Methanogenic Biocathode Microbial Community Development and the Role of Bacteria

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Supporting Information

ABSTRACT: The cathode microbial community of a methanogenic bioelectrochemical system (BES) is key to the efficient conversion of carbon dioxide (CO₂) to methane (CH₄) with application to biogas upgrading. The objective of this study was to compare the performance and microbial community composition of a biocathode inoculated with a mixed methanogenic (MM) culture to a biocathode inoculated with an enriched hydrogenotrophic methanogenic (EHM) culture, developed from the MM culture following pre-enrichment with H₂ and CO₂, as the only externally supplied electron donor and carbon source, respectively. Using an adjacent Ag/AgCl reference electrode, biocathode potential was poised at ~0.8 V (versus SHE) using a potentiostat, with the bioanode acting as the counter electrode. When normalized to cathode biofilm biomass, the methane production in the MM- and EHM-biocathode was 0.153 ± 0.010 and 0.586 ± 0.029 mmol CH₄/mg biomass-day, respectively. This study showed that H₂/CO₂ pre-enriched inoculum enhanced biocathode CH₄ production, although the archaeal communities in both biocathodes converged primarily (86−100%) on a phylotype closely related to Methanobrevibacter arborophilus. The bacterial community of the MM-biocathode was similar to that of the MM inoculum but was enriched in Spirochaetes and other nonexoelectrogenic, fermentative Bacteria. In contrast, the EHM-biocathode bacterial community was enriched in Proteobacteria, exoelectrogens, and putative producers of electron shuttle mediators. Similar biomass levels were detected in the MM- and EHM-biocathodes. Thus, although the archaeal communities were similar in the two biocathodes, the difference in bacterial community composition was likely responsible for the 3.8-fold larger CH₄ production rate observed in the EHM-biocathode. Roles for abundant OTUs identified in the biofilm and inoculum cultures were highlighted on the basis of previous reports.

INTRODUCTION

Biogas produced by anaerobic digestion contains a mixture of carbon dioxide (CO₂), methane (CH₄), and other trace gases. To increase the energy content (i.e., CH₄) of biogas, current methods separate or sequester CO₂, resulting in a waste product.¹ Instead, bioelectrochemical systems (BESs) may be utilized to directly convert biogas CO₂ to CH₄.² A methanogenic BES pairs an oxidation reaction in the bioanode with the overall reduction of CO₂ to CH₄ in the biocathode in a process termed electromethanogenesis.³ Methanogenesis in the biocathode may proceed via several known mechanisms. Hydrogen (H₂), produced at the cathode from the reduction of protons: 2H⁺ + 2e⁻ ⇌ H₂ (E° = −0.421 V versus standard hydrogen electrode, SHE; pH 7), may be utilized by hydrogenotrophic methanogens to reduce CO₂: CO₂ + 4H₂ ⇌ CH₄ + 2H₂O (ΔE° = 0.170 V). At an applied voltage sufficient to overcome thermodynamic limitations and losses, H₂ may be produced abiotically at a cathode. However, in biocathodes, H₂ production may also be catalyzed by extracellular enzymes, providing an electron donor for methanogenesis at a smaller applied potential.⁴,⁵ Electrons may also be transferred from the cathode to other mediators that, in turn, act as electron donors for the microbial reduction of CO₂. Alternately, direct electron transfer from the cathode to methanogens may occur: CO₂ + 8H⁺ + 8e⁻ ⇌ CH₄ + 2H₂O (E° = −0.224 V versus SHE; pH 7).⁶

Methanobrevibacterium and, in some cases, Methanobrevibacter dominate biofilms of methanogenic biocathodes.³,⁷−⁹ While Archaea are undoubtedly important in a methanogenic biocathode, Bacteria may also play a significant role. Some Bacteria may function as anchors for electrode biofilm development, particularly species that can withstand direct current and strongly adhere to surfaces, such as Sphingomonas spp. and Mycobacterium frederiksbergense.¹⁰−¹³ Fermentative Bacteria that produce H₂ and CO₂ could potentially recycle cell lysis products into substrate for methanogens, thus improving CH₄ yield. Furthermore, some Bacteria may produce mediators that can act as electron shuttles,¹⁴−¹⁶ potentially improving CH₄ production by biocathode methanogens. Bacteria may also

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be exoelectrogens capable of cathode biocatalytic H₂ formation. Although typically found in anodes, exoelectrogens could also potentially act as endogenous anodes that recycle electron equivalents from organic compounds within the biocathode.

Although many species may be beneficial to biocathode methanogenesis, some types of Bacteria could divert the electron flow within a system away from methanogenesis, reducing biocathode efficiency and CH₄ yield. If acetlastic methanogens are not present or not active in the biocathode, acetogens may act as H₂/CO₂ scavengers and divert electron equivalents away from the desired product, CH₄. Although fermentative Bacteria can recycle cell lysis organic compounds and enhance methanogenesis if H₂ or CO₂ are produced, other species transfer electron equivalents to volatile fatty acids (VFAs) that must undergo further fermentation, thus slowing the ultimate flow of electron equivalents to CH₄. For example, *Moorella thermoacetica* ferments glucose into acetate nearly stoichiometrically without producing H₂ or CO₂. Moreover, acetogens such as *Clostridium aceticum* may convert H₂ and CO₂ into acetate, removing substrate from hydrogenotrophic methanogens. In addition, biofilm-forming Bacteria may physically impede the mass transfer of gases to and from methanogens. Bacteria may also affect methanogenesis in other ways that are not currently understood.

While several studies have examined the predominant Archaea in methanogenic biocathodes, little is known about the role of Bacteria. Using anaerobic digester sludge and bag sediment as separate inocula, Siegert et al. showed that the archaeal community of methanogenic biocathodes converged on hydrogenotrophic methanogens, *Methanobacterium* and *Methanothermobacter*. Other studies have reported similar results. Bacterial communities have also been reported in biocathodes, but it is not yet understood how the bacterial community of a culture changes through inoculum pre-enrichment for hydrogenotrophic methanogens or biocathode inoculation. Regardless of inoculum pre-enrichment, biocathode archaeal communities tend to converge on specific hydrogenotrophic species over time and, thus, differences in the performance of fully established biocathodes inoculated with a mixed culture and the mixed culture following a hydrogenotrophic pre-enrichment step (i.e., a subset of the mixed culture microorganisms) may be explained, in large part, by the difference in the biocathode bacterial communities. Therefore, to investigate the effect of the bacterial community composition on methanogenic biocathode performance, two established biocathodes were compared: a biocathode inoculated with a mixed methanogenic (MM) culture, and a biocathode inoculated with an enriched hydrogenotrophic methanogenic (EHM) culture, developed from the MM culture by pre-enrichment with H₂ and CO₂ as the only externally supplied electron donor and carbon source, respectively. By understanding microbial community changes during pre-enrichment and the establishment of cathode biofilm, insights may be gained on how Archaea and Bacteria affect cathodic CH₄ production and devise means to guide microbial selection for the development of efficient biocathodes.

**MATERIALS AND METHODS**

**Bioelectrochemical System Setup.** Each batch BES was a dual chamber, H-type with two 300 mL (250 mL liquid volume) modified square glass bottles separated by a proton exchange membrane (PEM; Nafion 117, 6.16 cm²; DuPont, Wilmington, DE). Each chamber was sealed with a butyl rubber stopper and initially flushed with nitrogen (N₂) (anode) or CO₂ (cathode) gas. All BESs were maintained at room temperature (22 ± 2 °C) under continuous mixing using magnetic stirrers. The anode and cathode electrodes were porous carbon felt (five stripes, approximately 86 cm², 15 cm²; Alfa Aesar, Ward Hill, MA) attached to a stainless steel rod. An Ag/AgCl electrode (+0.199 V versus SHE) was placed adjacent to the carbon felt electrode in each chamber to allow for voltage measurements. The cathode Ag/AgCl electrode acted as the reference electrode and the anode and cathode carbon felt electrodes were the working and counter electrodes, respectively. The working, counter, and reference electrodes were attached to a Gamry Interface 1000 potentiostat (Warminster, PA) with shielded cables, and the cathode potential was poised at −0.8 V (versus SHE). The anode compartment was filled with 250 mL anolyte, consisting of phosphate buffer medium (14.2 g/L NaH₂PO₄·H₂O, 13.8 g/L Na₂HPO₄·2H₂O, 0.31 g/L NH₄Cl, 0.13 g/L KCl), mineral stock (12.5 mL/L), and vitamin stock (2.5 mL/L). The cathode compartment was filled with 250 mL catholyte, consisting of phosphate buffer medium, mineral stock (12.5 mL/L), and vitamin stock (5.0 mL/L). Anolyte and catholyte were completely exchanged with freshly made solutions each week, for a hydraulic retention time (HRT) of 7 days. Bioanodes were fed sodium acetate at an initial concentration of 2.5 g COD/L, a model compound known to serve as an electron donor for exoelectrogenic Bacteria such as *Geobacter sulfurreducens* sp. nov.11 Biocathodes were fed by flushing the headspace with CO₂ and then pressurizing to an absolute pressure of 1.65 atm. Each BES was monitored for approximately 1 month until a consistent performance was achieved. Following the seventh consistent feeding cycle, determined by CH₄ production measurements and the current density profile, samples of catholyte and biocathode felt were removed and analyzed for protein to estimate biomass.

**Inocula.** Bioanode inoculum was obtained from a stock microbial fuel cell (MFC) consisting of a carbon felt anode, 150 Ω resistor, and Pt-coated air cathode. The MFC anode contained 250 mL of anolyte, which was partially replaced weekly with fresh medium to achieve a HRT of 8.75 days. Sodium acetate was added weekly at an initial concentration of 1.2 g COD/L. Following a period of biofilm establishment (61 days, 7 HRTs), a piece of MFC bioanode felt was used as inoculum in the anode of the methanogenic BES.

Biocathode inoculum from two sources was used in this study: a mixed methanogenic (MM) culture and an enriched hydrogenotrophic methanogenic (EHM) culture. The MM culture was derived from a stock mixed methanogenic culture initially developed with inoculum from a mesophilic, municipal anaerobic digester, fed with a mixture of dextrin and peptone and maintained at 35 °C for several years. The MM culture (1.5 L) was developed with 500 mL of stock mixed methanogenic culture and 1 L of reduced medium and was maintained at 22 °C for 2 weeks before was used as biocathode inoculum. Like the stock culture, the MM culture was maintained with a HRT of 27 days fed with a mixture of dextrin and peptone, along with reduced medium.

A 1.5 L sample of the MM culture was used to develop the EHM culture, which was fed five times per week by adding a mixture of H₂ and CO₂ (80:20; v/v) to the headspace (0.7 L) of a glass reactor, pressurized to an absolute pressure of 1.65 atm. The EHM culture was partially wasted once per week for a
HRT of 21 days and the wasted culture volume replaced by catholyte. BES biocathode inoculation was conducted by anaerobically transferring 200 mL of MM or EHM suspended growth culture samples to the cathode and then adding 50 mL of catholyte medium.

**Microbial Community Analysis.** Bacterial DNA was extracted from the suspended growth cultures and biocathode felts using the UltraClean Soil DNA Kit and PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA), respectively, according to the manufacturer’s instructions (Text S1). Bacterial DNA was amplified using the 16S rDNA gene primers 519wF (5′-CAGCMGCCGCGGTAA-3′) and 909R (5′-TGCTGCTCTCAGGATT-3′). The analysis of archaeal communities is strongly dependent on cell lysis efficiency during extraction and primer set selection.22,23 A pair of methods of DNA analysis were used for the archaeal communities. In the first method (M1), archaeal DNA was extracted from suspended growth cultures and biocathode carbon felts according to the DNA extraction kit’s manufacturer’s instructions, with an additional step to enhance cell lysis that consisted of two cycles of heating of the cell dry weight.29

**Analytical Methods.** Total suspended solids (TSS), volatile suspended solids (VSS), and pH were measured as described previously.26 Total gas production was measured using a pressure transducer (resolution ±1.974 atm, accuracy to 0.002 atm). Gas composition (CO₂, CH₄, and H₂) and volatile fatty acids (VFAs, C₂–C₇) were determined by gas chromatography (GC) with thermal conductivity (TCD) and flame ionization detection (FID), respectively, as previously reported.27,28 Biofilm protein was extracted from the electrode carbon felt by vortexing with 1N NaOH and beads for 20 min. Suspended biomass protein was extracted by centrifuging a liquid sample to concentrate cells, followed by the addition of 1N NaOH. Protein was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and a BioTek Synergy HT 96-well microplate spectrophotometer (Winooski, VT). Biomass was estimated by assuming cell protein was 63% of the cell dry weight.29

**RESULTS AND DISCUSSION**

Performance of BES with MM- and EHM-Inoculated Biocathodes. The suspended growth EHM culture was developed as described in Text S2; methane production over three representative feeding cycles is shown in Figure S1. A pair of BESs were developed to compare the performance of an EHM-inoculated cathode with that of a MM-inoculated cathode, while each was paired with the same acetate-fed, pre-established bioanode. The EHM-inoculated biocathode was set up first with a 50 mM buffered anolyte and catholyte, which was insufficient to maintain a stable pH, necessitating an increase of the buffer concentration to 300 mM. Subsequently, the BES with the MM-biocathode was set up with 300 mM buffered anolyte and catholyte, which maintained a nearly constant pH of 6.8 in both the anode and cathode throughout each feeding cycle. Following buffer strength adjustment, the EHM-inoculated biocathode experienced temporary pH fluctuations before maintaining a nearly constant pH of 6.8 in the anode and a pH of 6.8–7.2 in the cathode for the duration of each feeding cycle during steady BES operation. Although ammonia was not measured in this study, the anode and cathode of a similar system had an ammonia concentration in the range of 67 to 73 mg N/L,30 an ammonia level that is not expected to be inhibitory to exoelectrogens31 nor to methanogens.32

Initially, the mean anode acetate removal over the first four feeding cycles was 83 ± 5% and 81 ± 6% while paired with the EHM- and MM-biocathodes, respectively. After steady operation (6–8 cycles for each biocathode) was established, the anode acetate removal was 81 ± 7% over three typical feeding cycles when paired with the MM-biocathode (Figure 1A). In contrast, the anode acetate removal was 69 ± 2% over three typical feeding cycles when paired with the EHM-biocathode (Figure 1A). VFAs were not detected in the biocathodes, with the exception of trace levels of acetate (≤25 mg COD/L), which could have originated from acetogenesis,30 fermentation of cell lysis products, or the transport of acetate from the anode across the PEM to the cathode.30,33 Immediately after inoculation, the BES current density was higher with the EHM-biocathode than with the MM-biocathode (Figure S2A). Following biofilm development, the current density in the BES with EHM-biocathode increased dramatically upon feeding (Figure 1B). In contrast, the BES with MM-biocathode maintained a relatively low current density upon feeding (Figure 1B). The BES Coulombic efficiency during a typical cycle was substantially lower with the MM-biocathode (11%) than with the EHM-biocathode (39%). Regardless, these Coulombic efficiency values are likely underestimated because they are based on an assumption of complete acetate oxidation, which does not account for acetate used for microbial growth in the anode or the possible conversion of acetate to microbial carbon storage molecules in the anode biofilm, as has been previously observed.34

Transient H₂ was observed in the headspace of the MM-inoculated cathode, peaking at 24% v/v on day 4 of the first feeding cycle and 9% on day 2 of the second feeding cycle; after the second feeding cycle, H₂ was not detected in the cathode headspace. In the EHM-inoculated cathode, H₂ was not detected in the headspace, even during start-up. Previous work has shown that although H₂ is not detected in the headspace of a biocathode, H₂ may still be available in the liquid phase, as evidenced by continued transport of H₂ across
the PEM from cathode to anode. Thus, H₂ may have contributed to the biocathode CH₄ production from the reduction of CO₂, even though it was not detected in the headspace.

Headspace CH₄ in the MM- and EHM-inoculated cathodes during the first three feeding cycles following start-up is shown in Figure S2B. The EHM-biocathode was very active upon inoculation and, during the initial three feeding cycles, produced 1.24 ± 0.16 mmol CH₄ per cycle. In contrast, the MM-biocathode produced 0.83 ± 0.33 mmol CH₄ per cycle during the first three feeding cycles, and its CH₄ yield declined following the second cycle. Thus, as the microbial community developed in the biocathodes, CH₄ production by the EHM-inoculated biocathode community increased, while CH₄ production by the MM-inoculated community decreased. Biocathode CH₄ production may occur through direct electron transfer or through H₂ production, which can be catalyzed by extracellular enzymes or other cell products. A wide-range cyclic voltammetry scan of the BES with enriched biocathode under inactive, unfed conditions (Figure S3) showed that abiotic H₂ evolution, catalyzed by the presence of inactive biomass or bioproducts on the cathode, occurred at −1.1 V, a more-negative applied potential than that used in this study (−0.8 V). Furthermore, our previous work with a similar system showed that the biocathode produced over 5-fold higher total CH₄ than the CH₄ equivalents of H₂ produced by an uninoculated, abiotic cathode. Therefore, direct electron transfer was likely one mechanism of CH₄ production in the enriched biocathode, alongside hydrogenotrophic methanogenesis with biologically catalyzed H₂.

At the end of experimentation, total biomass on the MM- and EHM-inoculated carbon felt biocathodes was 0.540 ± 0.067 and 0.639 ± 0.189 mg, respectively. Total suspended biomass, estimated by protein measurement, was also similar between the MM- and EHM-biocathode chambers, with 0.067 ± 0.006 and 0.078 ± 0.022 mg, respectively. Thus, the biofilm biomass made up 88.9% and 89.2% of the total biomass in the MM- and EHM-biocathodes, respectively. The headspace CH₄ in the MM- and EHM-biocathodes during three typical cycles following 56 days of cathode biofilm establishment is shown in Figure 1C. Over the course of three cycles, the CH₄ production rate in the MM- and EHM-biocathode was 0.083 ± 0.006 and 0.375 ± 0.019 mmol CH₄/day, respectively. Normalizing to cathode biofilm biomass, the MM- and EHM-biocathode CH₄ production rate was 0.153 ± 0.010 and 0.586 ± 0.029 mmol CH₄/mg biomass-d, respectively. Thus, the EHM-biocathode produced CH₄ at a rate 3.8 times higher than that of the MM-biocathode, indicating a far more productive biofilm in the EHM-biocathode. However, both CH₄ production rates were much higher than that of the EHM suspended growth culture (4.22 × 10⁻³ mmol CH₄/mg biomass-d). Compared with reported CH₄ production rates for the anaerobic digestion of mixed food waste (6.76 × 10⁻⁴ mmol CH₄/mg biomass-d), the EHM-biocathode CH₄ production rate was 21 mmol/m²-d and 603 mmol/m²-d. Other studies have reported higher biocathode CH₄ production rates at higher poised potentials or over shorter periods of operation (e.g., −0.9 V, 5 h). Compared to the initial feeding cycle, the established EHM-biocathode produced twice the CH₄ but the MM-biocathode produced only 3% more CH₄ than it did during the initial start-up cycle and substantially less than in the initial second and third cycles. This pattern in the CH₄ production in the MM-biocathode could be explained by the early enrichment of methanogens capable of utilizing H₂ or electrons directly from the cathode, followed by the growth of bacterial species that interfere with methanogenesis. In contrast, the EHM-biocathode community, which was pre-enriched under hydrogenotrophic methanogenic conditions, did not experience a later decline in CH₄ production. Thus, an enrichment step may be useful in not only selecting for hydrogenotrophic methanogens but also selecting for a bacterial community that is complementary and supports electromethanogenesis, as further discussed below. Indeed, the current capture efficiency (CCE), a measure of how efficiently the cathode biofilm uses electron equivalents from the cathode to reduce CO₂ to CH₄, was 98% during a typical BES feeding cycle with the EHM-biocathode. In contrast, the BES CCE with the MM-biocathode was only 62%, indicating a far less efficient biofilm. Cyclic voltammetry also confirmed a greater catalytic activity with the EHM-biocathode than with the MM-biocathode (Figure 2). The ionic strength of anolyte and catholyte may affect the shape and current magnitude of the BES voltammogram, as discussed in Text S3. Although a trend of increasing current with increasing ionic strength was observed in CVs conducted...
with an abiotic (i.e., uninoculated BES) at increasing anolyte and catholyte ionic strength from 1.21 to 1.41 M, the current increase at an applied potential of $-0.8$ V was $0.108 \pm 0.003$ mA in both the increasing (more-positive) and decreasing (more-negative) potential scan direction (Figure S4). In addition to the relatively low impact of ionic strength within the ionic strength range tested, all voltammograms in the present study were conducted under similar conditions (i.e., ionic strength, scan rate, temperature, etc.).

Overall, the pre-enrichment step selecting for hydrogenotrophic methanogens increased the methanogenic bio-cathode productivity over the use of a nonenriched, mixed methanogenic inoculum. Furthermore, inoculum enrichment allowed for faster BES start-up because of the greater methanogenic inoculum. Furthermore, inoculum enrichment allowed for faster BES start-up because of the greater abundance of hydrogenotrophic methanogens.

**Change in Microbial Community Structure.** The total number of OTUs detected in the MM culture, EHM culture, MM-biocathode, and EHM-biocathode was 285,968, 268,884, 216,080 and 278,850, respectively. A rarefaction curve for each community sample (Figure S5) indicates that most of the OTUs in each sample were sequenced. The bacterial Shannon indices (Table 1) for the suspended growth cultures, MM and EHM, were lower than those of their respective biocathode communities, more likely the result of a much higher biomass retention time in the biofilm, as well as less homogeneity in the biocathodes than in the well-mixed suspended growth cultures. The carbon felt and biofilm affect mass transfer and may allow for the development of microenvironments with conditions that can host a more diverse bacterial community. One study of a MFC anode found the bacterial community in the biofilm had a higher Shannon index (3.34) than that of the activated sludge used as inoculum (2.85). In contrast, inocula with highly diverse bacterial communities (Shannon indices of 4.7 and 4.9) used by another study to setup microbial electrolysis cells (MECs) resulted in lower cathode biofilm bacterial diversity (Shannon index of 2.0). Principal coordinate analysis (PCA; Figures S6–7) indicated a greater shift in bacterial community composition occurred due to biofilm development and increased buffer strength with the EHM-biocathode (EHM culture versus EHM-biocathode) than with the MM-biocathode (MM culture versus MM-biocathode). A redundancy analysis and canonical correspondence analysis (CCA) was performed to reveal the factors that most contributed to the change in microbial community (Figure S8). Redundancy analysis (Figure S8A) indicated that large changes in microbial community structure occurred during enrichment from MM culture to EHM culture (i.e., change to a lower temperature and a different, simple substrate) and with inoculation of biocathodes (i.e., biofilm development and higher buffer strength). CCA analysis (Figure S8B) indicated that substrate type and biofilm development were likely to be primary factors in the microbial community change.

The archaeal Shannon index for DNA samples extracted using method 1 (M1), which represented the hydrogenotrophic methanogen diversity, decreased between the suspended growth cultures and the resulting cathode biofilms (Table 1). This result is likely due to enrichment of methanogens capable of utilizing H₂ and electrons originating from the cathode. Thus, the bacterial diversity may have increased due to biofilm formation, but the archaeal diversity (M1 DNA extraction method) may have decreased because of electrochemical enrichment of hydrogenotrophic methanogens at the cathode. The archaeal diversity for DNA samples extracted using method 2 (M2), which represented acetoclastic methanogen diversity, declined substantially between the MM suspended growth culture, in which acetate would be available from dextrin and peptone fermentation, and the MM-biocathode biofilm, which was not fed complex organic substrates. As expected, the M2 archaeal Shannon indices were low in both the EHM suspended growth culture and EHM-biocathode biofilm; the EHM-biocathode had a slightly higher index than the EHM suspended growth culture, which could be due to minor diffusion of acetate across the PEM from the anode to the cathode, as has been previously reported. PCA showed that the MM culture and EHM culture archaeal community composition shifted similarly following biofilm development on the MM- and EHM-biocathodes (Figures S6–S7).

**Phylogeny of Suspended Growth Cultures and Cathode Biofilms.** The most abundant (≥1% relative abundance) bacterial and archaeal OTUs were placed with their closest defined species matches in GenBank into phylogenetic trees (Figures S9–S11). The relative abundance of Archaea in the MM and EHM suspended growth cultures and biocathode biofilms is given in Table 2. The first method of DNA analysis, M1, was more effective at detecting hydrogenotrophic methanogens, particularly *Methanoculleus*, *Methanolinea*, and *Methanobacterium*, whereas the second method of DNA analysis, M2, was more effective at detecting acetoclastic methanogens such as *Methanoaeta*. For the DNA samples extracted using method 1 (M1), *Methanomicrobiaceae* and *Methanoregulaceae* together represented 97% of sequenced Archaea in the MM culture. In contrast, *Methanobacteriaceae* represented 95% of sequenced Archaea in the EHM culture.

**Table 1. Bacterial and Archaeal Shannon Diversity Indices for Suspended Growth Cultures and Biocathode Biofilms**

<table>
<thead>
<tr>
<th>culture*</th>
<th>bacterial community</th>
<th>archaeal community (M1)*</th>
<th>archaeal community (M2)*</th>
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<tbody>
<tr>
<td>MM</td>
<td>1.466</td>
<td>1.132</td>
<td>1.578</td>
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<tr>
<td>MM-biocathode</td>
<td>2.120</td>
<td>0.114</td>
<td>0.035</td>
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<td>EHM</td>
<td>2.033</td>
<td>0.240</td>
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<td>EHM-biocathode</td>
<td>2.684</td>
<td>0.047</td>
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*Abbreviations: MM, mixed methanogenic suspended growth culture; EHM, enriched methanogenic suspended growth culture; MM-biocathode, MM-inoculated biocathode; EHM-biocathode, EHM-inoculated biocathode. M1 and M2: DNA analysis method 1 and method 2, respectively (see text).
This result indicates that the enrichment conditions in the EHM culture altered the hydrogenotrophic methanogen community composition. For example, in the MM culture, *Methanoculleus* was the most-abundant genus of the sequenced Archaea (77%, M1) but was the least abundant of detected Archaea in the EHM culture (86%, M1). Similarly, *Methanolina*, which had the second greatest abundance in the MM culture sequenced Archaea (20%, M1), was undetected in the EHM culture. Although *Methanoculleus* and *Methanolina* are hydrogenotrophic methanogens capable of converting H2 and CO2 to CH4, some species are known to require acetate as a carbon source or peptone as a growth factor. Thus, the EHM culture’s enrichment conditions favored the growth of other hydrogenotrophic methanogens, such as *Methanobrevibacter* and *Methanobacterium*, which do not require complex carbon sources and growth factors. Indeed, the most abundant M1 phylotype in the EHM culture was closely related to *Methanobrevibacter arborophilus*, a species that grows autotrophically and only requires B vitamins as growth factors.

Methanosarcinales is an order consisting of methylotrophic and acetoclastic methanogens, such as *Methanomethylovorans* and *Methanoseta*. Despite the feeding of a complex substrate (dextrin–peptone) to the MM culture, *Methanoseta* was only detected at trace levels and the methylotrophic genus *Methanomethylovorans* represented just 2% of sequenced Archaea (M1 DNA extraction method). However, using method 2 for DNA extraction, *Methanoseta* was the most abundant identified genus in the MM culture (Table 2), illustrating the large bias that may be introduced on the basis of the choices of the DNA extraction method and primers. As expected, due to a lack of complex substrate, *Methanosarcinales* was not detected in the EHM culture with either M1 or M2 DNA extraction method.

In both the MM- and EHM-biocathodes, the genus *Methanobrevibacter* was highly enriched following inoculation. *Methanobrevibacter* ssp. increased from 70% (M1) of detected Archaea in the EHM suspended growth inoculum to 100% (M1) in the established EHM cathode biofilm. Similarly, in the MM suspended growth culture, *Methanobrevibacter* ssp. represented only 0.5% (M1) of all detected Archaea but, following biocathode biofilm establishment, it represented 86% (M1) of detected Archaea in the MM cathode biofilm. Thus, *Methanobrevibacter* was highly enriched under biocathode conditions, regardless of the level of initial abundance in the two inocula. Furthermore, the dominant phylotype in the MM- and EHM-biocathodes was most closely related to *M. arborophilus*, representing 86% and 100% (M1) of detected Archaea in the MM- and EHM-biocathodes, respectively. In comparison, this phylotype represented only 0.003% and 68% of detected Archaea in the MM and EHM suspended growth inoculum, respectively. Biocathode enrichment of *M. arborophilus* has also been noted in other studies. Because complex substrates were not added to the cathodes, Methanosarcinales were either not detected or present only in trace amounts (<1% relative abundance) using M1 and M2 DNA extraction methods.

The bacterial microbial community composition and relative abundance of phyla and selected species detected in the MM and EHM suspended growth cultures and biocathode biofilms are shown in Figure 3 and listed in Tables 3 and S2. Enrichment of the suspended growth culture had a substantial impact on the bacterial community structure, which was also illustrated with PCA (Figures S6–S7). Bacteroidetes, Proteo-

Table 2. Relative Abundance (%) of Archaeal Community Members in Suspended Growth Cultures and Biocathode Biofilms

<table>
<thead>
<tr>
<th>family</th>
<th>genus</th>
<th>MM suspended culture</th>
<th>EHM suspended culture</th>
<th>MM-B biocathode biofilm</th>
<th>EHM-B biocathode biofilm</th>
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<td></td>
<td>M1</td>
<td>M2</td>
<td>M1</td>
<td>M2</td>
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<tr>
<td>Methanomicrobiaceae</td>
<td><em>Methanoculleus</em></td>
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<td>Methanobacteriaceae</td>
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<td>–</td>
<td>68</td>
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“Abbreviations: MM, mixed methanogenic suspended growth culture; EHM, enriched methanogenic suspended growth culture; MM-B, MM-inoculated biocathode; EHM-B, EHM-inoculated biocathode; M1, M2, DNA analysis method 1, method 2, respectively (see text). ND, not detected. T, trace with relative abundance <1%. TT, trace with relative abundance ≤0.1%.”

Figure 3. Bacterial community structure in suspended growth culture and biofilm samples. MM, mixed methanogenic suspended growth culture; MM-B, MM-inoculated biocathode; EHM, enriched methanogenic suspended growth culture; EHM-B, EHM-inoculated biofilm.
Table 3. Relative Abundance, Closest GenBank Match, and Role of Related Bacterial Species for OTUs Identified in Suspended Growth Cultures and Bio cathode Biofilms

<table>
<thead>
<tr>
<th>closest GenBank match and accession no.</th>
<th>relative abundance (%)</th>
<th>role</th>
<th>ref</th>
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<tr>
<td></td>
<td>MM</td>
<td>MM-B</td>
<td>EHM</td>
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<tr>
<td>Actinomyces georgii strain DSM 6843 (NR_026182)</td>
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<td>2</td>
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<td>Arcobacter mytilis strain T234 (FJ156092)</td>
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<td>Bacteroides MB7-1 (DQ453797)</td>
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<td>0</td>
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<td>Bacteroides sp. strain Z4 (AY949860)</td>
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<td>Citrobacter amalonaticus Y19 (LN564018)</td>
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<td>Cloacibacillus eryensis strain 158 (NR_115465)</td>
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<td>minor species (&lt;1% relative abundance)</td>
<td>72</td>
<td>21</td>
<td>12</td>
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Abbreviations: MM, mixed methanogenic suspended growth culture; EHM, enriched methanogenic suspended growth culture; MM-B, MM-inoculated biocathode; EHM-B, EHM-inoculated biocathode. Known roles of related bacteria: carbohydrate fermenter (CF); amino acid fermenter (AAF); hydrogen producer (HP); hydrogen scavenger (HS); acetogen (Ac); exoelectrogen (EE); mediator producer (MP); implicated in biofilm formation (BF); found in anaode biofilm (AB); found in cathode biofilm (CB); unknown function (Unk).

bacteria, Thermotogae, and Actinobacteria were enriched in the EHM suspended growth culture, relative to the MM culture. Similarly, Bacteroidetes and Proteobacteria were enriched following biofilm development in both the MM-biocathode and EHM-biocathode. However, Thermotogae increased in relative abundance between the MM culture (16%) and MM-biocathode (24%) but decreased from 53% in the EHM culture to below detection in the EHM-biocathode.

Bacteroidetes, which increased in relative abundance following H₂ and CO₂ enrichment and cathode biofilm formation, are capable of utilizing many different complex polysaccharides and are highly adaptable in changing environments due to their ability to widely regulate gene expression. While complex substrates were not added to the EHM culture or MM- and EHM-biocathodes, lysed microbial cells release organic substrates, which may be utilized by Bacteroidetes. Phylogenotypes in the EHM suspended growth culture and MM-biocathode biofilm were most closely related to Bacteroides sp. strain Z4 (Table 3). A similar strain, Bacteroides sp. W7, has previously been isolated from an anode biofilm. In the MM-biocathode, the composition of Bacteroidetes was similar to that of the MM suspended growth culture Bacteroidetes, indicating the enrichment primarily affected the abundance of Bacteroidetes as a whole relative to total Bacteria. In contrast, Bacteroidetes in the EHM-biocathode were different in both bacterial relative abundance and composition from the EHM suspended growth culture. The EHM suspended growth culture Bacteroidetes consisted predominantly of two phylogenotypes: Bacteroides spp. (49%) and Parabacteroides spp. (40%). Following biofilm development in the EHM-biocathode, a phylogenotype similar to Proteiniphilum sp. made up 98% of Bacteroidetes. In fact, this phylogenotype alone made up 43% of all detected Bacteria in the EHM cathode biofilm yet was undetected in the EHM suspended growth culture used as inoculum (Table 3). In contrast, the Proteiniphilum sp. was not well-studied, although two strains have been isolated from the granular sludge of an upflow anaerobic sludge blanket reactor treating brewery wastewater. Proteiniphilum sp. is known to ferment amino acids and anaerobically degrade polycyclic aromatic hydrocarbons (PAHs). Considering its substantial presence in the EHM-biocathode, Proteiniphilum spp. is a noteworthy candidate for future study as to its role in the cathode biofilm community.
Proteobacteria were also detected with a greater relative abundance in the EHM culture than the MM culture and were also present at a greater relative abundance in the two bioanodes than in their respective inocula (Table 3 and Figure 3). Members of the phylum Proteobacteria are highly diverse in their morphology, phenotype, and metabolism, with no one class serving as a model of the entire phylum.49 Because the Proteobacteria phylum consists of such diverse classes, a further analysis of the relative class abundance was conducted (Text S4 and Figure S12). The most-abundant Proteobacteria in the MM culture were δ-Proteobacteria, while in the EHM culture, γ-Proteobacteria were the most abundant, each representing 64% of their respective Proteobacteria. The MM and EHM cathode biofilms contained more α-Proteobacteria, β-Proteobacteria, and Campylobacterales and less δ-Proteobacteria than in their respective MM and EHM suspended culture inocula. However, γ-Proteobacteria, which were enriched in the MM-biocathode, declined between the EHM inoculum and the EHM-biocathode.

In the EHM-biocathode, a phylotype was identified that was most closely related to Ochrobactrum anthropi, a species of α-Proteobacteria that has been reported to have exoelectrogenic properties and was isolated from an MFC anode.60 A pair of phylotypes related to β-Proteobacteria species Propionibivrio limicola were also identified in the EHM-biocathode. P. limicola is an aerotolerant anaerobe known to ferment hydroaromatic compounds, producing only acetate and propionate. However, P. limicola does not ferment sugars, amino acids, or aromatic compounds.61 The EHM-biocathode also had a phylotype related to Thiomonas intermedia strain K12, a species capable of fermenting tetrathionate under anoxic conditions or oxidizing sulfur species under aerobic conditions.62 A relative, Thiomonas arsenivorans, is known to excrete exopolymeric substances that enhance biofilm stability.63 A total of three phylotypes of γ-Proteobacteria were also observed in the EHM-biocathode, most closely related to Citrobacter amalonaticus, Pseudomonas aeruginosa, and Pseudomonas grimonii. Several Citrobacter species are exoelectrogens capable of using a wide range of substrates.64,65 P. aeruginosa has been isolated from the microbial community of a MFC anode, found to produce pyocyanin, a redox mediator that facilitated electrochemical activity.66 P. aeruginosa may also produce extracellular polymeric substances that assist in biofilm formation.67 However, less is known about the related P. grimonii.68

While Proteobacteria were enriched in the EHM-biocathode, Spirochaetes were similarly enriched in the MM-biocathode to 35% of total detected Bacteria. In contrast, Spirochaetes represented less than 1% of the total detected Bacteria in the MM culture, EHM culture, and EHM-biocathode. Spirochaetes are Bacteria that utilize carbohydrates and produce H2 and CO2, along with other fermentation products such as acetate, lactate, and ethanol.69,70 Spirochaetes are normally low in abundance in most anaerobic reactors but have been found to persist under low-substrate conditions because of several mechanisms, including nutrient-seeking chemotaxis and the ability to derive energy from polymeric storage molecules, amino acids, or intracellular RNA.71 It is not likely that Spirochaetes were enriched in the MM-biocathode due to electrochemical conditions because a similar enrichment was not observed with the EHM-biocathode. Rather, it is probable that in the EHM-biocathode, Spirochaetes were outcompeted by other Bacteria such as Proteobacteria, which were more abundant in the EHM suspended growth culture than in the MM culture and include exoelectrogenic species. In the MM-biocathode, low substrate concentrations from cell lysis products and the relatively lower abundance of Proteobacteria in the MM suspended growth culture may have contributed to the enrichment of Spirochaetes. One phylotype, most closely related to Treponema spp., represented 34% of total Bacteria in the MM-biocathode. Treponema spp. are acetogens that use H2 and CO2,69,72 and could theoretically divert electron equivalents away from the desired product, CH4. Therefore, the substantial presence of acetogens in the MM-biocathode biofilm may have contributed to lower CH4 production, relative to the EHM-biocathode.

Thermotogae increased in relative abundance between the MM and EHM suspended growth cultures and was the most abundant phylum in the EHM culture (53%). Despite the high abundance in the EHM culture, Thermotogae were undetected in the EHM-biocathode. In contrast, the relative abundance of Thermotogae increased between the MM culture (16%) and MM-biocathode (24%). Thermotogae are anaerobic Bacteria that ferment a wide array of carbohydrates and amino acids, known to produce H2,64–67 While most known Thermotogae are thermophilic, some, such as Mesotoga prima, are mesophilic and grow at the temperature conditions of this study.67 Indeed, phylotypes most closely related to M. prima represented 31% and 99% of the total Thermotogae detected in the MM and EHM cultures, respectively. Furthermore, in the EHM culture, M. prima phylotypes represented 52% of the total Bacteria (Table 3), indicating H2 and CO2 enrichment conditions favored M. prima growth. MM-biocathode cultures also appeared to favor M. prima growth (20% relative abundance; 84% of total Thermotogae), but this phylotype was not detected in the EHM-biocathode.

Actinobacteria were slightly more abundant in the EHM and EHM-biocathode cultures than in the MM and MM-biocathode cultures (Figure 3). Phylotypes related to Actinomycyes georgiae were identified in the MM, EHM, and MM-biocathode cultures. A. georgiae is a carbohydrate fermenter,68 and similar species have been implicated in biofilm formation processes.69 In contrast, the EHM-biocathode had a phylotype most closely related to Mycobacterium frederiksenbergense, a PAH degrader.69 A relative, Mycobacterium sp. LB501T, was capable of growing on solid anthracene as a sole carbon source.71

Firmicutes had a similar relative abundance in each suspended growth culture and a similar, albeit smaller, relative abundance in each cathode biofilm. The EHM and MM suspended growth cultures, but not the respective biofilms, contained several phylotypes related to carbohydrate-fermenting, H2-producing Clostridium spp., which are exoelectrogenic.72,73 The MM culture contained a phylotype related to Lactobacillus crispatus, a carbohydrate fermenter and close relative to a species found within a MFC.74,75 A phylotype in the MM-biocathode was most closely related to Trichococcus pasteurii, which has previously been observed in the biofilm of a chromium (Cr(VI)) reduction cathode.76

The relative abundance of total identified OTUs that were closely related to a bacterial species with one of several known functions was presented in a heatmap for the EHM, MM, and bioanode cultures (Figure S13). While the function of many bacterial species within a methanogenic bioanode is not clear, this analysis suggests that amino acid fermenters, exoelectrogens, hydrocarbon degraders, and redox mediator producers may be the most beneficial to methanogenic bioanode performance. Further study is needed to understand how the
members of the biocathode microbial communities interact to influence CH₄ production. By understanding how to select for beneficial biocathode Bacteria, more-effective enrichment techniques may be developed to enhance biocathode CH₄ production for use in BES-based biogas upgrading.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04112.

Additional experimental details. Tables showing bacterial composition and closest GenBank match and relative abundance of identified bacterial OTUs. Figures showing headspace gas pressures, gas composition, time courses, cyclic voltammetry scans, rarefaction curves, PCA plots, redundancy and canonical correspondence analysis plots, phylogenetic trees, relative abundances of classes, and a relative abundance heatmap of OTUs. (PDF)

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**Notes**

The authors declare no competing financial interest.

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