BIOENERGY AND BIOFUELS

Analysis of the microbial community of the biocathode of a hydrogen-producing microbial electrolysis cell

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Abstract The microbial electrolysis cell (MEC) is a promising system for hydrogen production. Still, expensive catalysts such as platinum are needed for efficient hydrogen evolution at the cathode. Recently, the possibility to use a biocathode as an alternative for platinum was shown. The microorganisms involved in hydrogen evolution in such systems are not yet identified. We analyzed the microbial community of a mixed culture biocathode that was enriched in an MEC bioanode. This biocathode produced 1.1 A m^{-2} and 0.63 m^3 H$_2$ m^{-3} cathode liquid volume per day. The bacterial population consisted of 46% *Proteobacteria*, 25% *Firmicutes*, 17% *Bacteroidetes*, and 12% related to other phyla. The dominant ribotype belonged to the species *Desulfovibrio vulgaris*. The second major ribotype cluster constituted a novel taxonomic group at the genus level, clustering within uncultured *Firmicutes*. The third cluster belonged to uncultured *Bacteroidetes* and grouped in a taxonomic group from which only clones were described before; most of these clones originated from soil samples. The identified novel taxonomic groups developed under environmentally unusual conditions, and this may point to properties that have not been considered before. A pure culture of *Desulfovibrio* strain G11 inoculated in a cathode of an MEC led to a current development from 0.17 to 0.76 A m^{-2} in 9 days, and hydrogen gas formation was observed. On the basis of the known characteristics of *Desulfovibrio* spp., including its ability to produce hydrogen, we propose a mechanism for hydrogen evolution through *Desulfovibrio* spp. in a biocathode system.

Keywords *Desulfovibrio* G11 · MEC · Hydrogen · Exocellular electron transfer · Sulfate-reducing bacteria

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Introduction

The high-energy demands of our modern society in combination with the foreseeable depletion of fossil fuels call for the development of sustainable, green forms of energy. Biomass or the organic waste from wastewaters is a source of renewable energy. Recent advances in the use of organic matter for energy production include electricity generation in a microbial fuel cell (MFC) (Logan et al. 2006) and the production of hydrogen in a microbial electrolysis cell (MEC) (Liu et al. 2005; Rozendal et al. 2006; Logan et al. 2008). These kinds of systems are still under development, but they show great potential for green energy production.

Both MFC and MEC usually consist of two compartments containing an anode and a cathode separated by an ion exchange membrane (Rozendal et al. 2007). The two electrodes are connected through an electrical circuit. At

the anode, electrochemically active microorganisms are present that consume organic matter and transfer the electrons derived from metabolic processes to the electrode, either by direct or indirect extracellular electron transfer (Ieropoulos 2005; Lovley 2006; Stams et al. 2006; Torres et al. 2009; Lovley and Nevin 2011). An electron acceptor in the cathode liquid enables a current flow from anode to cathode. Typically, oxygen or Fe(III) is used as the electron acceptor in the MFC (Rabaey and Verstraete 2005; Logan and Regan 2006), while in the MEC, protons act as the sole electron acceptor to form hydrogen. For the MEC, a supply of electrical energy is required to make hydrogen gas production possible (Liu et al. 2005; Rozendal et al. 2006).

Acetate is often used as model substrate in MEC systems because it is an end product of fermentation. Theoretically, acetate oxidation yields a potential of −0.29 V (vs. standard hydrogen electrode (SHE), at pH 7, $pH_2 = 1$ bar), while for hydrogen production from protons, a potential of −0.41 V (vs. SHE, at pH 7, $pH_2 = 1$ bar) is required (Liu et al. 2005). Energy is added by applying enough voltage to render an exergonic reaction. Hence, the theoretically applied voltage required for hydrogen gas production in an MEC fed with acetate is 0.12 V. In comparison, for conventional water electrolysis, the theoretically applied voltage needed is 1.2 V at pH 7 (Liu et al. 2005). The lower energy requirement of the MEC makes it an attractive system for hydrogen gas production. In practice, however, a minimum applied voltage of 0.25 V is needed because of several potential losses in the system (Rozendal et al. 2006; Sleutels et al. 2009a, b). The total applied voltage demand in practice is for a great part dependent on the overpotential at the electrodes. The use of a good catalyst can decrease the overpotential significantly (Jeremiasse et al. 2009b). Conventionally, platinum is used as a catalyst for hydrogen gas production (Vetter 1967) and is therefore also applied at MEC cathodes (Rozendal et al. 2006). Because of the high costs and scarcity of platinum, alternative catalysts for hydrogen production are desirable. Microbial cathodes (biocathodes) form an alternative with great prospectives since they are low cost (both electrode material and catalyst) and self-generating. A biocathode can be defined as an electrode from cheap material (e.g., carbon) with a microbial population present at the electrode or in the electrolyte that catalyzes the cathodic reaction. To act as a biocathode in an MEC, microorganisms need to be able to take up electrons from the electrode material and use these electrons to produce hydrogen.

The uptake of electrons from a solid surface or cathode is known from corrosion studies, where metals (e.g., iron) are oxidized by microorganisms that use the electrons from this reaction for metabolic processes (Dinh et al. 2004; Mehanna et al. 2009). Furthermore, in MFCs, biocathodes have been successfully applied to reduce oxygen, fumarate, nitrate, perchlorate, or chlorinated compounds (Huang et al. 2011).

Microorganisms that can produce hydrogen are found in a large variety of environments (Schwartz and Friedrich 2006) and contain hydrogenases that catalyze the reversible reaction $2H^+ + 2e^- \leftrightarrow H_2$. Purified hydrogenases have been successfully used on carbon electrodes as a catalyst for hydrogen production (Vignais et al. 2001; Lojou and Bianco 2004; Lojou 2011; Vincent et al. 2007). The drawback for these systems is that the enzymes are relatively unstable and lose catalytic activity over time. The use of whole cells can help in maintaining enzyme stability. Immobilization of whole *Desulfovibrio vulgaris* cells (well known to contain hydrogenases) on an electrode was successful for hydrogen production, and the process was more stable than with enzymes only (Guiral-Brugna et al. 2001; Lojou et al. 2002). For continuous hydrogen production, the challenge is to generate a biocathode with living cells, able to survive and grow.

The microbial uptake of electrons from a cathode for the production of hydrogen in an MEC was shown for the first time by Rozendal et al. (2008). In their study, an MEC half cell with carbon felt electrodes was started up with a biological anode that was initially fed with acetate and hydrogen. Hexacyanoferrate(III) was reduced at the cathode. When stable anodic current was reached, the acetate and hydrogen supply was stopped, and the polarities of anode and cathode were reversed, resulting in a biocathode and chemical anode. The cathode potential was poised at −0.7 V vs. SHE resulting in an average current of 1.1 A m^{-2} and production of 0.63 m^3 H$_2$ m^{-3} cathode liquid volume per day. A similar setup that was not inoculated served as negative control and produced a current of 0.3 A m^{-2} and 0.08 m^3 H$_2$ m^{-3} cathode liquid volume per day. In the present study, we describe the microbial population present on the graphite felt cathode using scanning electron microscopy, denaturing gradient gel electrophoresis (DGGE), and cloning and sequencing of 16S ribosomal RNA genes.

Material and methods

Microbial electrolysis cell operation and sample collection

The setup and operation of the microbial electrolysis cell was described previously (Rozendal et al. 2008). From this experiment, samples were collected for the current study. The inoculum of this setup was a mixed microbial community previously enriched and sequentially transferred over a period of 4 years in MFC and MEC anodes amended with acetate. The original MFC anode was inoculated with anaerobic sludge from a paper mill wastewater treatment plant (Eerbeek, the Netherlands), anodic effluent from a

molasses-fed MFC, and *Geobacter sulfurreducens* strain PCA (U. Michaelidou, personal communication). Briefly, the system was started as a two-chamber microbial fuel cell fed with a mixture of acetate and hydrogen (at the bio-electrode) and with a mixed hexacyanoferrate(II) and hexacyanoferrate (III) solution as catholyte (at the chemical counter electrode). The electrode material was graphite felt of 6 mm thickness. After 8 days, the substrate for the bio-electrode was changed to bicarbonate and hydrogen. Following stable anodic current production at a bio-electrode potential of −0.2 V (vs. SHE), at day 11 the hydrogen supply was stopped, and the bio-electrode potential was lowered to −0.7 V. This resulted in consumption of electrons and production of hydrogen at the bio-electrode, now functional as biocathode. The current developed from 0.3 to 1.2 A m^{-2} projected electrode surface area over a period of 13 days. After this period, bicarbonate addition was stopped. In this layout, i.e., a biocathode with a chemical hexacyanoferrate(II) oxidizing anode, the system was run for over 40 days without addition of any carbon source. Subsequently, the system was disassembled, and samples were collected for our study. Graphite felt electrode material with attached biomass was cut into 7-mm-diameter disks originating from four different locations of the electrode (1) influent site: where medium enters the cathode compartment, (2) middle of the electrode, (3) effluent site: where medium exits the cathode compartment (4) non-flow site: part of electrode which did not have direct contact with the flow path of the medium. Additional liquid samples (2 ml) were taken from the inoculum material (I) and from the effluent (E) at the end of the run. Samples for DNA extraction were stored at −20°C until use.

Strains and cultivation

Desulfovibrio strain G11 (DSM 7057) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). It was cultured with 10 mM lactate as energy and carbon source and 15 mM sulfate as electron acceptor in anaerobic medium with H$_2$ headspace as additional energy source. The medium consisted of (millimolars) MgCl$_2$, 1; CaCl$_2$, 0.7; (NH$_4$)$_2$SO$_4$, 0.4; NH$_4$Cl, 4.7; KH$_2$PO$_4$, 6; and Na$_2$HPO$_4$, 9, supplemented with 1 mL/L trace elements (Zehnder et al. 1980), 1 mL/L selenite/tungstate solution (Widdel and Bak 1992), 2 mL/L vitamins (Wolin et al. 1963), and 0.5 mg/L resazurin. Na$_2$S (1 mM) was added to reduce the media.

Desulfovibrio G11 biocathode

One liter of a *Desulfovibrio* G11 culture, grown to the end log phase, was centrifuged (8,000 rpm, 15 min), and the pellet was resuspended in 10 mL anoxic phosphate-buffered saline (pH 7.4). The MEC design was as previously described (Jeremiasse et al. 2010) with the exception that both electrodes consisted of 2.5-mm-thick graphite felt, and the membrane was a Ralex cation exchange membrane. The anolyte (hexacyanoferrate(II)) and catholyte (phosphate-buffered medium, as used in Rozendal et al. 2008) recycle speed was 60 mL/min. The cathode was inoculated with 10 mL of cell suspension. After inoculation the cell suspension was pumped to the electrode compartment and left without pumping for 1 h. The phosphate-buffered medium was supplemented with 2 mM of bicarbonate and 0.1 mL/L selenium/tungstate solution; selenium is an essential trace element for some hydrogenases in *Desulfovibrio* species (Hensgens et al. 1994; Valente et al. 2006). Temperature was controlled at 303 K, and pH was controlled at 7 by dosing 1 M HCl. The cathode potential was manually adjusted to −0.7 V vs. SHE if needed. The current, pH, anode potential, and cathode potential were logged every 5 min (Memograph M, Endress + Hauser, Naarden, the Netherlands). Cathodic current is measured with a negative sign; all current measurements were multiplied by −1 and thereby indicated with a positive sign. When stable current was reached (day 9 after inoculation), a gas sample was taken from the headspace, and H$_2$, CO$_2$ and CH$_4$ were measured using gas chromatography (Varia CP-4900 microGC, TCD detector, MS5 and PPU columns in parallel).

DNA extraction and amplification of 16S rRNA gene

Genomic DNA was extracted from the graphite felt and the liquid samples using the Fast DNA spin kit for soil (Bio101, Vista, CA, USA). Bacterial 16S rRNA genes were amplified with the primers Bact27F and Univ1492R (Lane 1991). PCR settings were initial denaturation for 2 min at 95°C, followed by 25 cycles of 30-s denaturation at 95°C, 40 s annealing at 52°C, and 1.5 min elongation at 72°C. Post-elongation was 5 min at 72°C. The PCR samples were tested on a 1% agarose gel for amount and size of product. Partial bacterial 16S rRNA genes to be used for DGGE analysis were amplified using primers Bact968F (including GC clamp) and 1401R (Nübel et al. 1996). PCR conditions were as above, except that 35 cycles were applied, and an annealing temperature of 56°C was used.

Clone library construction and analysis

For clone library analysis, the electrode sample from the middle of the electrode (2) was used. PCR amplicons of almost complete bacterial 16S rRNA genes were purified using Nucleo Spin Extract II kit (Macherey-Nagel, Düren, Germany) and ligated into pGEM-T easy vector system I (Promega, Madison, WI, USA). After ligation, the vectors were transformed in XL-1 blue competent *Escherichia coli*

cells (Stratagene, Santa Clara, CA, USA) and grown on LB agar containing 100 mg/L ampicillin, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and 40 mg/L 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Fifty-seven white clones were used for further analysis. Clone inserts from lysed *E. coli* cells (95°C, 10 min) were amplified with primers T7 and SP6 (Promega, Madison, WI, USA) using 35 cycles and an annealing temperature of 55°C. The PCR products were purified using the DNA clean and concentrator-5 kit (Zymo Research, Orange, CA, USA).

Clone inserts were sequenced by the company Baseclear (Leiden, the Netherlands) using primers T7, SP6, and 1100R (Lane 1991). The DNA sequences were checked using Chromas (version 2.32, Technelysium Pty. Ltd.), and contigs were constructed from the partial sequences using DNAbaser (version 2.71.0, Heracle Software, Lilienthal, Germany) resulting in a sequence of (at least) the first 1,250 bp of the 16S rRNA gene. The obtained bacterial 16S rRNA sequences were checked for anomalies using Pintail online software (Ashelford et al. 2005) and compared to the GenBank database using the National Center for Biotechnology Information (NCBI) blastn tool to identify the most closely related sequences. The newly obtained sequences were deposited in the European Molecular Biology Laboratory nucleotide sequence database (accession numbers FR669194-FR669243 and FR675968-FR675974), aligned using the online Silva alignment tool (Pruesse et al. 2007), and merged with the ARB database using ARB software package version 5.1 (Ludwig et al. 2004). A phylogenetic tree was constructed using the ARB Neighbor-Joining Algorithm with bootstrapping (1,000 replicates) and Jukes Cantor correction.

Denaturing gradient gel electrophoresis

The bacterial communities populating the graphite electrode on four different spots of the electrode, as well as inoculum and effluent samples, were analyzed with DGGE. Amplicons were run on an 8% polyacrylamide gel containing a formamide and urea denaturant gradient of 30–60%, similar as described by Martín et al. (2007). Gels were run for 16 h at 60°C and stained with silver nitrate (Sanguinetti et al. 1994). In addition, all amplicons of the clones were run on DGGE to evaluate their positions on the gel in comparison to the band migration behavior of the total bacterial communities. The bands that were not identified from the clones were excised from the gel. This material was incubated in Tris–EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8) at 4°C for 2 days, and the extract was used for re-amplification with DGGE primers. The PCR product was checked for purity on DGGE, purified and sequenced with primers 968F by Baseclear (Leiden, the Netherlands). The obtained sequences were checked in Chromas and the

most closely related relatives identified using the NCBI blastn search tool.

Results

Bacterial community on the electrode

During operation the MEC produced 0.63 m^3 H$_2$ m^{-3} cathode liquid volume per day or 0.63×10^{-3} m^3 H$_2$ m^{-2} electrode surface per day at an applied voltage of −0.7 V vs. SHE, and scanning electron microscopic imaging showed that after more than 80 days of operation, microorganisms were present and attached to the electrode felt fibers (Rozendal et al. 2008). Analysis of the dominant members of the bacterial population on the graphite felt electrode by DGGE showed a complex pattern which did not differ between the different locations on the electrode (Fig. 1), but there was a large difference between inoculation material, effluent material, and electrode material. A large number of bands with different intensity were present, presumably reflecting the difference in abundance of different bacterial ribotypes. Subsequent cloning and sequencing of the bacterial community were done with the sample obtained from the middle of the electrode.

16S rRNA gene distribution among different phylogenetic groups

The sequences from the 16S rRNA gene clone library (total of 57 clones) were related to seven different phyla (Table 1). The majority (88%) of the clones clustered within three phyla, the *Proteobacteria* (26 clones, 46%, FR669218-FR669243), the *Firmicutes* (14 clones, 25%, FR669204-FR669217), and the *Bacteriodetes* (10 clones, 17%, FR669194-FR669203). The other 12% of the clones were phylogenetically related to *Tenericutes* (one clone, 2%, FR675972), *Spirochaetes* (one clone, 2%, FR675968), *Chlorobi* (one clone, 2%, FR675969), *Actinobacteria* (two clones, 3%, FR675973 and FR675974), and unclassified bacteria (two clones, 3%, FR675970 and FR675971).

The phylogenetic relation of the clones from our study, including several related uncultured and cultured species from the GenBank database, is shown in Fig. 2. Within the *Proteobacteria*, 81% (21 clones) belonged to the subclass of the *Deltaproteobacteria* of which 90% (19 clones) of the clones clustered with the genus *Desulfovibrio* (Fig. 2a). Moreover, 67% (14 clones) showed more than 98% identity to the species *D. vulgaris* Hildenborough, an anaerobic bacterium that is able to use hydrogen, organic acids, or alcohols as electron donor and sulfate as electron acceptor (Postgate and Campbell 1966). *D. vulgaris* is also known to be able to produce hydrogen (Carepo et al. 2002). The remaining 10% of *Deltaproteobacteria* (two clones) showed
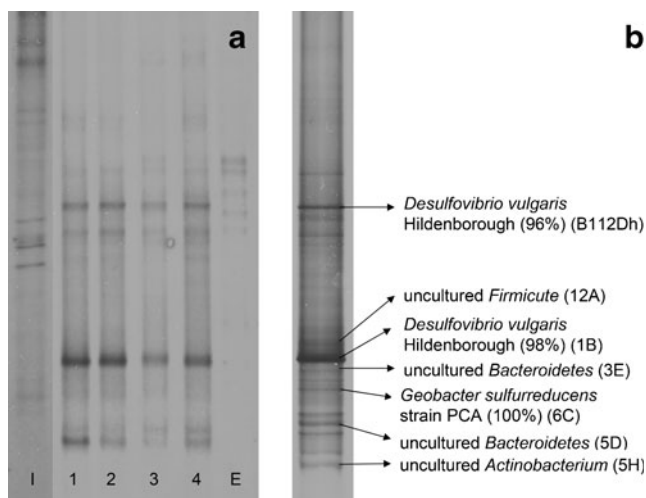
Fig. 1 DGGE profile of the total bacterial community on the biocathode. **a** Different locations and samples. *I* inoculation material, *1* influent site of electrode compartment, *2* middle of electrode, *3* effluent site of the electrode compartment, *4* non-flow site of the electrode, *E* effluent liquid. **b** Identification of the bands from sample 2. Identity of the bands is indicated with the matching clone named in brackets; B112Dh (FR669244) was identified by PCR amplification of the band cut from the DGGE profile. The indicated percentages are percentage identity of the found sequence with the indicated closest relatives found in GenBank

identical 16S rRNA gene sequences to *G. sulfurreducens* strain PCA, a bacterium which uses hydrogen or acetate as electron donor to reduce metals (Caccavo et al. 1994). *G. sulfurreducens* is able to use the electrode of an MFC as electron acceptor (Bond and Lovley 2003) but also as electron donor for fumarate reduction (Gregory et al. 2004; Dumas et al. 2008) and hydrogen production (Geelhoed and Stams 2011). The other 19% (four clones) of *Proteobacteria* belonged to the *Alpha-* (one clone), *Beta-* (two clones), and *Gammaproteobacteria* (one clone) and were most closely related to, respectively, *Mesorhizobium amorphae, Pelomo-*

nas saccharophila, Azonexus caeni, and *Dokdonellala koreensis* (Fig. 2a).

All 14 clones that clustered in the phylum of *Firmicutes* belonged to the *Clostridia* class. Within the *Clostridia*, one prominent group of nine clones (64% of the total *Firmicutes*) belonged to the family of unclassified *Clostridiales* (Fig. 2b). The clones in this group showed more than 98% identity to each other but did not belong to any cultured or uncultured genus listed in the GenBank database (using 95% identity in the 16S rRNA gene as the genus delineation value (Rosselló-Mora and Amann 2001)). The most similar GenBank sequence showed only 90% identity to this cluster of uncultured *Firmicutes* and was obtained from a dechlorinating flow column (FM178833) (Behrens et al. 2008). The most closely related cultured bacterium was *Desulfitobacterium hafniense*, with 88% 16S rRNA gene sequence identity. The other clones within the *Firmicutes* were all related to anaerobic species such as *Clostridium viride, Clostridium cylindrosporum,* or uncultured species found in anaerobic environments such as dechlorinating enrichment cultures (EF64459), anaerobic digesters (CU918499 and CR933290), mouse cecum (EU457310), a trichlorobenzene transforming culture (AJ009499), and a denitrifying community inoculated from rice paddy soil (AB486915).

Within the phylum of *Bacteroidetes*, seven clones (70%) belonged to the class of *Bacteroidia*. Within this class, there were five clones that clustered together as unclassified *Bacteroidetes* with more than 99% identity to each other. These five clones did not belong to any cultured genus. In the GenBank database, several sequences were present that clustered in the same taxonomic group (more than 95% identity). These sequences were derived from the following sources: chloraminated drinking water distribution system (EU808333), chromium-contaminated soil (EU037360), and high-carbohydrate and high-pH sludge (FJ5234992) (Fig. 2c). The closest related cultured microorganism was

Table 1 Distribution and abundance of 16S rRNA gene sequences in the biocathode clone library within the different phyla

Phylum	% of total (57 clones)	Cluster of clones (>97% similar to each other)	Similarity of cluster to cultured species
Proteobacteria	46	14 clones of *Desulfovibrio vulgaris*	>98% *D. vulgaris* str. Hildenborough
Firmicutes	25	9 clones of uncultured *Firmicutes*	<89% *Desulfitobacterium hafniense* DCB-2
Bacteroidetes	17	5 clones of uncultured *Bacteroidetes*	<92% *Rikenella microfusus* ATCC 29728
Tenericutes	2		
Actinobacteria	3		
Chlorobi	2		
Spirochaetes	2		
Unclassified bacteria	3		

The main cluster within the phylum is indicated for the major groups with the most closely related cultured species and its identity with the specific cluster
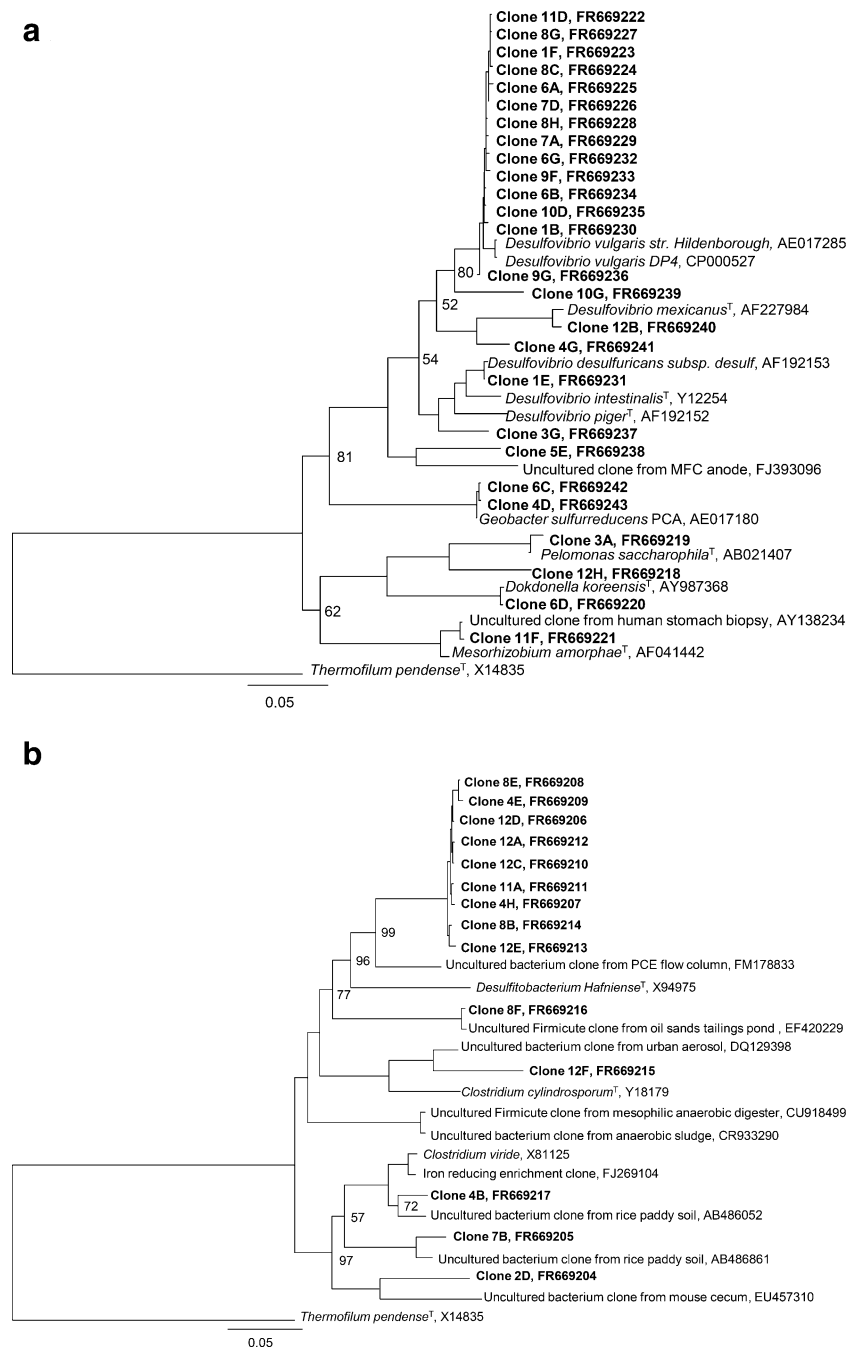
Fig. 2 Phylogenetic neighbor-joining bootstrap (1,000 replicates) consensus tree of the three major phyla detected on the biocathode. **a** *Proteobacteria* (accession numbers: FR669218–FR669243), **b** *Firmicutes* (accession numbers: FR669204–FR669217), **c** *Bacteroidetes* (accession numbers: FR669194–FR669203). The archaeon *Thermofilum pendense* is used as outgroup. Clones from our study are indicated in *bold*, and the rest represent 16S rDNA sequences imported from the GenBank database. The sequences in the tree are at least 1,250 bp long. The *scale bar* indicates the distance of 0.05 (or 5%) sequence identity

Rikenella microfusus with 92% identity of the 16S rRNA gene. *R. microfusus* is a fermentative bacterium isolated from feces of a Japanese quail (Kaneuchi and Mitsuoka [1978](#)). The other clones within the class of *Bacteroidia* were related to uncultured species from nitrobenzene-polluted river water (EF590019) and a bovine serum albumin digester (AB175369). The other clones within the

Bacteroidetes phylum belonged to the class of uncultured *Sphingobacteria* (two clones, 20%) and *Sediminibacteria* (one clone, 10%) and were most closely related to uncultured bacteria from thermophilic anaerobic sludge fed with methanol (AY526509) and *Sediminibacterium salmoneum*, an aerobic bacterium isolated from sediment of the Guanting reservoir in Beijing, China.
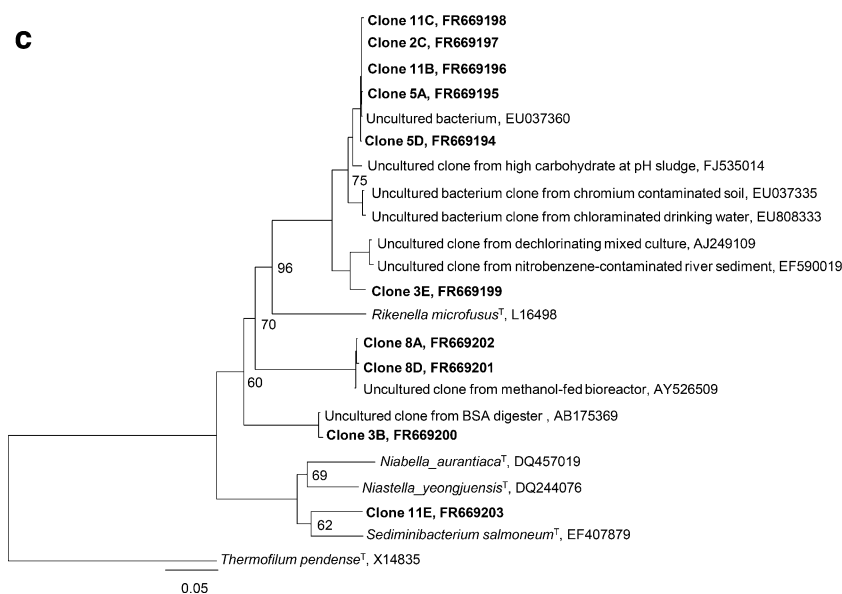
c

Clone 11C, FR669198
Clone 2C, FR669197
Clone 11B, FR669196
Clone 5A, FR669195
Uncultured bacterium, EU037360
Clone 5D, FR669194
Uncultured clone from high carbohydrate at pH sludge, FJ535014
75
Uncultured bacterium clone from chromium contaminated soil, EU037335
Uncultured bacterium clone from chloraminated drinking water, EU808333
Uncultured clone from dechlorinating mixed culture, AJ249109
Uncultured clone from nitrobenzene-contaminated river sediment, EF590019
96
Clone 3E, FR669199
Rikenella microfusus[T], L16498
70
Clone 8A, FR669202
Clone 8D, FR669201
60
Uncultured clone from methanol-fed bioreactor, AY526509
Uncultured clone from BSA digester , AB175369
Clone 3B, FR669200
Niabella_aurantiaca[T], DQ457019
69
Niastella_yeongjuensis[T], DQ244076
Clone 11E, FR669203
62
Sediminibacterium salmoneum[T], EF407879
Thermofilum pendense[T], X14835

0.05

Fig. 2 (continued)

Identification of cloned 16S rDNA in the bacterial DGGE profile

Comparison of the sequencing data and the DGGE profiles of the clones and the total bacterial community showed that the most dominant band represented *D. vulgaris* (clone 1B). The other identified bands represented other *Desulfovibrio* sp. (B112Dh, FR669244), *G. sulfurreducens* (clone 6C), uncultured *Bacteroidetes* (clone 3E and clone 5D), an uncultured *Firmicutes* sp. (clone 12A), and an uncultured *Actinobacterium* (clone 5H) (Fig. 1).

A pure culture of *Desulfovibrio* G11 in an MEC

After inoculation of *Desulfovibrio* G11 in the MEC cathode, the current increased from 1.7 (0.17 A m^{-2}) to 7.6 mA (0.76 A m^{-2}) over a period of 9 days (Fig. 3). During this period, it was visible that gas accumulated in
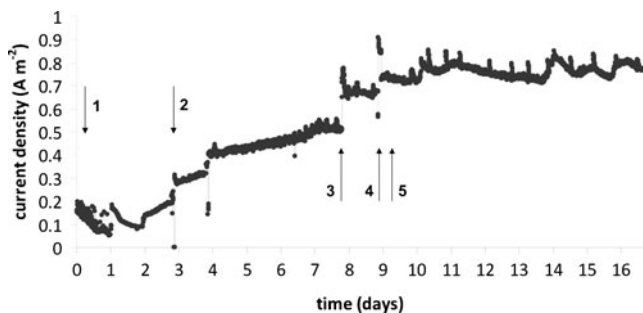
Fig. 3 Current production over time after inoculation of the cathode with pure culture of *Desulfovibrio* G11. The current increased from 0.17 to 0.76 A m^{-2} in 9 days. *Arrow 1* indicates the time of inoculation; *arrows 2, 3,* and *4* indicate an adjustment of the cathode potential to −0.7 V vs. SHE; and *arrow 5* indicates the time of gas sampling

the system. Analysis of the gas phase on day 9 showed the presence of hydrogen gas, whereas no methane or carbon dioxide was detected.

Discussion

In previous research, hydrogen production in an MEC with a biocathode has been shown (Rozendal et al. 2008; Jeremiasse et al. 2009a). Our research gives the first description of a microbial community of a hydrogen-producing biocathode in an MEC. The results showed high bacterial 16S rRNA gene diversity, with the dominant species belonging to the genus *Desulfovibrio*. Two other predominant clusters were found that were related to uncultured *Firmicutes* and uncultured *Bacteroidetes*. In addition to being the dominant ribotype in the MEC biocathode, progression of current was shown after inoculation of an MEC cathode with pure cultures of *Desulfovibrio* G11.

The dominance of *Desulfovibrio* spp. in the biocathode can be reasoned because *Desulfovibrio* species are well known for their ability to produce and consume hydrogen gas (Carepo et al. 2002). Coating of an electrode with immobilized *D. vulgaris* cells has been reported to catalyze the evolution of hydrogen at a cathode (Lojou et al. 2002). However, this catalytic effect occurred only in the presence of the electron shuttle methyl viologen. The application of living *Desulfovibrio* as hydrogen catalyst at a cathode without an added mediator, as in our system, was not shown before. This is not only fundamentally, but also practically, of great interest because it will allow low-cost and self-maintaining cathode systems for hydrogen production.

The second major group of bacteria found in this study, the uncultured *Firmicutes*, does not belong to any earlier described genus. Apparently, the conditions in the MEC created an environment in which bacteria belonging to a new taxonomic group were able to develop predominantly. Moreover, it is interesting to note that the closest related genus is *Desulfitobacterium*, of which at least one member, *D. hafniense* strain DCB2, was electrochemically active in an anode from an MFC (Milliken and May 2007). Furthermore, *Desulfitobacterium* spp. were found as the dominant population in a mixed culture that was producing hydrogen gas in a dechlorinating cathode system (Aulenta et al. 2008). Similar to the experiment with immobilized *D. vulgaris*, no hydrogen was produced in the absence of methyl viologen as a mediator.

The third major group of bacteria in the MEC biocathode belonged to the uncultured *Bacteroidetes* and also constitutes a novel group without cultured relatives at genus level (92% identity). Members of the *Bacteroidetes* phylum are found in a large variety of environments such as soil, sediments, human and animal gut, and seawater. The group of uncultured *Bacteroidetes* clones in our study was most closely related to clones from various environmental samples that presumably all originated from anaerobic sources. The closest related cultured bacterium, *R. microfusus*, grows fermentatively on carbohydrates (Kaneuchi and Mitsuoka 1978), but no other information about its metabolism is available.

The principle of electron uptake from a solid surface has been shown before, but the mechanisms are poorly understood. The reverse process of electron transfer to an anode has been studied in more detail, and those studies provided information on which mechanisms are possible (Rabaey et al. 2004; Lovley 2008; Nevin et al. 2009). Extracellular electron transfer can take place indirectly using electron shuttles such as methyl viologen, humic acid, sulfide, cysteine, riboflavin, phenazine, and quinones (Stams et al. 2006; Logan 2009). Membrane-associated proteins such as cytochromes and cell appendages or nanowires have been suggested to be involved in direct electron transfer (Kim 2002; Mehta et al. 2005; Reguera et al. 2005). For the most extensively studied species *G. sulfurreducens*, expression and deletion studies have shown that direct extracellular electron transfer to an electrode involves multicopper proteins (Holmes et al. 2008), several *c*-type cytochromes, (Holmes et al. 2006) and pillin structures which most likely are involved in the physical association with the electrode (Nevin et al. 2009). Furthermore, *G. sulfurreducens* can change from electron donating to electron uptake for hydrogen production after reversing the potential from anodic to cathodic (Geelhoed and Stams 2011). For extracellular electron uptake from an electrode, several mechanisms have been suggested

(Geelhoed et al. 2010; Rosenbaum et al. 2011). Recent findings suggest that *G. sulfurreducens* uses different cytochromes in the pathways for electron donating than for electron uptake (Strycharz et al. 2011). These authors suggest that this might reflect the optimal potential at which specific proteins can accept or donate electrons. With our findings that *Desulfovibrio* spp. are dominant microorganisms at the cathode together with our findings that *Desulfovibrio* G11 is electrochemically active at the cathode, the possible mechanisms of electron transfer and hydrogen production for this species can be inferred, as discussed below.

The genomes of *Desulfovibrio* species show several *c*-type cytochromes and multicopper proteins with homology to the proteins involved in electron donation in *Geobacter* species (NCBI search). Similar to the pillin structures in *Geobacter* spp., *D. vulgaris* flagellar appendages (genes *flgC*, *flgB*, and *flgL*) have been associated with physical association during syntrophic growth (Walker et al. 2009) and might also be involved in adherence to electrodes. These similarities suggest that the mechanism of extracellular electron transfer by *Desulfovibrio* spp. could be similar to previously described mechanisms of electron transfer involving extracellular appendage (pili or flagella)-like structures, cytochromes, or shuttle compounds. The electron transfer from an electrode to the microorganisms can possibly take place by reversed reaction of those previously described mechanisms. More research is needed to understand how electron transfer in cathode systems takes place.

Hydrogen production from protons is energetically costly. For microbial hydrogen production, energy needs to be added in the form of an electron donor with high energy (e.g., glucose or light) or in the MEC biocathode by the applied voltage. A putative mechanism for the conservation of energy from hydrogen production at the cathode may be comparable to hydrogen production from formate in methanogenic co-cultures (Dolfing et al. 2008; Stams and Plugge 2009). Energy gain and growth from production of hydrogen have been shown for *Desulfovibrio* G11 grown on formate in coculture with *Methanobrevibacter arboriphilus* AZ (Dolfing et al. 2008). Conservation of energy by *Desulfovibrio* spp. was proposed to involve an energy-conserving hydrogenase or a hydrogenase present at the cytoplasmic side of the membrane. In the genome of *D. vulgaris*, genes coding for both types of hydrogenases are present. The release of protons from formate by a formate dehydrogenase located at the periplasmic side of the membrane combined with proton consumption at the cytoplasmic side results in the generation of a proton gradient over the membrane that can be utilized by a membrane-bound ATPase. It has been suggested that in a similar way energy, could be gained from the production of

hydrogen from electrons derived from a cathode (Geelhoed et al. 2010). Our findings that *Desulfovibrio* species dominate the microbial community of the MEC support the idea that the mechanism of electron transfer from an electrode to the bacterium can take place like suggested before by Dolfing et al. (2008) and Geelhoed et al. (2010). Comparing MEC experiments to syntrophic growth, Geelhoed et al. (2010) calculated that the energy applied to MEC systems is enough to allow energy conservation and growth. However, in those calculations, no energy losses in the system were taken into account. The actual energy available at the cathode can be estimated from the potential of the cathode (−0.7 V vs. SHE) minus the energy needed to form hydrogen (−0.41 V vs. SHE) which gives the maximum theoretical energy available for the microorganisms (−0.29 V). The cathode losses, expressed as the concentration overpotential, can be calculated as described by Jeremiasse et al. (2009b). Under the conditions prevailing in the biocathode system studied here (Rozendal et al. 2008), using a pK_{a2} of 7.21 for phosphate buffer, the concentration overpotential can be estimated at −0.019 V. Hence, the actual energy available for the microorganisms is −0.29+0.019=−0.27 V or −52 kJ per mole H_2 produced (at pH 7 and pH_2=1 bar). In comparison, the Gibbs free energy change associated with conversion of formate to hydrogen and carbon dioxide is −17 to −19 kJ per mole H_2 (Dolfing et al. 2008). This shows that for the studied biocathode system, even if the overpotential is taken into account, there is sufficient energy available for the microorganisms to grow. The energetic limits for microbial hydrogen production and growth in an MEC biocathode still need to be explored.

Our findings that the dominant microorganism in the MEC biocathode is a *Desulfovibrio* sp., together with the knowledge about the hydrogen metabolism and potential for exocellular electron transfer of *Desulfovibrio* spp., give very strong indications that they are actively involved in the hydrogen production at the biocathode of the MEC. Since *Desulfovibrio* spp. are also able to consume hydrogen, it can be reasoned that the microbial community on the electrode developed during the anodic phase, in which acetate and hydrogen were the substrates. However, after switching the polarity, the production of hydrogen gas commenced only after several days, suggesting that microbial adaptation and possibly growth were necessary to start hydrogen production at the cathode. In addition, the potential electroactivity of *Desulfovibrio* in a cathode was supported by the observed current production and hydrogen production after inoculation of an MEC cathode with *Desulfovibrio* G11. Besides *Desulfovibrio*, two novel and abundantly present groups of bacteria were present. These bacteria need to be characterized further before their role in an MEC can be inferred.

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