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Bio-electrochemical analyses of the development of a thermophilic biocathode catalyzing electromethanogenesis

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Abstract

The use of thermophilic microorganisms as biocatalysts for electromethanogenesis was investigated. Single-chamber reactors inoculated with thermophiles and operated at 55°C showed high CH$_4$ production rates (max. 1103 mmol m$^{-2}$ day$^{-1}$ at an applied voltage of 0.8 V) with current-capture efficiencies >90%, indicating that thermophiles have high potential as biocatalysts. To improve the electromethanogenic activity, the developed biocathode was transferred to a two-chamber reactor and operated at a poised potential of −0.5 V vs. SHE. The CH$_4$ production rates of the biocathode were enhanced approximately six-fold in 160 h of poised-potential incubation, indicating that the acclimation of the biocathode resulted in performance improvement. Compositional alteration of the cathodic microbiota suggested that a *Methanothermobacter*-related methanogen and Synergistetes- and Thermotogae-related bacteria were selected during the acclimation. Cyclic voltammetry of the “acclimated” biocathode showed an augmented cathodic catalytic wave with a midpoint potential at ca. −0.35 V vs. SHE. Moreover, the biocathode was able to catalyze electromethanogenesis at −0.35 V vs. SHE. These results suggested that the ability of the biocathode to catalyze electromethanogenesis via direct electron transfer was enhanced by the acclimation. This study provides new technological and fundamental information on electromethanogenic bio-electrochemical systems (BESs) that may be extended to other BESs.
**Introduction**

Electromethanogenesis is a reaction in a bio-electrochemical system (BES) in which microorganisms catalyze the reduction of CO$_2$ to methane by electric current at the biocathode. At a biocathode, electrons can be captured in methane with an efficiency as high as 96%. Thus, electromethanogenesis permits the storage of electrical energy in a stable form as “carbon-neutral” methane. Such a technology is an attractive option for biogas production as well as energy storage and also facilitates the integration of variable renewables.

Electromethanogenesis is catalyzed at least partly by methanogenic archaea (methanogens) via two distinct mechanisms, which involve direct and indirect (H$_2$-mediated) electron transfer. Methanogens have been detected in all electromethanogenic biocathodes examined to date. Specifically, the methanogens dominating biocathode populations are hydrogenotrophic species, which perform hydrogenotrophic methanogenesis (CO$_2$ + 4H$_2$ → CH$_4$ + 2H$_2$O). A recent study with a hydrogenase-disrupted mutant of Methanococcus maripaludis has provided direct evidence that the hydrogenotrophic methanogen can accept electrons directly from a cathode and solely catalyze direct electron transfer (DET)-mediated electromethanogenesis (CO$_2$ + 8H$^+$ + 8e$^-$ → CH$_4$ + 2H$_2$O). Alternatively, when the cathode potential is negative enough for the hydrogen evolution reaction (HER) (2H$^+$ + 2e$^-$ → H$_2$), the molecular hydrogen generated at the cathode can be utilized by hydrogenotrophic methanogens for hydrogenotrophic methanogenesis. In addition, because the catalytic activity of the “cathodophilic” consortia was reported to be an order of magnitude higher than that of pure-cultured methanogens, microorganisms other than methanogen likely play auxiliary roles in electromethanogenesis. Based on a recent finding of interspecies DET in a syntrophic association, it is possible that bacteria on the biocathode surface mediate (or facilitate) DET from the electrode to methanogens. Similarly, as reported in the biocathodes...
of microbial electrolysis cells, certain cathodic microorganisms can catalyze HER at the cathode.\textsuperscript{16-20} However, the functional roles of biocathodic microorganisms (including methanogens) in electromethanogenesis remain largely unknown.\textsuperscript{1,8}

The catalytic ability of biocatalysts is one of the key factors influencing BES performance.\textsuperscript{13} To date, the exploitation of biocatalysts for electromethanogenesis is limited mostly to mesophilic species derived from anaerobic sludge or effluents from other BESs (which were originally inoculated with anaerobic sludge or soil).\textsuperscript{1,10,21} With the aim of improving electromethanogenic BES performance for applicability, the present study evaluated the potential of thermophilic microorganisms as biocatalysts. It has been reported that biotechnological systems using thermophiles have several advantages over mesophilic systems in performance, such as higher reaction activity, greater durability, and wider substrate range.\textsuperscript{22} For BESs, promising performances of microbial fuel cells (e.g. 1030 mW m\textsuperscript{−2})\textsuperscript{19} and a microbial electrolysis cell using thermophiles as biocatalysts have been reported.\textsuperscript{22-26} In the present study, to evaluate the capability of thermophilic microorganisms as biocatalysts, an electromethanogenic BES was constructed at 55°C using thermophiles as the inoculum. The developed thermophilic biocathode was further assessed with particular focus on the alteration of bio-electrochemical properties during operation at a poised potential (−0.5 V vs. SHE). It has been reported that the performance of an acetate-producing biocathode was improved after long-term operation at a poised potential (−0.59 V vs. SHE)\textsuperscript{3}. To identify the mechanism of the performance improvement, the acclimation process of the thermophilic biocathode was characterized.

\section*{Materials and methods}

\textit{Construction of bio-electrochemical reactors}
Single-chamber reactors were constructed using glass bottles of 250 ml volume as described in a previous study. Each two-chamber reactor consisted of two identical 300-ml glass bottles separated by a pre-treated proton exchange membrane (12.5 cm², Nafion 117, DuPont Co., Wilmington, DE, USA), as previously described. The anode and cathode were made of a plain carbon cloth (4 × 10 cm, TMIL Ltd, Ibaraki, Japan). Each electrode was connected to the circuit via a titanium wire (0.5 mm in diameter, Alfa Aesar, Ward Hill, MA, USA), which was directly fastened to an end of electrode without any glue. The internal resistances between the electrodes and titanium wires were less than 3.0 Ω. All reactors were sealed with butyl rubber stoppers and aluminum seals to maintain anaerobic conditions.

**Operation of the single-chamber reactors**

The effluent of the anode chamber of a thermophilic microbial fuel cell (MFC) was used as the inoculum. The MFC was inoculated with formation water (anaerobically collected from an oil reservoir formation, which is located in Akita, Japan, around 1293 to 1436 m under the surface, with *in situ* temperature of 40-82°C) and had been producing electricity at 55°C for more than three months. Twenty-five milliliters of inoculum and 125 ml of pre-sterilized anaerobic medium (0.136 g, KH₂PO₄; 0.54 g, NH₄Cl; 0.2 g, MgCl₂·6H₂O; 0.147 g, CaCl₂·2H₂O; 2.5 g, NaHCO₃; and 10 ml Wolfe’s mineral solution per liter) supplemented with sodium acetate (0.8 g/l) as the electron donor was added to each of the single-chamber reactors. The abiotic control reactors were filled only with 150 ml of the pre-sterilized anaerobic medium. Their headspaces were filled with N₂/CO₂ (80/20).

During the startup process, a constant voltage of 0.7 V was applied to the reactor with a digital power supply (Array 3645A, Array Electronics, Nanjing, China) with the positive pole connected to the anode and the negative pole connected to the cathode. A fixed external resistance (1.0 Ω) was connected to each circuit. The voltage across the resistance was
recorded every five minutes with a multimeter (34970A, Agilent Technologies, Santa Clara, CA, USA) to calculate the current using Ohm’s law. The reactors were incubated at 55°C in the fed-batch mode, in which the medium was exchanged with fresh medium when current production was attenuated to the background level. During the incubation, the medium was continuously stirred with a magnetic stirrer. Inoculated reactors without applied voltage (open-circuit controls) and non-inoculated reactors with an applied voltage of 0.7 V were used as negative controls. The experiment was conducted in triplicate.

Operation of two-chamber reactors

The biocathodes developed in the single-chamber reactors were transferred to the cathode chambers of the two-chamber reactors under anaerobic conditions (by using an anaerobic chamber). Upon transfer, each biocathode was gently rinsed with pre-sterilized anaerobic medium. Each of the anode and cathode chambers was filled with 200 ml pre-sterilized anaerobic medium (with no sodium acetate added) as the electrolyte. A fresh abiotic electrode was used as the anode. An Ag/AgCl reference electrode (1 M KCl) that had a potential of 0.201 V vs. SHE at 55°C was inserted into the cathode chamber. The biocathode, anode, and Ag/AgCl electrode were connected to a potentiostat (HSV-110, Hokuto Denko, Japan) as the working, counter, and reference electrodes, respectively. The biocathode was initially poised at −0.5 V vs. SHE. The reactors were incubated at 55°C in the fed-batch mode. The media of both chambers were continuously stirred with magnetic stirrers. Reactors harboring non-inoculated abiotic electrodes at the cathode chambers were used as negative controls. The experiment was conducted in triplicate. To assess the CO₂ dependency of the CH₄- and current-producing reaction at −0.35 V vs. SHE, the bicarbonate of the anaerobic medium was replaced with 50 mM phosphate buffer (pH 7.0).
Analytical measurements and calculations

The concentrations of H\textsubscript{2} and CH\textsubscript{4} in the headspaces of single-chamber reactors and the cathode chambers of two-chamber reactors were analyzed using a gas chromatograph [GC-2014 equipped with a Shincarbon ST column (6 m × 3 mm ID); Shimadzu, Kyoto, Japan]. The headspace volume (V) of the reactors was maintained at a constant volume (100 ml). The pressure (P) in the headspace of each reactor was measured with a digital pressure sensor (AP-C40 series, Keyence, Osaka, Japan). The detailed calculation methods were described in the supporting information.

Cyclic voltammetry

Cyclic voltammetry (CV) analyses were performed in the two-chamber reactors with a standard three-electrode system. The parameters for CV were as follows: equilibrium time 99 s, scan rate 1 mV s\textsuperscript{−1}, and scanning range −0.7 to −0.2 V vs. SHE. CV was also performed on the biocathode immediately after replacement with a fresh electrolyte. In this case, the biocathode was gently washed three times with the fresh electrolyte and the catholyte was changed to a fresh pre-sterilized anaerobic medium, as described by Marshall \textit{et al.}\textsuperscript{25} CV was also performed on a pre-sterilized cathode in pre-sterilized fresh anaerobic medium as well as the cell-free spent medium. The spent medium was collected from the cathode chamber harboring the biocathode and then anaerobically filtered through a pre-sterilized filter to remove planktonic cells. CV was also performed on the bioelectrodes of the single-chamber reactor, using an Ag/AgCl electrode inserted in the reactor as the reference electrode. All CV measurements were performed in triplicate.

Characterization of microorganisms on electrodes
For scanning electron microscopy (SEM) analysis of the electrode surfaces, the electrodes were fixed with 2.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). For molecular phylogenetic analysis of the microbial consortia associated with the bioelectrodes, 16S rRNA gene-clone libraries were constructed using community DNAs extracted from the aseptically crushed electrodes as the PCR template, as described previously. Clones of each library were sequenced until Good’s coverage estimator reached at least 96%. Phylogenetic trees were constructed with MEGA (ver. 4.0.2.), as described previously. The detailed procedure was described in the supporting information.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers KM819470-KM819499.

Results

Construction of thermophilic electromethanogenic BES

Figure 1A and B show profiles of current and CH₄ production in single-chamber BES reactors with an applied voltage of 0.7 V. In the non-inoculated control reactor, only background levels of current and no CH₄ were detected throughout the experiments. In the inoculated reactors, the level of current generation began to increase in an exponential manner at 10 h post-inoculation (hpi) and reached a stable level of 3125 mA m⁻² at 25 hpi (Figure 1A). In correspondence, CH₄ was produced in the inoculated reactor at a rate of 365 mmol m⁻² day⁻¹ (5840 mmol L⁻¹ day⁻¹) (Figure 1B). The current- and CH₄-generating activities were sustained at stable levels for more than 20 h and then attenuated drastically likely owing to the depletion of the electron donor (acetate). Upon the replacement of spent
with fresh medium, the activities recovered to the maximum levels in less than 1 h. Such rapid recovery of activities typically indicated the establishment of bioelectrodes, and the catalytic microorganisms attached on the electrodes were responsible for current and CH$_4$ production. The abilities of the bioelectrodes to catalyze electrochemical reactions were further confirmed by cyclic voltammetry (CV) (Figure S1). The CH$_4$ production rate of the inoculated reactor showed an applied-voltage dependence and increased from 84 mmol m$^{-2}$ day$^{-1}$ (1347 mmol L$^{-1}$ day$^{-1}$) to a maximum of 1103 mmol m$^{-2}$ day$^{-1}$ (17653 mmol L$^{-1}$ day$^{-1}$) with an increase in the applied voltage from 0.4 to 0.8 V (Figure 1C). The efficiency of capturing electrons in the produced CH$_4$ (the current capture efficiency) on average exceeded 90% at all the applied voltages tested. Notably, the maximum CH$_4$ production rate of the inoculated reactor was higher than the rates of mesophilic electromethanogenic BESs (e.g. 656 mmol m$^{-2}$ day$^{-1}$ at −1.2 V vs Ag/AgCl). Although the performances of the BESs have been evaluated under various reactor configurations (e.g. the reactor structures, electrode materials and operation modes varied among studies) and therefore cannot be simply compared with each other, these results indicate that thermophilic microorganisms have high potential as the biocatalysts for electromethanogenic BES. Based on both input electrical energy and acetate (heat of combustion), the overall energy efficiency of the inoculated reactor with an applied voltage of 0.7 V was 61%, which could be enhanced by using more optimized electrode materials and reactor structures in future studies. In the open-circuit inoculated control reactor, CH$_4$ production was also detected (Figure 1B). However, compared to the inoculated reactors with applied voltages, CH$_4$ was produced at a much slower rate (0.5 mmol m$^{-2}$ day$^{-1}$) in the open-circuit control. Thus, in the single-chamber reactors, acetoclastic methanogenesis and/or syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis also likely contributed to the CH$_4$ production, albeit to a lesser degree than electromethanogenesis.
Alteration of biocathode activities during incubation at a poised potential

The thermophilic biocathode developed in the single-chamber reactor was transferred to the cathode chamber of a two-chamber reactor and connected to a potentiostat for further analyses. This setting allowed more precise analyses of the biocathode performance. In the single-chamber reactor, acetate, which was added to the medium as an electron donor for the bioanode, could also act as the electron and carbon source for the biocathode. In contrast, in the two-chamber reactor, given that no acetate was present in the catholyte, the electrode and CO₂ were the sole electron and carbon sources, respectively, for the cathodic microorganisms. Under this condition, we attempted further performance improvement of the biocathode by incubating it at the poised potential of −0.5 V vs. SHE. This potential was selected because Cheng et al. reported that the highest potential at which the mesophilic biocathode could catalyze electromethanogenesis was −0.7 V vs. Ag/AgCl (ca. −0.5 V vs. SHE).¹

When the poised-potential incubation was initiated (0 hpi), the biocathode produced a cathodic current of ca. 25 mA m⁻² (Figure 2A). The current production gradually increased and reached a stable level of 175 mA m⁻² at 160 hpi, indicating that the reducing ability of the biocathode improved during the poised-potential incubation. Correspondingly, the CH₄ production rate increased from 9 mmol m⁻² day⁻¹ (147 mmol L⁻¹ day⁻¹) at 20 hpi to 65 mmol m⁻² day⁻¹ (1042 mmol L⁻¹ day⁻¹) at 215 hpi (Figure 2B). No current or CH₄ production was observed in the abiotic control (Figure 2). The current- and CH₄-producing activities decreased gradually with prolonged incubation (over 170 hpi). This decrease was likely due to the consumption of CO₂, because the current-producing activity immediately recovered to the original level after medium exchange followed by sparging the cathode chamber with CO₂ (Figure S-2). During the poised-potential incubation, the current capture efficiency averaged ca. 93%, comparable to the mesophilic biocathode in the previous study.¹ Neither
H₂ nor acetate was detected in the cathode chamber (data not shown), indicating that the thermophilic biocathode was highly specialized to catalyze electromethanogenesis. It has to be noted that, as the minimum detection limit of the gas-chromatographic assay for H₂ was ca. 3.0 µM, a low level of H₂ below the threshold might be present. These results suggested that the acclimation of the biocathode to the poised potential resulted in marked improvement of the electromethanogenic performance.

**Morphological and molecular phylogenetic analyses of the biocathodic microbial consortium**

To investigate the mechanism of biocathode acclimation (the improvement of its electromethanogenic performance), the microbial populations on the electrode surface were morphologically and phylogenetically analyzed at the early (12 hpi) and late (200 hpi) stages of poised-potential incubation in the two-chamber reactor. For comparison, the biocathode as well as the bioanode of the single-chamber reactor (operated for ca. 250 h) were also examined. Given that the biocathode was first developed in the single-chamber reactor and then transferred to the two-chamber reactor for the poised-potential incubation, the biocathode of the single-chamber reactor served as a “pre-culture” sample prior to the poised-potential incubation.

The morphology of microbial consortia on the electrode surfaces was investigated by SEM (Figure 3A-C, Figure S-3 and Figure S-4). Compared with a thick biofilm on the bioanode of the single-chamber reactor (Figure S-3A), relatively thin biofilms containing sparse aggregates of microbial cells were observed on the biocathodes of the single-chamber reactor (Figure S-3B) and two-chamber reactor at the early stage of the poised-potential incubation (Figure 3B and Figure S-4B). On the biocathode of the two-chamber reactor at the late stage, despite its improved current- and CH₄-generating ability, the cell density on the surface was markedly reduced (Figure 3C and Figure S-4C). No biofilm was observed on the
Compositions of the biocathode microbiota at the different incubation stages were investigated by constructing 16S rRNA gene-clone libraries (Figure S-5 and 6; for the bioanode bacterial microbiota, see Figure S-7). To evaluate the contributions of the detected phylotypes semiquantitatively, the relative abundances of clones representing the phylotypes were compared (Figure 3D and E). The archaeal compositions of the biocathode consortia showed limited diversity (representing two phylotypes belonging to the Euryarchaeota phylum, Figure S-5) and maintained relatively uniform profiles throughout the incubation stages (Figure 3D). Majority of the clones in the archaeal libraries were closely related to *Methanothermobacter thermoautotrophicus* strain Delta H, a hydrogenotrophic methanogen of the Methanomicrobia class. The bacterial compositions of the biocathode consortia represented in total 21 phylotypes (Figure S-6) belonging to nine phyla (Actinobacteria, Bacteroidetes, Chloroflexi, Coprothermobacter, Elusimicrobia, Deferribacters, Firmicutes, Synergistetes, and Thermotogae) (Figure 3E). The bacterial components of the biocathodes in the single-chamber reactor and at the early stage of the poised-potential incubation shared the dominant species: two closely related phylotypes (EMTBiocatB-1 and EMTBiocatB-2; Figure S-6). These species, closely related to the exoelectrogenic Firmicutes of the genus *Thermincola*,\textsuperscript{25,31} represented 24% and 44% of the clones in the libraries derived from the cathode at those two incubation stages. The phylotype EMTBiocatB-17 (Figure S-6), which was closely related to the uncultured Bacteroidetes clone (FJ462092.1), was another dominant species in both libraries (representing 39% and 27%, respectively, of the clones in the libraries of the biocathode in the single-chamber reactor and at the early stage in the two-chamber reactor). However, in the library derived from the biocathode at the late stage of
the poised-potential incubation, those three phylotypes represented minor proportions (3%, 0%, and 1%, respectively) of the clones. Instead, the phylotypes EMTBiocatB-16 and EMTBiocatB-21 (Figure S-6), which were closely related to the uncultured Synergistetes clone (AY426471.1), and *Thermotoga elfii* strain G1 were the dominant and second most dominant species (representing 30% and 14%), respectively.

**Electrochemical analysis of the biocathode at different stages**

The catalytic activity of the biocathode was evaluated by CV at different incubation stages: early (12 hpi) and late (200 hpi) stages of the poised-potential incubation (Figure 4). In the potential range examined (from −0.2 to −0.7 V), only a small current was generated in the abiotic control (Figure 4A). In contrast, the voltammograms of the biocathodes at the different stages showed profiles of pronounced current generation having two distinct inflection points in common. The cathodic current generation was first initiated at an onset potential of −0.32 V vs. SHE (the first inflection point) and plateaued from −0.45 to −0.65 V vs. SHE, followed by a further rise from the potential of −0.65 V vs. SHE (the second inflection point) (Figure 4A). However, the electrochemical property of the biocathode at the late stage differed markedly from that at the early stage (which was similar to the voltammogram of the biocathode in the single-chamber reactor: Figure S-1B). Although the voltammograms shared similar inflection points, the current generation at the potentials between them was enhanced in the biocathode at the late stage. In the potential range between −0.45 and −0.65 V vs. SHE, the biocathode at the late stage steadily produced a cathodic current of −1.6 mA, whereas the current production by the biocathode at the early stage was limited to −0.5 mA (Figure 4A). The enhanced catalytic current of the biocathode at the late stage further confirmed that the acclimation of the biocathode during the poised-potential incubation resulted in enhanced catalytic ability. It should be noted, however,
that the poised potential alone might not lead to the enhanced performance because there were other variables involved. For example, in the poised-potential incubation, the cathode potential was changed from the prior operation in the single-chamber reactor. Also, acetate was no longer provided, which resulted in a change in carbon source and therefore likely in an enrichment of a different microbial community with different catalytic ability.

Notably, in the CVs of the biocathodes (especially at the late stage), the current shapes between the potential of −0.32 and −0.65 V vs. SHE showed a typical cathodic catalytic wave with a midpoint potential at ca. −0.35 V vs. SHE (Figure 4B). No appreciable current or redox peaks were observed in the voltammogram of the abiotic electrode in the cell-free spent medium collected from the biocathode-harboring cathode chamber of the two-chamber reactor (Figure 4A). Moreover, CV was performed on the “acclimated” biocathode immediately after the replacement of the catholyte with fresh medium (Figure S-8). The voltammogram reproduced the catalytic wave similar to that observed before the medium replacement. These results indicated that the redox component(s) with a midpoint potential of ca. −0.35 V vs. SHE likely govern the electron transfer at the biocathode in a direct manner.

Characterization of the electromethanogenic reaction catalyzed by the “acclimated” biocathode

To characterize the electrochemical reaction(s) catalyzed by the “acclimated” biocathode, the electromethanogenic activity of the biocathode at the late stage was further evaluated at the potential range (from −0.3 to −0.8 vs. SHE) where the reductive catalytic currents were detected by CV (Figure 5A). At −0.3 V vs. SHE, no appreciable CH₄ production was detected. However, CH₄ production [13 mmol m⁻² day⁻¹ (206 mmol L⁻¹ day⁻¹)] was detected at a potential as high as −0.35 V vs. SHE, a point likely corresponding to the first inflection point of the catalytic wave in the cyclic voltammogram (Figure 4A). The CH₄ production rate was
enhanced at −0.4 V vs. SHE and gradually increased from 18 mmol m\(^{-2}\) day\(^{-1}\) (194 mmol L\(^{-1}\) day\(^{-1}\)) at −0.4 V vs. SHE to 69 mmol m\(^{-2}\) day\(^{-1}\) (1102 mmol L\(^{-1}\) day\(^{-1}\)) at −0.7 V vs. SHE. Further acceleration of the CH\(_4\) production \([242 \text{ mmol m}^{-2}\text{ day}^{-1} (3877 \text{ mmol L}^{-1}\text{ day}^{-1})]\) was detected at −0.8 V vs. SHE, likely corresponding to the second inflection point in the voltammogram (Figure 4A). Moreover, at all the potentials, the current capture efficiency on average exceeded 91%. Thus, the catalytic behavior of the “acclimated” biocathode detected at CV corresponded well with methane production, further indicating that the electrochemical reactions catalyzed by the biocathode were methanogenic (corresponding to electromethanogenesis). However, although the formation of molecular hydrogen was not detected, it was possible that H\(_2\) was formed at the biocathode and immediately consumed by hydrogenophilic methanogens on the cathode surface, resulting in cathodic depolarization.\(^{32}\)

The role of hydrogenophilic methanogens in cathodic depolarization was examined in the supporting text and Figure S-9. Given that the cathodic potential was much lower than the theoretical redox potential for HER, it was likely that the second inflection point (and the accelerated CH\(_4\) production at −0.8 V vs. SHE) was attributed to HER.

Under the thermophilic conditions (at 55°C, pH 7.0) used in this study, the theoretical redox potential for CO\(_2\) reduction \((\text{CO}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow \text{CH}_4 + 2\text{H}_2\text{O})\) was −0.287 V vs. SHE. Taken together with the above results (Figure 4 and Figure 5A), it was plausible that the electrochemical reaction with midpoint potential of −0.35 V vs. SHE, for which the catalytic ability was particularly enhanced during the acclimation of the biocathode, was DET-mediated electromethanogenesis. Thus, to further characterize the electrochemical reaction, electromethanogenesis by the acclimated biocathode was evaluated at potentials near the midpoint potential. At −0.35 V vs. SHE, the biocathode was able to catalyze CH\(_4\) production at the rate of 18 mmol m\(^{-2}\) day\(^{-1}\) (294 mmol L\(^{-1}\) day\(^{-1}\)), producing a current density of ca. −120 mA m\(^{-2}\) (Figure 5B). The current and CH\(_4\) production depended on the
presence of CO₂ (Figure 5C). No appreciable current or CH₄ was produced by the abiotic control cathode. Moreover, 12 mM of 2-bromoethanesulfonate, a structural analog of coenzyme M and therefore a methanogen-specific inhibitor, strongly inhibited current and CH₄ production by the biocathode at −0.4 V vs. SHE (Figure 6). Production of molecular hydrogen was not detected (data not shown). Thus, methanogenesis was likely the final electron-consuming step at the biocathode at the potentials of −0.35 and −0.4 V vs. SHE. Taken together, these results supported the possibility that the “acclimated” biocathode was capable of catalyzing DET-mediated electromethanogenesis.

Discussion

The thermophilic biocathode was first developed in a single-chamber reactor and then transferred to a two-chamber reactor for the further improvement of catalytic ability. Such a two-reactor-step procedure is likely an effective option for developing an electromethanogenic biocathode. The SEM analysis showed that microbial cell density on the cathode surface was markedly reduced during poised-potential incubation. This observation suggested that excess microorganisms had accumulated on the cathode in the single-chamber reactor and that the microorganisms catalyzing DET-mediated electromethanogenesis were selected (and/or enriched) during the poised-potential incubation in the two-chamber reactor. The higher colonization in the single-chamber reactor was possibly due to the presence of acetate in the medium and/or low cathodic potential (ca. −1.0 V vs. SHE, with an applied voltage of 0.7 V). Given that it remains unclear whether methanogens can propagate during DET-mediated electromethanogenesis,¹² such initial accumulation of excess microorganisms on the cathode surface is likely advantageous for the effective development of an electromethanogenic biocathode. Indeed, although we were able to develop electromethanogenic biocathodes in two-chamber reactors by directly inoculating fresh
electrodes with the effluent from the cathode chamber, the establishment of the biocathodes was failed at −0.5 V vs. SHE and required a poised-potential incubation at −0.7 V vs. SHE (Figure S-10). Moreover, the electromethanogenic performance of the resulting biocathode was lower than that of the biocathode developed via the two-reactor-step procedure (Figure S-10).

In previous studies, the electron-transfer mechanism(s) of electromethanogenesis remained in debate. More specifically, DET-mediated electromethanogenesis has remained to be a hypothetical reaction model. As mentioned earlier, the hydrogenase-disrupted mutant of a methanogen was shown to produce CH\(_4\) in a BES reactor in an applied-voltage-dependent manner. In this study, as a complementing approach, electromethanogenesis by an “acclimated” biocathode was demonstrated at a potential of −0.35 V vs. SHE, the midpoint potential of the catalytic wave of the biocathode, presenting supporting evidence for the DET-mediated mechanism. Moreover, CV on the “acclimated” biocathode showed a current-generation profile with a sigmoidal shape and plateau catalytic current, typical features indicating microbial catalysis of DET from the cathode to microorganisms on the surface. However, it has to be noted that, in theory, H\(_2\) can also be generated at −0.35 V vs. SHE. Thus, the involvement of HER in electromethanogenesis at −0.35 V vs. SHE could not be ruled out. If the biocathodic microbial consortium is efficient at rapidly removing the generated H\(_2\), then the H\(_2\) concentrations will remain low, and maintain favorable conditions for H\(_2\) evolution.

The microbial mechanism of catalysis of DET-mediated electromethanogenesis remains to be determined. Alteration of the electrochemical properties suggested that the ability of the biocathode to catalyze DET-mediated electromethanogenesis was enhanced during the poised-potential incubation. Based on the concomitant alteration in the bacterial component of the biocathode, it is tempting to hypothesize that *Methanothermobacter*-related
methanogen and Synergistetes- and/or Thermotogae-related bacteria have role(s) in DET-mediated electromethanogenesis. The cathode inoculated with *M. thermoautotrophicus* strain Delta H, which is closely related to the dominant methanogen, did not show the catalytic ability for the electrochemical reaction at −0.35 V vs. SHE (Figure S-9). However, as *M. thermoautotrophicus* strain Delta H and the dominant methanogen are not the same strain, it is a formal possibility that the methanogen enriched at the biocathode has the ability to solely catalyze DET-mediated electromethanogenesis. Given that Synergistetes- and Thermotogae-related bacteria can establish syntrophic associations with methanogens, those bacteria might play auxiliary roles at the biocathode. Interestingly, it has been reported that cell components of non-viable microorganisms (i.e. cell debris) can catalyze HER at a biocathode. Thus, cell components derived from the surface microorganisms, which had “disappeared” during the acclimation, could also contribute to the catalytic ability of the biocathode. Further studies on the mechanisms of catalysis as well as acclimation of the thermophilic biocathode will provide basic knowledge for improving and sustaining electromethanogenic BES performance for practical application.

**Supporting Information**

“This information is available free of charge via the Internet at http://pubs.acs.org/.”

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Figures

Figure 1. Current generation (A), CH₄ production (B) (with an applied voltage of 0.7 V), and effect of applied voltage on CH₄ production rate and electron capture efficiency (C) of a single-chamber thermophilic electromethanogenic BES reactor. Arrows represent medium-replacement events. Control 2 is an open circuit inoculated control reactor. The experiment was performed in triplicate and the figure shows a representative experiment. Error bars denote standard error.
Figure 2. Current generation (A) and CH$_4$ production (B) by a thermophilic biocathode operated at a poised potential of −0.5 V vs. SHE in a two-chamber reactor. The experiment was performed in triplicate and the figure shows a representative experiment.
Figure 3. Morphological and molecular phylogenetic analyses of a biocathodic microbial consortium: SEM images of the surfaces of the control electrode (A), the biocathode at 12 hpi (B) and 200 hpi (C) of a poised-potential incubation. Relative abundance of the archaeal (D) and bacterial (E) groupings among sequences detected in the 16S rRNA gene-clone libraries derived from the biocathode microbiota at different stages. (D) Distribution of archaeal library sequences in identified classes of *Euryarchaeota*. The euryarchaeotal classes are shown as their relative proportions within the libraries derived from the biocathodes of the single-chamber reactor (SC), at 12 hpi (12) and 200 hpi (200) of poised-potential incubation. (E) Distribution of the identified bacterial library sequences in bacterial phyla. The phyla are shown as their relative proportions within the libraries.
Figure 4. Cyclic voltammetry analyses of a biocathode under −0.5 V vs. SHE operation: (A) abiotic control cathode (red line), abiotic control cathode in cell-free spent medium (blue line), the biocathode at 12 hpi (black line) and at 200 hpi (green line) of poised-potential incubation; (B) The first derivative of CV of the biocathode at 12 hpi (black line) and 200 hpi (green line). Scan rate, 1 mV s⁻¹.
Figure 5. (A) CH$_4$ production and electron capture efficiency of an “acclimated” biocathode at different potentials; (B) CH$_4$ production and current generation of the “acclimated” biocathode operated at −0.35 V vs. SHE in the presence of CO$_2$; (C) CH$_4$ production and current generation of a biocathode operated at −0.35 V vs. SHE in the absence of CO$_2$. Error bars denote standard error.
Figure 6. Effect of a methanogen inhibitor on current generation (black line) and CH$_4$ production (blue line) by an “acclimated” biocathode operated at −0.4 V vs. SHE. The arrow indicates the addition of the methanogen inhibitor 2-bromoethanesulfonate.