Comparison of Exoelectrogenic Bacteria Detected Using Two Different Methods: U-tube Microbial Fuel Cell and Plating Method

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In a microbial fuel cell (MFC), exoelectrogens, which transfer electrons to the electrode, have been regarded as a key factor for electricity generation. In this study, U-tube MFC and plating methods were used to isolate exoelectrogens from the anode of an MFC. Disparate microorganisms were identified depending on isolation methods, despite the use of an identical source. Denaturing gel gradient electrophoresis (DGGE) analysis showed that certain microorganisms became dominant in the U-tube MFC. The predominant bacterium was similar to each isolate after isolation from plates produced lower current (peak current density: 3.6–16.3 mA/m²). Although the isolates produced low currents, various bacterial groups were found to be involved in current production.

Key words: electricity, exoelectrogen, dilution to extinction, U-tube microbial fuel cell

Microbial fuel cells (MFCs) have been considered to be a promising technology in the field of sustainable and renewable energy. An MFC is a system for generating electricity from organic compounds using microorganisms as a biocatalyst. In the anode chamber of MFCs, microorganisms degrade organic compounds, such as glucose, acetate and ethanol, etc., and the electrons produced from this degradation are transferred to the anode as an electron acceptor. The microorganisms capable of transferring electrons to the anode are called “exoelectrogens”. Exoelectrogens function as electrochemically active bacteria, capable of transferring electrons from their cell body to outside of the cell, and play an important role in electricity generation (7, 8). In general, there are direct and indirect ways for exoelectrogens to transfer electrons. Microorganisms transfer electrons directly by developing a biofilm on the anode surface or indirectly through electron shuttles that exist in the anodic suspension. Current can be generated from differences in the potential due to the movement of electrons; they contribute to the production of electricity in both ways; however, such information on electron transfer mechanisms is still insufficient to understand the physiology of the exoelectrogen, the ecology of anodic microbial communities on the electrodes, and the relationship between the exoelectrogen and other bacteria. Therefore, the identification and characterization of exoelectrogens are the most significant factors for increasing the efficiency of transfer electrons and producing higher power via MFCs (7, 8).

Methods for the isolation of exoelectrogens from the anode of MFCs can be categorized as follows: dilution to extinction and plating methods. The plating method is known as a generally convenient method to isolate exoelectrogens from MFC anodes. So far, a great number of exoelectrogens isolated from MFC anodes have been reported. There have also been many investigations on these bacteria, such as Clostridium butyricum (10), Aeromonas hydrophila (13), Rhodoseudomonas palustris (17), Aeromonas sp. (2) and Acrobacter butzleri (3). The main advantage of the plating method for their isolation is the conventional and relatively convenient experimental process. With plating, however, it is possible that it will discover not only exoelectrogens but also other bacteria that are not able to transfer electrons extracellularly on an MFC anode.

The exoelectrogens identified by the plating method are also known as dissimilatory Fe (III) reducing bacteria, which are able to reduce insoluble iron (7). One reason is that the media for metal-reducing bacteria have been used to isolate exoelectrogens, even though not all of the microorganisms growing in the media are exoelectrogens. Therefore, microorganisms were generally identified by their electrochemical activity after isolation (by plating). It was impracticable to observe the electrochemically active activity of bacteria discovered in the microbial community in MFCs. Moreover, the cultivation-dependent method, the plating method, is well known for significant limiting the numbers and populations of bacteria that represent the entire microbial community.

The dilution to extinction method, a different and cultivation-independent method for the isolation of exoelectrogens devised in previous studies, is an alternative method that enables exoelectrogens to be isolated by continuous monitoring of the electricity produced in MFCs. With this method, dominant strains of anode respiring bacteria or the electrochemically active microbial community can be isolated. Ochrobactrum anthrophi (20) and Comamonas denitrificans (16) have been reported as microorganisms...
isolated via the dilution to extinction method. *O. anthrophi* YZ-1 isolated from U-tube MFCs using the dilution to extinction method showed lower maximum current density of 708 mA/m², but higher coulombic efficiency of 93% than the mixed culture (1730 mA/m²). They produced current using a wide range of substrates (acetate, lactate, propionate, butyrate, glucose, sucrose, cellobiose, glycerol, and ethanol) (20).

Most of the defined strains isolated by the plating method have revealed the capability of reducing metals. It is unclear whether electrochemically active metal-reducing bacteria can significantly affect electricity generation; thus it should be considered that metal-reducing bacteria are really major exoelectrogens in MFC. However, previous studies have generally been carried out using a single method, or with different studies using different inocula, which have made it difficult to collectively compare studies. Thus, in this study, we used two methods, the dilution to extinction and plating methods, to identify and to isolate exoelectrogen. Through dilution to extinction experiments using a U-tube MFC, we tried to reduce the diversity of the exoelectrogenic community and to identify exoelectrogen. Then we isolated iron-reducing bacteria from the anodic microbial community of U-tube MFC by the plating method, and they were compared to the exoelectrogen identified using U-tube MFC.

**Materials and Methods**

**U-tube MFC construction**

A U-tube MFC was constructed so that exoelectrogens could be easily enriched and settled on the anode. The anode chamber (10 mL) in the MFC was a cylindrical rod-shape and the cathode chamber (30 mL) an asymmetrical U-shape (20). Sodium acetate (1 g/L) was used as the electron donor, and the composition of the medium used for the isolation of Fe(III)-reducing bacteria was as follows (per liter): NH₄HCO₃ 30 mL, NaH₂PO₄·4H₂O 2 g, KH₂PO₄ 6.8 g, KH₂PO₄ 8.8 g, NaCl 0.5 g, and 1 mL of a trace metal solution. In the cathode chamber, 100 mM ferrocyanide, with 100 mM PBS, was used as the catholyte. All solutions were autoclaved before injection and the pH was typically controlled to 7. The anode and cathode chambers were separated by a proton exchange membrane (Nafion 117; DuPont, USA). Gr christian felt and graphite cloth were used as the anode and cathode, respectively. Titanium wire was set to function as the current collector. The circuit between the anode and cathode was connected by a 1000 Ω external resistor. 

**Isolation using U-tube MFC**

The initial inoculum was a cell suspension separated from a piece of the anode of a lab-scale two-chamber MFC fed with acetate for 1 year. The excised anode from the MFC was transferred to a test tube, which also contained glass beads and 10 mL of 50 mM phosphate-buffered saline (PBS), using the tube, and then mixed by vortexing to obtain the cell suspension from the anode electrode (1 cm³). The cell suspension in the tube was serially diluted 10-fold in steps to 10⁻⁸. The diluted suspensions were added to 8 U-tube MFCs, which were operated with 3 times in batch mode as the 1st cycle. Then, the anode cell of U-tube MFC inoculated with the dilution suspension of 10⁻⁸ was serially diluted from 10⁻⁸ to 10⁻¹⁷ again. U-tube MFCs were operated in the same way (Fig. 1). The experimental process was repeated 10 times. A U-tube MFC, with only sterile medium and without an inoculum in the anode chamber, was used as the control. U-tube MFCs were operated in the same way as in Zuo et al. (20) at a constant temperature of 30°C. The medium in the U-tube MFC was refreshed when the voltage decreased below 10 mV. The medium in the reactor showing the minimum current for that dilution among all the U-tube MFC reactors was used in the next dilution step.

**Isolation using plating method**

The inoculum used in isolation plating was obtained from the anode cell suspension of the 10th cycle of the U-tube MFCs. The cell suspension was diluted to 10⁻¹⁰ and 10⁻¹⁷, and the diluted solutions spread onto agar plates. Sodium acetate (1 g/L) was used as the electron donor, and the composition of the medium used for the isolation of Fe(III)-reducing bacteria was as follows (per liter of distilled water): NaHCO₃ 2.5 g, CaCl₂·2H₂O 0.1 g, KC10.1 g, NH₄Cl 1.5 g, NaH₂PO₄ 0.6 g, Na₂CO₃ 0.1 g, MgCl₂·6H₂O 0.1 g, MgSO₄·7H₂O 0.1 g, MnCl₂·4H₂O 0.005 g, NaMoO₄·2H₂O 0.001 g, and yeast extract 0.05 g. Amorphous ferric oxyhydroxide was added to provide ca. 250 mmol Fe(III) per liter. Samples were cultured in an anaerobic chamber at 30°C. After 25 days, the dominant colonies were classified morphologically and then streaked onto other agar plates. The cultured isolates were transferred to 1.5 mL tubes and stored with 40% glycerol at −80°C.

**Electrical analysis**

The voltage output was measured using a data acquisition system (Model 7700; Keithley Instruments, OH, USA) every 2 min. The voltage, current density and power density were calculated using the following equations:

\[ V = IR, \quad Pd = VI/A, \quad Cd = V/A, \quad R = \text{ext. resistance} \]

where \( V \) is the voltage; \( I \), current; \( R \), external resistance; \( Cd \), current density; \( A \), anode surface and \( Pd \), power density. Current density and power density were normalized to the anode’s projected surface area.

**16S rRNA gene analysis**

After each dilution step had been completed, genomic DNA from

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**Table 1.** PCR primers and conditions used in this study

| Primer | Sequence (5' to 3') | PCR conditions | Target |
|--------|---------------------|----------------|--------|
| Eub 27F(GC) | AGT TTG ATC CTG GCT CAG | 2 min 95°C, followed 30 cycles of 20 s at 95°C, 40 s at 55°C, 30 s at 72°C followed by a 3 min final extension at 72°C | Bacteria |
| Eub 518R | ATT ACC GCG GCT GGT | | |

F: forward primer, R: reverse primer, GC: GC clamp (CGC CCG CCG CCG CCG GCG GCG GGG GGG GCA GGG GGG G) was attached to the 5' end for DGGE.
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Anodic microbial community analysis

Several bands showed higher intensities on the DGGE gel with respect to the dilution steps (Fig. 3). The microorganisms were assumed to represent bands of higher intensity and therefore would have the potential to transfer electrons and generate electricity in a U-tube MFC. Band UM1 was dominant in the U-tube MFC and was affiliated with *Ochrobactrum* sp., which has been reported as an exoelectrogen (Table 2). This exoelectrogen has not previously been reported as an Fe (III)-reducing bacterium. Bands UM2 and UM4 vanished during the early dilution step in serial U-tube MFCs. Band UM3 disappeared after 8 cycles. Band UM 2 was related to *Achromobacter* sp., which has been detected in an MFC. Zhu *et al.* (19) reported that *Achromobacter* sp. was isolated in enrichment culture tolerating and reducing Cr (VI) at extremely high concentrations. The closest bacterium to band UM 3 also belonged to *Ochrobactrum* sp. Band UM 4 was closely related to *Acinetobacter* sp., which has been reported as a community member in previous MFC studies (1, 5).

Isolated microorganism using plating method

The anode cell suspension of the 10th cycle was diluted to 10⁻¹ and 10⁻³, and the diluted solutions spread onto agar plates. The plates were cultured in an anaerobic chamber at 30°C. After 25 days, the dominant colonies were classified morphologically and then streaked onto other agar plates. Among the colonies on the plates, three (PM 1, PM 2 and PM 3) were differentially selected due to their morphologies, with all identified as Gram-positive bacteria from their 16S rRNA sequence analysis. So far, there have been a few MFC studies in which Gram-positive bacteria were reported as exoelectrogens; Rabaey *et al.* (14, 15) reported that *Enterococcus* spp., Gram-positive bacteria, were detected in an MFC and *Brevibacillus* spp. were dominant in the anodic microbial community of the MFC (12). In this study, PM 1, 2 and 3 were identified as *Bacillus sonorensis*, *Paenibacillus pabuli* and *P. amylolyticus*, respectively (Table 3).

Current production by isolates

A current production test was conducted to confirm the electrochemically activities of PM1, 2 and 3. The culture solution of the PMs (3×10⁸ cells/mL) was inoculated into the U-tube MFCs, which were operated in batch mode. All
the isolates produced relatively lower peak current densities of 3.6–16.3 mA/m² than the U-tube MFC inoculated with mixed cultures (Fig. 4). Of the isolates, PM 3 exhibited the highest peak current density, 16.3 mA/m². Rabaey et al. (14, 15) reported that Enterococcus spp., Gram-positive bacteria, generated a very low current in a pure culture with acetate as the electron donor.

Discussion

We used two different methods, the U-tube MFC and plating method, to compare exoelectrogen isolated from identical inoculum U-tubes. To identify exoelectrogens that produced a current, a cultivation-independent technology, PCR-DGGE, was used, and then DNA band fragments on the DGGE-gel were sequenced; however, we could not isolate exoelectrogens using U-tube MFC and 3 species, Ochrobactrum sp., Achromobacter sp. and Acinetobacter sp. were observed in U-tube MFCs. Among them, the most dominant bacterium was closely related to Ochrobactrum sp. belonging to the Alphaproteobacteria. Achromobacter sp. is a Gram-negative genus bacterium belonging to the Betaproteobacteria and can use a variety of organic acids and amino acids as carbon sources. Acinetobacter sp. is a Gram-negative bacterium included in the Gammaproteobacteria and is an important soil bacterium (4). Interestingly, the isolates obtained via the plating method were affiliated with Bacillus sp. and Paenibacillus sp., belonging to the Firmicutes. Bacillus spp. and Paenibacillus spp. are groups of Gram-positive, spore-forming, and rod-shaped bacteria. In particular, Paenibacillus spp. were predominant in iron-reducing consortia in contaminated sediments. In a previous study, it was reported that Bacillus sp. can generate current in MFCs (9); however, it has not been reported whether Paenibacillus sp. has the ability to function as exoelectrogens in MFCs. All U-tube MFCs inoculated with each isolate after isolation from plates produced a lower current (peak current density: 3.6–16.3 mA/m²) than those in U-tube MFCs with mixed culture (48.3–62.6 mA/m²). This shows that the exoelectrogenic activity of a complex microbial community is more effective than that of pure isolates in MFCs. Some MFC studies have reported that the power generated from pure cultures was much lower than from mixed cultures (12, 14, 20). If some strains directly transfer electrons to the electrode, current could be immediately generated in MFC when substrate was added to MFC. In this study, however, U-tube MFC inoculated with the isolated strain showed a long lag phase before current generation.

Table 2. 16S rRNA gene sequences of DGGE band

| Band name | Phylum         | Closest 16S rRNA sequence                          | Accession No. | Similarity (%) |
|-----------|----------------|---------------------------------------------------|---------------|----------------|
| UM 1      | Alphaproteobacteria | Ochrobactrum antrophi                             | EU187487      | 100            |
|           |                 | Ochrobactrum sp. OTU29                             | HM159984      | 100            |
|           |                 | Ochrobactrum tritici                               | EU301689      | 100            |
| UM 2      | Betaproteobacteria | Achromobacter sp.                                 | HM103444      | 100            |
|           |                 | Achromobacter sp. QXH                             | JN043371      | 99             |
|           |                 | Achromobacter xylodesulans                        | HQ540558      | 99             |
| UM 3      | Alphaproteobacteria | Ochrobactrum sp. OTU29                            | EU187491      | 97             |
|           |                 | Ochrobactrum tritici                               | HM159984      | 96             |
|           |                 | Uncultured alphaproteobacteria bacterium           | CU918533      | 96             |
| UM 4      | Gammaproteobacteria | Acinetobacter sp.                                | AJ812014      | 96             |
|           |                 | Uncultured Acinetobacter sp.                      | EU705310      | 96             |
|           |                 | Acinetobacter berezinaea                          | FM177774      | 96             |

Table 3. Identification of isolates by a plating method using sequences of partial 16S rRNA genes

| Name | Phylum         | Closest 16S rRNA sequence                          | Accession No. | Similarity (%) |
|------|----------------|---------------------------------------------------|---------------|----------------|
| PM 1 | Firmicutes     | Bacillus sonorensis                               | DQ993679      | 100            |
| PM 2 | Firmicutes     | Paenibacillus pabuli                              | AM260978      | 99             |
| PM 3 | Firmicutes     | Paenibacillus amyloyticus                         | FJ944661      | 96             |

Fig. 4. Current density from U-tube inoculated with PM1, PM2 and PM3 isolated using the plating method; the arrows indicated substrate feeding.
generation, which indicated that the strain might produce electron mediators for the transfer of electrons to the electrode. Some Paenibacillus sp., such as P. polymyxa, produce antibiotics, which could be used as exogenous mediators, as is the case with phenazines produced by Pseudomonas aeruginosa (6, 15). In general, Bacillus sp. and Paenibacillus sp. use glucose as a carbon source. In this study, however, acetate was used as the electron donor in MFC. Thus, U-tube MFC might produce a low current because acetate is not an appropriate carbon source.

The phylogenetic tree showed that the isolated/identified exoelectrogens can differ significantly, although an identical inoculum source was used (Fig. 5). Isolates from the conventional plating method were included in the Firmicutes, while those identified by DGGE from U-tube MFCs belonged to proteobacterial groups. This is the first study to show that Paenibacillus sp. has the ability to generate electricity, which indicates that many bacteria might be related to those that produce electricity in MFCs; however, additional study is required to elucidate further information about the metabolism, the electron transfer mechanism within Paenibacillus sp. and their role in the anodic microbial community.

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