Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2017

www.elsevier.com/locate/jbiosc

Bacterial communities adapted to higher external resistance can reduce the onset potential of anode in microbial fuel cells

Kei Suzuki,[1] Yutaka Kato,[2] Arashi Yui,[2] Shuji Yamamoto,[2] Syota Ando,[2] Owen Rubaba,[3] Yosuke Tashiro,[2] and Hiroyuki Futamata[1,2,4,*]

Graduate School of Science and Technology, Shizuoka University, Hamamatsu 432-8561, Japan,[1] Department of Applied Chemistry and Biochemical Engineering, Graduate School of Engineering, Shizuoka University, Hamamatsu 432-8561, Japan,[2] College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa,[3] and Research Institute of Green Science and Technology, Shizuoka University, Suruga-ku, Shizuoka, Shizuoka 422-8529, Japan[4]

Received 5 May 2017; accepted 20 December 2017
Available online xxx

We investigated how bacterial communities adapted to external resistances and exhibited the performance of electricity production in microbial fuel cells (MFCs) with external resistance of 10 Ω (LR-MFC) and 1000 Ω (HR-MFC). The HR-MFC exhibited better performance than the LR-MFC. The power densities of the LR-MFC and the HR-MFC were 5.2 ± 1.6 mW m^{-2} and 28 ± 9.6 mW m^{-2} after day 197, respectively. Low-scan cyclic voltammetry analyses indicated that the onset potential of the HR-MFC was more negative than that of the LR-MFC, suggesting that the higher external resistance led to enrichment of the highly current producing bacteria on the anode surface. All clones of *Geobacter* retrieved from the LR-MFC and the HR-MFC were members of the *Geobacter metallireducens* clade. Although the population density of *Geobacter* decreased from days 366–427 in the HR-MFC, the current density was almost maintained. Multidimensional scaling analyses based on denaturing gradient gel electrophoresis profiles indicated that the dynamics of the biofilm and anolytic communities changed synchronously in the two MFCs, but the dynamics of the bacterial communities in the LR-MFC and the HR-MFC were different from each other, reflecting different processes in adaptation to the different external resistances. The results suggest that the microbial community structure was formed by adapting to higher external resistance, exhibiting more negative onset potential and higher performance of the HR-MFC through collaborating with anode-respiring bacteria and fermenters.

[**Key words:** Microbial fuel cell; External resistance; Adaptation of microbial community; Extracellular electron transfer; Onset potential]

Chemical and biological approaches to sustainable energy production, such as using methane, ethanol, and hydrogen, have been developed. However, many of these approaches have encountered technical and economical hurdles (1,2). Microbial fuel cells (MFCs) represent an alternative strategy capable of directly converting organic waste to electricity (3,4). MFCs are devices that exploit numerous and diverse microorganisms as biocatalysts to generate electric power from organic waste such as wastewater and garbage. It is important for the practical application of MFCs to improve harnessing structure, including electrode and proton exchange membranes (5), and to control the microbial ecosystem in the anode chamber of the MFC (5–8).

Microbial communities in MFCs are formed corresponding to the electron donors (9–12). Therefore, how do we control the microbial community for efficient production of electricity in practical MFCs supplied with complex organic wastes? It is controversial for effects of external resistances on the performance of MFCs: the external resistance (R_{ext}) affects not only the anode potential (E_{an}) but also the anode biofilm communities, affecting current generation (13–16). For example, Aelterman et al. reported that E_{an} (0, −0.20, and −0.40 V vs. Ag/AgCl) did not affect the start-up time or the final power outputs during a period of approximately 1 month (17). Although anode-respiring bacterial (ARB) communities were grown at different E_{an} (−0.06 to 0.62 V vs. Ag/AgCl), their current outputs were similar under all conditions (18). Further, constant positive potential enables effective acclimatization of ARBs in MFCs, resulting in a faster start-up faster (19). In contrast, a more positive E_{an} (+0.37 V vs. standard hydrogen electrode [SHE]) generates highly diverse communities on the anode, with a low proportion of *Geobacter sulfurreducens*, and produces low current density, whereas more negative E_{an} (−0.15 and −0.09 V vs. SHE) preferentially selects *G. sulfurreducens* and results in high current density (15). Thus, it appears that a more negative E_{an} generates a high proportion of *Geobacter* and low-diversity communities on the anode, resulting in effective production of electricity from MFCs.

Since in the practical application MFCs are connected to devices for supplying electricity, it is important to understand the effects of external resistance on the performance of MFCs. The external resistance constrains the flux of electrons, which has significant impacts on the both of performance of MFCs and on its bacterial communities. The objective of this study was to evaluate the effects of external resistance on the electrochemical performance of MFCs and on their microbial community structures. We constructed two MFCs, namely a low resistance-MFC (LR-MFC) and a high-resistance

* Corresponding author at: Department of Applied Chemistry and Biological Engineering, Graduate School of Engineering, Shizuoka University, Hamamatsu 432-8561, Japan. Tel.: +81 53 478 1178; fax: +81 53 476 0095.
E-mail address: futamata.hiroyuki@shizuoka.ac.jp (H. Futamata).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2017.12.018

MFC (HR-MFC), with external resistance of 10 Ω and 1000 Ω, respectively. We discuss why the performance of the HR-MFC was better than that of the LR-MFC from the perspective of microbial adaptation.

MATERIALS AND METHODS

MFC configuration and operation A mediator-less air-cathode MFC (8) (Fig. S1) was used to evaluate power generation by microbial communities derived from the sediment of Lake Sanaru (Hamamatsu City, Shizuoka, Japan). A carbon paper electroplated with platinum (0.5 mg cm^{-2}) on one side was used as the cathode electrode (CHEMIX Co., Ltd., Sagamihara, Japan), thereby providing a total projected cathode surface area (on one side) of 4 cm^2. A proton exchange membrane (Nafion 117, DuPont, Wilmington, DE, USA) was placed between the anode and the cathode. Graphite felt strips (SOHGOH-C Co., Ltd. Yokohama, Japan) were used as the anode (4 cm × 4 cm × 0.5 cm) and were packed in the anode chamber (36 mL capacity) to provide a projected anode surface area of 40 cm^2 without a headspace.

The lake sediment (0.4 g) was inoculated into a MFC containing BE medium (5), which is a modification of DHE2 medium (20) and the medium reported by Ishii et al. (21). The BE medium contained 0.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.15 g $CaCl_2{\cdot}2H_2O$, 0.5 g NH_4Cl, 2.5 g $NaHCO_3$, 20 mM sodium lactate, 1.0 mL trace element SL8 solution (22), 1.0 mL Se/W solution (23), and 1.0 mL vitamin solution PV1 (24) per liter. Lactate (electron donor) was added to 20 mM in the anode whenever the cell voltage decreased to baseline. The MFC was incubated under batch conditions with stirring. To compare the effect of external resistance on the generation of electricity, two types of MFCs were constructed, with a different external resistances 10 Ω (called the LR-MFC) and 1000 Ω (the HR-MFC), respectively. Construction of the MFCs was otherwise the same.

Electrochemical analyses MFC voltage (V) was recorded every 5 min across a resistance (R) using a data logger (GL200A, Graphtec, Tokyo, Japan) connected to a computer. To evaluate MFCs performance, a polarization curve was determined using a potentiostat (HAV-110, Hokuto Denko Co. Ltd., Japan) set to 2 mV min^{-1} of a slope range within an appropriate interval. MFC performance indices (open-circuit voltage [V_{OC}], short-circuit current density per projection surface area (40 cm^2) of the anode electrode [I_{max}], maximum power density per the projection surface area of the anode electrode [P_{max}], and internal resistance [R_{int}]) were calculated from the slopes of the polarization curves.

In chronopotentiometry (CP) and low-scan cyclic voltammetry (LSCV) analyses, an Ag/AgCl reference electrode (HX-R6; 0.199 V corrected to an SHE; Hokuto Denko Co. Ltd.) was placed into the anode chamber to determine the electrode potential. When the E_{an} was measured by CP analysis using a potentiostat (HAV-110, Hokuto Denko Co. Ltd., Japan), the anode and cathode were used as the working and the counter electrodes, respectively. CP analysis was performed at appropriate intervals of current using the potentiostat. Simultaneously, CP analysis is able to evaluate the performance of electrodes known as limiting current density, which is able to distinguish which electrodes is the limiting factor for producing electricity in a MFC (21). When the cathode was evaluated by CP analysis, the cathode and anode were used as the working and the counter electrodes, respectively. When LSCV analysis was conducted, the anode and cathode were used as the working and counter electrodes, respectively. LSCV was performed at a scan rate of 1 mV s^{-1} between −500 mV and 700 mV vs. SHE. Onset potential was defined as the most negative potential in a Tafel plot (Fig. S2), indicating the most negative potential in stable extracellular electron transfer from microbial cells to the anode. When a sigmoidal curve such as the Nernst–Monod model (7,25) was observed in the LSCV, a half-saturation potential (E_{KA}), which is the potential at half-maximum current density (13), was estimated from the LSCV curve. The Ag/AgCl reference electrode was placed in the MFC 30 min before performing the CV and CP analyses to allow the electrode to stabilize.

Bacterial community analyses The anolytic culture (1.0 mL or 2.0 mL) was directly sampled from the anode compartment of the MFC and bacterial cells were collected by centrifugation for 5 min at 4°C and 20,000 ×g. Sections of anode (5 mm × 5 mm × 5 mm) were cut off for bacterial community analyses of biofilm on the anode. The total projection surface area of the cut off portion of the anode was 1.5 cm^2. These sections were washed gently with sterilized sodium-phosphate buffer solution (10 mM, pH 7.0) and were stored at −20°C until DNA extraction, which was used. DNA was extracted according to the conventional method (20).

Bacterial community structures were analyzed using a library of cloned 16S rRNA genes. The sediment of Lake Sanaru was used as the inoculum and analyzed on day 0. Anolytic cultures (1 mL or 2 mL) and anodes were collected from MFCs on days 197, 333, 427, and 564. Two sections (5 mm × 5 mm × 5 mm) were cut off from the anode in a glove box in anaerobic conditions. After taking the sections of the anode, new sections of the graphite felts were attached to original anode with a platinum wire. Fragments of 16S rRNA genes were amplified using the primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (corresponding to the *Escherichia coli* 16S rRNA gene nucleotide positions 8–27 (26)) and 5′-AAGGAGGTGATCCAGCC-3′ (corresponding to *E. coli* 16S rRNA gene nucleotide positions 1525–1542). Amplification was performed using a thermal cycler PC320 (ASTEC, Osaka, Japan) in a 50 μL mixture containing 0.5 U of KOD FX DNA polymerase (TOYOBO Co., Ltd, Osaka, Japan), buffer solution included with the PCR kit, 400 μM each deoxynucleoside triphosphate, 15 pmol each primer, and 50 ng template DNA. The PCR conditions were 2 min for activation of the polymerase at 94°C and then 25 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C, and finally 10 min extension at 72°C. The PCR products were checked using electrophoresis through 1.5% (w/v) agarose gels in TAE buffer (27); gels were stained with GelRed (Wako, Japan). PCR products were cloned into the vector pTA2 and introduced into competent *E. coli* DH5α cells using a TArget Clone-Plus kit (TOYOBO Co. Ltd., Osaka, Japan) according to the manufacturer's recommendations. Clones were isolated by screening for blue or white phenotypes of bacteria that were incubated in TB medium supplemented with kanamycin (50 mg L^{-1}). Plasmid DNA was extracted using a Wizard Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's directions. The DNA was digested with *Eco*RI and electrophoresed to confirm the expected sizes of the amplicons. In total, 956 clones were analyzed.

Bacterial community structures were analyzed using denaturing gradient gel electrophoresis (DGGE) analysis targeting 16S rRNA genes. The variable region V3 of the bacterial 16S rRNA gene (corresponding to nucleotide positions 341–534 in the *E. coli* sequence) was amplified using primers P2 and P3 (containing a 40-bp GC clamp (28)) and a thermal cycler PC320 as described previously (20). A Dcode DGGE system (Bio-Rad Laboratories, Inc., CA., USA) was used as recommended by the manufacturer. The PCR-amplified mixture (10 μL) was subjected to electrophoresis through a 10% (w/vol) polyacrylamide gel at 200 V for 3.5 h at 60°C. Gel gradients used for separation, which were applied in parallel to the direction of migration, were 35%–55%. After electrophoresis, the gel was stained with SYBR Green I (FMC Bioproducts) for 30 min as recommended by the manufacture.

The intensity of bands in the DGGE gel was measured using a Gel Doc XR+ system (Bio-Rad), and band intensities were subjected to multidimensional scaling (MDS) analysis. DGGE analysis is not necessarily reproducible. Therefore, the intensities and locations of the DGGE bands were compensated by comparing them with the intensities and locations of common samples electrophoresed through different DGGE gels (Fig. S3). MDS analysis based on the Bray–Curtis index was used to analyze the dynamics of the bacterial community structure, because this index is recognized as one of the most useful methods for evaluating the differences among populations (29,30). The equation used to calculate the Bray–Curtis index was as follows:

$$\delta_{AB} = \left(\sum |n_A - n_B|\right) \Big/ \left[\sum (N_A + N_B)\right] 0 \le \delta_{AB} \le 1,$$

where δ_{AB} represents the dissimilarity index between communities A and B, n_A and n_B represents the intensities of DGGE bands in clusters of A and B, respectively, and N_A and N_B represent the total intensities of DGGE bands in A and B, respectively (30–32). For example, "the dissimilarity index of the anolytic community in the LR-MFC" means the average of dissimilarity indices among all communities in the anolytic community in the LR-MFC. MDS analysis and cluster analysis were conducted using the R v2.12.1 (The R Project for Statistical Computing: http://www.r-project.org; University of Tsukuba, Japan: http://cran.md.tsukuba.ac.jp) (33). Commands used in R v2.12.1 are shown in Fig. S4. The 3D graph was generated using the RINEARN Graph 3D v.5.2.0 software.

Nucleotide sequence and phylogenetic analyses Cloned genes were sequenced using an ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit and analyzed using an ABI PRISM 3100-*Avant* genetic analyzer (Applied Biosystems, CA, USA). Sequence data were compiled using the GENETYX-MAC program (GENETYX Corporation, Tokyo, Japan). 16S rRNA gene sequence data of chimeras was analyzed using the CHIMERA_CHECK version 2.7 and compared with those retrieved from the Ribosomal Database Project II (34). Sequence data were compared using the BLAST homology search system with those deposited in databases. Multiple sequence alignments and calculations of the nucleotide substitution rate using Kimura's two-parameter model (35) were performed using the CLUSTAL W program (36). Distance-matrix trees were constructed using the neighbor-joining method (37), and the topologies of the trees were evaluated by bootstrapping with 1000 resamples (38).

Real-time PCR analysis of *Geobacter* spp. A real-time PCR assay was applied to genomic DNA to measure 16S rRNA gene copy numbers of *Geobacteraceae* in biofilm on the anode. The DNA extracted for bacterial community analyses was used as template DNAs in this experiment. Standard DNA fragments were produced using a cloned DNA affiliated with the *G. metallireducens* clade. All *Geobacteraceae* clones detected in this study were classified into the *G. metallireducens* clade (Fig. S5). Therefore new specific primers were designed according to the alignment of the *Geobacter* 16S rRNA gene sequences obtained from these experiments with those deposited in GenBank; New *Geo*-f (5′-CGTACCATTAGCTAGTTGGTG-3′) and New *Geo*-r (5′-GATCAAGAGGTATTAGCTCC-3′). Since this set of primers could amplify 16S rRNA genes from cloned DNA affiliated with the *G. metallireducens* clade but could not amplify the 16S rRNA genes of *G. sulfurreducens* PCA which is closest related strain to the *G. metallireducens* clade, the specificity of the set of primers was confirmed (Fig. S6). Real-time quantitative PCR was performed as follows: 95°C for 10 min, then 40 cycles of denaturation at 95°C for 10 s, annealing at 65°C for 5 s and extension at 72°C for 15 s. Fluorescence was detected at 86°C for 1 s during each

Please cite this article in press as: Suzuki, K., et al., Bacterial communities adapted to higher external resistance can reduce the onset potential of anode in microbial fuel cells, J. Biosci. Bioeng., (2017), https://doi.org/10.1016/j.jbiosc.2017.12.018