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Methanobacterium Dominates Biocathodic Archaeal Communities in Methanogenic Microbial Electrolysis Cells

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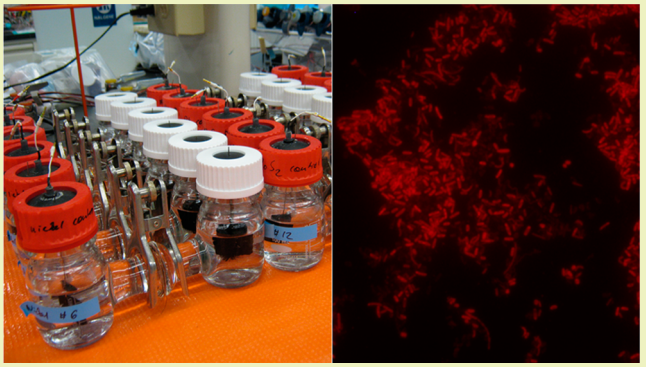
Supporting Information

ABSTRACT: Methane is the primary end product from cathodic current in microbial electrolysis cells (MECs) in the absence of methanogenic inhibitors, but little is known about the archaeal communities that develop in these systems. MECs containing cathodes made from different materials (carbon brushes, or plain graphite blocks or blocks coated with carbon black and platinum, stainless steel, nickel, ferrihydrite, magnetite, iron sulfide, or molybdenum disulfide) were inoculated with anaerobic digester sludge and acclimated at a set potential of −600 mV (versus a standard hydrogen electrode). The archaeal communities on all cathodes, except those coated with platinum, were predominated by *Methanobacterium* (median 97% of archaea). Cathodes with platinum contained mainly archaea most similar to *Methanobrevibacter*. Neither of these methanogens were abundant (<0.1% of archaea) in the inoculum, and therefore their high abundance on the cathode resulted from selective enrichment. In contrast, bacterial communities on the cathode were more diverse, containing primarily *δ-Proteobacteria* (41% of bacteria). The lack of a consistent bacterial genus on the cathodes indicated that there was no similarly selective enrichment of bacteria on the cathode. These results suggest that the genus *Methanobacterium* was primarily responsible for methane production in MECs when cathodes lack efficient catalysts for hydrogen gas evolution.



KEYWORDS: *Electrotroph, CO_2 valorization, Hydrogenotrophic methanogenesis, Methanobrevibacter, Microbially influenced corrosion, Power-to-gas, Carbon capture and sequestration, Methane catalyst*

INTRODUCTION

The ability of certain microorganisms to donate or accept electrons from electrodes has made it possible to develop different microbial electrochemical technologies, such as microbial fuel cells (MFCs) for electrical power generation,[1] and microbial electrolysis cell (MECs) for biofuel production.[2] Many bacterial genera are capable of electrical current generation in these bioelectrochemical systems,[3] but high current densities are associated with the presence of *Geobacter* species.[4,5] When MFCs or MECs were fed acetate as the main source of carbon, anode biofilms become enriched with bacteria highly related to the genus *Geobacter*.[6−8] Much less is known about the microorganisms that can develop on the cathodes in these bioelectrochemical systems. In MECs, energy is added using an external source of power to make it possible to evolve hydrogen gas from the cathode. However, when the cathode is exposed to a diverse mixture of microorganisms, the main gaseous product is methane.[9−11] Thus, MECs can be used as a method to convert electricity into hydrogen or methane gases. Although methane has a lower energy density than hydrogen, it is easier to store and transport it. If a renewable source of energy is used, such as solar or wind energy, then an MEC can be used as a sustainable method to convert electricity into a transportable fuel.

The archaea that develop on cathodes in MECs are usually hydrogenotrophic rather than acetoclastic methanogens. Two-chamber MECs have a membrane separating the electrodes, and therefore it is possible to cultivate microbes on the cathode under conditions that are unaffected by solutions or microorganisms on the anode. The methanogen first identified to be most predominant on the cathode of a methanogenic two-chamber MEC was most closely related to *Methanobacterium*

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palustre.[12] Another hydrogenotrophic methanogen of the same genus, *Methanobacterium* strain IM1, that facilitated iron corrosion under methanogenic conditions,[13] was also recently shown to produce methane using the cathode in a two-chamber MEC.[14] A detailed taxonomic classification of this methanogen was not conclusive, as this isolate was also related to the genus *Methanobrevibacter*.[13] In single-chamber MECs both the anode and cathode are exposed to the same electrolyte, and therefore the microorganisms on the cathode are exposed to the organic substrate in the medium used for growing anode bacteria. Even under these conditions, single-chamber MECs fed acetate contained cathodes enriched with hydrogenotrophic methanogens most similar to the genera *Methanobacterium* and *Methanobrevibacter*, suggesting that methane was not derived from acetate.[6,15] Notably, hydrogenotrophic methanogens were highly enriched on cathodes in single-chamber MECs even when the inoculum contained almost exclusively acetoclastic *Methanosaeta*.[6] All other reports on nonthermophilic MECs have identified *Methanobacterium* or *Methanobrevibacter* as the predominant archaea.[16,17] Other, less abundant methanogens identified in these MECs were *Methanocorpusculum*, *Methanosarcina*, and *Methanoculleus* species.[17,18]

The materials,[19−21] surface area,[22] and set cathode potentials[20] are known to affect abiotic rates of hydrogen gas production, and it is unclear to what extent these reactor materials or operational conditions contribute to the enrichment of hydrogenotrophic methanogens. Methane production using ten different cathode materials was recently examined in the absence of organic substrates using two-chamber MECs.[23] Rates of methane production were only consistent with abiotic measurements of hydrogen gas production when platinum was used as a catalyst to reduce the cathode overpotential for hydrogen gas production. However, abiotic hydrogen gas production was too low to explain methanogenesis rates. To determine whether there were different genera of methanogens associated with these various materials, we examined the archaeal communities that developed on those cathodes, as well as on new cathodes following transfer of the solution or biofilms from these MECs into new reactors. The bacterial communities present on the cathodes were also examined to determine whether there was any consistent relationship between the development of archaeal and bacterial communities.

MATERIALS AND METHODS

Operation of MECs. Two-chamber MECs were operated in fed-batch mode for several months (6−7 batch cycles, with 4−5 weeks per cycle, as previously reported.[23] A potentiostat (MPG2, Bio-Logic, Knoxville, TN, USA) was used to set the cathode potential to −600 mV (vs a standard hydrogen electrode, SHE). Duplicate reactors were incubated at 30 °C in the dark without shaking or stirring. The cathodes were originally inoculated with 1% (v/v) anaerobic digester (AD) sludge from the Pennsylvania State University wastewater treatment plant. The two chambers (100 mL electrolyte each) were separated by a Nafion membrane. All anodes were carbon fiber brushes (4 × 4 cm, 740 m^2 effective surface) wound using a titanium wire current collector. Cathodes were made from bare graphite blocks (2 × 2 × 0.32 cm), graphite blocks coated only with carbon black and a Nafion binder, or blocks additionally containing (10% w/w): platinum, stainless steel, nickel, ferrihydrite, magnetite, iron sulfide (FeS), or molybdenum disulfide (MoS_2). Cathodes were also tested that were identical to the carbon fiber brushes used as the anodes. Controls (single reactors) were constructed and inoculated as described above, except they were operated under open circuit conditions. A bicarbonate medium (30 mM) was used at a pH of 7 prior to use.

New reactors were inoculated 1/10 using effluent from the existing MECs (parent reactors) into fresh reactors containing fresh medium (generation 1). Following operation of these reactors, a second set of new reactors was inoculated (generation 2) using either effluent or material scraped-off the cathodes of the generation 1 reactors. Gas production was examined weekly for the generation 1 and 2 reactors, and compared to that previously reported for the parent reactors.

DNA Extraction and Microbial Community Analysis. Cells were collected by scraping the electrode surfaces using sterile scalpels or by using 0.5 mL of the catholyte. For the brush cathodes, the titanium lead was unwound and fibers were removed using sterile tweezers. Half the fibers were used for extraction. PowerSoil DNA Isolation kits (MoBio Laboratories, Inc., Carlsbad, CA, USA) were used according to the manufacturer's instructions. Extracted DNA of the parent generation was preamplified for pyrosequencing using the 16S rRNA gene primers 341F (5′-CCTAYGGGGYGCASCAG-3′) and 1000R (5′-GAGARGWRGTGCATGGCC-3′) for archaea[24] as well as 27F (5′-AGAGTTTGATCCTGGCTCAG-3′)[25] and 519R (5′-GTNTTACNGCGGCKGCTG-3′)[26] for bacteria. For the generation 2, DNA was preamplified for MiSeq sequencing using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) for archaea and bacteria.[27] Raw read data were processed using the mothur software package version 1.34.4 according to the standard operating procedures[28] and classified against the SILVA database version 119 NR99.[29] Individual taxa that fell below the 10% abundance cutoff are not displayed. They may sum up to more than 10% as more than one taxon may have fallen below that cutoff. Raw read files were deposited online in the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) database under the BioProject accession number PRJNA256170. Ecological indices were calculated using the mothur software package as well. Two DNA extracts of low archaeal Shannon diversity indices (platinum cathode 1, graphite cathode 2) were selected for cloning to obtain nearly full-length 16S fragments for better classification and phylogenetic tree construction. A pGEM-T Easy cloning kit with JM109 *Escherichia coli* chemically competent cells (Promega, Madison, WI, USA) was used to obtain fragments of about 1.4 kb size. Because the employed primer pair A2Fa (5′-TTCCGGTTGATCCYGCCGGA-3′)[30] and U1406R (5′-GACGGGCGGTGTGTRCA-3′)[31] also amplified unspecific DNA, 1.4 kb fragments were excised and extracted from agarose gels prior to cloning. The PCR program was cycled 30 times with the following steps: 95 °C 0.5 min, 57 °C 1 min, and 72 °C 1 min. Cloned fragments of the correct size were Sanger-sequenced. The obtained sequences had a length between 1299 and 1350 base pairs. After alignment using the SINA online aligner,[32] the sequences were incorporated into the existing SILVA tree version 115 NR99 using the "quick parsimony add" function of the ARB software package.[29] Chimeric sequences were removed using ARB's chimera detection tool. For species level classification distance matrices were calculated using Felsenstein for DNA correction. All cloned 16S rRNA genes had >98% similarity with their next neighbor. Sequences obtained from cloning were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KM235284−KM235287 for clones obtained from platinum cathode 1 and KM235290 for the graphite cathode 2.

The same DNA extracts were used to absolutely quantify microbial communities by quantitative PCR (qPCR) targeting archaeal[33] and bacterial[34] 16S rRNA genes. Numbers of 16S rRNA genes were used to approximate cell numbers. General *mcrA* genes were quantified using a qPCR assay published previously.[35] An Applied Biosystems (Grand Island, NY, USA) StepOne Plus instrument was used for qPCR.

Microscopy. Microscopic images of cells were prepared by using a fluorescent *in situ* hybridization (FISH) probe (MB1174, 5′-TACCGTCGTCCACTCCTTCCTC-3′).[12] This probe binds to *Methanobacterium*, *Methanobrevibacter*, and *Methanothermobacter* 16S rRNA. For staining cells not specific for this probe, we used 4′,6-diamidino-2-phenylindole (DAPI). The resulting two images were overlain to obtain a composite image. For bright field images, cells were grown on an indium tin oxide (ITO) coated glass slide used as cathode.

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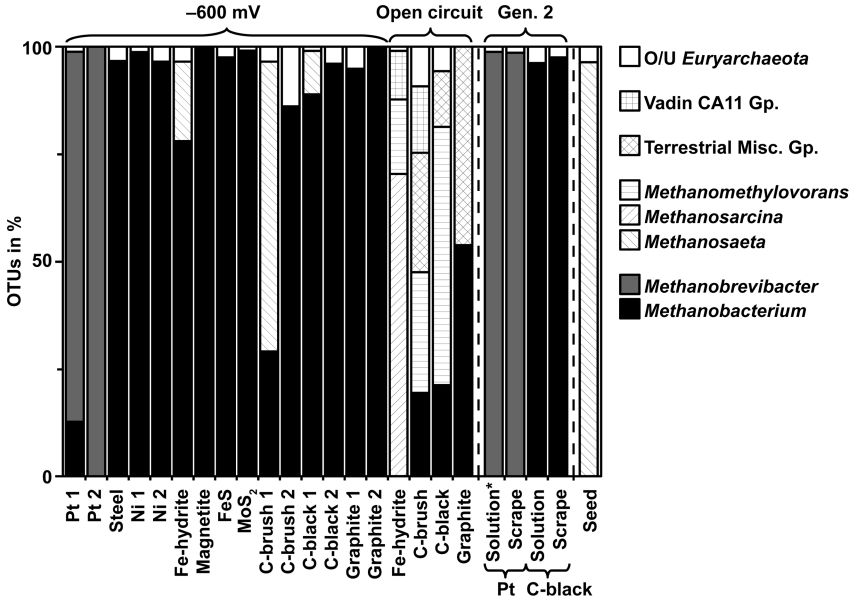

Figure 1. Archaeal communities that populated the surfaces of biocathodes in the parent generation reactors. Vadin group reference;[39] * "Solution" and "Scrape" indicate the source of inoculum of generation 2; OTU, operational taxonomic unit; O/U, other (<10% each) and unclassified OTUs; gen., generation; Gp., group; Misc., miscellaneous; Fe-hydrite, ferrihydrite.

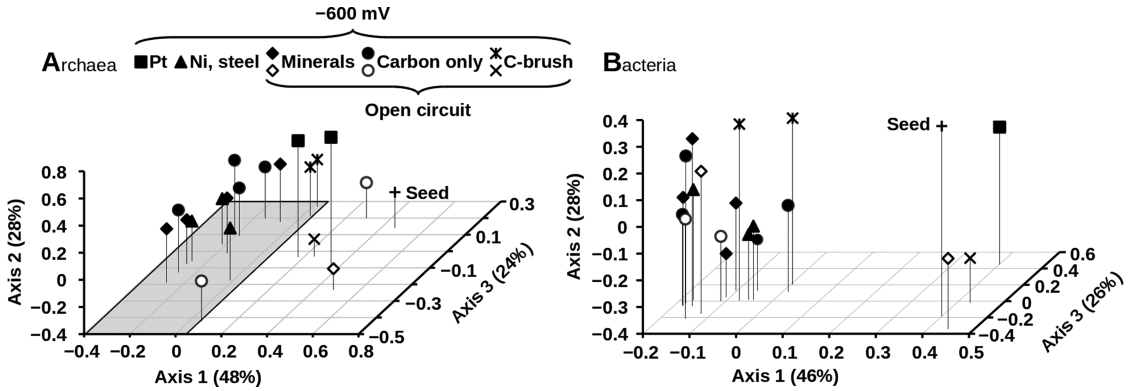

Figure 2. Principal component analysis archaea (A) and bacteria (B) of the parent generation. The first three dimensions in (axes 1−3) account for 42% (archaea) or 28% (bacteria) of the community variation. The variation percentages of the three axes are relative to the sum of the forst three axes (100%). Axis 1 of the archaea correlates with the condition poised (filled symbols) vs open circuit (open symbols). The shaded area highlights this correlation where *Methanobacterium* was the dominant genus (see also Figure 1).

Environmental scanning fluorescent microscopic (ESEM) images were produced using a FEI Quanta 200 instrument (FEI company, Hillsboro, OR, USA) using standard ESEM preparation procedures.

Gas Analysis. Methane concentrations in the headspace were measured using a gas chromatograph SRI 310C (SRI Instruments, Torrance, CA, USA) equipped with a 6-foot long mol-sieve column running continuously at an oven temperature of 80 °C. The methane production rate was calculated using the linear regression of weekly measurements over a period of 4−5 weeks (defined as a single fed-batch cycle). Rates of the parent generation (before the potentials were split) were correlated to cell numbers using the analysis of variance (ANOVA) and regression tools included in the Microsoft Excel software package.

RESULTS

Archaeal Communities. Microbial communities of the cathodes of all MECs except those containing platinum, and one of the carbon brush duplicates, all contained archaea belonging to the genus *Methanobacterium* (median of 97% in abundance of all archaea; Figure 1). The one different carbon brush reactor (C-brush 1) that did not have a high percentage of *Methanobacterium* instead contained mostly *Methanosaeta* (67%), and this cathode was previously found to also have lower methane production rates relative to the duplicate reactor (C-brush 2; Supporting Information Table S1). *Methanosaeta* was also found on one of the duplicates of the ferrihydrite cathodes (19%), and plain carbon black cathodes (10%; Figure 1). The biofilms of the platinum cathodes were comprised primarily of the genus *Methanobrevibacter* (86% for Pt 1, and 100% for Pt 2), with one of the platinum cathodes also containing a smaller amount of *Methanobacterium* (13% for Pt 1). The archaeal communities of these electrodes were different from the inoculum, which contained primarily the genus *Methanosaeta* (95%). The composition of the archaeal community was relatively stable on the cathodes throughout the subsequent transfers independent of the inoculum source (solution or scraped off cathode material).

The diversity of archaea in the reactors operated at a set potential increased in all reactors relative to the initial inoculum, as shown by an average Shannon index of 1.0, compared to 0.4 for the inoculum. Controls operated under

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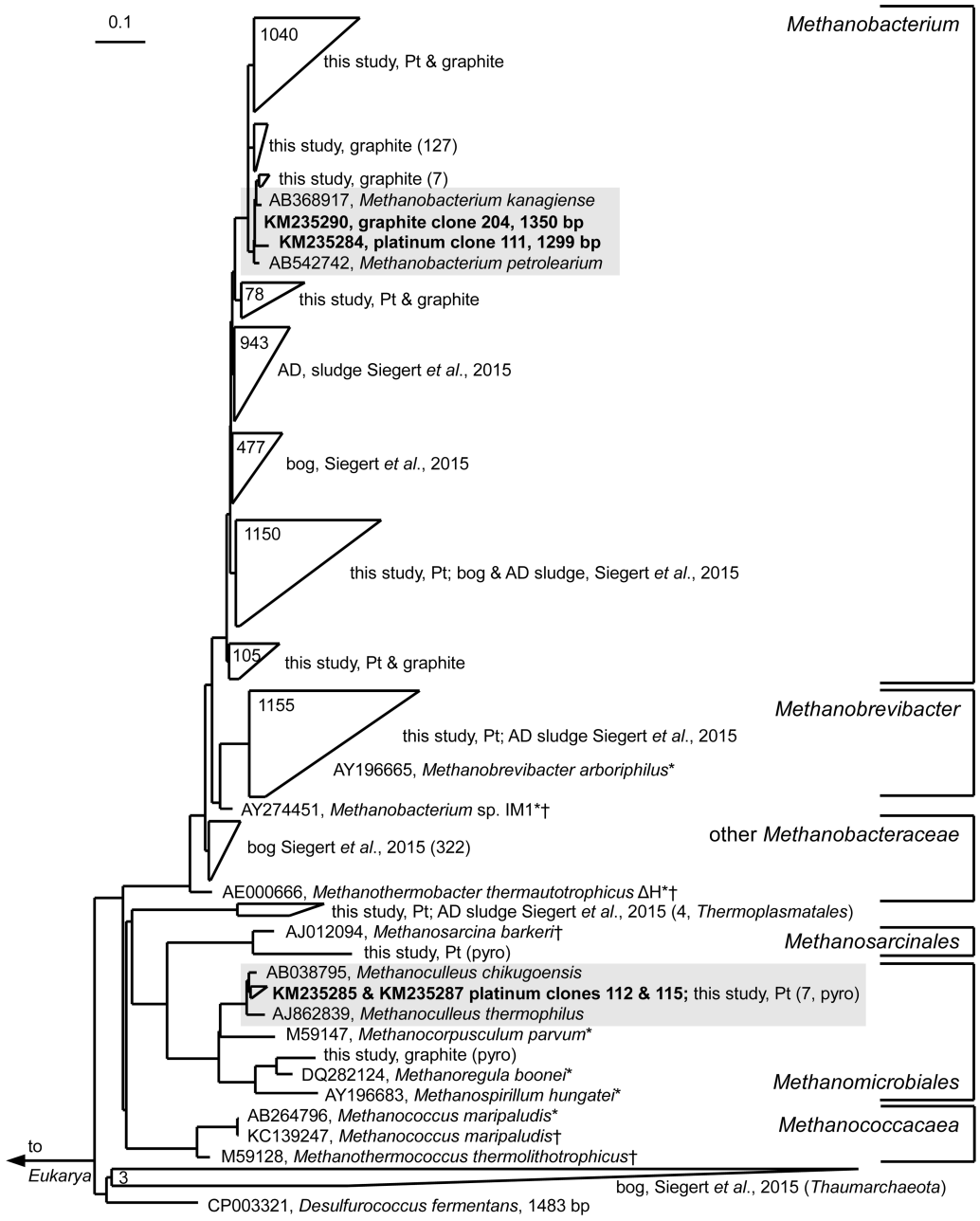

Figure 3. Maximum parsimony tree based on the SILVA tree 115[29] with sequences obtained from cloning (bold, shaded areas) as well as pyrosequencing of DNA extracted from cathodes of the parent generation. Asterisks (*) indicate MEC associated strains and † iron corroders (references are *Methanobacterium palustre, Methanocorpusculum parvum, Methanoregula boonei;*[12] *Methanobrevibacter abroriphilus;*[17] *Methanobacterium* IM1;[13,14] *Methanothermobacter thermautotrophicus;*[47,48] *Methanosarcina barkeri, Methanothermococcus thermolithotrophicus;*[47] *Methanococcus maripaludis;*[49,50]). "Sludge" and "bog" name sequences were from cathodes of single-chamber MECs published previously.[6] Numbers inside clusters or in brackets indicate the amount of sequences obtained from pyrosequencing (pyro).

open circuit conditions harbored an even more diverse archaeal community (median of Shannon diversity index of 1.4) than reactors operated at a fixed potential. A principal component analysis revealed that the dominance of *Methanobacterium* correlated with set potential conditions in the first dimension (*x*-axis, Figure 2A). This dimension also accounted for 46% of the variation between data points. For the platinum cathodes, which were predominantly *Methanobrevibacter*, a distinct cluster in the second dimension (*y*-axis) formed which was separated also in the first dimension compared to open circuit reactors and the *Methanobacterium* dominated clusters. In the generation 2 reactors, the average archaeal diversity of the platinum cathodes remained constant at 0.3 whereas for the carbon black cathodes it slightly decreased to 1.0 (parent: 1.2).

The numbers of archaeal and bacterial cells in the reactors were determined using qPCR. The absolute number of archaeal cells in the total populations (11 cm^2 cathode + 100 mL catholyte) at a median of 2×10^8 cells per reactor was the same as the 2×10^8 cells in 1 mL of seed used for inoculation (Supporting Information Figure S1). The relative increase from 4% archaeal cells in the inoculum, to a median of 41% at the end of the parent generation tests (reactors originally inoculated with AD sludge), was largely due to a decrease in the total number of bacterial cells in the reactors (Supporting

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Information Figure S2). Of the total population, more archaea were usually found on the cathodes (43% of all cells) whereas they were little less abundant in the solution (31% of all cells). The total number of archaeal cells was about 10^8 (median) on the cathodes and 7×10^7 in the catholyte, resulting in a median cell ratio of cathode:catholyte of 2:1 (Supporting Information Figure S1). However, this ratio varied for the different cathode materials. The cathode:catholyte ratios ranged from ∼18:1 for FeS (1×10^8 cathode, 7×10^6 catholyte) and ∼16:1 for 10% steel (2×10^8 cathode, 1×10^7 catholyte), to ∼1:1 for the graphite reactors (8×10^7 cathode and catholyte). The only exceptions were one molybdenum disulfide cathode with 1:5 (3×10^7 cathode, 1.5×10^8 catholyte) and a plain carbon black cathode with 1:20 (1.5×10^7 cathode, 3×10^8 catholyte). The 10% platinum electrodes had the largest ratio of ∼120:1 compared with the other cathodes, and the carbon brush electrode was 2:1, within the median range. On the platinum cathodes the absolute number of archaeal cells was the highest of all reactors with 5×10^9. Electrodes operated at a set potential had $\sim 10^4$–10^5 times more archaeal cells than open circuit controls (Supporting Information Figure S1). In generation 2 tests, the fraction of archaeal cells remained approximately stable with an average of 58% on the platinum cathodes (parent 66%) and 25% on carbon black (parent 31) cathodes based on MiSeq data. Total cell numbers were not determined for the generation 2 reactors.

There was an apparent linear correlation between archaeal cell numbers and methane production ($R^2 = 0.82$, $p = 4 \times 10^{-8}$; Supporting Information Figure S3). However, this correlation was primarily due to the much higher methane rates for the reactors with the platinum cathodes compared to all other materials, as there were no significant correlation for the other cathodes and methane generation rates if the platinum data were removed from the analysis ($R^2 = 0.13$, $p = 0.1$).

Nearly complete (∼1300 bp) 16S rRNA gene sequences were obtained from clone libraries prepared from communities of one plain graphite cathode (*Methanobacterium* majority) and one platinum cathode (*Methanobacterium* minority). These sequences, as well as short >400 bp fragments obtained from pyrosequencing of biofilms in these two reactors, were incorporated into an existing maximum parsimony tree based on the SILVA database version NR 115 (Figure 3). 16S rRNA gene clone sequences derived from the platinum and graphite cathodes were closely related (>98% similarity) to *Methanobacterium* kanagiense (accession number AB368917, rice field isolate)[36] and *Methanobacterium petrolearium* (accession number AB542742, petroleum tank isolate).[37] *Methanobrevibacter* sequences could not be captured using the primers employed here.[38] The *Methanobacterium* cells were rod shaped, based on FISH staining of cells obtained from a plain carbon black electrode using a *Methanobacteraceae*-specific probe (Figure 4). No cells were observed using ESEM on the cathodes of the open circuit controls.

Bacterial Communities. There was no clearly predominant bacterial genus on the non-platinum cathodes in set potential MECs (Figure 5), although all cathodes (except platinum) were more enriched in *δ-Proteobacteria* than other bacteria. One platinum cathode was populated mainly by *Bacteroidetes*, comprising about 50% the Vadin BC27 group.[39] On 12 out of 15 set potential cathodes, *Desulfuromonas* was found in higher percentages (median of 38% on these 12). The plain graphite electrode containing *Desulfuromonas* performed better as it had higher methane production rates than the other

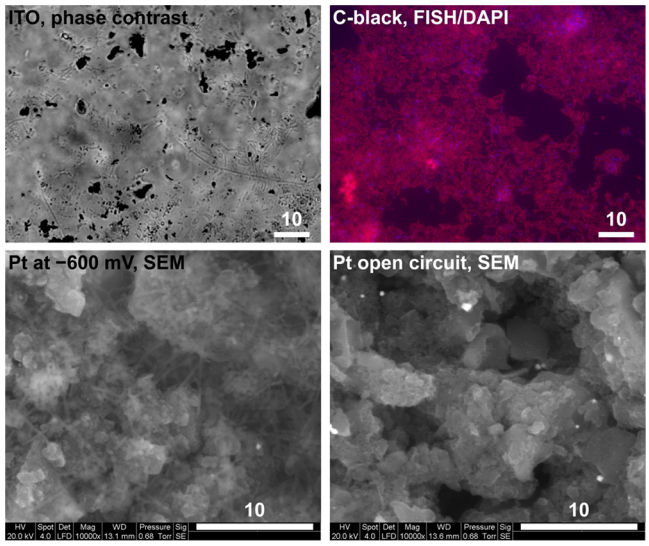

Figure 4. (A) Brightfield and (B) FISH/DAPI microscopic, and (C, D) SEM images of methanogenic cathodes poised at −600 mV. The white scales are 10 μm. Top left, an indium tin oxide (ITO) coated glass slide was used as electrode. Top right, carbon black particles were scraped off a neat carbon black electrode of generation 1 and stained with 4′,6-diamidino-2-phenylindole (DAPI, blue color) and a *Methanobacterium*-specific FISH probe (red color). Bottom left, environmental scanning electron microscopic (ESEM) image of a poised potential electrode containing platinum particles (bright white spots). Bottom right, the same material but incubated in open circuit. The white bars indicate 10 μm length.

graphite electrode, but the opposite was observed for the two nickel-doped electrodes (Supporting Information Table S1). The better performing nickel electrode contained *Desulfovibrio*, a close relative of *Desulfuromonas*, which was also absent from the worse performing plain graphite electrode (Figure 5). Both genera were also present in the generation 2 carbon reactors. A number of unclassified bacteria were observed in the seed, as well as in many set potential reactors.

The Shannon diversity index of bacteria in the inoculum was very high (5.00 ± 0.08) relative to those for bacteria on the different cathodes, consistent with the finding that there were many genera that were present in numbers below the 10% abundance cutoff. The platinum cathode had a bacterial diversity index of 2.03 ± 0.03, which was within the range of the other cathodes that varied from 0.79 ± 0.06 (steel cathode) to 3.73 ± 0.07 (plain graphite cathode). Overall, the set potential MECs had a median Shannon index of 3.2, which was similar to that of the open circuit reactors (3.1). The principle component analysis of the communities did not reveal any clustering of the communities based on set potential or open circuit conditions, or types of cathode materials (Figure 2B).

The total number of bacterial cells in the reactors (except platinum) decreased relative to the inoculum ($\sim 10^{10}$ cells mL^{-1}) to a median of 4×10^8 cells (Supporting Information Figure S2). For platinum cathodes, the number of cells was 4×10^9. The partitioning of bacterial cells between cathode surface and the catholyte was similar to that observed in the archaeal community. The median cathode:catholyte ratio was 2:1 (2×10^8 cathode, 10^8 catholyte), ranging from ∼13:1 for the graphite cathodes (3×10^8 cathode, 2×10^7 catholyte) and ∼10:1 for the FeS cathodes (3×10^8 cathode, 9×10^7 catholyte) to ∼1:1 for MoS_2 (8×10^7 cathode, 9×10^7 catholyte). For the platinum cathodes, the ratio was ∼3:1 ($3 \times$

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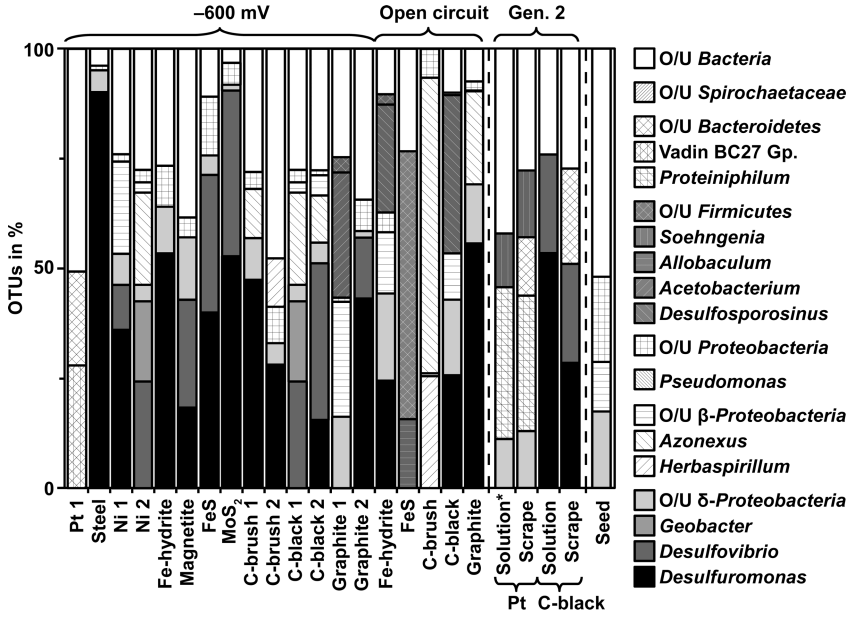

Figure 5. Bacterial communities that populated the surfaces of biocathodes in the parent generation reactors. Vadin group reference;[39] * "Solution" and "Scrape" indicate the source of inoculum of generation 2; OTU, operational taxonomic unit; O/U, other (<10% each) and unclassified OTUs; gen., generation; Gp., group, Fe-hydrite, ferrihydrite.

10^9 cathode, 9×10^8 catholyte) and for the carbon brush it was ~1.5:1 (2×10^8 cathode, 1×10^8 catholyte). Consistent with the archaea results, the ratio for the carbon black cathodes was below 0, at ~1:3 (3×10^7 cathode, 9×10^7 catholyte). Compared to open circuit controls, the set potential reactors contained 1−3 orders of magnitude more cells.

There was a significant correlation between bacterial cell numbers and methane gas production ($R^2 = 0.78$, $p = 3 \times 10^{-7}$), but as observed for archaea, this was primarily due to the platinum cathodes (Supporting Information Figure S3). When platinum was removed from the calculation, there was no significant correlation of cell numbers and methane production ($R^2 = 0.05$, $p = 0.4$).

Gas Production and Coulombic Recoveries. Methane gas production was further monitored for selected cathode materials (platinum, carbon brush, carbon black, and plain graphite) in two serial transfers after the parent generation (generation 1, generation 2). The inoculation strategy, using cathode material versus the catholyte as seed for generation 2, had no impact on performance (Supporting Information Figure S4). Methane production in MECs with the 10% platinum cathodes remained relatively stable during the subsequent transfers, with 250 ± 30 nmol cm^{-3} d^{-1} in the parent MEC, 220 ± 10 in generation 1 (first transfer) and 210 ± 50 nmol cm^{-3} d^{-1} in generation 2 (all rates in detail in Supporting Information Table S1). There was no consistent trend in methane production rates for the carbon brushes with successive transfers as gas production in the parent reactors (110 ± 30 nmol cm^{-3} d^{-1}) decreased with the first transfer (70 ± 40 nmol cm^{-3} d^{-1}) and then increased with the second transfer (290 ± 4 nmol cm^{-3} d^{-1}). The lowest methanogenesis rates measured throughout the entire experiment were obtained using plain carbon black cathodes (31 ± 11 nmol cm^{-3} d^{-1} parent generation, 18 ± 9 nmol cm^{-3} d^{-1} generation 1, 24 ± 10 nmol cm^{-3} d^{-1} generation 2) and graphite electrodes (29 ± 10 nmol cm^{-3} d^{-1} parent generation, 34 ± 14 nmol cm^{-3} d^{-1} generation 1, 17 ± 5 nmol cm^{-3} d^{-1} generation 2).

Coulombic recoveries were initially higher than 100% for some of the parent reactors, which indicated cathode corrosion as previously reported (Supporting Information Table S2).[23] However, the Coulombic recoveries decreased in most cases to around 100% in generation 2 (Supporting Information Figure S5). The only reactors that were always stable in terms of methane production and Coulombic recoveries below 100% were those with platinum cathodes.

DISCUSSION

MECs operated using cathodes that did not contain platinum, a highly efficient hydrogen-forming catalyst, were consistently colonized by methanogens having the greatest similarity to the genus *Methanobacterium* (Figure 1). In contrast, when platinum was used on the cathode, the archaeal community of the reactors was dominated by *Methanobrevibacter*. These two genera are close relatives and belong to the same family of *Methanobacteraceae*. They were the only methanogens detected based on tests using 15 poised potential cathodes, with only three exceptions where *Methanosaeta* were also found in varying amounts (67−10%). These two hydrogenotrophic genera were clearly enriched through operation of the MECs, as only 14 sequences belonging to *Methanobacterium* and 6 to *Methanobrevibacter* out of 20 000 sequences were recovered from analysis of the AD inoculum. The vast majority of the archaea present in the AD inoculum belonged to the genus *Methanosaeta*.

The very high enrichment of either *Methanobacterium* or *Methanobrevibacter* in the cathode communities is remarkable given their low abundance in the seed. Over 5 batch cycles, the number of these two genera in the microbial community increased 10 000-fold from 4×10^5 to 5×10^9 for 10% platinum and 500-fold to 2×10^8 for the rest. Because of the decrease in the numbers of bacteria on the cathode, the relative fraction of archaea in the total population increased 10-fold (Supporting Information Figure S2). *Methanobacterium* and to a lesser extent also *Methanobrevibacter*, have previously been found to

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be the predominant genera in numerous MEC studies confirming the reproducibility of this convergence on MEC cathodes.[6,9,12,15,16,40] These results show that these two genera may be uniquely adapted for growth in methanogenic MECs compared to conditions that favored other methanogens, such as those in ADs. The AD inoculum contained mostly *Methanosaeta*, which was expected as acetoclastic methanogens usually predominate over hydrogenotrophic methanogens in these systems.[35] *Methanobacterium* and *Methanobrevibacter*, however, are both hydrogentrophic methanogens.

There was no hydrogen gas detected (detection limit ~10 nmol mL^{-1}) when the reactors were originally inoculated with AD sludge, except for reactors containing a platinum cathode catalyst, as noted in our previous study of hydrogen and methane gas production in these reactors.[6] In the following two generations, no hydrogen gas was detected except at the start of batch cycle 3 and the following cycles (for platinum, beginning with batch cycle 1) where low concentrations of hydrogen were found (between 10 and 50 nmol mL^{-1}) the day after the new batch cycle was started. Hydrogen was not subsequently detected until a new cycle was started. Although abiotic hydrogen production rates were much lower than those measured for all of the cathode materials except platinum,[23] it is possible that association of microorganisms with the cathode surfaces altered the propensity of these materials for hydrogen gas evolution. It was recently shown that addition of certain microorganisms (*Geobacter* or *Methanosarcina*), or their cell debris, to an electrode enhanced abiotic hydrogen gas evolution rates relative to uninoculated controls.[41] The measured rate of 120 nmol mL^{-1} d^{-1} of hydrogen gas from the graphite electrodes in that study corresponds well with the measured rate of methane on graphite here of 30 nmol mL^{-1} d^{-1} (Supporting Information Table S1) as four moles of hydrogen are required per mole of methane produced. Hydrogenases and other redox-active enzymes, their remnants, or degradation products, can adsorb to a cathode surface and release hydrogen at rates orders of magnitude higher than "abiotic" controls.[41,42] Other chemicals, such as acetate, have also been shown to be produced by biocathodes,[43−45] and thus it is not possible in this study to identify the mechanism by which these methanogens use the cathode to produce methane.

Cathodes and anodes of the single-chamber MECs have recently been shown to become dominated by *Methanobacterium* and *Methanobrevibacter* when inoculated with an anaerobic bog sediment or AD sludge.[6] The operation of these single-chamber MECs, however, was different from that used here for the two-chamber systems in several ways. First, a whole-cell voltage of 700 mV was applied to the single-chamber MEC, whereas a set cathode potential of −600 mV was used here for the two-chamber MECs. More negative potentials increase the rate of abiotic hydrogen gas evolution, and in single-chamber MECs the cathode potential will vary based on current production and substrate concentrations. For example, previous single-chamber tests showed that the cathode potential became more negative than −600 mV when a whole-cell voltage was applied using a power source as opposed to a set potential.[46] Thus, the cathode in the single-chamber MECs could easily have reached more negative potentials than the set potential of −600 mV in the two-chamber MEC tests, resulting in an increase in hydrogen gas production rates. This shift to greater abiotic hydrogen gas rates would be consistent with our findings of *Methanobrevibacter* on platinum cathodes with good abiotic hydrogen gas evolution rates, and *Methanobacterium* on poor abiotic hydrogen catalysts. Second, more negative potentials can also catalyze the production of toxic byproducts such as hydroxyl radicals and peroxides. A better adaptation of *Methanobrevibacter* to cope with these compounds may explain their greater abundance as well. Third, another difference between the single- and two-chamber tests was that acetate was added in the single-chamber system to sustain current production from the anode. However, the presence of acetate did not result in a noticeable increase in the abundance of acetoclastic methanogens, such as *Methanosaeta*. Fourth, there was no membrane in the single-chamber system, allowing microorganisms present on one electrode to reach and colonize the other electrode. Even in the absence of the membrane, the methanogens on the both electrodes were similar to that of the cathodes in the two-chamber experiments.[6]

The lack of bacterial clusters in principal component analysis (Figure 2) and the lacking correlation between bacterial cell numbers and performance (Supporting Information Figure S3) suggest that specific genera of bacteria were not directly involved in methane gas production. However, as all bacteria were not removed from the reactor it is still possible that they had some role in either production of intermediates used by the methanogens, or the release of materials that helped to catalyze hydrogen gas production. The role in methane production might not be phylogenically restricted, and thus could perhaps be filled by numerous taxa. Whether bacteria might "pave the way" for methanogens, or whether methanogens alone are sufficient to modify the electrode to enhance methane production rates compared to abiotic and not previously colonized electrodes, remains to be elucidated in future studies.

■ ASSOCIATED CONTENT

Supporting Information

Methane and hydrogen production rates for individual reactors and cycles, cell numbers and their correlation with methane production rates, and Coulombic recoveries at different potentials. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.5b00367.

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Notes

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