Influence of setup and carbon source on the bacterial community of biocathodes in microbial electrolysis cells.

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Running Headline: “MEC biocathode biodiversity”

Highlights

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1. a large variety of bacteria is able to catalyze H$_2$ evolution; hydrogen evolution is a trait that is shared by guilds rather than by specific species.

2. cytoplasmic H$_2$ production by Hox-type hydrogenases is a potential key factor in the mechanism of microbial H$_2$ production and growth in bio-electrochemical systems.

The design of the compartments greatly affects the microbial populations that develop at the biocathode.

Abstract

The Microbial Electrolysis Cell (MEC) biocathode has shown great potential as alternative for expensive metals as catalyst for H$_2$ synthesis. Here, the bacterial communities at the biocathode of 5 hydrogen producing MECs using molecular techniques were characterized. The setups differed in design (large versus small) including electrode material and flow path and in carbon source provided at the cathode (bicarbonate or acetate). A hydrogenase gene-based DNA microarray (Hydrogenase Chip) was used to analyze hydrogenase genes present in the 3 large setups. The small setups showed dominant groups of Firmicutes and two of the large setups showed dominant groups of Proteobacteria and Bacteroidetes. The third large setup received acetate but no sulfate (no sulfur source). In this setup an almost pure culture of a Promicromonospora sp. developed. Most of the hydrogenase genes detected were coding for bidirectional Hox-type hydrogenases, which have shown to be involved in cytoplasmatic H$_2$ production.

Keywords: Microbial Electrolysis Cell (MEC), hydrogen, bioelectrochemical system (BES), bicarbonate, acetate, Hydrogenase Chip
1. Introduction

Hydrogen gas ($H_2$) is a valuable product as a renewable energy carrier and as a reductant in the chemical industry [1]. $H_2$ can be formed by steam-reforming of natural gas, gasification of fossil or renewable materials and by water electrolysis. Water electrolysis, however, is energetically costly. An interesting alternative is microbial electrolysis. In a microbial electrolysis cell (MEC) microorganisms degrade organic compounds (e.g. acetate) at the anode to $CO_2$, protons and electrons. At the cathode, protons and electrons derived from the anode and energized by a power supply are combined to $H_2$. The anode and the cathode are generally separated by an ion selective membrane [2]. By using this technology, the fossil energy requirements for $H_2$ production can be diminished more than 5 times compared to $H_2$ production from direct water electrolysis [3, 4].

Platinum is generally used to catalyze $H_2$ production at the cathode, as it significantly reduces the cathode overpotential. However, platinum is expensive, non-renewable, and very ineffective in catalyzing $CO_2$ reduction, and it is susceptible to poisoning by sulfur and carbon [5] monoxide. A low cost alternative for a platinum cathode is the biocathode.

A biocathode can be defined as an electrode made of cheap material (e.g. carbon or graphite) at which microorganisms catalyze the cathodic reaction (in this case: $H_2$ production). In a microbial cell, $H_2$ formation is thermodynamically confined and dependent on the available energy [6]. Microorganisms can produce $H_2$ through dark fermentation, with a low conversion efficiency of substrate to $H_2$, or by addition of energy in the form of light or heat [7, 8]. The required energy can alternatively be provided by an electrode. In MEC cathodes inoculated with biomass originated from a wastewater treatment plant, the possibility of electron transfer from the electrode to microorganisms for the production of $H_2$ was demonstrated [9, 10]. Little information is available on the types of microorganisms that develop at a biocathode in a microbial electrolysis cell and on the mechanism of electron transfer from the cathode to the microorganism to produce $H_2$. 


Biocathode microorganisms have been studied mainly in cathodes for oxygen reduction [11], but also fumarate [12] and nitrate reduction, dechlorination and product formation (e.g. methane or acetate) with an electrode as electron donor have been shown [13, 14]. Only a few studies describe the microbiology in H₂ producing biocathodes [15, 16, 17, 18, 19]. Microbial H₂ production involves hydrogenases, the enzymes that catalyze the reversible reaction \(2\text{H}^+ + 2e^- \leftrightarrow \text{H}_2\). Hydrogenases are categorized according to their (redox active) metal site. The three groups of hydrogenases are 1) nickel-iron (NiFe)-hydrogenases of which a sub-group contains also selenium, 2) iron-iron (FeFe)-hydrogenases and 3) iron (Fe)-hydrogenases. The last group was previously characterized as iron-free hydrogenases because they don’t contain a redox active iron. The catalysis of H₂ production has been mostly associated with the FeFe-hydrogenases and H₂ oxidation (consumption) mostly with NiFe-hydrogenases. Nevertheless, some NiFe-hydrogenases, mainly the cytoplasmic ones, can also catalyse H₂ production \textit{in vivo} [20, 21]. The Fe-hydrogenases, which are found in several methanogens, are involved in methane formation from CO₂ and H₂ rather than in H₂ production [22]. No knowledge is currently available on the hydrogenases involved in H₂ production in the MEC biocathode.

For growth, biocathodic bacteria require a carbon source. Carbon dioxide is a low cost carbon source but in lab scale experiments acetate is often used as carbon source because it is an end product of dark fermentation. Acetate seems to be a preferred energy and carbon source for high efficiencies in Microbial Fuel Cell (MFC) anodes [23] and recently it was also shown that in MEC biocathode systems acetate is a preferred carbon source over bicarbonate, for rapid startup of a biocathode [24]. The effect of different carbon sources on the growth and further development of the active microbial population of an MEC biocathode has not been studied before.

In the present study 5 MEC biocathode samples from an experiment that was described before by Jeremiasse \textit{et al.} [24] were analyzed. The microbial communities were determined by 16S rRNA gene analysis and the hydrogenases of 3 of the samples were analyzed by using a
Hydrogenase Chip developed previously [25]. Two small setups were operated of which one was supplemented with acetate (AcS) and the other with bicarbonate (BicS) and three large setups of which one received acetate (AcL), one bicarbonate (BicL) and the third one contained catholyte with acetate but without any sulfate (AcnSL), this to prevent loss of electrons by reduction of (the low amounts of) sulfate present in the growth medium. We hypothesize that the carbon source will have a major impact on the development and composition of the microbial population.

2. Material and methods

2.1 Operational conditions of the microbial electrolysis cell cathode

All setups were operated as described by Jeremiasse et al. [24]. In short, two different setups were used. The small setup, described by Ter Heijne et al. [26], consisted of a 22 cm² graphite paper cathode, and the large setup, described by Jeremiasse et al. [27], consisted of 100 cm² projected surface area of a 0.25 cm thick graphite felt cathode. The cathode was fed with anaerobic mineral salts medium containing (g L⁻¹) KH₂PO₄, 0.68; K₂HPO₄, 0.87; KCl, 0.74; NaCl, 0.58; NH₄Cl, 0.28; CaCl₂·2H₂O, 0.1; MgSO₄·7H₂O, 0.01 and 0.1 mL L⁻¹ of a trace element mixture [28], supplemented with either 0.01 mol L⁻¹ sodium bicarbonate or 0.001 mol L⁻¹ sodium acetate. The cathode and anode were separated by a cation exchange membrane (Ralex CMH-PES, Mega A.S., Prague, Czech Republic). In the anode compartment 0.1 mol L⁻¹ potassium hexacyanoferrate(II) was used, circulated from a 5 L tank which was refreshed weekly. No crossover of cyanoferrate was observed between compartments in all experiments. First the optimal potential for operation was determined in two series of small setups with a total cathodic circulation volume of 192 mL which was constantly refreshed with medium at a rate of 36 mL h⁻¹. The small setups were inoculated with 10 mL of biomass from the effluent and the biofilm of previously operated MEC anodes and cathodes. The two series of the small setups consisted of 4 MECs that were operated at potentials of -0.5, -0.6, -0.7 and -0.8 V (vs SHE) for more than 60 days. Those values were chosen because at those cathode potentials no significant chemical H₂ is produced.
produced at carbon electrodes in those systems [24]. A cathode potential of -0.7 V (vs. SHE) resulted in the highest catalytic activity. After operation, samples were collected from the electrode material of the -0.7 V setups (AcS and BicS). The biomass including electrode material was resuspended in catholyte solution and used as inoculum for the large setups (10 mL per setup). The large setups contained a 100 cm² (projected surface area) flow-through graphite felt electrode, a total volume of 100 mL and nutrient solution dosed at a rate of 156 mL h⁻¹. The large setups were operated at -0.7 V and supplemented with acetate (AcL, inoculated from AcS) or bicarbonate (BicL, inoculated from BicS). A third large setup (AcnSL) was inoculated from the AcL setup (10 mL of electrode biomass resuspended in catholyte) and run without any added sulfate to exclude that sulfate was used as an electron acceptor instead of protons. For this setup the MgSO₄ in the nutrient solution described above was replaced by MgCl₂. H₂ production was determined in a 48 h yield test for the BicL and AcnSL setup and in a 6 h yield test for the AcL setup as described previously [24]. After operation 1 cm² of a representative part of the electrode material (visual inspection) was cut from all 5 cathodes (AcS, BicS, AcL, BicL and AcnSL). The samples were stored at -20°C for DNA analysis or processed further for SEM imaging.

2.2 Scanning electron microscopy (SEM)

Electrode samples were fixed in 2.5% glutaraldehyde (w/v) for 2 h at room temperature and washed twice with 0.01 mol L⁻¹ PBS buffer (pH 7.4). Subsequently, the samples were dehydrated in a graded series of ethanol (10%, 25%, 50%, 75%, 90% and twice in 100% during 20 minutes for each step) and dried in a desiccator. The samples were coated with gold and examined in a JEOL JSM-6480LV Scanning Electron Microscope (acceleration voltage 6 kV, HV-mode, SEI detector).

2.3 DNA extraction and amplification of 16S rRNA genes
Genomic DNA was extracted from the electrode samples using the Fast DNA spin kit for soil (Bio101, Vista, CA, USA) using the manufacturer’s instructions. Bacterial 16S rRNA genes were amplified using the primers Bact27F and Univ1492R [29]. PCR reaction mixtures consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 mM each of the four deoxynucleoside triphosphates (dNTP), 2.5 U of Taq polymerase (Invitrogen), 200 nM of each primer and 2 µL of appropriately diluted template DNA in a final volume of 50 µL.

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 products were tested on a 1% agarose gel for amount, integrity and size of the amplicon. For DGGE analysis partial bacterial 16S rRNA genes were amplified using primers Bact968F (including GC clamp) and 1401R [30] and partial archaeal 16S rRNA genes were amplified using primers Arch109(T)F and GC515R (including GC clamp) [31, 29]. PCR conditions were as above, except that 35 cycles were applied and an annealing temperature of 56°C was used.

2.4 Clone library construction and analysis

For all 5 cathode samples 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 cloned in XL-1 blue competent Escherichia coli (Stratagene, Santa Clara, CA, USA) and grown on LB-agar containing 100 mg L⁻¹ ampicillin, 0.001 mol L⁻¹ isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 40 mg L⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). After blue-white screening, ninety six white colonies were transferred to 1mL liquid LB medium with 100 mg L⁻¹ ampicillin. After overnight incubation at 37°C all clones were transferred to a GATC 96 well nutrient agar plate with 100 mg L⁻¹ ampicillin and sent to GATC (GATC Biotech AG, Konstanz, Germany) for sequencing.
Electropherogram analysis and sequence assembly were performed with DNAbaser version 2.71.0 (Heracle Software, Lilienthal, Germany) and phylogenetic affiliation of the sequences was examined with an NCBI BLAST identity search. All sequences were aligned using the online Silva alignment tool [32] and merged with the ARB database using ARB software package version 5.1 [33]. A Chimera check was performed using the Ribosomal Database Project website [34]. A phylogenetic tree was constructed using the ARB Neighbour Joining Algorithm with Jukes Cantor correction.

The microbial diversity per setup was calculated using Shannon’s diversity index [35] (Bianchi & Bianchi, 1982) using:

\[ H' = -\sum_{i=1}^{S} p_i \ln p_i \]

where \( S \) represents the number of ribotypes (defined by >97% gene identity) per sample (richness) and \( p_i \) represents the proportion of a specific ribotype relative to the sum of all clones in a sample. Rarefaction curves were created to estimate sampling coverage using DOTUR 1.53 [36] with identity of 97% or more considered as one operational taxonomic unit. Good’s coverage was calculated using the estimate for sampling coverage \( \tilde{C} \) described by Good [37, 38] using:

\[ \tilde{C} = 1 - \frac{N1}{n} \]

where \( \tilde{C} \) is the sampling coverage of a random sample size \( n \) and \( N1 \) is number of classes observed exactly once.

2.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Partial 16S rRNA genes of the bacterial communities populating the 5 different graphite cathodes were visualized using DGGE. Amplicons were separated on an 8% polyacrylamide gel containing a formamide and urea denaturant gradient of 30-60%, similar as described by Martín et al. [39].
Gels were run for 16 hours at 60°C and stained with silver nitrate [40] after which the band profiles were compared.

2.6 DNA Microarray Analysis

The three large setups (AcL, BicL and AcnSL) of this study were further analyzed using a hydrogenase DNA microarray, Hydrogenase Chip version 4.0, designed and synthesized according to the same protocol as Hydrogenase Chip versions 1.0-3.0 [25]. Hydrogenase gene sequences were taken from Integrated Microbial Genomes and Metagenomes (IMG/M) version 3.4 [41] and several hydrogenase gene clone libraries [42, 43]. Tiling probes were designed for single-fold coverage of each gene.

DNA was amplified via multiple displacement amplification, labeled with fluorescent Cy3 dye, and hybridized to the Hydrogenase Chip version 4.0 as previously described [25]. Microarray data analysis was performed using the TilePlot package version 1.3 in R version 2.13.1 (http://www.r-project.org/). Gene presence/absence was determined independently for each sample using the tileplot.single() function. Bright Probe Fraction (BPF) cutoffs for each hybridization were determined using the default method within TilePlot. For each sample, the section of the BPF curve with the sharpest drop-off was used to determine the BPF threshold. Samples BicL and AcnSL were loess-normalized to sample AcL using the tileplot.double() function for semi quantitative comparisons.

For each gene on the array, a bright-segment length dependent score (BSLDS) was calculated based on the length of each continuous section of the gene with bright probe (or “bright segment length”). The sum of squares of all bright segment lengths for a given gene was said to be the BSLDS. The BSLDS is a method of differentiating results not just based on the fraction of bright probes, but rather on the length of continuous bright segments.

2.7 Nucleotide sequence accession numbers
All nucleotide sequences obtained in this study were deposited in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database under accession numbers HE582784 to HE583182.

3. Results

3.1 Performance of the MEC biocathode

The performance of the biocathodes that were sampled was partly described before [24]. The small setups produced 1 A m\(^{-2}\) (projected cathode area) for the acetate setup and 0.8 A m\(^{-2}\) (projected cathode area) for the bicarbonate setup. \(\text{H}_2\) was detected but not quantified for the small setups. An non-inoculated control of the small setup produced 0.4 A m\(^{-2}\) [16]. The startup time (time until current was stable and no further current increase or decrease was observed) for the AcS setup was 30 days and for the BicS setup was 60 days. This startup time was similar for the large setups, namely 28 days for the AcL setup, 63 days for the BicL setup and 47 days for the AcnSL setup. The large setups produced an average current of: 2.7 A m\(^{-2}\) (AcL), 2.3 A m\(^{-2}\) (BicL) and 2.2 A m\(^{-2}\) (AcnSL) with a \(\text{H}_2\) yield of 2.4 (AcL), 2.7 (BicL) and 2.2 (AcnSL) m\(^3\) \(\text{H}_2\) per m\(^3\) reactor volume per day. A control large setup (non-inoculated) produced 0.8 A m\(^{-2}\) with 0.32 m\(^3\) \(\text{H}_2\) per m\(^3\) reactor liquid per day, most likely due to minimal growth of biomass towards the end of the test, since the setups were not operated in a sterile mode. The higher current production in the large setups was initially explained for a major part by the difference in cell design and by the higher surface area of the porous graphite felt electrode [24].

3.2 Microbial community composition

SEM revealed that on all electrode samples microorganisms were attached. The electrode paper from the small setup showed a dense packed biofilm, whereas on the electrode felt from the large setups, which had a much larger surface area available for biofilm attachment, a less dense biofilm was visible (Figure 1).
Denaturing gradient gel electrophoresis (DGGE) analysis revealed that the bacterial communities in the small setups were similar, suggesting that the carbon source had little effect on the community that was enriched. The bacterial communities in the large setups were different from the ones in the small setups, even when supplied with the same carbon source. The DGGE profiles of the large setups showed two dominant bands that were present in both the AcL and BicL setups, but also several unique bands per setup. The AcnSL setup showed one dominant band that was not abundant in the samples of the other setups (Figure 2). Analyzing multiple samples from the same electrode did not reveal significant differences in DGGE profiles of the microbial population (data not shown). PCR amplification of 16S rRNA genes from archaea was not successful, indicating that archaea were not present, or at very low levels.

Sequence analysis of the 475 clones (95 per sample) revealed that 76 clones had low quality or small insert. These were excluded from further community analysis. No chimeras were detected. Phylogenetic analysis of the remaining 399 clones revealed large bacterial diversity differences in the MEC biocathode samples. However, predominant groups of clones were present in each setup. At the phylum level both small setups consisted of mainly clones affiliated with the Firmicutes (BicS: 93%, AcS: 92% of total clones) but for both large setups the majority of the clones affiliated with the Proteobacteria (BicL: 91%, AcL: 57%). The AcL setup also contained a predominant group affiliated with Bacteroidetes (37%). The AcnSL setup consisted of almost exclusively Actinobacteria (98%) (Figure 3).

At the species level, the composition of the bacterial communities of the biocathode of the two small setups was very similar, but for the large setups the communities differed more from each other (Table 1). The clones obtained from the AcS setup (total 81 clones) consisted of two predominant ribotypes. The first ribotype (32 clones, 40% of total) clustered with uncultured Clostridiaceae. The closest related uncultured clone (AY261814) was derived from an UASB reactor (99% identity). The closest related cultured species was Clostridium cylindrosporum (Y18179, 91% identity). The second predominant ribotype (32 clones, 40% of total) belonged to
the family of *Peptococcaceae*. The closes related clone (GQ921447, 95% identity) was derived
from fracture water from a gold mine. The closest related cultured species was *Desulfotomaculum*
sp. Ox39 (AJ577273, 91% identity).

The microbial community of the BicS biocathode (total 85 clones) consisted of the same
predominant ribotypes as the AcS setup, but in different proportions. 19% (16 clones) belonged to
the uncultured *Clostridiaceae* with 100% identity to the clones from the AcS setup. In addition
59% (50 clones) belonged to the family of *Peptococcaceae* again with 100% identity to this
ribotype from the AcS setup.

In the AcL setup most clones affiliated with the *Proteobacteria*, however the dominant
ribotype was found in the *Bacteroidetes*. 23% (18 clones of a total of 77 clones) belonged to the
species *Kaistella* (*Chryseobacterium*) *koreensis* (AF344179, 99% identity), an aerobic, non-spore
forming rod, isolated from industrial phenolic compound-degrading waste water [44]. Further, 4
clones were related to *Leptothrix* sp. MOLA 523 (AM990747, 99% identity) isolated from
*Petrosia ficiformis* sponge. 36% of the *Proteobacteria* belonged to the *Gammaproteobacteria* of
which most clones (12 of total 18 clones) were related to several *Pseudomonas* species.

*Pseudomonas* species have shown to be electrochemically active in MFCs [45, 46, 47]. The other
clones affiliated to the *Proteobacteria* in this sample clustered with several different genera and
did not group within predominant ribotypes of more than 4 clones.

For the BicL setup, the predominant ribotypes within the *Proteobacteria* all belonged to
the *Betaproteobacteria*. Of the total 71 clones, 13% (9 clones) grouped with the genus
*Hydrogenophaga*. Closest related was an uncultured *Hydrogenophaga* sp. (GU560177, 99%
identity) detected in a biofilm of a reactor for treatment of pharmaceutical wastewater. The
closest related cultured species was *Hydrogenophaga flava* strain 2 (NR_028718, 97% identity).

*Hydrogenophaga* ssp. are Gram-negative, aerobic bacteria that grow chemolithoautotrophically
or chemoorganotrophically with H₂ [48]. Another 13% (9 clones) grouped with *Desulfovibrio*
vulgaris Miyazaki F (NC_011769, 99% identity). *Desulfovibrio* ssp. are sulfate-reducing species
well known to consume and produce $\text{H}_2$ [49, 50]. A further 13% (9 clones) grouped with the
genus *Azonexus*, with the closest related clone (AJ009452, 99% identity) that was derived from a
trichlorobenzene-degrading microbial consortium. The closest related cultured species was
*Azonexus caeni* (AB166882, 97% identity) a denitrifying bacterium isolated from a wastewater
treatment plant [51]. 7% (5 clones) grouped with the species *Azospira oryzae* (NR_024852, 99%
identity) a Gram-negative, highly motile, nitrogen-fixing bacterium isolated from anaerobic soil
[52].

The AcnSL setup showed a biocathode microbial community of almost exclusively
*Actinobacteria* of which 96% (82 of the 85 clones) belonged to one ribotype and showed 99%
identity with the species *Promicromonospora* sp. CPCC100077 (FJ529706). *Promicromonospora*
spp. are Gram-positive, spore forming bacteria. Strain CPCC100077 was isolated from soil at
3000 m altitude at the Qinghai–Tibet plateau in China [53]. For more detailed information on the
less dominant ribotypes found in the clone library we refer to the phylogenetic tree in the
Supplement S1.

### 3.3 Bacterial diversity and sampling coverage estimation

Statistical analysis of the clone libraries showed a lower diversity for the small setups with a
Shannon’s diversity index of 1.41 for AcS and 1.23 for BicS and a higher diversity for the large
setups with a Shannon’s diversity index of 2.91 for AcL and 2.65 for BicL. The diversity of
AcnSL was very low (0.19). The Goods coverage ranged from 77% to 96% (see Table 1) and
rarefaction curves showed levelling off of the curves which indicated that sufficient samples were
taken to be able to draw conclusions on which ribotypes were dominantly present in the samples.

### 3.4 Hydrogenase Chip

Several different hydrogenase genes were detected in the samples from the large setups. 41 of
2275 genes on the array showed BPF values above the identified thresholds (0.958, 0.917, 0.979,
for samples AcL, BicL, and AcnSL respectively) in at least one of the samples analyzed. Of those 33 genes with above-threshold BPF values, 8 were from genome sequences of bacterial isolates and 33 were from metagenomic sequences. Of these 33 genes from metagenomes above the BPF threshold, only 4 had a BSLDS greater than 100 (equivalent to 10 bright probes across a contiguous section of the gene). Those 4 genes with a high BSLDS values were considered the most likely to be truly present, rather than the result of cross hybridization to the relatively small number of probes targeting most metagenomic genes.

Within the used detection limit 6 hydrogenase genes were considered present in the AcL setup (Table 3) of which 4 were from bacterial isolates namely: 1) NiFe hydrogenase large subunit (Lcho_1459) from *Leptothrix cholodnii* SP-6, 2) FeFe hydrogenase (DMR_02480) from *Desulfovibrio magneticus* RS-1, 3) NiFe hydrogenase large subunit (Anae109_4306) from *Anaeromyxobacter* sp. Fw109-5 and 4) NiFe hydrogenase large subunit (RPC_3774) from *Rhodopseudomonas palustris* BisB18. Furthermore, two coenzyme F420-reducing hydrogenases (the alpha subunit) genes were found from metagenomic sequences, one from Sludge/Australian, Phrap Assembly (IMG identifier: 2000494750) and one from a methylotrophic community from Lake Washington sediment characterized after methanol enrichment (IMG identifier: 2006298366).

The BicL setup contained 3 paralogs of the NiFe hydrogenase large subunit genes from *D. vulgaris* Miyazaki F (DvMF_1733, 0270 and 0273). No other hydrogenase gene matches were found with significant bright probe intensity (Table 3). The AcnSL setup gave positive matches with the NiFe hydrogenase large subunit gene (Anae109_4306) from *Anaeromyxobacter* sp. Fw109-5 and probable ferredoxin hydrogenase, large subunit gene (azo3808) from *Azoarcus* sp. BH72. In this sample also two FeFe hydrogenase large subunit, C-terminal domains were found as match with metagenome data from Guerrero Negro salt ponds hypersaline mat 06(P) and 07(S) (IMG identifiers: 2004316449 and 2004332277). All probes on the chip and its bright probe
intensities (raw data and normalized data) for the samples AcL, BicL and AcnSL are available as
supporting information (Supplement S2).

Statistical analysis of the median of the probe intensity ratios showed that the probe
intensities of the probes for DvMF_1733, 0270 and 0273 in the BicL sample and Anae109_4306
and azo3808 in the AcnSL sample were significantly more abundant in those samples than in the
other samples. There was no statistically significant difference in abundance of the genes
Lecho_1459, DMS_02480 and RPC_3774 between the different samples (see Supplement S3).

4. Discussion

4.1 Effect of the carbon source on microbial population

The microbial populations of H\(_2\)-producing biocathodes from five MECs which differed in setup
design (including size and cathode material) and carbon source, were analyzed. In contrast to our
hypothesis that the carbon source would have a major impact on the development of the microbial
population, our results suggest that other factors had greater influence on the development of the
biofilm than the carbon source. This might include factors such as setup geometry, nutrient
distribution, flow path, electrode material or local H\(_2\) partial pressure. The different setup types
are referred to as large and small setup, with this naming we do not tend to imply that the actual
volume has the largest influence. When we inoculated biomass from small setups AcS to AcL
and from BicS to BicL we anticipated further enrichment of dedicated populations dependent on
the carbon sources (acetate versus bicarbonate). Instead, different populations developed. Our
results showed that on both small setups mainly *Firmicutes* and on both large setups mainly
*Proteobacteria* were enriched on the cathodes. Because the results of the AcnSL setup are very
different from all the other setups this setup is discussed separately. The detectable bacterial
diversity was much lower in the small setups than in the large setups. Since the large setup was
inoculated with biomass from the small setup fed with the same carbon source, one would expect
selection for species able to use the specific carbon source (acetate or bicarbonate) and thus a
decrease or no change in bacterial diversity. However, as indicated by Shannon’s diversity index, the bacterial diversity is higher in both large setups compared to both small setups. This also indicates that one of the parameters from the setup had a greater influence on the development of the microbial community than the carbon source. In contrast, when we compare the microbial populations in the same type setups, the Shannon’s diversity index shows higher values for the setups fed with acetate which can be used by (facultative) heterotrophic microorganisms, than setups fed with bicarbonate, which can only be used by autotrophic growing microorganisms. This was found for both the small and the large setups. On species level the two small setups were very similar, whereas the AcL and BicL setups showed more diversity of species. This difference was confirmed with the Hydrogenase Chip data which showed that in the AcL sample genes coding for NAD(P)\(^+\) dependent NiFe, FeFe hydrogenases, and a coenzyme F420-reducing hydrogenase were found to be abundantly present. In the BicL sample, genes coding for three different NiFe hydrogenases from \textit{D. vulgaris} Miyazaki F were abundantly present. A semi quantitative analysis of the samples showed that the gene coding for NiFe hydrogenase from \textit{D. vulgaris} Miyazaki F was present in higher abundance in sample BicL than in any of the other samples. These differences between samples AcL and BicL suggests that the carbon source does have some effect on the microbial population as was recently also suggested by Jeremiasse \textit{et al.} [24]. Nevertheless, no clear conclusions on the effect of the carbon source can be made, as no exclusive autotrophic bacteria were identified in the bicarbonate setups or heterotrophic species in the acetate setups. Although no acetate was measured in the bicarbonate systems (data from [24]), it cannot be excluded that acetogenic bacteria produce acetate from bicarbonate [13] and that this acetate is used by other microorganisms for heterotrophic growth. Also decaying biomass could have been used as a carbon source for heterotrophic growth.

The major effect on the microbial diversity was caused by something different than the carbon source and seemed to have more relation to the type of setup used. This effect on the microbial population might be affected by several components in the setup. The shape and
surface area of the electrode material was different, flat paper versus three dimensional felt, and may have influenced the attachment of bacteria to the electrode. This could also explain the differences in bacterial species diversity of the large compared to the small setups. The relatively small and flat electrode surface of the small setups might facilitate attachment of the initial biomass to the electrode [54], preventing further attachment or growth of enriched biomass. In the large setups there is sufficient surface available for initial attachment and other bacteria that are slower in colonization of the electrode. This was supported by the SEM images (Figure 1), revealing whole surface colonization of the cathode in the small setup versus only partial colonization of large setup biocathode. The current production in the large setups with the graphite felt electrodes reached a steady state which indicates that the microbial coverage was not the limiting factor for current production. The differences in flow path of the nutrient solution might have influenced the microbial population because of differences in mixing, mass transfer in the electrode compartment and shear forces at the electrode surface. Poor mixing and mass transfer limitation might create local high concentration of metabolic or electrochemical products (e.g. H$_2$ pressure or pH) or gradients in nutrient availability that will influence the growth of microorganisms. Shear forces at the electrode surface remove microorganisms that are not able to attach sufficiently strong to the electrode.

Although several ribotypes from seemingly aerobic species were detected, it is unlikely that presence or leakage of oxygen influenced the difference in microbial populations between de systems because the cathode conditions are reduced conditions and all oxygen that might be present will, under the applied conditions, chemically reduce to water.

4.2 Effect of S-limitation on microbial population

The bacterial community of the AcnSL setup was very different from all the other setups. At the electrode of the AcnSL setup an almost pure culture of *Promicromonospora* sp., a member of the *Actinobacteria*, was found to be dominant. The sulfate, although only present in trace amounts,
was initially left out from the medium to ensure that no electrons were lost in dissimilatory sulfate reduction. However, sulfur is an essential element and although very little sulfur is needed to sustain growth [55] the lack of sulfur might limit growth of a diversity of bacteria. Interestingly, after startup, this setup performed similar to the large setups fed with additional sulfate as source of sulfur. In the other setups no clones related to *Promicromonospora* spp. were detected. The lack of sulfate clearly has a major influence on the type of microorganisms that grow at the cathode. Limiting factors like this could be a good strategy for isolation of single species that can catalyze H₂ production in a cathode for further studies.

The two hydrogenase genes of known species that were detected in the AcnSL setup did not belong to an *Actinobacterium*. Possibly the detected hydrogenase genes were from species that were present but not detected in the clone library. At least one of the genes (Anae109_4306) was present, although less abundant, in the AcL setup from which this setup was inoculated. The other AcnSL detected hydrogenase gene (azo3808) was also present in the AcL sample but just below the significant bright probe fraction detection limit. This similarity suggests those hydrogenase genes belong to species also present in the AcL setup. The hydrogenase genes from metagenomic data found in the AcnSL setup showed two FeFe-hydrogenases which did not have a match with any cultured species and might be derived from the dominant *Promicromonospora* sp. in this sample. To our knowledge nothing is known about H₂ production by *Promicromonospora*. This could be a possible novel characteristic of the *Promicromonospora* strain detected in our biocathode, but this could not been extracted from the draft genome (http://genomesonline.org/cgi-bin/GOLD/bin/GOLDCards.cgi?goldstamp=Gi05674) of *Promicromonospora kroppenstedtii* which was isolated from garden soil [56] and only 97% related (based on 16S rRNA) to the phylotype in our reactor. A PROSITE scan [57,25] of the draft genome, using the PROSITE motive as described by [58], and an IMG/GEBA search revealed three putative hydrogenases (IMG/GEBA identifiers: 2507527031, 2507526009, and...
2507525797) which are, however, not related to any of the hydrogenases found in this study and also are not present on the Hydrogenase Chip.

### 4.3 Bacteria involved in $H_2$ evolution

In this study very distinct bacterial populations with members of different dominant phylogenetic groups (Firmicutes, Proteobacteria and Actinobacteria) were detected in setups that all catalyzed the production of $H_2$ at the biocathode. In one of the first studies on the microbial community of the biocathode for $H_2$ production a dominant group of *D. vulgaris* related species was found (and active) when the biocathode was enriched as anode fed with $H_2$ and acetate [17]. This suggested that *Desulfovibrio* species might be the major players in $H_2$ production at the cathode. In the present study we only found *Desulfovibrio* in the large setups and only predominant in the BicL setup. Apparently, a wide range of phylogenetically different bacteria can grow in the biocathode which presumably catalyze, or are involved in $H_2$ production at the MEC biocathode.

Several mechanisms for electron transfer and $H_2$ production in cathode systems have been proposed [14]. Hydrogenases are present in many bacteria from different phylogenetic groups [59], and also in many of the bacteria that we identified in the biofilms on the biocathodes in the present study. It is not known if bacteria are able to conserve energy by formation of $H_2$ through reduction of protons with electrons derived from the electrode. It has been suggested that bacteria are able to grow in $H_2$ producing biocathode systems through the activity of energy converting hydrogenases (Ech) or via cytoplasmic hydrogenase activity resulting in a proton gradient that can be utilized by a membrane-integrated ATPase [6, 17].

Using a hydrogenase DNA microarray, we tried to identify the hydrogenase genes that are present in the biocathode samples. The genes coding for hydrogenases from several species that were identified in the clone library were identified with the Hydrogenase Chip. First of all, *D. vulgaris* Miyazaki F which was identified in the BicL clone library and its NiFe hydrogenase genes were identified using the Hydrogenase Chip. The NiFe hydrogenases in *D. vulgaris*
Miyazaki F have not been linked to H₂ production before but no other hydrogenase genes for H₂ production are known for this species, although it has been recorded to be able to produce H₂ [60] (Tsuji and Yagi 1980). In the AcL setup one clone of Desulfovibrio was found and also a FeFe hydrogenase gene was identified from D. magneticus. Furthermore, 4 Leptothrix clones were detected and one Leptothrix NiFe hydrogenase gene was identified using the chip. This hydrogenase belongs to the Hox-type or bidirectional NAD(P)⁺ dependent hydrogenases which can be involved in cytoplasmic H₂ production and consumption [61, 21, 62]. The other detected hydrogenase genes are from bacteria in the Proteobacteria phylum but did not match with any of the detected clones. Some of the dominant species that were detected in the clone library were not detected using the Hydrogenase Chip because either the abundance of their hydrogenase genes was too low to be able to detect them with the microarray or there are no hydrogenase genes known and/or sequenced for those species (e.g. Promicromonospora, Hydrogenophaga, Azospira, Azonexus, Kaistella).

In general, the detected hydrogenase coding genes seem to be mostly cytoplasmic, bidirectional, NAD(P)⁺ dependent Hox-type hydrogenases. Interestingly, the Hox-type hydrogenases, although of the NiFe-type, have been associated with, (mostly fermentative) H₂ production before [20, 61].

Concerning the mechanism of biocathodic H₂ production, if the Hox-type hydrogenases are mostly involved, this would suggest a predominant role of cytoplasmic hydrogenases rather than of membrane integrated energy converting hydrogenases. In that case electrons need to be shuttled from the outside to the cytoplasm by, thus far unknown, electron mediators. Although a microbial biofilm developed on the cathodes which catalyzed H₂ production, it cannot be excluded that part of the microbial community consumed H₂ and the hydrogenases originated from those species. Furthermore, from our study we cannot fully exclude the possibility that free hydrogenases (derived from lysed cells) play a role in biocathodic H₂ formation. Immobilized hydrogenases were successfully applied in fuel cells [63, 15] but were shown to be unstable and...
needed regeneration. Active microbial cells can facilitate continuous regeneration of hydrogenase activity in an MEC.

In conclusion, we uncovered the dominant bacterial species at 5 MEC biocathodes and herewith enhanced the general knowledge on composition, diversity and activity of microorganisms in bio-electrical systems. Furthermore we showed that design had a significant effect on the development and composition of the biofilm on the biocathode of these MECs. This effect was larger than the effect of differences in carbon source (autotrophic or heterotrophic) and therefore setup design needs to be carefully considered when designing experiments and especially when comparing the microbiology from experiments using different setup designs. Which specific parameters from the setup design (e.g. electrode material, flow path) have the greatest effect on the microbial population is not known and is a challenge for further studies. We have revealed that several microbial populations were electrochemically active and involved in the production of H₂ in the cathode of these MEC. From an applied perspective this is very beneficial because mixed and diverse microbial communities rather than specific pure cultures can be used at the biocathode. To unravel the mechanisms underlying the production of H₂ in an MEC biocathode, more in depth research is essential. The Hydrogenase Chip offers an excellent platform for further MEC biocathode studies.

Acknowledgements

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The authors like to thank Peer Timmers, Mark Levisson, Ágnes Janoska and Astrid Paulitsch-Fuchs for technical assistance and the participants of the Wetsus research theme “Bio-energy” for the fruitful discussions and their financial support.
**Tables**

**Table 1** Overview of the dominant ribotypes on the different MEC biocathode samples with the closest relatives and the closest cultured species as found in GenBank. BicS, AcS, BicL, AcL and AcnSL as explained in methods section.

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>MEC</th>
<th>#clones (total)</th>
<th>Closest relative (GenBank accession #, identity)</th>
<th>Closest cultured (GenBank accession #, identity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcS 1</td>
<td>Small acetate</td>
<td>32 (81)</td>
<td>uncultured <em>Clostridiaceae</em> clone derived from UASB reactor (AY261814, 99%)</td>
<td><em>Clostridium cylindrosporum</em> (Y18179, 91%)</td>
</tr>
<tr>
<td>AcS 2</td>
<td>Small acetate</td>
<td>32 (81)</td>
<td>Clone derived from a gold mine (GQ921447, 95%)</td>
<td><em>Desulfotomaculum</em> sp. Ox39 (AJ577273, 91%)</td>
</tr>
<tr>
<td>BicS 1</td>
<td>Small bicarbonate</td>
<td>16 (85)</td>
<td>uncultured <em>Clostridiaceae</em> clone derived from UASB reactor (AY261814, 99%)</td>
<td><em>Clostridium cylindrosporum</em> (Y18179, 91%)</td>
</tr>
<tr>
<td>BicS 2</td>
<td>Small bicarbonate</td>
<td>50 (85)</td>
<td>Clone derived from a gold mine (GQ921447, 95%)</td>
<td><em>Desulfotomaculum</em> sp. Ox39 (AJ577273, 91%)</td>
</tr>
<tr>
<td>AcL 1</td>
<td>Large acetate</td>
<td>18 (77)</td>
<td><em>Kaistella</em> (<em>Chryseobacterium koreensis</em>) (AF344179, 99%)</td>
<td></td>
</tr>
<tr>
<td>BicL 1</td>
<td>Large bicarbonate</td>
<td>9 (71)</td>
<td>uncultured <em>Hydrogenophaga</em> (GU560177, 99%)</td>
<td><em>Hydrogenophaga flava</em> strain 2 (NR_028718, 97%)</td>
</tr>
<tr>
<td>BicL 2</td>
<td>Large bicarbonate</td>
<td>9 (71)</td>
<td><em>Desulfovibrio Miyazaki</em> F (NC_011769, 99%)</td>
<td></td>
</tr>
<tr>
<td>BicL 3</td>
<td>Large bicarbonate</td>
<td>9 (71)</td>
<td>Clone from trichlorobenzene-transforming microbial consortium (A009452, 99%)</td>
<td><em>Azonexus caeni</em> (AB166882, 97%)</td>
</tr>
<tr>
<td>BicL 4</td>
<td>Large bicarbonate</td>
<td>7 (71)</td>
<td><em>Azospira oryzae</em> (NR_024852, 99%)</td>
<td></td>
</tr>
<tr>
<td>AcnSL 1</td>
<td>Acetate no sulfate</td>
<td>82 (85)</td>
<td><em>Promicromonospora</em> sp. CPCC100077 (FJ529706, 99%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 Good’s coverage and Shannon’s diversity index of the bacterial communities of 5 different setups.

<table>
<thead>
<tr>
<th>Setup</th>
<th>No. of sequenced clones</th>
<th>Good’s coverage (%)</th>
<th>Shannons diversity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcS</td>
<td>81</td>
<td>96</td>
<td>1.41</td>
</tr>
<tr>
<td>BicS</td>
<td>85</td>
<td>93</td>
<td>1.23</td>
</tr>
<tr>
<td>AcL</td>
<td>77</td>
<td>97</td>
<td>2.91</td>
</tr>
<tr>
<td>BicL</td>
<td>71</td>
<td>85</td>
<td>2.65</td>
</tr>
<tr>
<td>AcnSL</td>
<td>85</td>
<td>96</td>
<td>0.19</td>
</tr>
</tbody>
</table>
Table 3 Hydrogenase genes identified as being present using the Hydrogenase Chip. The function and extra information (remarks) were obtained from the IMG database.

<table>
<thead>
<tr>
<th>Setup</th>
<th>Function</th>
<th>Locus tag and species or IMG identifier</th>
<th>Remarks</th>
<th>Present in 16S rRNA gene clone library?</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcL</td>
<td>NAD(P)⁺ dependent, NiFe hydrogenase (Hox type)</td>
<td>Lcho_1459 from <em>Leptothrix cholodnii</em> SP-6</td>
<td>Bidirectional, cytoplasmic, involved in both H₂ consumption and production</td>
<td>Yes (4/77 clones)</td>
</tr>
<tr>
<td>AcL</td>
<td>NAD(P)⁺ dependent FeFe hydrogenase</td>
<td>DMR_02480 from <em>Desulfovibrio magneticus</em> RS-1</td>
<td>Cytoplasmic, involved in H₂ production</td>
<td>Yes (1/77 clones)</td>
</tr>
<tr>
<td>AcL</td>
<td>nickel-dependent (NiFe) hydrogenase large subunit NAD⁻-reducing hydrogenase subunit (Hox type)</td>
<td>Anae109_4306 from <em>Anaeromyxobacter</em> sp. Fw109-5</td>
<td>Bidirectional, cytoplasmic, involved in both H₂ consumption and production</td>
<td></td>
</tr>
<tr>
<td>AcL</td>
<td>nickel-dependent (NiFe) hydrogenase, large subunit</td>
<td>RPC_3774 from <em>Rhodopseudomonas palustris</em> BisB18</td>
<td>Cytoplasmic, membrane bound H₂ase. Has interaction with cytochrome b and can also act on soluble electron donors.</td>
<td></td>
</tr>
<tr>
<td>AcL</td>
<td>Coenzyme F420-reducing hydrogenase</td>
<td>Metagenome data IMG identifier 2000494750 and 2006298366</td>
<td>Coenzyme F420-reducing H₂ases can act in a similar way as NAD(P)⁺-dependent hydrogenases</td>
<td></td>
</tr>
<tr>
<td>BicL</td>
<td>nickel-dependent (NiFe) hydrogenase large subunit (hya/hyb type)</td>
<td>DvMF_1733, 0270 and 0273 from <em>Desulfovibrio vulgaris</em> Miyazaki F</td>
<td>No evidence that hya/hyb type H₂ases are involved in H₂ production.</td>
<td>Yes (9/71 clones)</td>
</tr>
<tr>
<td>AcnSL</td>
<td>nickel-dependent (NiFe) hydrogenase large subunit NAD⁻-reducing hydrogenase subunit (Hox type)</td>
<td>Anae109_4306 from <em>Anaeromyxobacter</em> sp. Fw109-5</td>
<td>Same as in sample AcL. Bidirectional, cytoplasmic, involved in both H₂ consumption and production</td>
<td></td>
</tr>
<tr>
<td>AcnSL</td>
<td>probable ferredoxin NiFe hydrogenase, large subunit</td>
<td>azo3808 from <em>Azoarcus</em> sp. BH72</td>
<td>Homology to hoxC which is a H₂ sensor</td>
<td></td>
</tr>
<tr>
<td>AcnSL</td>
<td>iron only (FeFe) hydrogenase large subunit, C-terminal domain</td>
<td>Metagenome data IMG identifier 2004316449 and 2004332277</td>
<td>Not related to anything known but in general FeFe H₂ases are involved in H₂ production</td>
<td></td>
</tr>
</tbody>
</table>
Figures

Fig. 1 Scanning Electron Micrograph of the paper (a) and felt (b) cathode surface after operation as H\text sub{2} producing biocathode. All setups were examined and representative pictures are shown here ((a) from BicS, (b) from BicL). Scale bars indicate the actual size of the items on the image.

Fig. 2 Denaturing gradient gel electrophoresis profiles of bacterial communities on the electrodes from setup BicS, AcS, bicL, AcL and, AcnSL (names as explained in method section).
**Fig. 3** Distribution of different phyla per MEC setup. On the x-axis the different setups are plotted. BicS, AcS, BicL, AcL and AcnSL are as explained in methods section. On the y-axis the percentage of clones within a phylum relative to the total amount of clones in the sample is plotted. (Black (1) indicates the *Firmicutes*, dark gray (3) the *Proteobacteria*, light gray (2) the *Bacteroidetes* and very light gray (4) the *Actinobacteria*. Clones belonging to other groups (only 1% in the BicL setup) are indicated in very dark gray (5)).

**Appendix Supplementary data**

**Supplement S1** Phylogenetic tree of all clones from the biocathodes including closest related cultured species. Numbers indicate amount of clones within that cluster. *Sulfolobus tokodaii* was used as out-group.

**Supplement S2** Hydrogenase microarray data. Including probe names of used probes, raw data of the (median) measured probe intensities and non-normalized and normalized probe intensity ratio’s.

**Supplement S3** Log median probe intensity ratios of the Loess-normalized data of the genes from bacterial isolates that showed above-threshold BPF values for samples BicL and AcnSL to sample AcL using the tileplot.double() function for quantitative comparisons.
References


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