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Electrochemical Characterization of *Geobacter sulfurreducens* Cells Immobilized on Graphite Paper Electrodes

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ABSTRACT: Bacteria able to transfer electrons to conductive surfaces are of interest as catalysts in microbial fuel cells, as well as in bioprocessing, bioremediation, and corrosion. New procedures for immobilization of *Geobacter sulfurreducens* on graphite electrodes are described that allow routine, repeatable electrochemical analysis of cell–electrode interactions. Immediately after immobilizing *G. sulfurreducens* on electrodes, electrical current was obtained without addition of exogenous electron shuttles or electroactive polymers. Voltammetry and impedance analysis of pectin-immobilized bacteria transferring electrons to electrode surfaces could also be performed. Cyclic voltammetry of immobilized cells revealed voltage-dependent catalytic current similar to what is commonly observed with adsorbed enzymes, with catalytic waves centered at −0.15 V (vs. SHE). Electrodes maintained at +0.25 V (vs. SHE) initially produced 0.52 A/m^2 in the presence of acetate as the electron donor. Electrical Impedance Spectroscopy of coatings was also consistent with a catalytic mechanism, controlled by charge transfer rate. When electrodes were maintained at an oxidizing potential for 24 h, electron transfer to electrodes increased to 1.75 A/m^2. These observations of electron transfer by pectin-entrapped *G. sulfurreducens* appear to reflect native mechanisms used for respiration. The ability of washed *G. sulfurreducens* cells to immediately produce electrical current was consistent with the external surface of this bacterium possessing a pathway linking oxidative metabolism to extracellular electron transfer. This electrochemical activity of pectin-immobilized bacteria illustrates a strategy for preparation of catalytic electrodes and study of *Geobacter* under defined conditions.

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Introduction

The *Geobacteraceae* family of *δ-proteobacteria* are capable of reducing insoluble Fe(III) and Mn(IV) oxyhydroxides at circumneutral pH as part of their energy-generating strategy (Lovley, 2000). To obtain electrons for this metabolism, members of the *Geobacteraceae* completely oxidize fermentation products, including; acetate, ethanol, propionate, butyrate, and lactate, as well as aromatics such as benzoate, toluene, and phenol (Lovley, 2000). Because of their ubiquitous nature and demonstrated role in hydrocarbon bioremediation (Lovley and Anderson, 2000; Rooney-Varga et al., 1999; Snoeyenbos-West et al., 2000), dechlorination (Sung et al., 2003, 2006), and heavy metal bioimmobilization (Anderson et al., 2003; Holmes et al., 2002; Wilkins et al., 2006), the mechanisms used by *Geobacteraceae* to oxidize electron donors and reduce extracellular metals are of great interest.

Recent work has demonstrated that members of the *Geobacteraceae*, as well as other metal-reducing bacteria, can transfer electrons to electrodes (Bond and Lovley, 2003; Bond et al., 2002; Chang et al., 2006; Holmes et al., 2004a,b). While this phenomenon is typically studied in devices able to produce electrical power (microbial fuel cells), the ability to interact with an electrode suggests broader applications. Electrodes can serve as tools for detecting electron transfer processes between bacteria and conductive surfaces, and can be used to influence biochemical processes by serving as electron donors in metabolism (Gregory and Lovley, 2005; Gregory et al., 2004).

Geobacter sulfurreducens strain PCA, which often serves as a model for the *Geobacteraceae*, produces multiple membrane-bound cytochromes that have been implicated in electron transfer to external metals and electrodes (Chin et al., 2004; Khare et al., 2006; Kim et al., 2005; Leang and Lovley, 2005; Leang et al., 2003, 2005; Magnuson et al., 2004; Mehta et al., 2005). In addition, pili (Reguera et al., 2005, 2006, 2007) have been described which may contribute to attachment or electron transfer over larger (>1 μm) distances. Multiple investigations have failed to detect a secreted soluble compound that would assist *G. sulfurreducens* in the transfer of electrons to metals (Nevin and Lovley, 2000, 2002; Straub and Schink, 2003).

Immobilization of bacteria has increased reaction rates and longevity of biocatalysts (Fidaleo et al., 2006; Flickinger et al., 2007; Gosse et al., 2007; Lyngberg et al., 1998, 2000, 2001, 2005). However, many aspects of *Geobacter* metabolism could prevent adaptation of such techniques. For example, proteins required for extracellular respiration may not be expressed when *Geobacter* is cultured with soluble electron acceptors prior to cell harvesting (Chin et al., 2004; Mehta et al., 2005). Delicate membrane proteins could be easily sheared from membrane surfaces (Mehta et al., 2005; Reguera et al., 2005). Also, polymers could prevent proteins from contacting electrodes.

Before any *Geobacter* immobilization strategy can be evaluated for biotechnology applications, it must be shown that electrical contact can be established between entrapped cells and electrodes. In this report, we describe methods for immobilizing *Geobacter* on graphite paper electrodes, and measuring voltammetric and impedance characteristics of electricity-generating films. These results support a model of electron transfer via a direct mechanism by *Geobacter*, and demonstrate how electrochemical capabilities of intact organisms can be directly measured using voltammetry.

Materials and Methods

Bacterial Strain and Culture Media

G. sulfurreducens strain PCA (ATCC #51573) was subcultured in our laboratory at 30°C using a standard anaerobic medium containing per 1 L: 0.38 g KCl, 0.2 g NH_4Cl, 0.069 g $NaH_2PO_4 \cdot H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 10 mL of a mineral mix (containing per 1 L: 1.5 g NTA, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.3 g $FeSO_4 \cdot 7H_2O$, 0.17 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnCl_2$, 0.04 g $CuSO_4 \cdot 5H_2O$, 0.005 g $AlK(SO4)_2 \cdot 12H_2O$, 0.005 g H_3BO_3, 0.09 g Na_2MoO_4, 0.12 g $NiCl_2$, 0.02 g $NaWO_4 \cdot 2H_2O$, and 0.10 g Na_2SeO_4).

Where indicated, the electron acceptors fumarate (40 mM) or Fe(III) citrate (55 mM) were included in the medium. Acetate was provided as an electron donor at 20 mM. All media were adjusted to pH 6.8 prior to addition of 2 g/L $NaHCO_3$, and flushed with oxygen-free N_2/CO_2 (80/20, v/v) prior to sealing with butyl rubber stoppers and autoclaving. Mid-exponential phase cells were harvested at half-maximal optical density (<0.3 OD^{600}).

Pectin Coating Procedure

Genu pectin X 920-02 (CPKelco, Atlanta, GA) was mixed in a blender to a final concentration of 4%, while adjusting the pH to 8.5 with NaOH. This was autoclaved, during which time the pH dropped to 6.5. 10 mL of *G. sulfurreducens* ($\sim 1 \times 10^9$ cells) in mid exponential phase were centrifuged at 4,900*g* for 3 min to pellet cells, and were gently resuspended in 1 mL sterile wash buffer containing (per liter) 0.38g KCl, 0.2 g NH_4Cl, 0.069 g $NaH_2PO_4 \cdot H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 3.5 g NaCl. The suspension was pelleted at 4,900*g* for 3 min and the supernatant aseptically removed.

The remaining cell slurry (~15 μL) was pipetted onto both sides of a sterile piece of 0.4 × 2 cm × 0.3 mm prewetted graphite paper (TGPH-120 E-Tek, Somerset, NJ), to cover a geometric area of 0.6 cm^2. The suspension diffused into the porous weave of the graphite within 30 s. Ten microliter of 4% pectin was distributed on both sides of the paper, and immediately cross-linked with a spray of 25 mM $BaCl_2$. The coatings were soaked in a medium supplemented with 25 mM $BaCl_2$ for 0.5 h in an anaerobic chamber to crosslink the polymer (Coy Laboratories, Ann Arbor, MI) under $N_2/CO_2/H_2$ (75/20/5, v/v/v).

Electrochemical Cell

A 20 mL capacity cone-bottom electrochemical cell (Bioanalytical Systems, West Lafayette, IN) was used for the 3-electrode bioreactor. Teflon caps were constructed to accommodate all wires, electrodes, and gas fittings. A standard calomel electrode (SCE) was used as the reference electrode, connected to the cell via a saturated KCl salt bridge ending in a 5 mm Vycor glass membrane (Bioanalytical Systems). A 0.1 mm Pt wire was used as the counter electrode, while carbon electrodes (pectin-coated paper for most experiments, or planar glassy carbon for long-term growth experiments) attached via Pt wire served as the working electrode. Planar electrodes were used in long term growth experiments to reduce capacitive current in non-pectin coated electrodes.

During voltammetry, 10 mL of HEPES-NaCl-sodium bicarbonate buffer (10, 100, and 20 mM, respectively, pH 6.8) was used as the supporting electrolyte, to minimize the influence of electroactive minerals or vitamins. Cells were flushed for 10 min prior to each electrochemical analysis with humidified, sterile N_2/CO_2 (80/20, v/v) which had been passed over a heated copper catalyst to remove oxygen, and were immersed in a 30°C water bath. When incubated under growth conditions, electrodes were incubated in growth medium to which appropriate electron donor or acceptor was added. This medium was removed and replaced with two changes of supporting electrolyte before electrochemical analysis.

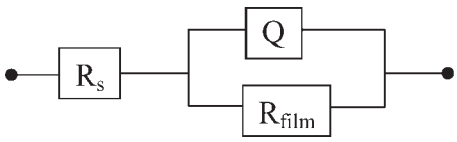
Cyclic Voltammetry (CV)

CV data acquisition and analysis was performed with a CH potentiostat 840B (CH Instruments, Austin TX). In a typical experiment, supporting electrolyte in a temperature- controlled cell with electrodes was purged for 10 min with oxygen-free N_2/CO_2 (80/20, v/v). Electrodes were incubated for 10 min after electron donors or acceptors were added, before performing another CV analysis.

Unless otherwise indicated, CV was carried out at 0.01 V/s scan rate from 0.240 to −0.56 V versus standard hydrogen electrode (SHE) while the headspace was continuously flushed with the same gas. Three CV cycles were performed for each measurement and the average recorded. The limiting current was defined as the average current observed at 0 V (vs. SHE) taken from both forward and reverse scans. By using an excess of electron donor, CVs at low scan rate (<10 mV/s) were assumed to represent an "enzyme controlled regime" (Heering et al., 1998).

Electrochemical Impedance Spectroscopy

Potentiostatic EIS was performed with a PCI4 Femtostat integrated with a Frequency Response Analyzer (Gamry Instruments, Warminster, PA), and driven by Gamry Framework software (Version 5.1). In a typical experiment, open circuit potential was monitored until it reached a constant value. The amplitude of the overimposed AC signal for impedance was 10 mV, and frequency was varied between 100 kHz and 0.01 Hz. Data fitting was performed with Gamry Analyst software (Version 5.04), which embeds a Levenberg–Marquardt non-linear least square fit algorithm. A simple 3-element equivalent circuit (see below) was used for data fitting.



(1)

The circuit consisted of solution resistance (R_s), and a distributed element Q [known as constant phase element (CPE)] (Macdonald, 1992), in parallel with a charge transfer resistance (R_{film}). The CPE is suited to describe the behavior of a non-ideal capacitance, and it is commonly used in impedance analysis of coatings (Vanwesting et al., 1994).

Additional Treatments

After initial CV and EIS analysis, coated electrodes were incubated anaerobically for 24 h at 30°C in 10 mL of growth medium containing: 20 mM acetate, 2 mM fumarate, and 40 mM NaCl (electron acceptor limiting conditions); 40 mM fumarate and 40 mM NaCl (electron donor limiting conditions), 20 mM acetate and 40 mM fumarate (growth conditions); or 60 mM NaCl (no donor, no acceptor).

When electrodes were incubated under conditions of constant oxidizing potential, coated electrodes were suspended in electrochemical cells with growth medium containing 20 mM acetate, but lacking electron acceptor. The working electrode was connected to the negative pole of a Agilent E3646A DC power supply (Agilent, Palo Alto, CA), while a SCE (Fisher Scientific, Hanover Park, IL), connected to the electrolyte via a saturated KCl salt bridge, was connected to the positive pole of the power supply. The potential was set at +10 mV, for a working electrode potential of +0.25 V versus SHE at 30°C. Current flux from the working electrode was monitored with a Keithley 6485 picoammeter (Keithley Instruments, Cleveland, OH). Unless otherwise stated, all potentials refer to SHE.

Control coatings with heat-killed and sonicated *G. sulfurreducens* were also prepared. Cells were heat-killed by exposing the culture to 80°C for 15 min before washing cells and preparing the coatings. Cells were treated with glutaraldehyde by pelleting 10 mL of mid-exponential phase cells and resuspending them in wash buffer containing 1% glutaraldehyde for 10 min before being washed and used for coatings. *Escherichia coli* K12 was grown anaerobically in LB medium for 12 h. *E. coli* coatings were prepared in a manner similar to those of live *G. sulfurreducens*.

Fe(III) Reduction Assay

Whole coatings or cells recovered from immobilization experiments were incubated in Fe(III) citrate medium, and time course samples of Fe(III) media were diluted 1:10 in 0.5 N HCL. Samples were then mixed with a 2 g/L Ferrozine + 200 mM HEPES pH 7.0 reagent (1:7) in a clear 96 well plate (Nunc, Rochester, NY). Absorbance was read at 650 nm and compared with known Fe(II) standards. Sterile Fe(III)-citrate medium served as a control.

Results

Immobilization

A requirement of any polymer used for entrapping cells near an electrode was adhesion, and preliminary screening revealed that low methyl-ester pectin preparations bound well to graphite paper electrodes. Entrapment of cells required Mg^{+2}, Ca^{+2}, or Ba^{+2} ions to cross-link the polymer. Barium produced coatings with the strongest adhesive and mechanical properties, and when $BaCl_2$ was added to standard growth medium at levels as high as 25 mM, growth rate or yield of cells was unaffected. Activity assays based on Fe(III) reduction revealed that cell suspensions immobilized on electrodes reduced Fe(III) at rates similar to cell suspensions entrapped in pectin (<10% change after entrapment). An average of 1 mg concentrated cell protein

(10^9 cells), was routinely applied to 0.6 cm^2 of 0.3 mm thick graphite paper, immediately coated on both sides with 4% pectin. Following polymerization and incubation in an anaerobic (5% H_2, 20% CO_2, 75% N_2) chamber, the thickness of the pectin film extending beyond the paper surface was 35 μm (SD = 14 μm), based on profilometry measurements.

Cyclic Voltammetry of Immobilized Bacteria

A technique based on voltammetry in 3-electrode microbioreactors was developed to immediately detect catalytic properties of intact bacteria. First, sterile electrodes were studied. After equilibration in a N_2/CO_2 (80/20, v/v)-flushed 30°C electrochemical cell in NaCl-HEPES-bicarbonate supporting electrolyte, CV demonstrated negligible background current from sterile electrodes and sterile electrodes coated with pectin (Fig. 1). Addition of acetate did not increase current flow. The separation between reducing and oxidizing scans during CV showed that pectin affected the conductivity and double layer capacitance of graphite paper to only a small extent (see also EIS results below) (Fig. 1).

Electrodes were then functionalized using *G. sulfurreducens* cells that were washed twice in anaerobic buffer, entrapped near electrode fibers using cross-linked pectin, and soaked in anaerobic electrolyte to remove soluble compounds. Bacteria-coated electrodes established a moderately reducing (<−0.1 V vs. SHE) potential immediately after preparation and polymerization in an anaerobic, H_2-containing chamber. *G. sulfurreducens* was not capable of metabolizing or oxidizing pectin [consistent with its known inability to oxidize hexoses, pentoses, or even most short-chain acids (Caccavo et al., 1994)]. As this and subsequent activity was visualized via brief CV assays (not long-term incubation), this current flux was most likely due to oxidation of internal energy storage compounds.

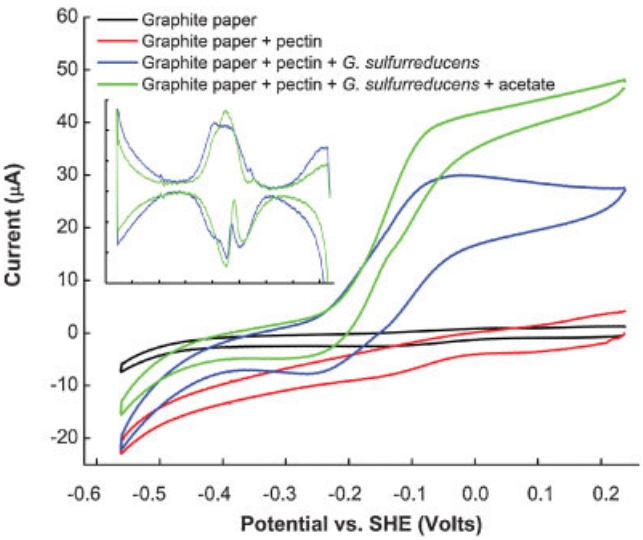


Figure 1. Typical experiment showing current production by strips of 0.6 cm^2 of 0.3-mm-thick macroporous graphite paper functionalized with pectin-entrapped *Geobacter sulfurreducens* cells, as assayed by cyclic voltammetry (CV, 10 mV/s) in 10 mL 3-electrode reactors at 30°C. Inset: First derivative analysis of current observed in the presence of live cells in the presence and absence of acetate shows similar midpoint potentials of catalytic waves (centered at −0.15 V).

CV of these electrodes (10 mV/s) revealed a sigmoidal anodic current profile characteristic of catalytic activity (Fig. 1). This positive current observed above a potential of approximately −0.2 V reflected flow of electrons from bacteria to the working electrode. The addition of 10 mM acetate to electrodes with immobilized bacteria increased the limiting current during CV sweeps, from 25 μA (±4 μA) to 36 μA (±7 μA) (mean of 23 observations) (Fig. 1).

Identical results, most notably the magnitude of the catalytic current, were obtained regardless of whether the cell washing and electrode coating procedure was conducted under aerobic or strict anaerobic conditions, as long as electrodes were incubated under anaerobic conditions for >30 min prior to analysis. First derivative analysis of voltammetry results (Fig. 1, inset) allowed estimation of the potential at which the rate of increase of the catalytic wave reached a maximum [similar to what is typically termed "E_{CAT}" in purified protein studied (Armstrong, 2005)]. This potential (−0.15 V ± 0.01V) was consistent across all experiments. The steepness of the wave was also stable across multiple experiments and treatments, as determined by the width of the derivative peak at half-maximal height (Anderson et al., 2000).

Cell Lysis and Metabolic Inhibition

When cell suspensions of *G. sulfurreducens* were treated with 1% glutaraldehyde, heat-killed at 80°C, or lysed via sonication prior to immobilization onto carbon electrodes, no oxidative currents or redox peaks were observed. These preparations were allowed to incubate for 24 h under anaerobic conditions, in the presence and absence of electron donors, but no catalytic current developed during this time (Fig. 2A). When aerobically and anaerobically-grown cultures of *E. coli* were used to prepare pectin-immobilized coatings on carbon electrodes, no electroactive species or catalytic current could be observed (Fig. 2A).

Effect of Prior Growth Conditions and Cell Concentration on Electron Transfer

Coatings were also prepared from cells grown with Fe(III)-citrate as an electron acceptor, from cells harvested at mid-exponential phase, and from stationary phase cells. Prior culture conditions did not significantly affect the shape of voltammetry profiles, alter the location of E_{CAT} maxima, or increase the limiting current compared to what was observed with exponential phase fumarate-grown cells. Based on these experiments (and in light of possible effects of contaminant Fe(III) from Fe(III)-grown cultures), the

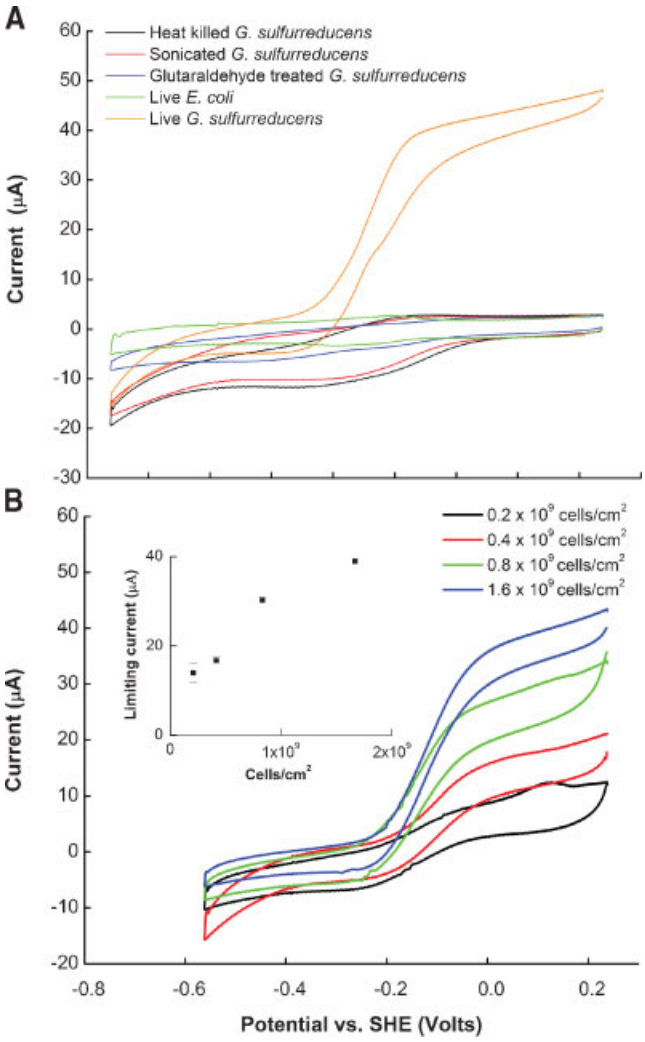


Figure 2. **A**: Current production by live versus killed *G. sulfurreducens* and *Escherichia coli* cells, as assayed by CV (10 mV/s), and (**B**) effect of increasing cell number at graphite electrodes (Inset: limiting current observed with increasing cell number, mean of three observations).

most reliable procedure for coating electrodes was to harvest cells utilizing fumarate as an electron acceptor during the mid-exponential phase of growth. Addition of lower or higher amounts of cell biomass to electrodes prior to coating with pectin directly affected rates of current production, which was best quantified by the limiting current of the catalytic wave. In these experiments, limiting current increased with increasing biomass in the coatings and approached saturating values as $>1 \times 10^9$ cells/cm^2 were added (Fig. 2B).

Incubation of Electrodes Coated With Immobilized Bacteria

Shear forces may harm delicate extracellular structures (Reguera et al., 2005, 2006, 2007), and expression of these structures may be triggered by growth conditions (Childers et al., 2002). To investigate these effects, electrodes containing immobilized bacteria were assayed for initial electrochemical activity, incubated under four different conditions, then assayed again after being soaked in electrolyte to remove all donors and acceptors and restore the original conditions, to avoid a direct competition between exogenous acceptors and the electrode (Esteve-Nunez et al., 2004). The most striking effects were observed with electrodes incubated under electron acceptor-limited conditions (20 mM acetate, 2 mM fumarate). CV revealed an increase in both catalytic current compared to initial coatings (''Fumarate-limited'' treatment in Fig. 3A), and quasi-reversible features at higher potentials. These results indicated that, even at the high cell densities applied to electrodes, incubation conditions could induce cells to increase contact with the electrode surface.

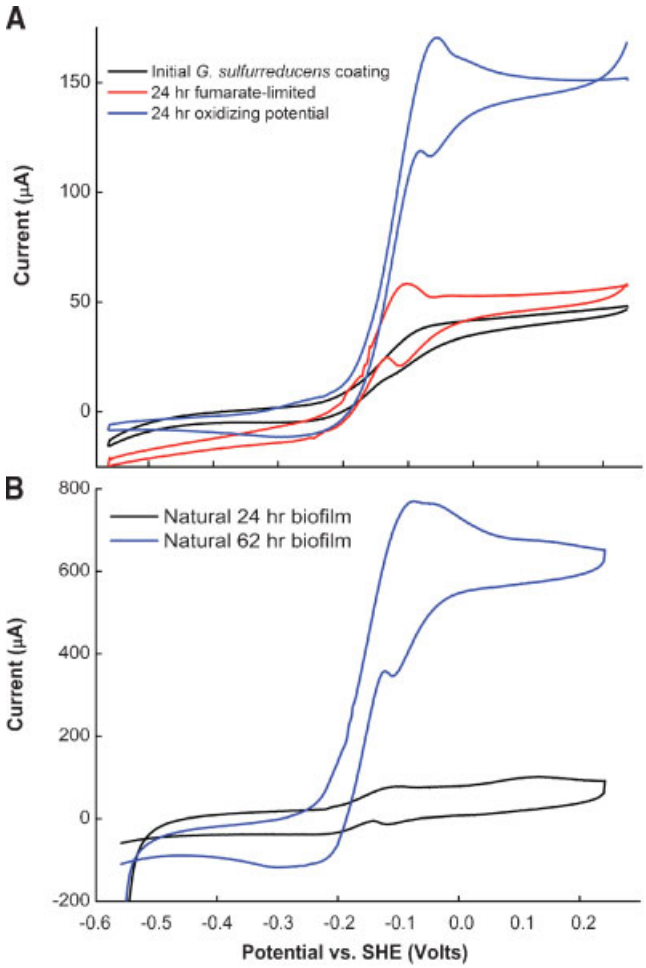


Figure 3. Typical current increase and change in CV features (10 mV/s) following incubation of electrodes containing (**A**) pectin-entrapped *G. sulfurreducens* for 24 h in the presence of acetetate (10 mM) and limiting fumarate (2 mM) or under oxidizing conditions (at +0.24 V vs. SHE) (**B**) changes in voltammetry after growth of natural *G. sulfurreducens* biofilms on flat carbon electrode surfaces (+0.24 V vs. SHE) with no pectin entrappment.

Incubation of Coated Electrodes Under Current-Generating Conditions

Electrodes prepared with *G. sulfurreducens* were also poised at +0.24 V versus SHE for 24 h in growth medium containing 20-mM acetate. In the absence of electron donor, current rapidly declined, but when electron donor (acetate) was added, a constant current of 45 μA (±6 μA, mean of nine observations) was achieved, reflecting immediate sustained use of the electrode as an electron acceptor. Within 24 h, this rate of electron transfer increased fourfold, to 150 μA (±5 μA, mean of nine observations). CV of electrodes after 24-h incubation under poised conditions also measured a nearly fourfold increase in catalytic current, while the catalytic wave remained centered at −0.15 V (Fig. 3A).

Comparison With Naturally Grown Biofilms

To ensure these experiments reflected native electron transfer processes, and not artifacts of the entrappment procedure, planar carbon electrodes were also incubated at oxidizing potentials (+0.24 V vs. SHE) with *G. sulfurreducens* to grow biofilms of naturally attached cells utilizing the electrode as an electron acceptor. Immediately after inoculation with *G. sulfurreducens*, the rate of current production at electrodes increased exponentially, but required a longer period to reach a plateau (60 h). At this point, the medium surrounding electrodes could be removed and replaced with fresh medium without affecting current production, demonstrating that cells had become attached. CV of these electrodes produced nearly identical catalytic features, particularly; the potential at which positive current was observed, the midpoint potential of the catalytic wave, and the wave steepness (Fig. 3B). These features were similar in early-stage (24 h) and longer-term (60 h) biofilms. These results indicated that the features observed in entrapped cells were similar to those expressed by naturally-grown electrode biofilms.

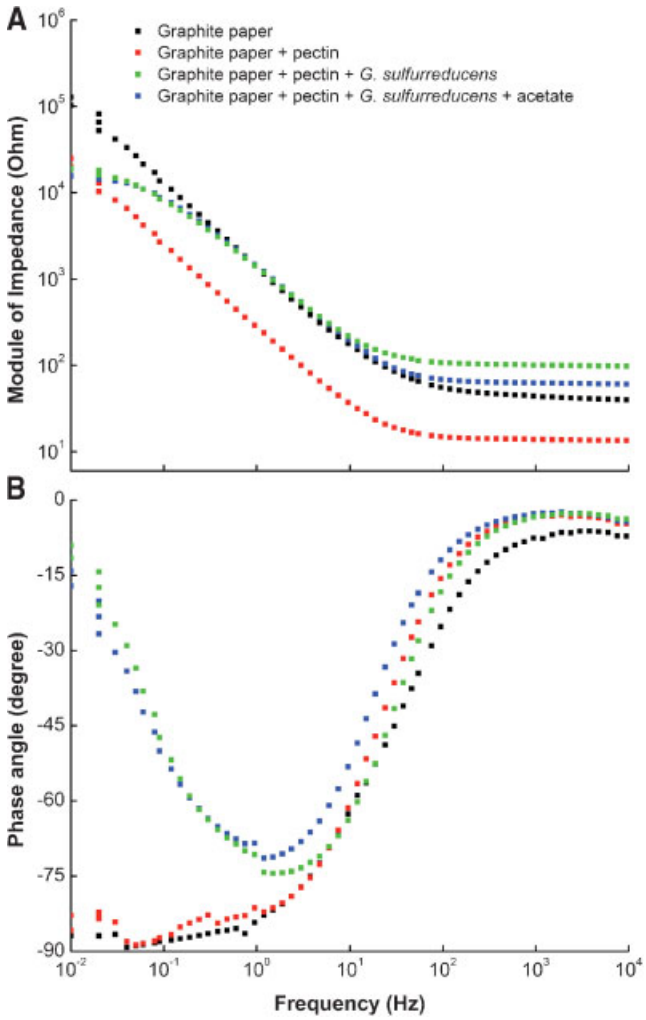


Figure 4. Bode graph (**A**) of graphite paper showing pure capacitive behavior of electrodes lacking cells, and changes in resistive behavior at low frequencies typical of charge transfer processes following addition of cells. Phase diagrams (**B**) for the same set of samples shows the effects of adding live cells at low frequencies (<10 Hz).

Electrochemical Impedance Spectroscopy of Electrodes

Another non-destructive method for monitoring coatings on conductive surfaces is the AC-based technique of electrochemical impedance spectroscopy (EIS) (Gonzalez et al., 1998; Lee et al., 2006; Moreno et al., 2004). Impedance of bare electrodes and electrodes coated with pectin is shown in Figure 4. As graphite is not redox-active toward acetate in these conditions, EIS of bare electrodes showed a very high charge transfer resistance (R_{film}) (Filipe and Brett, 2004). When electrodes were coated in sterile pectin, the charge transfer rate was still negligible (Fig. 4).

When *G. sulfurreducens* was entrapped on electrodes, EIS revealed a charge transfer process with one dominant time constant (based on the equivalent circuit described in methods). The decrease in charge transfer resistance was indicative of an active electron transfer process, while the increase in capacitance was consistent with the presence of a conductive bacterial film. Addition of acetate to bacteria-coated electrodes, which increased the electron transfer rate in CV measurements, decreased the charge transfer resistance slightly (19.4–16.1 kΩ) as measured by EIS (Fig. 4).

Incubation of electrodes at oxidizing potentials for 24 h was shown to lead to a fourfold increase in oxidative current. While electrodes clearly demonstrated increased electron transfer activity after growth at oxidizing potentials, the system became more complex, and non-ideal behavior of the electrode surfaces increased (Fig. 5). For example, apparent charge transfer resistance increased to 105 kΩ. In addition, capacitance of the microbial film increased from 188 to

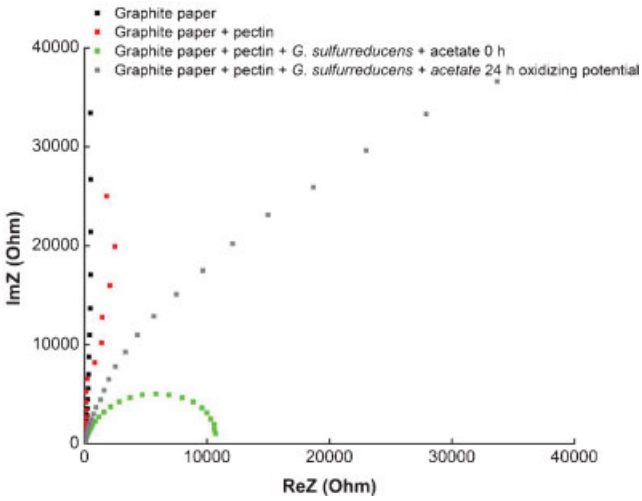


Figure 5. Nyquist graphs of graphite electrodes freshly coated with pectin and *G. sulfurreducens*, and the same electrodes after 24 h of incubation at +0.24 V versus SHE. While graphite paper in the absence of cells shows only diffusion-related resistance, the impedance of samples coated with living cells is typical of a charge transfer controlled process.

435 μF. These results also reflected an increased level of interaction between the biofilm and the electrode surface.

Discussion

G. sulfurreducens is a biocatalyst able to convert organic compounds into a flow of electrons (Bond and Lovley, 2003). This depends upon a suite of intracellular enzymes (Galushko and Schink, 2000; Mahadevan et al., 2006), linked to macromolecules at the outer membrane that have an ability to interface with insoluble Fe(III) minerals and conductive surfaces (Kim et al., 2005; Leang et al., 2005; Magnuson et al., 2001; Mehta et al., 2005; Reguera et al., 2005). With the molecular basis, and understanding of pili and cytochromes in this chemical–electrical interface still evolving (Holmes et al., 2006; Lovley, 2006; Reguera et al., 2006), the goal of this initial investigation was to prove that cells entrapped near electrode surfaces could establish electrical contact. This led to a series of electrochemical observations regarding the coupling between cytoplasmic oxidation and electron transport at the outer surface of *G. sulfurreducens*.

A key observation was that data obtained from electrodes coated with *Geobacter* was similar to what is often observed in catalytic protein film voltammetry, where thin films of enzymes are adsorbed to electrode surfaces (Armstrong, 2002, 2005; Elliott et al., 2004; Heering et al., 1998; Leger et al., 2003; Mondal et al., 1998). The sigmoidal catalytic wave was consistent with the hypothesis that the oxidative capacity of *G. sulfurreducens* was in excess, and that charge transfer from the adsorbed cell layer to the electrode was the primary reaction controlling electrical current flux (Kang et al., 2001; Rusling and Nassar, 1993; Udit et al., 2005). Similar voltammetry profiles are also evident in data from anodes in microbial fuel cells (Liu et al., 2005), although such analyses are typically performed at more rapid scan rates (20 mV/s), where the capacitive current of high surface area electrodes makes the catalytic nature less evident.

An unexpected finding was that washed cells of *G. sulfurreducens* demonstrated electrochemical activity, and generated electrical current, immediately after preparation. When *Geobacter* isolates grown with soluble electron acceptors are washed and incubated with Fe(III)-oxides, metal reduction is typically very slow unless a soluble mediator is added (Lovley et al., 1996; Nevin and Lovley, 2000), an observation that supports the hypothesis that delicate extracellular proteins make contact with Fe(III)-oxides (Mehta et al., 2005; Reguera et al., 2005). Because cells grown with soluble electron acceptors were repeatedly washed before immobilization, our data suggest *G. sulfurreducens* always possesses a rudimentary pathway for electron transfer to the outer surface, allowing at least a percentage of cells to immediately interface with an electrode. The consistency of the data from the point of immobilization onward suggests that the interface between cells and electrodes only increases in capacity over time.

The relationship between driving force (electrode potential) and electron transfer rate (current) by *G. sulfurreducens* at electrode surfaces suggested a dominant rate-limiting step, with an average midpoint potential (during catalysis) of −0.15 V (Armstrong, 2005). This is the first in situ measurement of the apparent potential of an electron transfer reaction in *Geobacter*, and it is likely that this reflects the potential of the site or species involved in cell-to-electrode electron transfer. This potential can be compared to average (and non-turnover) midpoint potentials of solubilized multiheme cytochromes implicated in *Geobacter* electron transport; PpcA purified from *G. sulfurreducens* (−0.167 V) (Lloyd et al., 2003), PpcA expressed in *E. coli* (−0.136 V) (Pessanha et al., 2004), and OmcB purified from *G. sulfurreducens* (−0.190 V) (Magnuson et al., 2001).

The principle of immobilization of microbial cells in thin films (e.g., 5 to <75 μm) has been demonstrated as method for achieving high reaction rates using a diverse range of organisms, such as *Gluconobacter oxydans* (Fidaleo et al., 2006), *Thermotoga maritima* (Lyngberg et al., 2005), *E. coli* (Lyngberg et al., 1998, 2001), and *Rhodopseudomonas palustris* (Gosse et al., 2007). When thin films are fabricated from polymers with high porosity and mechanical strength, diffusional limitations are minimized, while cells are protected and retained at the reaction site. Work in this report shows that active *Geobacter* coatings could be prepared and immediately monitored for electrical interaction with electrode surfaces, using electrochemical analysis. In comparison, growth of naturally-attached films required longer periods of adaptation. Based on these observations, future work to reduce the thickness and increase the strength of cell-

polymer coatings may improve electrical output, tolerate high rates of fluid flow, and provide a mechanism for fabrication of biocatalyst-functionalized electrode surfaces.

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