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Influence of inoculum and anode surface properties on the selection of *Geobacter*-dominated biofilms

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HIGHLIGHTS

- Dry soil MFC had longest start-up time (28 days) and poor current output.
- COO^- anodes had longest start-up times (6.3 days) but highest power output (118 mW m^{-2}).
- All biofilms selected were dominated by *Geobacter* sp.
- *Geobacter* sp. is widespread in soils, even those frequently exposed to oxygen.
- *Geobacter* is very much better at growing in MFC conditions than any other bacteria.

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ABSTRACT

This study evaluated the impact of inoculum source and anode surface modification (carboxylate $-COO^-$ and sulfonamide $-SO_2NH_2$ groups) on the microbial composition of anode-respiring biofilms. These two factors have not previously been considered in detail. Three different inoculum sources were investigated, a dry aerobic soil, brackish estuarine mud and freshwater sediment. The biofilms were selected using a poised anode (−0.36 V vs Ag/AgCl) and acetate as the electron donor in a three-electrode configuration microbial fuel cell (MFC). Population profiling and cloning showed that all biofilms selected were dominated by *Geobacter* sp., although their electrochemical properties varied depending on the source inoculum and electrode surface modification. These findings suggest that *Geobacter* sp. are widespread in soils, even those that do not provide a continuously anaerobic environment, and are better at growing in the MFC conditions than other bacteria.

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1. Introduction

Exoelectrogens are found in anaerobic sediments and soils where they have access to both reduced organic compounds, for use as electron donors, and insoluble inorganic electron acceptors including manganese and iron oxides (Lovley, 1993; Weber et al., 2006). Many locations meet these requirements while varying in other environmental parameters. Previous work has confirmed the presence of exoelectrogenic bacteria in various different environments including freshwater sediments (Chae et al., 2009; Holmes et al., 2004), marine sediments (Bond et al., 2002; Tender et al., 2002), salt-marshes (Holmes et al., 2004), anaerobic sludge from potato processing (Rabaey et al., 2004), wastewater treatment plants (Kan et al., 2011; Lefebvre et al., 2010), and recently in mangrove swamp sediments (Salvin et al., 2012). *Geobacteraceae* are usually the predominant microorganisms colonizing the anodes introduced in such environments, with a higher abundance of *Desulfuromonas* species in marine and salt-marsh sediments; while in freshwater sediments, *Geobacter* species are the most common *Geobacteraceae* (Holmes et al., 2004). Following the Baas-Becking hypothesis (1934) that "*Everything is everywhere, but the environment selects*", we should expect to select for exoelectrogenic biofilms dominated by *Geobacteraceae* whatever the inoculum used. Indeed, Yates et al. (2012) showed that the predominance of *Geobacter* sp. in acetate-fed MFCs (microbial fuel cells) was independent of the inoculum source, after testing

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three inocula (two wastewaters from different locations and an anaerobic bog sediment). However, other researchers found that the inoculum makes a difference in the selection of anode-respiring biofilm in MFCs (Miceli et al., 2012). Miceli et al. (2012) tested thirteen samples from locations around the world and placed them in MFCs with electrodes poised at −0.30 V vs Ag/AgCl in acetate medium. Only 7 out of 13 samples produced sufficient current (>1.59 A m^{-2}) after 21 days of selection. They found that bacteria related to the genus *Geobacter* dominated only two of the seven biofilm communities producing a high current; the other biofilm communities contained different known and/or novel exoelectrogenic bacteria (Miceli et al., 2012). Few studies have looked at the effect of inoculum source on the composition of exoelectrogenic biofilms selected in MFCs either with or without fixed anode potentials. To bring more consistency in the results, it is recommended to test inocula in MFCs held at the same fixed potential (e.g., −0.08 V vs SHE), as the anode potential is likely to influence the composition of the anodic biofilm (Commault et al., 2013). The inocula tested in previous studies are typically from rich, moist anaerobic environments likely to contain *Geobacter* sp. In this study three very different inocula are tested: a saline estuary mud; a freshwater sediment; and a dry, exposed, low fertility basalt/loess soil thought to be unlikely to contain *Geobacter* sp. Each inoculum was placed in an MFC with the anode held at −0.36 V vs Ag/AgCl (−0.08 V vs SHE) as an electron acceptor and provided acetate as an electron donor. The selected anodic biofilms were compared for current production, biofilm/electrode interaction, and dominant microbial community composition.

We also investigated the impact of electrode surface properties on the selection of electro-active biofilms in MFCs. The anode surface chemical and physical properties affect bacterial adhesion and electron transfer process between bacteria and electrodes (Guo et al., 2013). Modification of electrode surfaces aiming to improve the efficiency of MFCs has recently emerged as a new field of research (Kumar et al., 2013; Wei et al., 2011). Although some studies have proven that certain anode modifications lead to more efficient MFCs (Lapinsonniere et al., 2013; Picot et al., 2011), the influence of surface modifications for biofilm growth and maintenance is not well understood. In this study, the effect of two different chemical groups: negatively charged carboxylate group ($-COO^-$) and sulfonamide group ($-SO_2NH_2$) neutral at physiological pH were tested on electro-active biofilms selected in MFCs using the same inoculum and same anode potential (−0.36 V vs Ag/AgCl). The sulfanilamides are characterized by their lipophilicity and their amine groups partly protonated at pH 7. Note however that the amine group is lost in the modification process so that the resulting modifier bears a neutral charge (phenylsulfonamide). The lipophilicity of sulfanilamides favors their interactions with the lipid bilayer of the bacterial cell membrane and the polymeric lipophilic compounds of EPS (extracellular polymeric substances). The presence of phenylsulfonamide at the electrode surface is therefore likely to encourage the attachment of bacteria via lipophilic attachment. The carboxylates ($-COO^-$) are negatively charged at pH 7 (pKa($-COOH/-COO^-$) ~ 4), which could potentially repulse bacteria. The bacterial community composition of biofilms selected on modified electrodes was investigated along with their electrochemical properties.

This paper examines whether two independent factors, inoculum source and electrode surface modification, could alter the composition and electrochemical properties of anodic biofilms selected in MFCs. This question is of importance for the discovery of new anode-respiring bacteria and new metabolic pathways for higher current production in MFC. The two factors were tested independently starting with three different microbial inoculum sources.

2. Methods

2.1. Electrode modification procedures

Carboxylate and sulfonamide groups were grafted onto graphite rod electrodes using the electrochemical reduction of aryl diazonium salts, as described by Picot et al. (2011). The process involved two steps, the formation of aryl diazonium salts from their corresponding amines followed by *in situ* electro-reduction of the diazonium, by cyclic voltammetry with monitoring of the charge consumed in the process to control the amount of molecules grafted on the electrode (Picot et al., 2011). Diazonium salts were generated *in situ* in a total volume of 75 mL of acidic aqueous medium (0.1 M HCl) containing the starting aryl amine (4 mM of 4-aminobenzoic acid for $-COO^-$ and 2 mM of 4-aminobenzenesulfonamide for $-SO_2NH_2$) and sparged with argon for 10 min to remove oxygen. Then sodium nitrite ($NaNO_2$) was added at a final concentration of 10 mM. The mix was kept on ice in the dark to stabilize the generated aryl diazonium salt. This solution served as the electrolyte for the modification of the previously sandpapered graphite electrode by electrochemical reduction of the diazonium salts using a potentiostat (model EA164 QuadStat). A three-electrode cell configuration was used with an Ag/AgCl, NaCl (3 M) reference electrode (0.28 V vs SHE, BASI Electroanalytical Chemistry, MF-2052) and a second graphite electrode as the counter electrode, as described by Commault et al. (2013). Electrochemical reduction of the diazonium salts was achieved by recurrent cyclic voltammetry sweeps starting at zero-current potential (around +0.2 V vs Ag/AgCl) and decreasing to −0.2 V vs Ag/AgCl. Several scans at a rate of 0.05 V s^{-1} were needed to reach a global charge density (*Q*) of 15–20 mC cm^{-2} (projected anode area of 5.81 cm^2). To probe the effect of the modification on the electrode properties, cyclic voltammetry was performed at a scan range of −0.1 V to 0.4 V and a scan rate of 0.1 V s^{-1} in a solution of potassium ferricyanide $K_3[Fe(CN)_6]$: 2 mM of ferricyanide, 0.1 M KCl and 10 mM of phosphate buffer pH 7. The voltammograms obtained were compared to an unmodified graphite electrode.

2.2. Anode-respiring biofilm growth and selection

All the anode-respiring biofilms presented in this paper were selected in 100 mL MFCs as previously described by Commault et al. (2013). The anode potentials were maintained at −0.36 V vs Ag/AgCl (i.e. −0.08 V vs SHE) using a three-electrode arrangement. The counter electrode (carbon cloth, Fuel Cell Earth LLC, Ma, USA) was separated from the anolyte by an Ultrex CMI-7000 cation-exchange membrane (Membranes International Inc., NJ, USA) in a chamber containing 0.1 M phosphate buffer (pH 7.5). The anode, a (modified or unmodified) graphite rod of 5.81 cm^2, was maintained at a fixed potential by a 4-channel potentiostat (model EA164 QuadStat) connected to an e-corder 1621 unit (eDAQ Pty Ltd, NSW, AUS). The same inoculum was used for the experiment comparing the effects of two chemical groups grafted on anodes. The COO^- and SO_2NH_2 MFCs were both inoculated with 50 mL of water-saturated soil collected in Lincoln (Christchurch, NZ). For the experiment comparing the effect of three different inocula on the growth and selection of anodic biofilms, 50 mL of soils from diverse environments were added to three different MFCs with unmodified working electrodes. The inocula were referred to as (i) "Crater Rim" (CR) a dry soil collected on the hillside of a Banks Peninsula walking track (Canterbury, NZ); (ii) "Church Bay" (CB) a wet saline estuary mud (Canterbury, NZ); and (iii) "Halswell River" (R) a wet soil from the bed of a freshwater stream (Canterbury, NZ). Once inoculate, the 100 mL MFCs were

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