Microbial diversity and population dynamics of activated sludge microbial communities participating in electricity generation in microbial fuel cells

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ABSTRACT

In this study, we performed microbial community analysis to examine microbial diversity and community structure in microbial fuel cells (MFCs) seeded with activated sludge from a municipal wastewater treatment plant in South Korea. Because anode-attached biofilm populations are particularly important in electricity transfer, the ecological characteristics of anode-attached biofilm microbes were explored and compared with those of microbes grown in suspension in an anode chamber. 16S rDNA-based community analysis showed that the degree of diversity in anode-attached biofilms was greater than that of the originally seeded activated sludge as well as that of the suspension-grown microbes in the anode bottle. In addition, *Bacteroidetes* and *Clostridia* grew preferentially during MFC electricity generation. Further phylogenetic analysis revealed that the anode biofilm populations described in this work are phylogenetically distant from previously characterized MFC anode biofilm microbes. These findings suggest that a phylogenetically diverse set of microbes can be involved in the electricity generation of MFC anode compartments, and that increased microbial diversity in anode biofilms may help to stabilize electricity production in the MFC.

Key words | MFC, microbial community structure, microbial diversity

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INTRODUCTION

A microbial fuel cell (MFC) is an electrochemical device that converts chemical energy to electrical energy with electrochemically active microbes as biocatalysts (Park & Zeikus 2000; Kim *et al.* 2002). Microbes, as the electron donor in the anode compartment, oxidize fuel (organic matter), generating electrons and protons. Electrons and protons are consumed in the cathode compartment, reducing oxygen to water. Electricity generation using wastewater, marine sediments, and anaerobically digested sludge with MFCs is one of the main focuses of clean energy technology at the moment (Angenent *et al.* 2004).

Recently, a number of Fe(III)-reducing bacteria and sulfate-reducing bacteria, used as biocatalysts for electron transfer, were studied by several groups and isolated from MFCs (Park *et al.* 2001; Chaudhuri & Lovley 2003; Kim *et al.* 2006). These microbes are able to use energy efficiently for growth by coupling the oxidation and reduction processes. These include the following species: *Geobacter* (Lovley *et al.* 1993; Bond *et al.* 2002), *Shewanella* (Lovley *et al.* 1992; Hyun *et al.* 1999) and *Desulfotomaculum reducens* (Tebo & Obraztsova 1998). Various studies of microbial populations in MFC biofilms have been conducted using conventional culture-dependent and culture-independent methods, and have resulted in the isolation of the only specific species in the MFC biofilm community (Bond *et al.* 2002; Lee *et al.* 2003; Holmes *et al.* 2004; Kim *et al.* 2008). However, there

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are a very limited number of studies comparing biofilm and suspension-grown microbial communities in anode compartments (Kim *et al.* 2006).

In this study, glucose-fed mediatorless MFCs, inoculated with activated sludge, were operated at the laboratory scale, and molecular ecological techniques were used to analyze microbial communities in electricity-generating MFCs. This molecular ecological investigation compared the diversity and community structure of biofilm communities with those of suspension-grown microbial communities in anode compartments. Molecular ecological analysis was also performed for microbial communities from cathode compartments in which an organic carbon source (glucose) was added as an electron donor. To obtain information on microbial diversity, bacterial 16S rDNA-based T-RFLP (terminal restriction fragment length polymorphism) fingerprinting analysis was performed. Microbial community structures of the MFC samples were characterized using 16S rDNA amplification and sequencing techniques. Phylogenetic analysis was performed to examine whether the anode biofilm populations were phylogenetically different from previously characterized MFC-anode biofilm bacteria.

MATERIALS AND METHODS

MFC operational conditions

A two-chambered MFC system (with anode and cathode compartments) was operated in fed-batch mode using transparent polyacrylic plastic bottles (1 L). Each bottle was filled with 900 mL of wastewater. The two bottles were connected with a proton exchange membrane (Nafion™ 117, Dupont Co., USA). Soft graphite felt (National Electric Carbon, USA) was used for the electrodes, which varied the external resistance over a range of 0–5 kΩ. The medium contained the following (per L): 0.2 g of NH_4Cl, 0.15 g of $CaCl_2{\cdot}2H_2O$, 0.33 g of KCl, 0.3 g of NaCl, 3.15 g of $MgCl{\cdot}6H_2O$, 1.26 g of K_2HPO_4, 0.42 g of KH_2PO_4, and 0.5 g of yeast extract. Glucose, to be used as the sole electron donor for microorganisms, was added to the anode compartment (1,000 mg/L COD) and also to the cathode compartment (500 mg/L COD). The MFC was inoculated with activated sludge (2,000 mg/L MLSS) from a domestic municipal wastewater treatment plant in Jeonju, South Korea.

Seven sets of MFCs were operated in fed-batch mode in a laboratory. When using new electrodes, initially no biofilm was produced at the surface of anode electrodes, generating little electricity (Test 1). During the second test (Test 2), biofilm was formed, and a sufficient amount of electricity was generated from the MFCs. After Test 2, the MFC set that exhibited the greatest electricity production power was chosen, and its anode-attached biofilm and suspension-grown microbe samples were taken for molecular ecological analysis as described below. From the same MFC set, suspension-grown microbial samples were also taken from the cathode chamber. The biomass samples were stored at −80°C prior to microbial community analysis.

DNA extraction and 16S rDNA amplification

From the collected biomass samples, genomic DNA was extracted using the Fast DNA SPIN Kit for Soil (Q-BIO Gene, USA) according to manufacturer recommendations. If needed, the extracted DNA samples were stored at −20°C prior to the following analysis. Bacterial 16S rDNA genes were amplified from the extracted DNA using polymerase chain reaction (PCR) with a set of universal bacterial 16S rDNA primers, i.e., 27F (AGAGT TTGAT CATGG CTCAG) and 1492R (TACGG TTACCT TGTTAC GACTT). For bacterial 16S rDNA T-RFLP analysis, fluorescence labeled 27F-FAM (AGAGT TTGATC ATGGC TCAG) primer was used instead of the normal 27F primer. Each PCR reaction (25 μL) contained 0.1 ng of template DNA, 1X PCR buffer, 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP), 100 pM of each primer, and 0.025 U of *Taq* enzyme (Invitrogen, Inc., USA). An MJ Mini Thermocycler (BIO-RAD, USA) was used to run the PCRs through (i) an initial denaturation step consisting of 94°C for 3 min, (ii) 25 cycles of annealing (each cycle consisted of 94°C for 1 min followed by 55°C for 25 s), and (iii) an extension step at 72°C for 2 min. Under the optimal PCR conditions, the size of PCR products was ~1400 base pairs (bp). The PCR products were purified using the DNA Purification Spin Column Kit (Qiagen Inc., USA).

16S rDNA T-RFLP

Approximately 200 ng of the prepared PCR products were digested for 12 h at 37°C with either *Hha* I or *Msp* I restriction enzyme in a 20 μL reaction mixture. Each reaction mixture contained 2 μL of 10 × restriction enzyme buffer (New England Biolabs, Inc., USA), 1 μL of restriction enzyme (New England Biolabs, Inc., USA), 0.2 μL of 100 × BSA, 5 μL of DNA template, and 11.8 μL of ultrapure water. The enzyme-digested samples were purified using the DNA Purification Spin Column Kit (Qiagen Inc., USA), and then the fingerprints of terminal fragments were analyzed with an ABI 377 sequencer (PE Applied Biosystems, USA). Terminal fragments smaller than 50 bp (lower detection limit) or larger than 600 bp (upper detection limit) were not considered in the microbial community analysis. The fragment sizes were calibrated with the spiked controls, and each peak area was normalized to the total peak area from a single T-RFLP fingerprint. These adjustment procedures ensured valid quantitative comparison of various T-RFLP fingerprint profiles. As a measure of microbial diversity, Shannon index (H), richness (d), and evenness (e) were calculated from the adjusted T-RFLP profiles as described elsewhere (Tiquia 2005).

16S rDNA gene cloning, sequencing, and phylogenetic analysis

For 16S rDNA gene cloning, the prepared PCR products were inserted into the pCR4-TOPO cloning vector using TOPO TA Cloning Kit for Sequencing (Invitrogen, Inc., USA). After insertion into each *E. coli* host cell, the individual PCR product in each vector was cloned via the growth of the host cells on kanamycin-supplemented LB medium. After the vectors containing PCR products were isolated, the 16S rDNA PCR products were sequenced by MACROGEN Inc. (Seoul, South Korea). For the following phylogenetic analysis, the newly obtained 16S rDNA sequences (test sequences) were aligned together with the 16S DNA sequences of previously characterized MFC anode-attached bacteria (reference sequences). Alignment of the test and reference sequences was conducted using CLUSTALW (EBI, http://www.ebi.ac.uk/clustalw/). The average size of the aligned sequences was ~700 bp. Using the multiple-aligned sequences, a phylogenetic tree was constructed by MEGA4 program (Tamura *et al.* 2007). For the phylogenetic tree inference, the neighbor-joining algorithm was used, and a bootstrapping test (500 replicates and 64238 random seed) was run. For population identification of new sequences, Classifier from the Ribosomal Database Project II (http://rdp.cme.msu.edu/) and BLAST from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) were used.

RESULTS AND DISCUSSION

Electricity generation from microbial fuel cell

When the new electrodes were inoculated with the activated sludge microbial communities (Test 1), V_{oc} (open-circuit voltage) increased from 0.2 V to approximately 0.5 V and became unstable as time passed (Figure 1). Meanwhile, after formation biofilm at the anode (Test 2), the V_{oc} values immediately increased to around 0.4 V and remained stable for a period of 20 days. Similar outcomes were observed in all seven sets of MFCs. This indicates that the stability of electricity generation in Test 2 might be attributed to the development of biofilm in the anode. After biofilm formation became stabilized, the most efficient MFC set could generate approximately 0.39 V at its maximum voltage, and the generated power density was 21.34 mW/m^2. According to the literature, the values of power density have a wide range between 1 mW/m^2 and 3,600 mW/m^2 (Kim *et al.* 2002; Rabaey *et al.* 2003; Angenent

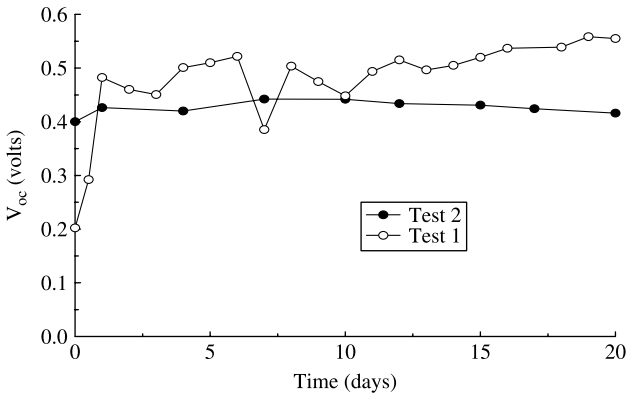

Figure 1 | V_{oc} change in response to time. (V_{oc} = open-circuit voltage. Test 1 indicates that V_{oc} does not change before biofilm formation, and Test 2 indicates that V_{oc} changes after biofilm formation).

et al. 2004). Since the power density generated from this work is within the typical range of power generation using MFC equipped with carbon electrodes, i.e., 10 – 100 mW/m^2 (Liu *et al.* 2004), it was concluded that our MFC system was optimally operated within the previously reported range of power density values for typical MFCs, especially for carbon-electrode-installed MFCs. Electricity production may depend upon (i) the amount and source of a seeding inoculum, (ii) electron donor, (iii) biofilm formation, and (iv) the configuration and operational characteristics of MFC chambers and electrodes (Angenent *et al.* 2004; Liu *et al.* 2004). These criteria may explain why the previously reported power density values varied so widely.

Microbial diversity

The diversity analysis from T-RFLP profiles showed that the degree of diversity (Shannon index and richness) in the anode-attached biofilm (AB in Table 1) was the highest among the tested samples. The richness was approximately two times greater than that of the activated sludge used for seeding and the suspension-grown microbes in the anode chamber. The Shannon index in the anode-attached biofilm was also higher than that of the activated sludge used for seeding and suspension-grown cells from the anode chamber. The higher diversity for the anode-attached biofilm might have resulted in the stability of electricity production since the degree of diversity tends to positively correlate with the stability of microbial communities (Colwell 1997; McGrady-Steed *et al.* 1997; Naeem & Li 1997; Tilman 1997).

Table 1 | Microbial diversity in MFC

Sample	AcS	AB	AS	CS
Shannon Index (*H*)	2.85	3.34	2.75	2.96
Richness (*d*)	4.29	8.06	4.07	6.47
Evenness (*e*)	2.19	2.09	2.15	1.97

Note: AcS indicates the seeding Activated sludge; AB, anode biofilm; AS, anode suspended; and CS, cathode suspended.

Because there was an insufficient amount of biofilm on the surface of the cathode electrode, the information on the diversity of cathode biofilm remains unclear. Nevertheless, the distribution of biomass observed could suggest that the microbes in the cathode chamber conditions preferred a suspension-grown state rather than a biofilm state. A possible explanation for the suspension-grown preference is that free-swimming microbes may be more efficient at utilizing dissolved oxygen than biofilm microbes. The values of the Shannon index and richness for suspension-grown cells in the cathode compartment (CS in Table 1) were slightly greater than those for the seeding activated sludge. This increased microbial diversity may be attributable to primary substrate (carbon and energy source) limitation in the cathode chamber. In the late phase of the MFC operation (when the biomass samples were taken for

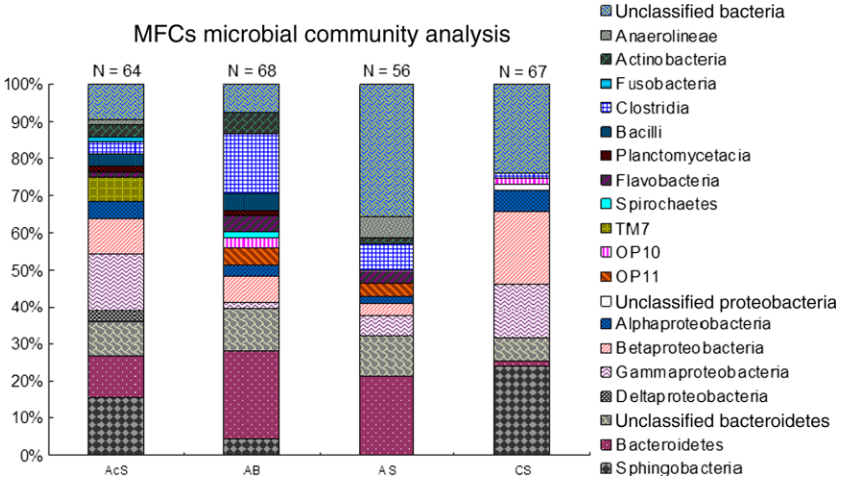

Figure 2 | Microbial community structures (class level) in MFCs fed with activated sludge. (N indicates the number of clones in each library; AcS Activated sludge; AB Anode biofilm; AS Anode suspended; and CS Cathode suspended). Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from http://www.iwaponline.com/wst.

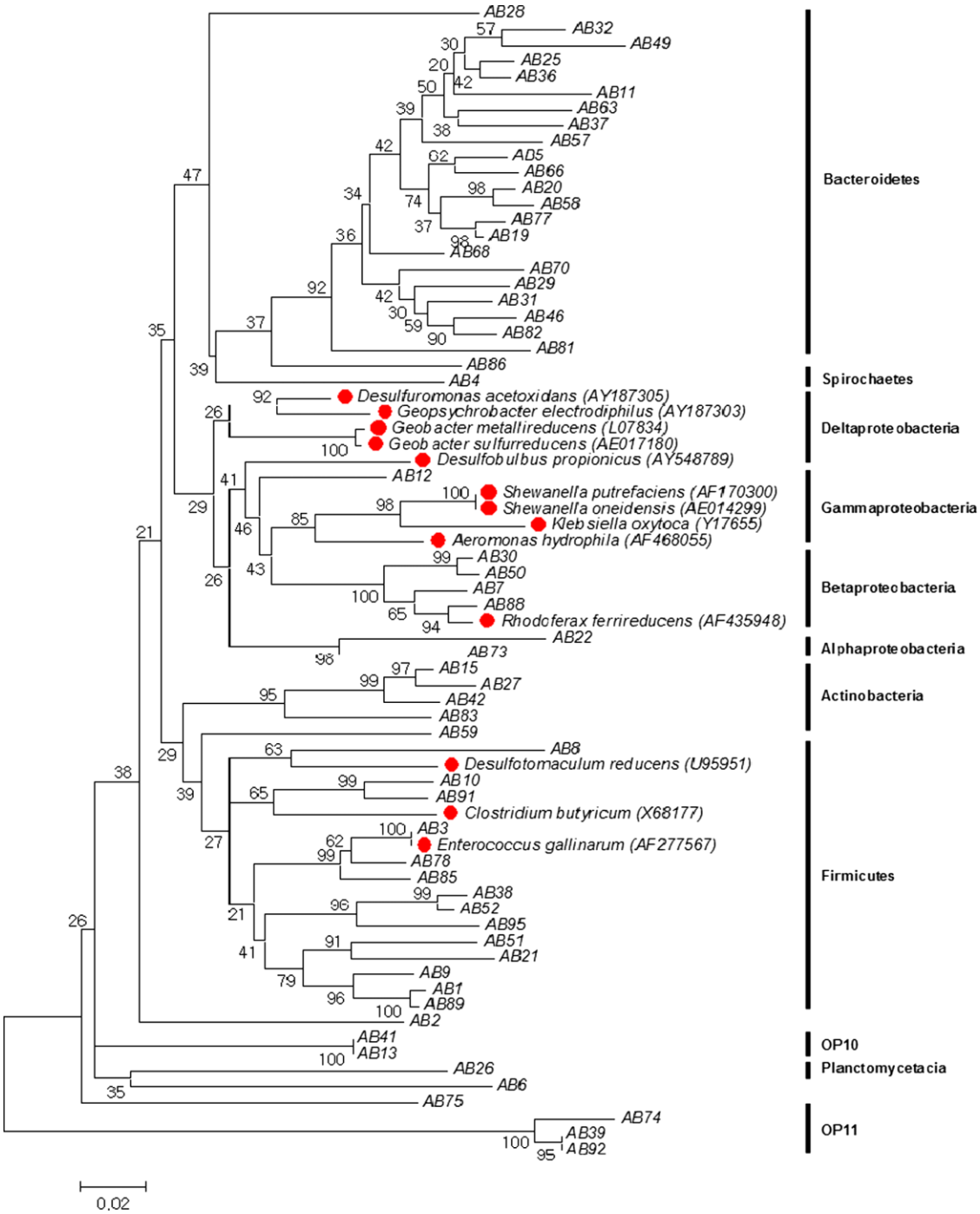

Figure 3 | Phylogenetic tree showing 16S rDNA gene sequences of the bacterial community from anode-attached biofilm. 16S rDNA gene sequences were aligned using CLUSTALW, and the tree was constructed by the neighbour-joining method using MEGA 4. The scale bar at the bottom indicates the distances between sequences, and red dots (●) indicate bacterial species known to participate in electron transfer in MFC.

microbial community analysis), the primary substrate (glucose) had been completely consumed in the fed-batch mode, and the supply of electrons from the cathode electrode may have been insufficient for the cathode microbes to grow. In general, the degree of microbial diversity is increased when microbial communities are starved for carbon and energy. Increased diversity and little biofilm formation seem to be advantageous for MFC cathode operation. Increased microbial diversity may result in enhanced stability in electricity generation, and little biofilm formation on the cathode electrode may be beneficial for efficiently transferring electrons to the most efficient terminal electron acceptor, namely dissolved oxygen in the cathode compartment.

Community structure and population dynamics in MFCs

During the operation of the electricity-generating MFCs, the microbial community structures in the anode chambers were shifted away from the original seeding microbial communities. In the seeding activated sludge, the major populations identified were *Gammaproteobacteria* (15.6%), *Sphingobacteria* (15.6%), and *Bacteroidetes* (11.0%) (Figure 2). In anode suspension-grown cells (AS), uncultured bacteria (35.7%) were the most predominant, followed by *Bacteroidetes* (21.4%), unclassified *Bacteroidetes* (10.7%), and *Clostridia* (7.1%). In the anode-attached biofilm (AB), *Bacteroidetes* (23.5%) was the most abundant division, followed by *Clostridia* (16.2%) and *Betaproteobacteria* (7.4%).

Because the anode biofilm has to be directly involved in electricity generation in anode compartments, our microbial community analysis paid special attention to anode biofilm populations. In the anode biofilm communities, *Bacteriodetes* was found to be the predominant population, preferentially growing from the original activated sludge. Among the anode biofilm populations, *Clostridia* was found to be an additional major population that was enriched from the original activated sludge microbial communities. These results show that *Bacteriodetes* and *Clostridia* are the major anode biofilm populations that may be actively involved in electricity generation in the MFCs.

In the literature, most previously reported anode biofilm microbes belong to the *Proteobacteria* phylum, especially the *Delta- and Gamma-groups* (Bond *et al.* 2002; Lee *et al.* 2003). In our study, however, *Deltaproteobacteria* and well-known electricity generating members such as *Geobacter* and *Shewanella* were not observed in the anode biofilm (AB) or anode suspended (AS) samples. In addition, the proportion of *Proteobacteria* was only 11.9% in the AB sample while in a previous study (Kim *et al.* 2006) *Proteobacteria* composed approximately 60% of the anode biofilm under operational conditions similar to those used for this work. According to a previous community structure analysis by Kim *et al.* (2006), the relative abundance of *Gammaproteobacteria* was increased in anode biofilm communities compared to suspension-grown communities in MFCs. However, our results indicated a different trend, i.e., that the relative abundance of *Gammaproteobacteria* in the anode biofilm was less than in anode suspension-grown cells. These findings suggest that the microbial structure and population dynamics contributing to electricity generation in this work may be different from those described in previous reports.

Phylogenetic tree analysis for anode biofilm populations

A phylogenetic tree was constructed with the 16S rDNA sequences from the anode-attached biofilm (using 59 total clones), and their phylogenetic distribution was compared with that of previously reported anode biofilm species (using 13 total isolates) (Figure 3). While most of the known anode biofilm species belong to *Proteobacteria*, only a limited number of our isolated clones belong to *Proteobacteria*. Furthermore, except for one clone (AB3) that is close to *Enterococcus gallinarum*, all of the isolated clones are phylogenetically distant from known anode biofilm species (Kim *et al.* 2008). These results indicate that novel anode biofilm populations were found in this work.

CONCLUSIONS

In this study, we conducted a microbial community analysis to compare the characteristics of anode-attached biofilm communities with those of anode suspension-grown microbial communities. For this purpose, microbial diversity and community structures were explored using 16S

rDNA-based, culture-independent methods. The major findings from this study are as follows:

1. In the MFC anode compartment, the microbial diversity of anode biofilm was higher than that of suspension-grown cells. The anode-electrode biofilm also exhibited a higher degree of microbial diversity than the original activated sludge used for seeding. The increased microbial diversity in the anode-electrode biofilm may have had positive effects on the stability of electricity production in the MFC.
2. In the anode biofilm communities, *Bacteroidetes* and *Clostridia* grew preferentially from the original activated sludge microbial communities in the course of generation of electricity. This suggests that these anode-stimulated biofilm populations may have participated in electricity-generation in the MFC.
3. According to the phylogenetic tree analysis, novel anode biofilm populations were found in this work. The presence of a wide range of bacterial phylogenetic groups in the anode-attached biofilm implies that a variety of microbes can participate *n* electricity generation.

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