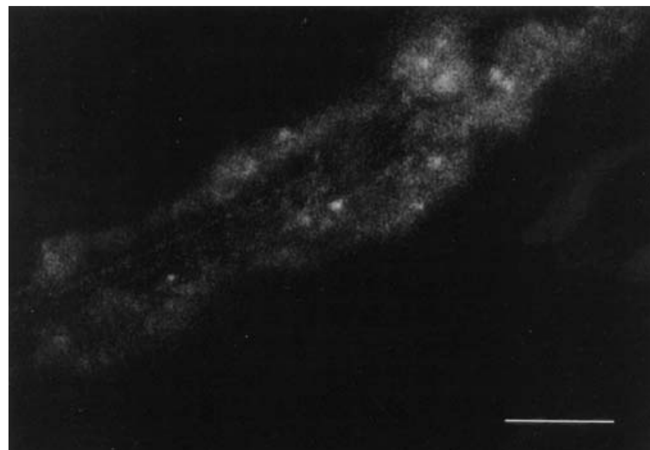

Fig. 2 A confocal scanning laser micrograph of the biofilm developed on the anode enriched for a year. The electrode was stained with LIVE BacLight bacterial gram stain kit (L-7005; Molecular Probes). *Red* Gram positive, *green* Gram negative, *bar* 3 μm)

Confocal scanning laser microscopy

The enriched electrode was observed using CSLM after fluorescent staining to distinguish the Gram reactions of the bacteria (Fig. 2). CSLM showed that Gram-negative and Gram-positive bacteria formed microcolonies throughout the electrode surface. The microbial clumps produced similar micrographs. The dense staining suggests that the majority of the bacteria present in the biofilm and the bacterial clumps were alive.

LVEM and SEM

A similar sample was used for LVEM and SEM after coating with gold (Fig. 3a–e). LVEM revealed a thick film on the electrode surface with particles of different sizes on its surface (Fig. 3b, c). Some particles were about the same size as normal bacteria and others were smaller (0.2–0.3 μm). The biofilm was separated from the electrode by vortexing and collected on a nucleopore filter (0.2 μm). SEM observation confirmed the existence of small particles in the size range of 0.2–0.3 μm (Fig. 3e).

Transmission electron microscopy

The suspension prepared from the electrode was used for transmission electron microscopic (TEM) observation (Fig. 3f). TEM showed bilayer membrane structures in the small particles. These results show that the enriched electrode contains a large community of small bacteria and normal-sized organisms.

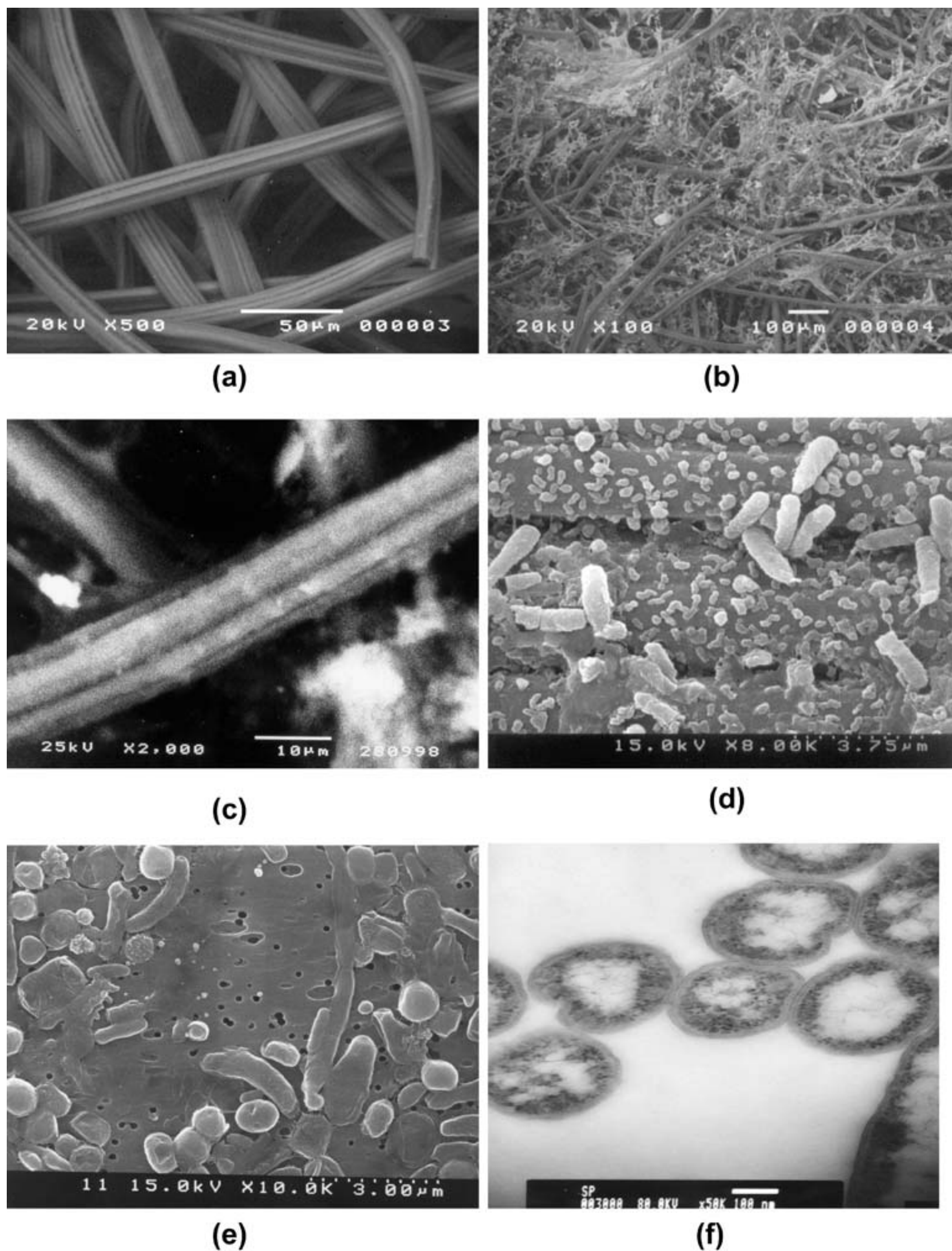

Fig. 3a–f Electron micrographs of the electrode and particles separated from the electrode. The control electrode (**a**) and enriched electrode (**b**, **c**) were observed under a low-vacuum electron microscope. **d**, **e** Scanning electron micrographs of electrode and cells retrieved from the electrode, respectively. **f** Transmission electron micrograph of cells retrieved from the electrode shown in (**d**)

DGGE analysis of the microbial population in the MFC

DNA extracted from the electrode was amplified by PCR using primers for 16S rDNA; and the amplicons were compared by DGGE with those of the sludge used as the inoculum. Figure 4 shows different patterns suggesting that the electrode contained a bacterial population different from that of the inoculum. This result clearly shows that the bacteria had been enriched during the operation of the fuel cell.

Analysis of 16S ribosomal DNA

A total of 25 clones were selected from the restriction fragment length polymorphism analysis of 195 clones containing 16S rDNA amplified from the anode of the MFC. The nearly complete 16S rRNA gene sequences compared with those of the database showed homologies of 85–99%. They were analyzed to belong to the α-Proteobacteria (six clones), β-Proteobacteria (nine clones), Chlorobia (two clones), Flavobacteria (two clones), Spingobacteria (one clone), Planctomycetacia (one clone), and Fermicute (one clone). The remaining three clones could not be classified. The clones classified as Chlorobia showed 16S rDNA homology of 88% with

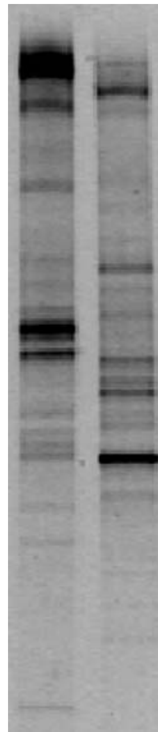

Fig. 4 16S rDNA denaturing gradient gel electrophoresis fingerprints of sludge used to inoculate the fuel cell (*lane 1*) and an electrode enriched for over a year (*lane 2*), separated using a denaturing gradient from 30% to 60%

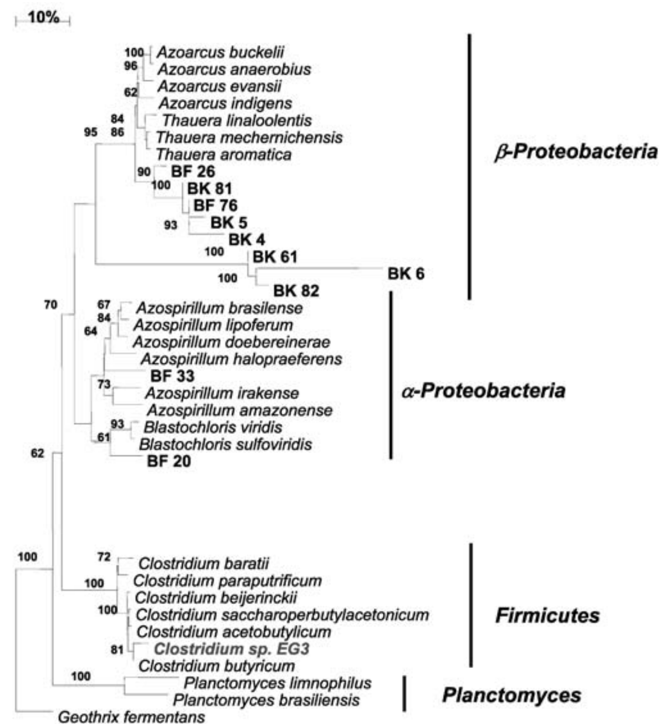

Fig. 5 Phylogenetic relationship of bacteria enriched with corn-processing wastewater, based on near full-length 16S rDNA

the nearest strain within the class. This result is different from Bond et al. (2002), who reported that δ-Proteobacteria comprised the majority of the bacteria in a MFC enriched with acetate in seawater. This difference might be due to differences in the electron donors or in the salt concentration used.

Figure 5 shows a phylogenetic tree of the clones. The tree contains 11 out of 25 clones. The remaining 14 clones could not be allocated to exact positions in the phylogenic tree due to incomplete sequencing results, even though they could be identified to the level of phyla.

Effects of metabolic inhibitors

The fuel cells operated for over a year were challenged with various metabolic inhibitors (Fig. 6). The current generation was reduced to varying degrees depending on the inhibitor used. Current generation fell gradually with increasing concentrations of the NADH reductase inhibitors HQNO and rotenone and the iron/sulfur protein inhibitor *p*-CMPS. Antimycin A (0.12 mM) and azide (1.2 mM) showed no effects. Current generation increased slightly with cyanide at low concentrations (up to 1.5 mM). The ATPase inhibitor DCCD (0.6 mM) and the uncoupler dinitrophenol (0.24 mM) inhibited the electrochemical activity completely. Increased current generation was expected in the presence of the uncoupler, which increases the oxygen consumption rate during aerobic respiration.

Effects of electron acceptors

The fuel cells operated for over a year were used to test the effects of electron acceptors by supplying air-saturated wastewater or by adding nitrate, nitrite, and sulfate (Fig. 7). The fuel cells generated less current when they were supplied with air-saturated wastewater or when nitrate or nitrite was added. The effects of sulfate addition were less significant than those of other electron acceptors. The rate of COD decrease was not significantly affected by the treatment. The high redox potential electron acceptors might have been consumed in the fuel cell. These results show that the microbial population in the fuel cell reduces oxygen, nitrate, and nitrite (but not sulfate) instead of transferring electrons to the electrode.

Discussion

Microbes enriched for 30 days in a fuel cell removed organic contaminants in wastewater almost completely, with the concomitant generation of electricity. Further experiments revealed that the current generation was stable for over 5 years (data not shown). DGGE analysis showed that the bacterial population in the anode of the MFC was different from the sludge used to inoculate the

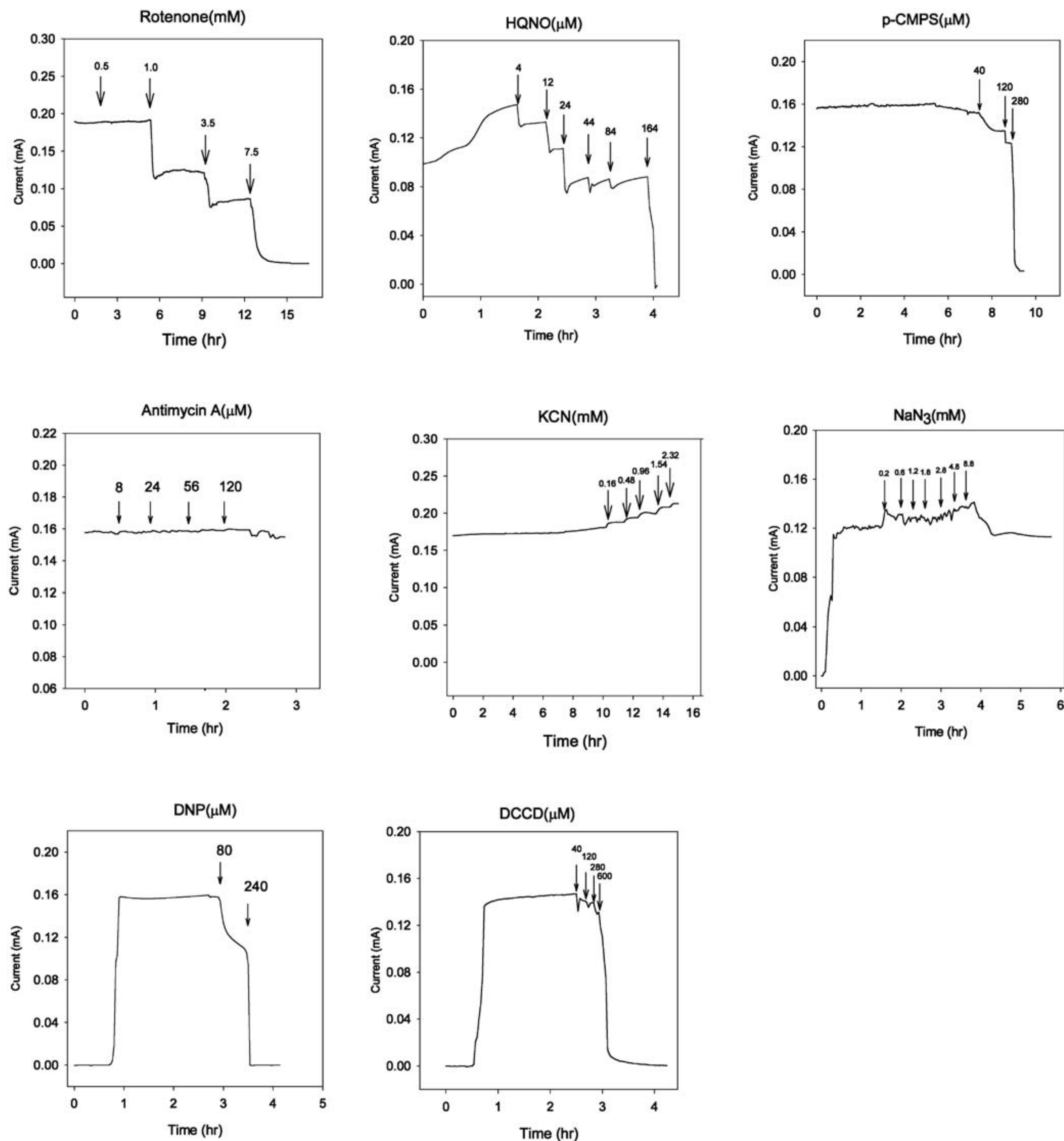

Fig. 6 The effects of metabolic inhibitors on the performance of the microbial fuel cell using corn-processing wastewater. The inhibitors were added to microbial fuel cells enriched for over a year, increasing the concentration progressively at the points *marked by arrows*. *HQNO* 2-Heptyl-4-hydroxyquinolone-*N*-oxide, *p-CMPS* *p*-chloromercuriphenylsulphonate, *DNP* 2,4-dinitrophenol, *DCCD* dicyclohexylcarbodiimide

fuel cell at the beginning of the enrichment. Some of the isolates from the MFC were electrochemically active; and current was generated from a fuel cell using one of the isolates identified as a strain of *C. butyricum* (Park et al. 2001). These results clearly show that electrochemically active microbes had been enriched in the MFC. The cell suspensions of the isolates were electrochemically active when measured by cyclic voltammetry (Park et al. 2001), suggesting that soluble electron carriers are not involved in electron transfer to the electrode, as reported by Bond et al. (2002).

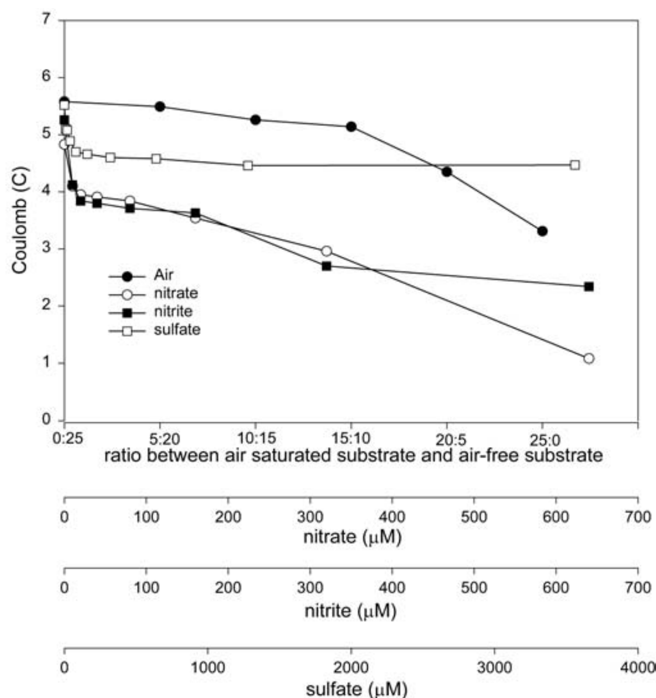

Fig. 7 Effects of electron acceptors on current generation by the enriched microbial fuel cell. The anode compartment was fed with mixtures of air-saturated and anaerobic wastewater at the ratios shown. Nitrate, nitrite, and sulfate were added to the wastewater before feeding

The current generation from the fuel cell was inhibited by various metabolic inhibitors and by high redox potential electron acceptors. The NADH reductase inhibitors HQNO and rotenone and the iron/sulfur protein inhibitor *p*-CMPS inhibited current generation by the MFC. Antimycin A (0.12 mM) and azide (1.2 mM) showed no effects. These results show that the electron transport system in the electrochemically active microbes utilizes NAD, iron/sulfur proteins, and quinone as the electron carriers, but does not use site 2 of the electron transport chain or the terminal oxidase. Current generation increased slightly with cyanide at low concentrations (up to 1.5 mM). The increase in current generation by cyanide suggests that a small portion of the electron donors is metabolized through a cyanide-sensitive terminal oxidase in the absence of an inhibitor.

The ATPase inhibitor and uncoupler inhibited current generation completely. Increased current generation was expected in the presence of the uncoupler, which increases the oxygen consumption rate during aerobic respiration. These results suggest that electron transfer to the electrode requires a reverse electron-transport step. Another explanation is that the chemicals inhibited the fermentative metabolism which plays a crucial role in the MFC.

Media with various combinations of electron donors and acceptors resulted in a maximum CFU count of $3.5\pm0.5\times10^7$ CFUs g^{-1} electrode, which is four orders of magnitude lower that the predicted bacterial number from the chemical analyses of the enriched electrode. The reason for this discrepancy might be that the bacteria in the fuel cell grew in a syntrophic association which could not be maintained on a solid medium, or that the conditions employed were not favorable for the electrochemically active microbes. Regardless of the reason for the discrepancy, it is certain that the MFC contains a complex microbial community including electrochemically active bacteria. They might grow via a novel form of anaerobic metabolism.

It is not clear whether the electrochemically active bacteria enriched in the fuel cell are Fe(III)-reducers. Some of the isolated bacteria showed Fe(III)-reducing activities, but the number was very small in comparison with the number predicted from the chemical analyses. It is possible that the electrochemically active bacteria are Fe(III)-reducers which do not grow under the conditions employed for isolation, or that their electrochemical activities are not related to Fe(III)-reduction.

16S ribosomal RNA gene sequence analyses revealed that the majority of the amplified clones were unidentified bacteria (35.9%), followed by β-Proteobacteria (25.0%), α-Proteobacteria (20.1%), and the Cytophaga/Flexibacter/Bacteroides group (19.0%). This result is different from Bond et al. (2002), who reported that δ-Proteobacteria were the major bacteria in a MFC enriched with acetate in seawater. This difference might be due to differences in the electron donors or in the salt concentration used.

LVEM showed a biofilm formed on the electrode surface and microbial clumps scattered around the electrode (Fig. 3b, c). A similar sample was used for SEM after coating with gold (Fig. 3d, e). SEM revealed a thick film on the electrode surface with particles of different sizes on its surface. Some particles were about the same size as normal bacteria and others were smaller (0.2–0.3 μm). TEM observation of biofilm separated from the electrode showed a bilayer membrane structure in the small particles (Fig. 3f). These results suggest that the small particles are nanobacteria. At this point, the function of the nanobacteria in the fuel cell is not known. To the author's knowledge, this is the first evidence of nanobacteria in biofilms in a man-made environment. CSLM showed Gram-negative and Gram-positive bacteria forming microcolonies throughout the electrode surface (Fig. 2). The microbial clumps produced similar micrographs. The dense staining suggests that majority of the bacteria present in the biofilm and the bacterial clumps were alive. Electrode enriched with a non-fermentable fuel, acetate, did not show microbial clumps (unpublished data). It might be plausible that the microbes in the clumps ferment the complex fuel and the electrochemically active microbes in the biofilm oxidize the fermentation products.

MFCs enriched in a similar manner could be used to develop a novel wastewater-treatment process (Jang et al. 2003). This would convert the major part of the energy of the organic contaminants into electricity, leading to a significant reduction in sludge production. It would be

interesting to see whether this MFC can be used to oxidize xenobiotics, including aromatic hydrocarbons which are known to be oxidized completely under anaerobic Fe(III)-reducing conditions (Anderson et al. 1998; Dolfing 1996; Kazumi et al. 1995; Lovley and Lonergan 1998; Lovley and Phillips 1986). Biochemical oxygen demand (BOD) sensor is another area of application for this microbial device (Kim et al. 2003). Because the current generated from the fuel cell is directly proportional to the amount of electron donor utilized, the MFC can be used as a sensor to determine BOD.

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