

Use of acetate for enrichment of electrochemically active microorganisms and their 16S rDNA analyses

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Abstract

A fuel cell-type electrochemical device has been used to enrich microbes oxidizing acetate with concomitant electricity generation without using an electron mediator from activated sludge. The device generated a stable current of around 5 mA with complete oxidation of 5 mM acetate at the hydraulic retention time of 2.5 h after 4 weeks of enrichment. Over 70% of electrons available from acetate oxidation was recovered as current. Carbon monoxide or hydrogen did not influence acetate oxidation or current generation from the microbial fuel cell (MFC). Denaturing gradient gel electrophoresis showed that DNA extracted from the acetate-enriched MFC had different 16S rDNA patterns from those of sludge or glucose+glutamate-enriched MFCs. Nearly complete 16S rDNA sequence analyses showed that diverse bacteria were enriched in the MFC fed with acetate. Electron microscopic observations showed biofilm developed on the electrode, but not microbial clumps observed in MFCs fed with complex fuel such as glucose and wastewater from a corn-processing factory.

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1. Introduction

A mediator-less microbial fuel cell (MFC) is a device that converts chemical energy to electrical energy with the aid of the catalytic reaction of electrochemically active microorganisms [1]. Electron mediators are not used in this device since the electrochemically active microorganisms can transfer electrons to the electrode directly. An Fe(III)-reducing bacterium, *Shewanella putrefaciens*, can grow on lactate in the anode compartment of a fuel cell-type electrochemical device without electron acceptors [2]. A similar device was successfully used to enrich a microbial consortium that reduced the chemical oxygen demand (COD) from around 1500 mg l⁻¹ to 30 mg l⁻¹ of wastewater collected from a corn-processing plant [3]. A biofilm was observed on the surface of the anode enriched with wastewater, and microbial clumps loosely associated with

the electrode through electron microscopic observation [3].

Acetate is the most abundant fatty acid in anaerobic ecosystems and is used as an electron donor by anaerobic respiratory bacteria [4]. Methanogens and sulfidogens are the best-known acetate-oxidizing anaerobes, and acetate can be also used by metal reducers belonging to the genus *Geobacter*. Acetate is metabolized either directly by these anaerobic bacteria or syntrophic associations [5]. An acetate-oxidizing syntrophic bacterium has been characterized [6]. Acetate was used as the fuel in an MFC using marine sediment as the inoculum [7]. The family Geobacteraceae were the predominant bacteria in that system.

In the present study, the mediator-less MFC was used to enrich electrochemically active microbes on acetate as the sole electron donor. The microbial community enriched in the fuel cell was characterized.

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2. Materials and methods

2.1. Mediator-less MFC and enrichment

MFCs used were a sensor-type up-flow continuous feeding system described previously [8]. The internal volume of the anode and cathode compartments was 26 ml each. MFCs were placed in a temperature-controlled chamber at 30°C. They were inoculated with activated sludge collected from the Jungyang sewage plant (Seoul, Korea), and fed continuously with artificial wastewater containing 5 mM acetate to the anode as fuel at a rate of 9 ml h⁻¹, and air-saturated tap water to the cathode at a rate of 8 ml min⁻¹. Peristaltic pumps (Watson-Marlow, Campel, UK) were used to feed the liquids pre-warmed to 30°C. The external resistance between anode and cathode was set at 500 Ω during the enrichment. The potential between anode and cathode was measured using a multimeter (Keithley Instruments, Cleveland, OH, USA) and recorded on a personal computer through a data acquisition system (Testpoint®, Capital Equipment, Richmond, VA, USA) every 5 min [1,2]. The experiment was conducted using 6 MFCs before two of them were sacrificed for electron microscopy and DNA extraction, and the results presented are mean values or typical current monitored.

For the comparisons in denaturing gradient gel electrophoresis (DGGE) and electron microscopy, MFCs enriched with different fuels were used. They were enriched with artificial wastewater containing glucose and glutamate [8], and with wastewater collected from a corn-processing plant [9].

2.2. Artificial wastewater and analyses

Artificial wastewater containing acetate was prepared by dissolving 410 mg sodium acetate and inorganic salts (KH₂PO₄, 15 mg; (NH₄)₂SO₄, 30 mg; MgSO₄·7H₂O, 50 mg; CaCl₂, 3.75 mg; FeCl₃·6H₂O, 0.25 mg; MnSO₄·H₂O, 5 mg; NaHCO₃, 105 mg) in 985 ml of 5 mM phosphate buffer (pH 7.0) to which was added 10 ml trace mineral solution (nitrilotriacetic acid, 1.5 g; FeSO₄·7H₂O, 0.1 g; MnCl₂·4H₂O, 0.1 g; CoCl₂·6H₂O, 0.17 g; ZnCl₂, 0.1 g; CaCl₂·2H₂O, 0.1 g; CuCl₂·2H₂O, 0.02 g; H₃BO₃, 0.01 g; Na₂MoO₄, 0.01 g; Na₂SeO₃, 0.017 g; NiSO₄·6H₂O, 0.026 g; NaCl, 1 g in 1 l of distilled water) and 5 ml vitamin solution (biotin, 2 mg; folic acid, 2 mg; pyridoxine HCl, 10 mg; thiamine HCl, 5 mg; riboflavin, 5 mg; nicotinic acid, 5 mg; pantothenic acid, 5 mg; cyanocobalamin, 0.1 mg; *p*-aminobenzoic acid, 5 mg; lipoic acid, 5 mg in 1 l of distilled water). The salt solution was autoclaved and cooled under a nitrogen atmosphere before mixing with the filter-sterilized vitamin solution. Finally, nitrogen gas was purged for 30 min to remove oxygen. The reservoir was kept under a nitrogen atmosphere by connecting to a nitrogen-containing gas-tight bag with a volume of 5 l (SKC, Eight Four, PA, USA).

Acetate was quantified by gas chromatography (Varian 3400, Varian, San Francisco, CA, USA) equipped with a flame ionization detector. The current yield was calculated as follows: (observed current/theoretical current) × 100 (%), where observed was monitored and theoretical current was calculated from acetate consumed [8].

Electrochemical activity was measured using cyclic voltammetry [1], and a standard method was employed to measure COD using chromate as the oxidant [9].

2.3. Hydrogen- and carbon monoxide-saturated fuel

In some experiment MFCs were fed with artificial wastewater saturated with either H₂ or CO. The artificial wastewater reservoir was gassed at least for 90 min after sterilization and connected a gas-tight bag (SKC) containing the gas used.

2.4. Electron microscopy

The electrode from MFCs operated over a year was observed using electron microscopy. Low vacuum electron micrographs were taken using a JSM 5410 (Jeol, Tokyo, Japan). For the scanning electron microscopy samples were fixed with 2% (v/v) glutaraldehyde in 100 mM HEPES buffer (pH 6.8) containing 2 mM MgCl₂ for 1 h before being stained with 1% (w/v) OsO₄ for 2 h. The fixed specimens were dehydrated with increasing concentrations of ethanol isoamyl acetate and coated with gold. The scanning electron microscope (S-4200 FE-SEM, Hitachi, Tokyo, Japan) was operated at 15 kV [10].

2.5. Respiratory inhibitors

MFCs were challenged with metabolic inhibitors including heptyl-4-hydroquinoline-*N*-oxide (HQNO, 4–164 μM), dicyclohexylcarbodiimide (DCCD, 50–500 μM), rotenone, antimycin A, sodium cyanide (CN⁻), sodium azide (N₃⁻), 2,4-dinitrophenol (DNP) and oligomycin. They were filter-sterilized and added into the artificial wastewater containing acetate.

2.6. DNA extraction and PCR amplification of 16S rDNA

Total chromosomal DNA was extracted from anodes of MFCs fed with different fuels including corn-processing wastewater [9] and artificial wastewater containing acetate, and from activated sludge used to inoculate the MFCs for the enrichment. DNA was purified using GeneClean® Turbo (Q-BIO gene, Carlsbad, CA, USA) before use as a template in polymerase chain reaction (PCR) amplification. For the 16S rDNA gene sequence, the purified DNA was amplified using a forward primer (27f; 5'-AGA GTT TGA TCM TGG CTC AG) and a reverse primer (1492r; 5'-TAC GGY TAC CTT GTT ACG ACT T). Agarose gel electrophoresis confirmed the ampli-

fication product as a 1500-bp DNA fragment. For the diversity analysis using DGGE, the extract was amplified using a forward primer (GC-341f; 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CGA CAG) and a reverse primer (534r; 5'-ATT ACC GCG GCT GCT GG) as described earlier [11]. Numbers given for the primers are those of the nucleotide sequence of *Escherichia coli* 16S rDNA.

2.7. DGGE

DGGE was performed using a DCode system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. PCR amplicons were loaded onto 10% polyacrylamide gel with a denaturing gradient range from 30 to 60% consisting of urea and formamide. The gel was stained using SYBR Green I (TaKaRa, Tokyo, Japan) for 30 min and destained in 1×TAE buffer (pH 7.5) for 15 min before DNA bands were observed by a Gel-Doc image analyzer (Bio-Rad) under UV illumination.

2.8. 16S rDNA sequencing and taxonomic analysis

Amplicons generated using primer pair 27f-1492r were cloned into pGEM-T® Easy Vector System I (Promega, Madison, WI, USA) and transformed into competent *E. coli* DH5 α (TaKaRa). The transformed culture was plated on LB agar medium containing ampicillin and X-gal and isopropyl- β -D-thiogalactopyranoside. Ampicillin-resistant and β -galactosidase-negative clones were transferred to a liquid medium of the same composition.

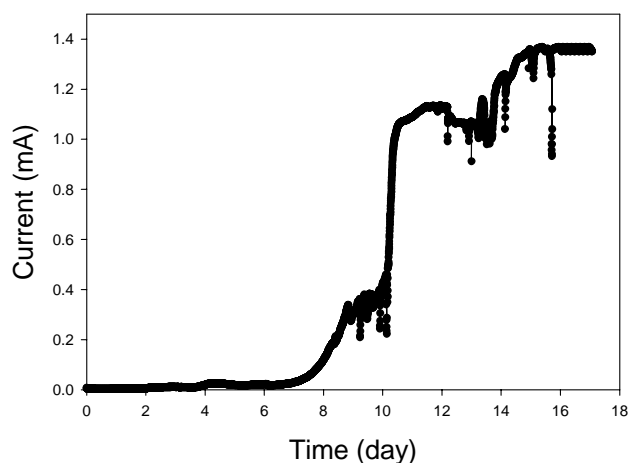


Fig. 1. Typical current generation from an MFC during the enrichment process using acetate as the fuel. MFCs were inoculated with activated sludge and fed continuously with artificial wastewater containing 5 mM acetate as the fuel at a hydraulic retention time of 2.5 h and the current was monitored through a resistance of 500 Ω after open circuit operation for 10 h. Initially six MFCs were used, and they gave similar results.

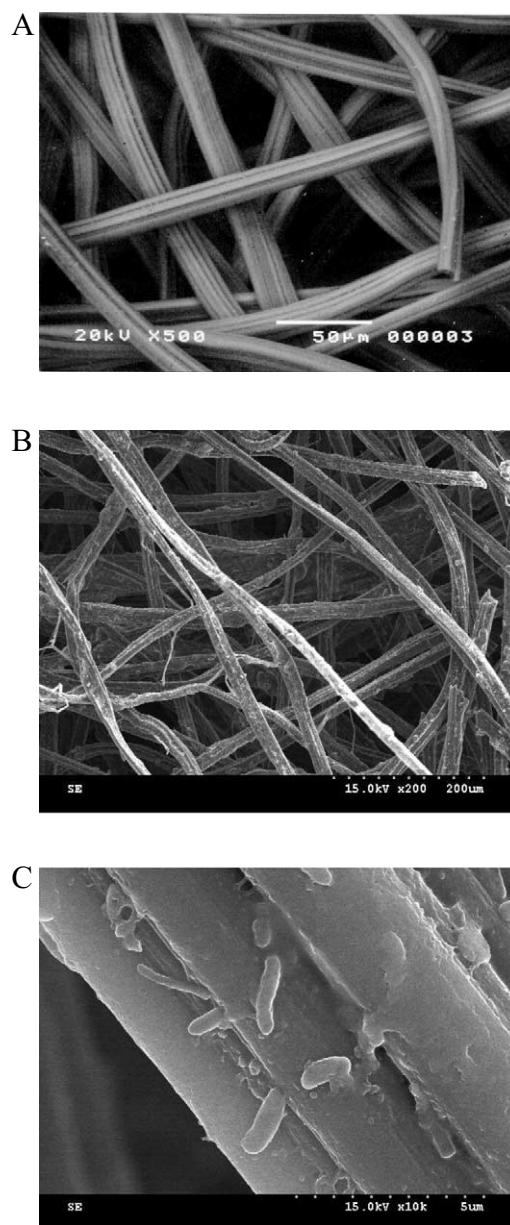


Fig. 2. Low vacuum (A,B) and scanning electron (C) micrographs of electrodes retrieved from MFCs enriched with acetate. A: Graphite felt before use. B,C: Electrode enriched with acetate.

Plasmids were purified and analyzed for restriction fragment length polymorphism (RFLP) using enzyme pairs (*Hae*III/*Xho*I or *Hap*II/*Eco*RI) to select different clones. Selected clones were sequenced using an automatic sequencer system (ABI 310, Applied Biosystems, Foster City, CA, USA) after purification using a plasmid purification kit (Nucleogen, Ansan, Korea) at Macrogen Co. (Seoul, Korea). The sequences were compared with those of the NCBI BLAST GenBank nucleotide sequence databases. Parsimony phylogenetic trees were constructed by the neighbor-joining method using the MegAlign program (DNASTAR, Madison, WI, USA) [12].

3. Results

3.1. Enrichment of acetate-using bacteria in the MFC

MFCs were inoculated with activated sludge and the artificial wastewater containing 5 mM acetate was continuously fed at a rate of 0.15 ml min^{-1} for the enrichment of the microbial consortium oxidizing acetate with concomitant current generation. The open circuit potential between the two electrodes reached around 0.7 V in 10 h. The current increased slowly during the first week after the electrodes were connected through a resistance of 500Ω . Within 3 weeks after the inoculation MFCs generated a stable current of around 1.5 mA (Fig. 1). MFCs were run under the same conditions for another 4 weeks before the resistance was lowered to 10Ω . Under these conditions the current was around 5 mA, and the acetate concentration in the effluent was less than the detection limit ($100 \mu\text{M}$), and about 70% of electrons available from acetate oxidation was recovered as current. The effluent from MFCs was centrifuged and the supernatant showed a COD value

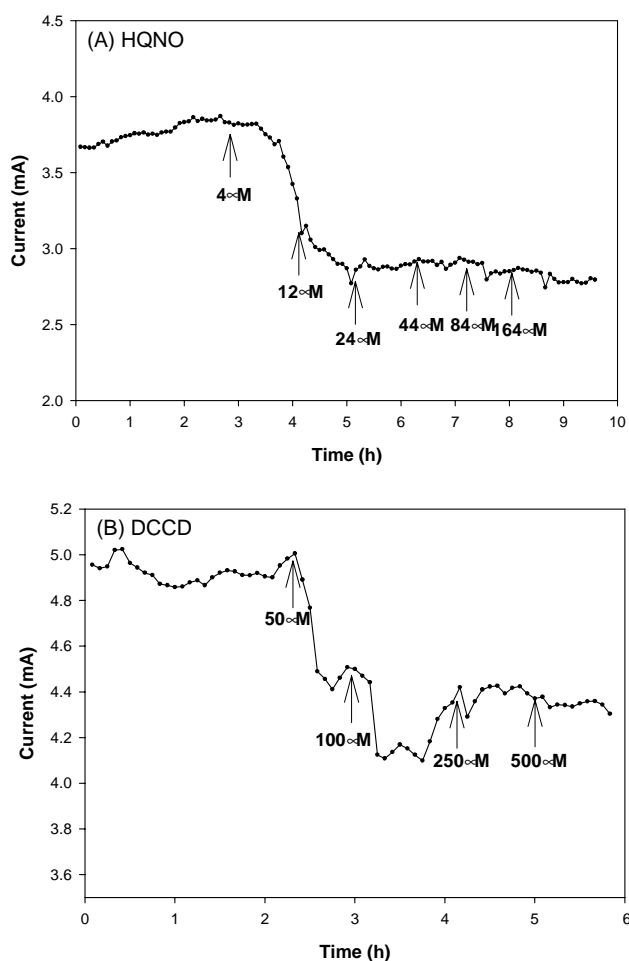


Fig. 3. The effects of HQNO (A) and DCCD (B) on the current generation from the MFC continuously fed with acetate. Concentration of the inhibitors used is indicated in the figure. Oligomycin gave a similar result at concentrations from $20 \mu\text{M}$.

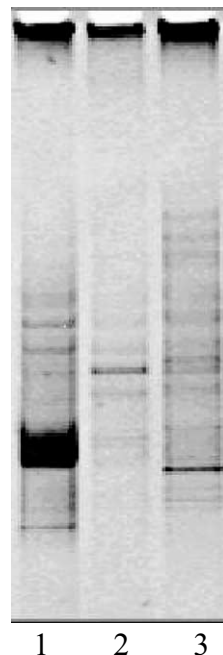


Fig. 4. Comparison of bacterial communities in the MFCs enriched with acetate or glucose+glutamate by DGGE. The denaturing gradient used was from 30 to 60%. Lane 1: Activated sludge (inoculum). Lane 2: Enriched with acetate. Lane 3: Enriched with glucose+glutamate.

of around 17 mg l^{-1} . These results show that acetate is completely oxidized to carbon dioxide. When the artificial wastewater feeding to the anode was stopped the current decreased gradually to less than 0.1 mA and the current increased immediately after the feeding was resumed.

3.2. Electrochemical activity of the effluent from MFCs

The effluent from MFCs fed with acetate was collected and centrifuged before being tested for electrochemical activity with cyclic voltammetry. The cyclic voltammogram showed no oxidation or reduction peaks as the artificial wastewater (data not shown).

3.3. Electron microscopy

Low vacuum electron micrographs showed a well-formed biofilm on the surface of the electrode retrieved from acetate-enriched MFC (Fig. 2B), but not the microbial clumps observed on the electrode enriched with corn-processing wastewater. High magnification scanning electron microscopic observations showed rod-shaped bacterial cells on the surface of the biofilm (Fig. 2C), but not particles with a diameter of around $0.2 \mu\text{m}$ that were observed on the surface of the biofilm formed on the electrode enriched with the complex wastewater [3].

3.4. Effect of H_2 - and CO -saturated media

MFCs were fed with H_2 - or CO -saturated artificial wastewater and the current was monitored. The current

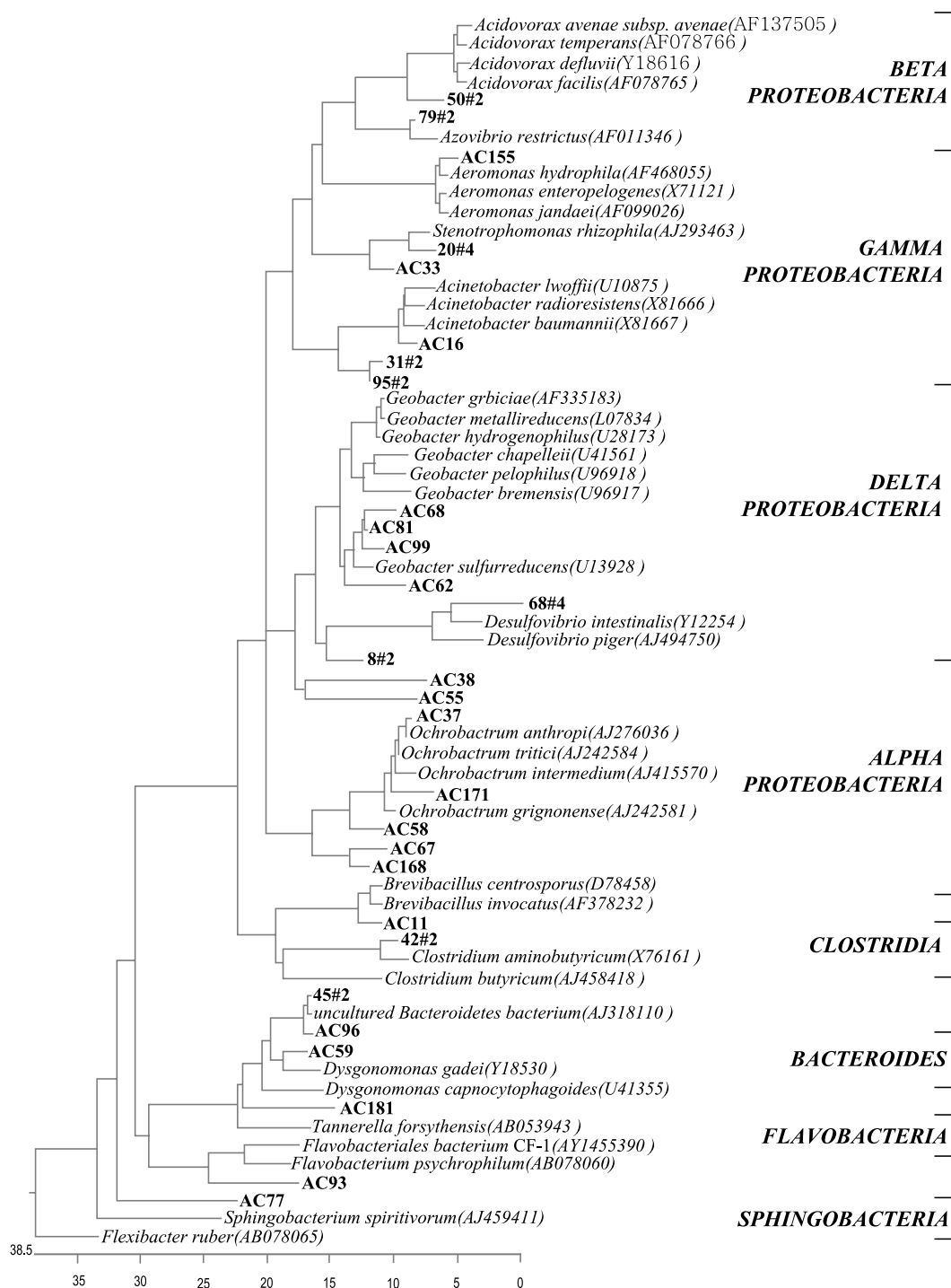


Fig. 5. Phylogenetic tree showing 16S rDNA gene sequences of the bacterial community from MFC enriched with acetate. The scale at the bottom indicates the distances between sequences.

neither decreased nor increased for over 10 h (data not shown).

3.5. Respiratory inhibitors

MFCs were fed with artificial wastewater containing various metabolic inhibitors, and the current was moni-

tored. As shown in Fig. 3, HQNO inhibited current generation at 4 μ M and at higher concentrations the current did not decrease further. DCCD and oligomycin (20 μ M) gave a similar result (Fig. 3). On the other hand, the current did not change when MFCs were fed with artificial wastewater containing antimycin A (340 μ M), CN⁻ (4 mM), and N₃⁻ (8 mM) (data not shown).

3.6. DGGE

Amplicons of DNA extracted from the electrode enriched with acetate and from one with glucose+glutamate were analyzed using DGGE with that where activated sludge was used as the inoculum. As shown in Fig. 4, the DGGE pattern of the electrode enriched with acetate was different from that of the electrode enriched with a complex carbon source and from that of the inoculum. Some bands were present in all cases especially between the electrodes, but the major bands in each sample were different. The electrode enriched with acetate showed the simplest bacterial diversity.

3.7. Phylogenetic analysis of bacterial population in MFC fed with acetate

A total of 330 colonies of *E. coli* DH5 α transformed with plasmid containing 16S rDNA from the acetate-enriched electrode were picked and the cloned sequences were analyzed by RFLP. Out of 330 clones 81 were selected for sequencing. Nearly complete sequences of 16S rDNA of 45 clones were obtained and compared with those of the database. Representatives (29 clones) were used to construct a phylogenetic tree. The remaining 16 clones that showed similarity through BLAST were not used for phylogenetic tree construction. The majority were proteobacteria consisting of seven α -proteobacteria, two β -proteobacteria, six γ -proteobacteria, six δ -proteobacteria. Others were two firmicutes and six others (Fig. 5).

4. Discussion

The current increased gradually from MFCs inoculated with activated sludge and fed with acetate continuously for about 2 weeks. Once the current reached a stable level, acetate was consumed completely, and current generation was dependent on the acetate supply. DGGE showed that bacterial populations in the MFCs were different from the activated sludge that was used to inoculate the MFC. These results show that MFCs could be used to enrich a bacterial consortium that oxidizes acetate with concomitant electron transfer to the electrode. Electron microscopic observations showed a well-formed biofilm on the electrodes enriched with acetate, similar to that with complex fuel. But the acetate-enriched electrode did not show microbial clumps that were observed in the electrode enriched with the complex fuel. This difference might be due to the fact that the microbial community in the clumps ferments complex fuel and the fermentation products are oxidized by the microbial community in the biofilm. This hypothesis is substantiated by DGGE analysis that showed more diverse bacterial species in the electrode

enriched with complex fuel than that with acetate, a non-fermentable fuel.

Acetate can be oxidized through a syntrophic association under certain anaerobic conditions such as methanogenesis [6] and metal reduction [7]. Neither hydrogen nor carbon monoxide influenced the current generation from MFCs fed with acetate. These results suggest that hydrogen (possibly syntrophic association) is not involved in current generation. Acetate was consumed nearly completely with an electron recovery of over 70%. The remaining 30% of electrons might be consumed through aerobic respiration reducing oxygen diffusing from the cathode compartment in the MFCs [13].

The acetate-enriched electrode showed a well-formed biofilm, and the effluent from MFCs was not electrochemically active. These results suggest that the electron transfer from the bacterial cells to the electrode does not involve soluble mediators, but requires direct contact between the bacterial cells and the electrode [7]. It is not known if the matrix forming the biofilm is electrically conductive.

Small ribosomal RNA gene analyses revealed that the acetate-enriched electrode contains various bacteria including δ -proteobacteria, which comprised 21% of the population. This is much lower than reported by Bond et al. [7]. They reported that δ -proteobacteria occupied over 70% of the bacterial population in an MFC enriched with acetate using marine sediment. The difference might be due to the salt. This study employed a low salt concentration whilst Bond et al. [7] used seawater. Another possibility is that our system might have a high redox potential for anaerobic Geobacteraceae due to the oxygen diffusion mentioned above.

The current generation was inhibited by HQNO, an NADH dehydrogenase/coenzyme Q inhibitor, but not by antimycin A, cyanide, or azide. These results show that the electron transfer to the electrode shares an early part of the aerobic respiration, and electrons are diverted from coenzyme Q to the cell surface electron carrier(s) through unknown intermediates. The partial inhibition even at high concentrations of the inhibitors might be due to the characteristics of microbial communities in biofilms [14]. The uncoupler DNP neither inhibited nor enhanced current generation. The uncoupler was expected to increase the current generation as they increase oxygen consumption in aerobic respiration. It is not clear how the ATPase inhibitors DCCD and oligomycin inhibit the current generation. It is interesting to note that the electrode enriched with complex fuel was observed to contain ultramicrobacteria-like particles which were not found in the acetate-enriched electrode.

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