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GENETIC IDENTIFICATION OF THE BIOANODE AND BIOCATHODE OF A MICROBIAL ELECTROLYSIS CELL

IDENTIFICACIÓN GENÉTICA DEL BIOÁNODO Y BIOCÁTODO DE UNA CELDA DE ELECTROLISIS MICROBIANA

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Abstract

The aim of this study was to identify the microorganisms present on the graphite cloth of the bioanode and biocathode of a Microbial Electrolysis Cell (MEC) using 16S ribosomal RNA gene cloning and sequencing. The results obtained indicated that the bioanode clones were related with the phyla: *Firmicutes* (15.3%), *Proteobacteria* (7.6%), *Bacteroidetes* (30.7%), and Ignavibacteriae (7.6%). Conversely, the biocathode clones were related with the phylum *Proteobacteria* (38.4%). The bioanode clones were related with species identified previously in MECs and Microbial Fuel Cells (MFCs). However, the biocathode clones were related with *Rhodopseudomonas palustris*, which have not been reported for hydrogen production by MEC. R. palustris probably should be involved into hydrogen production of 0.011 m^3 H_2/m^3 cathode liquid volume per day with an applied voltage of 1 V.

Keywords: exoelectrogenic bacteria, hydrogen generation, *Rhodopseudomonas palustris*, hydrogenase, MEC.

Resumen

El objetivo de este trabajo fue la identificación de microorganismos presentes en la tela de grafito del biocátodo y bioánodo de una Celda de Electrólisis Microbiana (CEM), utilizando clonación y secuenciación del gen 16S del RNA ribosomal. Los resultados obtenidos indicaron que las clonas del bioánodo se relacionaron con los filos *Firmicutes* (15.3%), *Proteobacteria* (7.6%), *Bacteroidetes* (30.7%), and *Ignavibacteriae* (7.6%). Mientras que las clonas del biocátodo se relacionaron con el filo *Proteobacteria* (38.4%). Las clonas del bioánodo se relacionaron con especies identificadas previamente en CEM y Celdas de Combustible Microbiana (CCM). Sin embargo, las clonas del biocátodo se relacionaron con *Rhodopseudomonas palustris*, la cual no había sido reportada para la producción de hidrógeno a través de CEM. Los resultados sugieren la participación de esta especie en la producción de 0.011 m^3 H_2/m^3 por volumen líquido de cátodo al día aplicando un voltaje de 1.

Palabras clave: bacteria exoelectrogénica, generación de hidrógeno, *Rhodopseodomonas palustris*, hidrogenasa, CEM.

1 Introduction

Microbial reactors have opened up new possibilities and are attracting a lot of attention for producing electricity with Microbial Fuel Cells (MFCs) (Logan and Regan, 2006) and hydrogen with Microbial Electrolysis Cells (MECs) (Rozendal *et al*., 2007), by their beneficial to environment into using waste waters (Domínguez-Maldonado *et al*., 2014).

Hydrogen production with MECs involves separating the anode and cathode compartments with an ion exchange membrane, and connecting them

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via an external electrical circuit (Rozendal *et al*., 2007). At the anode (also called the bioanode), microorganisms oxidize organic compounds, transferring electrons by direct contact between the bacterial cell and electrode (nanowires or membrane-bound c-type cytochrome) or by intermediate electron carrier molecules (endogenous or exogenous) (Alfonta, 2010). The electrons are conducted by the external circuit to the cathode to generate H_2 through the reduction of H+ ions (Drapcho *et al*., 2008). However, bacterial cells possess energetic limitations to complete the oxidation of organic substrates for hydrogen formation, requiring the application of an additional voltage to convert organic substrate to CO_2 and hydrogen (Geelhoed *et al*., 2010; Liu *et al*., 2010).

The hydrogen production reaction can be catalyzed using platinum (Rozendal *et al*., 2007), but its high cost limits scale-up (Liu *et al*., 2010). Furthermore, its susceptibility to poisoning has led researchers to examine non-precious metals (Selembo *et al*., 2010) and other alternatives. In this regard, purified hydrogenase enzymes isolated from microorganisms have been used to replace chemical catalysts in MFCs, because they catalyze the reversible $H_2 \longleftrightarrow 2H^+ + 2e^-$ reaction (Lojou *et al*., 2011). Nevertheless, whole immobilized cells of *Desulfovibrio vulgaris* (well known for its hydrogenase activity) have demonstrated more stable and maintained catalytic activity for hydrogen production than enzyme immobilization (Lojou *et al*., 2002).

Likewise, biocathodes have been conceived that consist of microorganisms covering an electrode made of material (e.g., carbon) or dispersed in the electrolyte to catalyze cathodic reactions in MECs (Rabaey and Rozendal, 2010). The biocathode activity in a MEC therefore depends on the microorganisms' ability to take up electrons from the electrode material and use them to produce hydrogen (Croese *et al*., 2011). The microorganisms active on the biocathode have scarcely been researched for hydrogen production in MECs. In this regard, Croese *et al*. (2011) identified *Desulfovibrio vulgaris* as a dominant ribotype in the biocathode of a MEC, but this is the only report so far. The challenge lies in biocathode identification in order to further contribute to knowledge about the reduction process. The aim of this study was to identify the microorganisms present on the graphite cloth of the biocathode and bioanode using 16S ribosomal RNA gene cloning and sequencing.

2 Methodology

2.1 Microbial Electrolysis Cell (MEC)

A double-chamber MEC system was constructed from poly-(methyl-methacrylate) with an operational volume of 0.57 L, and the chambers were separated by Nafion® 117 (22.2 cm^2). The electrodes were graphite cloth (32 cm^2), connected by an external circuit with stainless steel and covered with termofit® (Chae *et al*. 2008). System startup and operation were carried out in accordance with Rozendal *et al*. (2007) and Jeremiasse *et al*. (2010).

2.2 Biocathode startup

This electrode is referred to as the bioelectrode (during biocathode startup) or biocathode (after biocathode startup). The bioelectrode chamber was inoculated with a mixed culture of microorganisms. The composition of inoculums was soil (30 g/L), cow manure (300 g/L), pig manure (150 g/L), Na_2CO_3 (1.5 g/L), commercial sucrose (5 g/L), and tap water. The inoculum was pretreated to eliminate methanogenic microorganisms. This consisted of thermal treatment at 100 ºC for 15 min (Logan *et al*., 2008).

The bioelectrode was inoculated and subjected to a three-phase biocathode startup as follows. After inoculation, the bioelectrode was started up as a bioanode. This occurred in phase 1, which meant that the bioelectrode chamber was initially operated in batch mode to allow the microorganisms to adapt to the bioelectrode chamber without being washed out immediately. During this phase, 15 mM sodium acetate was used as the carbon source. After 63 hrs, anodic current generation started and operation was switched from batch to continuous mode. At this point of phase 1, the headspace chamber was flushed with H_2 gas (20 L of metal hydride, 40 mL/min) to achieve H_2 saturation.

In the transition from phases 2 to 3, a polarity reversal scan was performed to determine the suitable bioelectrode potential for biocathode operation. During this scan, the bioelectrode potential was lowered from -0.2 to -0.8 V at a scan rate of 0.025 mV/s using a potentiostat (Potentiostat, Biologic). At this point, H_2 gas flushing was stopped and the carbon source was replaced with sodium bicarbonate (10 mM) every 24 hrs. The theoretical potential of hydrogen formation at pH 7 is -0.414V. Finally, a potential of -0.7 V was applied for 45 hrs, because it generated greater current density (0.011 A/m^2). For

the final biocatalyzed MEC arrangement, the bioanode of a conventional MFC started up previously and the obtained biocathode were used.

2.3 Sample collection from MEC graphite cloth and 16S rRNA gene analysis

The MEC was disassembled in an anaerobic chamber equipped with nitrogen flow. Subsequently, 1 cm^2 of graphite cloth was taken from the bioanode and biocathode and used to extract the DNA of the attached microbial community, as described by Canto-Canché *et al*. (2013). The bacterial 16S rRNA gene was amplified with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3'), amplifying the 880 pb fragment of the V1 to V5 regions (Luna *et al*., 2006). V3-V5 nested PCR was performed using primer 341F (5'-CCTACGGGAGGCAGCAG-3') (Muyzer *et al*., 1993) in combination with primer 907R, yielding ~600pb. Each PCR reaction was carried out in a total volume of 10 μL. The cocktail mix consisted of Buffer 1X (200 mM Tris pH 8.4, 500 mM KCl), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, 0.065 U Taq DNA polymerase (Invitrogen) and 0.4 μL of DNA (2 ng/μL). The conditions for first PCR were 94 °C for 5 min for denaturation, followed by 35 cycles of 30 s at 94 °C, 1.5 min annealing at 55°C, a 30 s extension at 72 °C, and a final extension of 4 min at 72 °C. The conditions for second PCR were similar, but annealing temperature was 64 °C. The amplicons were run on 1.5% agarose gel. The amplicons were purified following PureLink^{TM} Quick Gel Extraction (K2100-12, Invitrogen) and cloned using pGEM-T Easy Vector System (A1360, Promega). A total of 413 colonies were recovered, of which 77 were white clones: 16 were recovered, and 8 were sequenced from the bioanode. For the biocathode, 11 were recovered and 5 were sequenced. The clones were sequenced (Macrogen, Korea). DNA sequences were checked with the VecScreen program available on the NCBI page, and the vector sequences removed. DNA sequences were then used as queries to retrieve homologous sequences from the GenBank database using the Microbial Genome Basic Local Alignment Search Tool (BLAST) algorithm. The sequences obtained were compiled and aligned by Clustal W (Mega 5.05). The Maximum Likelihood method was used for tree reconstruction, using the nucleotide substitution model (Kimura 2-parameter) as the best model for substitution. The phylogenetic tree was obtained by the neighbor-joining method (Saitou and Nei, 1987) with 1,000 resampling bootstrap analysis implemented in Mega 5.0.1 software (Tamura *et al*., 2011). The sequences were deposited in the NCBI database. 16S rRNA gene sequences from the biocathode were KF528148, KF528149 and KF528151-KF528153; and from the bioanode they were KF528150 and KF528154-KF528160.

3 Results

3.1 Microbial electrolysis cell production

The operation of MEC using bioelectrodes on both sides of the cell yielded 0.011 m3 H_2/m^3 cathode liquid volume/day at 1.0 V. At the end of the experiment, the microorganisms covered the electrode, generating a biofilm or bioelectrode.

3.2 Genetic identification of microbial consortium in a MEC

This research provides the first description of a microbial community that thrives in the bioanode and biocathode of a MEC. The tentative closest relative according to the NCBI from the 16S rRNA gene clone library (total of 13 clones) is shown in Table 1 and Fig. 1. Bioanode sequences were affiliated with the following four phyla: *Firmicutes* (2 clones, 15.3%, KF528158 and KF528156), *Proteobacteria* (1 clone, 7.6%, KF528154), *Bacteroidetes*, (4 clones, 30.7%, KF528155, KF528157, KF528159, KF528160), and *Ignavibacteriae* (1 clone, 7.6%, KF528150). Within the *Proteobacteria*, only one clone (20rA clone) was identified, belonging to the class *β-Proteobacteria*, *Advenella kashmirensis* species. *A. kashmirensis* is an aerobic, gram-negative chemolithoautotroph that oxidizes thiosulfate (Ghosh *et al*., 2011). The *A. kashmirensis* WT001 strain (NC017964) was isolated from bulk soil of a temperate orchard in the sub-tropical Himalayas, in India.

Within the *Firmicutes*, one clone belonged to the class *Clostridia*, and the other to the class *Negativicutes*. From the class *Clostridia*, one clone (22rA clone) was related to *Clostridium cellulovorans* 734B (NC014393). This organism is an anaerobic spore-forming bacterium and was isolated from a wood chip pile. This bacterium's ability to utilize cellulose and other carbon sources has been used in a biotechnological process for efficient degradation of plant cell wall polysaccharides to obtain fermentation

Table 1. Closets relatives of clones obtained from bioanode and biocathode biofilm.

Clones' name	Gene bank accession number	Nearest BLAST species	Phylum/ class	Identidy (%)
2C, 13C, 20C, 19C, 14C	NC005296	*Rhodopseudomonas palustris* CGA009	*Proteobacteria (Alphaproteobacteria)* (38.4%)	95-99
4A, 22rA	NC015437	*Selenomonas sputigena*	*Firmicutes (Clostridia)* (15.3%)	82-84
	NC014393	*Clostridium cellulovorans* 734B		
20rA	NC017964	*Advenella kashmirensis* WT001	*Proteobacteria (Betaproteobacteria)* (7.6%)	98
8A	NC017770	*Solitalea caadensis* DSM3403	*Bacterioidetes/Flavobacteriia* (30.7%)	87-89
24rA, 21rA, 7A	NC016599	*Owenweeksia hongkongensis*DSM17368		
14rA	NC017464	*Ignavibacterium album* JCM16511	*Ignavibacteria (Ignavibacteria)* (7.6%)	92

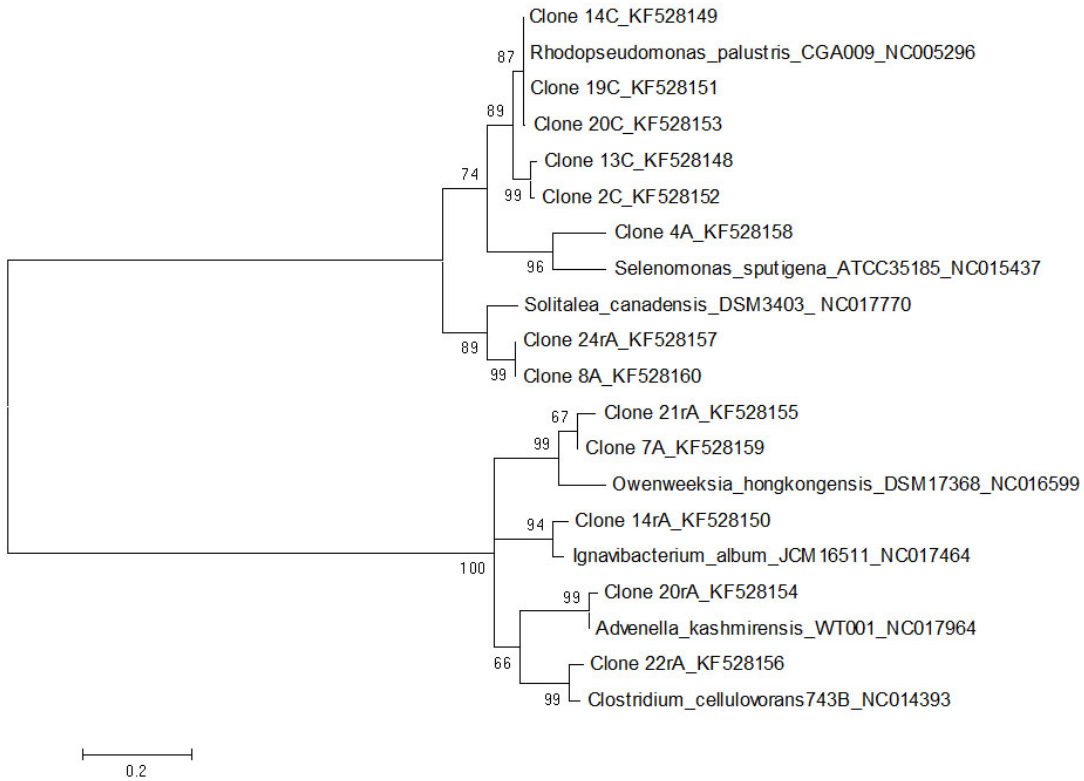

Fig. 1. Phylogenetic tree analysis of clones studied from the bioanode and biocathode, as well as their related species retrieved from GenBank based on 16S rRNA gene sequence. The maximum likelihood method was used to construct the tree. Bootstrap values shown next to nodes were calculated using 1,000 replicates.

products such as H_2, CO_2, acetate, butyrate, fumarate, lactate, and ethanol (Tamaru *et al*., 2010).

From the class *Negativicutes*, one clone (4A clone) was related to *Selenomonas sputigena* ATTC-35185 (NC015437). This species was isolated from human oral microflora and is free-living, anaerobic, gram-negative, rod-shaped and motile (McCarthy *et al*., 1981).

Within the *Bacteroidetes*, four clones (24rA clone, 8A clone, 21rA clone and 7A clone) belonged to the classes *Sphingobacteria* and *Flavobacteria. Sphingobacteria* (24rA clone and 8A clone) were related to *Solitalea canadensis* DSM3403 (NC017770). This species is gram-negative, motile, anaerobic, mesophilic and presents positive results for glucose fermentation (Hang-Yeon *et al*., 2009). It was isolated from soil in Canada. Within the *Flavobacteria* class (21rA clone and 7A clone), two clones were related to *Owenweeksia hongkongensis* DSM 17368 (NC016599). This species is an aerobic, mesophilic, gram-negative, motile, non-flagellated, and rod-shaped bacterium. The colonies are orange, convex, smooth, glistening and translucent. It was isolated from sea water (sand filtered) (Riedel *et al*., 2012).

Within the phylum, only one clone (14rA clone) was identified belonging to the class *Ignavibacterium album* JCM 16511 (NC017464). This species is a chemoheterotroph with a versatile metabolism, capable of organoheterotrophy under both oxic and anoxic conditions (Liu *et al*., 2012).

Biocathode sequences were only affiliated with the phylum *Proteobacteria* (5 clones, 38.4%, KF528148, KF528149, KF528151-KF528153). All five clones (14C clone, 19C clone, 20C clone, 13C clone and 2C clone) were related to the class *Alpha Proteobacteria* (Figure 1). Within this class, all clones were related to *Rhodopseudomonas palustris* CGA009 (NC005296). This species is a rod-shaped phototrophic bacterium, a member of the phototrophic purple non-sulfur (PPNS) bacteria which reproduces by budding (Okubo *et al*., 2006; Carlozzi and Sacchi, 2001) and proliferates in different environments due to its versatile metabolisms. *R. palustris* exhibits four different types of metabolism: photoautotrophic, photoheterotrophic, chemoheterotrophic and chemoautotrophic (Larimer *et al*., 2004). It can also grow by oxidation of organic compounds such as organic acids, alcohols or aromatic substances, as well as inorganic electron donors, carbon and nitrogen. It degrades plant biomass and chlorinated pollutants and it generates hydrogen as a product of nitrogen fixation. Its ability to produce hydrogen is highly exploited in industrial-scale fermentative processes (Chalam *et al*., 1996; Chen *et al*., 2006; Lee *et al*., 2011; Adessi *et al*., 2012).

4 Discussion

This research refers about the microbial diversity in the biocathode and bioanode of a MEC. The results showed bacterial diversity at the bioanode, with clones related to the phyla *Firmicutes* and *Bacteroidetes*. The clones related to *Firmicutes* were *Clostridium cellulovorans* 743B, which uses cellulose as a carbon source for growth. In this regard, *Clostridium* sp. has been identified in the anode of numerous MFCs fed precisely with cellulose (Kiely *et al*., 2011) to produce electricity, and has also been reported as an effective H_2 producer (Venkata *et al*., 2011). Its relation is therefore in keeping with the literature on active microorganisms in the bioanode of MFCs. *Selenomonas sputigena* was also identified from the *Firmicutes*. Although this species has not previously been identified in an MEC, another *Selenomonas* species, *Selenomonas ruminantium*, has been reported for H_2 production (Scheifinger, 1975). The phylum *Bacteroidetes* included bacteria species apparently unrelated to exoelectrogens. Both of the phyla related at the bioanode were identified by Croese *et al*. (2011) as the dominant ones in the biocathode of a MEC.

Rhodopseudomonas palustris was clearly dominant at the biocathode. This can be explained by the fact that *Rhodopseudomonas* species are known for their ability to generate hydrogen. The ability of the biocathode to generate hydrogen resides in the hydrogenase activity which catalyzes the reversible conversion of hydrogen from protons and electrons (Geelhoed *et al*., 2010). A recent study by Croese *et al*. (2011) reported the dominance of *Desulfovibrio vulgaris* at the biocathode of a MEC. Both *Desulfovibrio vulgaris* and *Rhodopseudomonas* species are highly notable given their remarkable ability to generate hydrogen, and therefore possess biotechnological relevance (Carepo *et al*., 2002).

However, despite the fact that *R. palustris* possesses this ability its presumable relation at the biocathode in a MEC had not been reported before this study. On the contrary, *R. palustris* strains such as DX-1 have demonstrated electrochemical activity in an anode of an MFC (Xing *et al*., 2008; 2009). The mechanisms involved in transferring the electrons generated from organic compound oxidation

to the bioanode by the electrochemical activity of microorganisms are just beginning to be understood, mainly through genetic analysis, such as microarray analysis. An analysis of *Geobacter sulfurreducens* demonstrated the involvement of genes encoding outer-membrane c-type cytochromes, multicopper proteins and pili as the electrical contact between *G. sulfurreducens* and the electrode surface to transfer electrons to the electrode in a MFC (Holmes *et al*., 2006). However, the mechanisms involved in the electron uptake process from the electrode are still under discussion. Recent studies on *G. sulfurreducens* suggest that the mechanisms for accepting electrons from electrodes are substantially different to those for transferring electrons to electrodes (Strycharz *et al*., 2011).

On the other hand, the mechanisms involved in hydrogen formation by MECs are poorly understood (Geelhoed *et al*., 2010; Croese *et al*., 2011). However, there are a series of requirements that active microorganisms on the biocathode should show. The active microorganisms should be able to resist the voltage applied to overcome thermodynamic barriers, and to take up electrons from the cathode (solid surface), to generate molecular hydrogen by enzymatic activity (Geelhoed and Stams, 2011).

R. palustris possesses three [NiFe]-hydrogenases (Larimer *et al*., 2004; Vignais, 2008): 1) uptake hydrogenase; 2) cytoplasmic H_2 sensors; and 3) energy-conserving, H_2 evolving, membrane-associated hydrogenases (Vignais, 2008). However [FeFe]-hydrogenase is also present in *R. palustris* (Larimer *et al*., 2004). Hydrogenase synthesis of *R. palustris* responds to several types of signals: (1) molecular hydrogen, as a substrate for activation of hydrogenase gene expression; (2) molecular oxygen, as an inhibitory signal for most hydrogenases; (3) nickel ions, as a cofactor for catalytic functions; and (4) metabolites as electron donors or acceptors such as formate, carbon monoxide, nitrate or sulfur. Hydrogenase synthesis is therefore under negative control of the redox-responding global two component-regulatory systems, RegB/PrrB/RegS-RegA/Prra/RegR (Vignais, 2008). This suggests that hydrogenase activation depends on the redox environment, because this Reg/Prr system controls metabolic processes involved on CO_2 assimilation, nitrogen fixation, hydrogen metabolism and the energy-generation pathway (Vignais, 2008).

The hydrogenase activity for hydrogen production in the biocathode may also be coupled to energy conservation. This will support bacterial growth and confer a continuous catalytic activity. The mechanism for the conservation of energy is via a cytoplasmic hydrogenase together with a membrane-bound ATPase, by proton reduction to hydrogen (Geelhoed *et al*., 2010; Rozendal *et al*., 2007).

In this regard, *R. palustris* possesses P-type ATPases, which pump up ample proton supplies from ATP, supplying the energy necessary to thermodynamically catalyze unfavorable reactions (Larimer *et al*., 2004). Protons supplied by ATPases are possibly reduced by energy-conserving, H_2 evolving, membrane-associated [NiFe]-hydrogenases (Vignais, 2008), and the resulting proton gradient over the membrane is utilized by a membrane-integrated ATPase to generate ATP (Geelhoed *et al*., 2010).

This study provides an indication of the potential of *R. palustris* in MECs. Clearly, further research on the growth of *R. palustris* as a pure culture must be performed to investigate the genes involved in the reduction process.

Conclusions

This research refers to the microbial diversity in the bioanode and biocathode of a MEC. The clones were related at those previously identity in MEC bioanode, whilst the clones in the biocathode were related to *Proteobacteria*, with *R. palustris* as a related ribotype. This suggests their probable involvement on hydrogen production. Genetic studies on pure cultures of this species are therefore required in order to discover which genes are involved in electron uptake from the electrode and the reduction process.

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Nomenclature

pb	pares de bases
MFC	Microbial Fuel Cell
MEC	Microbial Electrolysis Cell

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