Microbial Biofilm Voltammetry: Direct Electrochemical Characterization of Catalytic Electrode-Attached Biofilms

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While electrochemical characterization of enzymes immobilized on electrodes has become common, there is still a need for reliable quantitative methods for study of electron transfer between living cells and conductive surfaces. This work describes growth of thin (<20 μm) Geobacter sulfurreducens biofilms on polished glassy carbon electrodes, using stirred three-electrode anaerobic bioreactors controlled by potentiostats and non-destructive voltammetry techniques for characterization of viable biofilms. Routine in vivo analysis of electron transfer between bacterial cells and electrodes was performed, providing insight into the main redox-active species participating in electron transfer to electrodes. At low scan rates, cyclic voltammetry revealed catalytic electron transfer between cells and the electrode, similar to what has been observed for pure enzymes attached to electrodes under continuous turnover conditions. Differential pulse voltammetry and electrochemical impedance spectroscopy also revealed features that were consistent with electron transfer being mediated by an adsorbed catalyst. Multiple redox-active species were detected, revealing complexity at the outer surfaces of this bacterium. These techniques provide the basis for cataloging quantifiable, defined electron transfer phenotypes as a function of potential, electrode material, growth phase, and culture conditions and provide a framework for comparisons with other species or communities.

Anaerobic metal-reducing Geobacteraceae catalyze the transfer of electrons from reduced electron donors, such as acetate, to oxidized electron acceptors such as Fe(III). While electron transport is confined to the inner membrane of most respiratory bacteria (66), Fe(III) and Mn(IV) exist primarily in the environment as insoluble precipitates at circumneutral pH. To reduce minerals of various crystallinity, redox potential, and surface charge, metal-reducing bacteria must possess a complete mechanism for transporting electrons across the inner membrane, periplasm, outer membrane, and outer surfaces (23, 38, 48, 54, 57, 58). Soluble redox-active shuttles or chelators can facilitate respiration beyond the outer membrane in some organisms (12, 34, 55, 56, 63), but no evidence for the secretion of such a compound has been observed in Geobacteraceae.

This electron transfer capability has recently been utilized in devices known as microbial fuel cells (MFCs), where electrodes serve as electron acceptors to support growth (11, 18, 35, 36, 62). However, variations in electrode surfaces, operating temperatures, electron donor concentrations, and culture conditions limit comparison between electrode-reducing bacteria. In addition, MFCs aim to maximize electrical power production per unit volume, using large anodes consisting of carbon felt (19, 39, 50) or carbon paper (43) in small reactors (with surface/volume ratios between 25 and 640) (19, 25, 29, 43). In such systems, the actual surfaces accessible to bacteria are not known, multiple “dead zones” or diffusion-limited regions exist (19), high-surface-area electrodes increase capacitive current and reduce the sensitivity of analyses (53, 68), and irregular surfaces prevent mathematical modeling or imaging of attached biomass (22).

Electrochemical techniques typically used to study purified redox proteins (3, 4, 7) and cell extracts (26, 45) could likely be adapted for the study of metal-reducing bacteria. While protein voltammetry is limited by the success of purification and immobilization of enzymes (17, 49), metal-reducing bacteria represent a self-assembling multi-enzyme system that naturally interfaces with electrodes. In this report, we describe procedures for analysis of G. sulfurreducens biofilms on small carbon electrodes of defined roughness in potentiostat-controlled three-electrode cells. Several direct current and alternating current techniques could be applied to the characterization of the biofilm-electrode interface and have produced insights into electron transfer mechanisms between living cells and electrodes. Application of these techniques in routine characterization of other living bacteria and biofilm communities will allow quantitative comparisons between electron transfer rates and mechanisms used by these organisms.

MATERIALS AND METHODS

Bacterial strain and culture media. G. sulfurreducens strain PCA (ATCC 51573) was subcultured in our laboratory at 30°C using a vitamin-free anaerobic medium containing the following per liter: 0.38 g KCl, 0.2 g NH₄Cl, 0.069 g NaH₂PO₄ 7H₂O, 0.1 g MnCl₂ 4H₂O, 0.04 g CaCl₂ 2H₂O, 0.2 g MgSO₄ 7H₂O, and 10 ml of a mineral mix containing 0.1 g MnCl₂ 4H₂O, 0.3 g FeSO₄ 7H₂O, 0.17 g CoCl₂ 6H₂O, 0.1 g ZnCl₂, 0.04 g CuSO₄ 5H₂O, 0.005 g AlK(SO₄)₂ 12H₂O,
0.005 g H₃BO₃, 0.09 g Na₂MoO₄, 0.12 g NiCl₂, 0.02 g NaWO₄ · 2H₂O, and 0.10 g Na₂SeO₄ per 1 liter. Acetate was provided as an electron donor at 20 mM. All media were adjusted to pH 6.8 prior to the addition of 2 g/liter NaHCO₃ and were flushed with oxygen-free N₂-CO₂ (80:20 [vol/vol]) prior to sealing with butyl rubber stoppers and autoclaving. All experiments were initiated by inoculating with 40% (vol/vol) of cells within 3 h of reaching maximum optical density (0.6) in the medium described.

**Electrode preparation.** Glassy carbon (Toko America, New York, NY) was machine cut into 2- by 0.5- by 0.1-cm electrodes. Freshly cut glassy carbon electrodes were polished using aluminum oxide-silicon carbide sandpaper with a grit designation of between P400 and P4000 (3M, Minneapolis, MN), as indicated in the ISO/FEPA standard (http://www.fepa-abrasives.com/). Mirror-polished electrode surfaces were obtained with 0.05-μm alumina powder (CH Instruments, Austin, TX). Polished electrodes were sonicated to remove debris, soaked overnight in 1 N HCl to remove metals and other contaminants, washed twice with acetone and deionized water to remove organic substances, and stored in deionized water. After each experiment, electrode surfaces were cleaned with an additional 1 N NaOH treatment (to remove biomass), and the entire surface was refreshed through sandpaper polishing and cleaning as described above to remove immobilized electron transfer agents. These working electrodes were attached to 0.1-mm Pt wires via miniature nylon screws that ensured electrical contact throughout the experiment.

**Electrode cell assembly.** Platinum wires from the working electrode were inserted into heat-pulled 3-mm glass capillary tubes (Kimble, Vineland, NJ) and soldered inside the capillary to copper wires. Counter electrodes consisted of a 0.1-mm-diameter Pt wire (Sigma-Aldrich, St. Louis, MO) that was also inserted into a 3-mm glass capillary and soldered to a copper wire. The resistance of each electrode assembly was measured, and electrodes with a total resistance of higher than 0.5 Ω were discarded. Reference electrodes were connected to bioreactors via a salt bridge assembled from a 3-mm glass capillary and a 3-mm Vycor frit (BioAnalytical Systems, West Lafayette, IN). Electrode capillaries were inserted through ports in a custom-made Teflon lid that was sealed with an O ring gasket. This lid fit onto a 20-ml conical electrochemical cell (BioAnalytical Systems, West Lafayette, IN), which had been previously washed in 3 N HNO₃ (Fig. 1A).

After the addition of a small magnetic stir bar, the cell was autoclaved for 15 min. Following autoclaving, the salt bridge was filled with 0.1 M Na₂SO₄ in 1% agar. A saturated calomel reference electrode (Fisher Scientific, Pittsburgh, PA) was placed at the top of this agar layer and covered in additional Na₂SO₄ to ensure electrical contact (60). The reactors were placed in an in-house-built water bath to maintain cells at 30°C (which introduced less electrical noise than large circulators, incubators, or temperature-controlled rooms) (Fig. 1B). To maintain the strict anaerobic conditions required by bacteria, all reactors were operated under a constant flow of sterile humidified N₂-CO₂ (80:20 [vol/vol]), which had been passed over a heated copper column to remove trace oxygen. Each reactor was located above an independent magnetic stirring unit.

Autoclaved bioreactors flushed free of oxygen, filled with sterile growth medium, and incubated at 30°C were analyzed before each experiment to verify anaerobicity and the absence of redox-active species. Electrochemical cells showing residual peaks in differential pulse voltammetry (DPV), anodic current in cyclic voltammetry (CV), or baseline noise were discarded as having possible electrode cleanliness or connection noise issues. These autoclaved, verified bioreactors were then used for growth of *G. sulfurreducens* cultures.

A typical bioreactor was inoculated with 40% (vol/vol) of stationary phase *G. sulfurreducens* cells. After inoculation, a potential step of 0.24 V versus the standard hydrogen electrode (SHE) was applied and the reactors were incubated until the current stabilized (~72 h). The potential step was ~200 mV higher than the midpoint potential, sustaining a half-maximal anodic current (as determined by preliminary CV; see below) (9).

**Electrochemical instrumentation.** A 16-channel potentiostat (VMP, BioLogic, Knoxville, TN) was connected to the three-electrode cells described above (Fig. 1B). Software from the same producer (EC-Lab v9.41) was used to run simultaneous multitechnique electrochemistry routines, which include CV, DPV,
and chronoamperometry (CA). Scan rates higher than 100 mV/s and all electrochemical impedance spectroscopy (EIS) experiments were performed using a Gamry PC14 Femtostat (Gamry Instruments, Warminster, PA), and EIS data fitting was performed with E-Chem Analyst v5.1. Postacquisition analysis of CV data was performed with the software Utilities for Data Analysis (UTILS), kindly provided by D. Heering (v1.0; University of Delft, The Netherlands [http://www.tudelft.nl/]). All measurements, with the exception of CA, were performed in succession without stirring enabled. The parameters for DPV were as follows: 50 mV; pulse width, 300 ms; step height, 2 mV; step time, 500 ms; scan rate, 4 mV/s; current averaged over the last 80% of the step (1 s, 12 points); accumulation time, 5 s. The parameters for CV were as follows: equilibrium time, 5 s; rate, 1 mV/s; 0.242 V versus SHE; 0.558 V versus SHE; 0.24 V versus SHE; current averaged over the whole step (1 s, 10 points). For CA, the parameters were $E_{\text{app}} = 0.242$ V versus SHE.

EIS was conducted while maintaining a direct current voltage between the working electrode and the reference electrode (potentiostatic EIS) as electron transfer processes by *Geobacter* isolates were observed to be voltage dependent (62). EIS was performed at four potentials, 0.04, 0.06, 0.16, and 0.26 V versus SHE, with a perturbation amplitude of 10 mV. The frequency was varied from 10$^3$ to 0.01 Hz, as most biological charge transfer phenomena are observed in this range (10). Data were then fitted with a simple empirical model (62), using a constant phase element to account for irregularities in the morphology and chemistry of the glassy carbon surface. EIS was typically performed only at the end of incubations as extensive characterization required electrodes to be held at multiple potentials for long periods of time.

**Confocal and SEM analyses.** A S3500N scanning electron microscope (SEM) (Hitachi, Japan) was used to image freshly polished (SEM) electrodes. After being thoroughly cleaned with deionized water, 2- by 0.5- by 0.1-cm graphite electrodes freshly polished with P400 and P4000 sandpapers were air dried and fixed through graphite tape to a sample stub. The sample stubs were placed directly into the SEM vacuum compartment and examined at an accelerating voltage of 5 kV.

A Nikon C1 spectral imaging confocal microscope (Nikon, Japan) with a ×60 lens was used to image biofilm-covered electrodes. Immediately following harvesting, the biofilm-covered electrodes were gently washed in growth medium to remove planktonic cells and then stained with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen Corp., Carlsbad, CA), incubated for 15 min in the dark, rinsed with growth medium, and placed on a microscope slide. The coverslip was supported by glass spacers thicker than the electrode to prevent damage to biofilm structure. Two laser wavelengths, 488 and 561 nm, were used to excite the two stains.

**RESULTS AND DISCUSSION**

**Colonization of electrodes.** In MFCs, the half-cell potential at the anode varies with the activity of the bacterial biofilm and the chosen external resistance (37, 43). Potentiostat-controlled electrodes at a sufficiently positive potential are equivalent to anodes in which the electron acceptor is nonlimiting, reducing the technical complexity and simplifying the conceptual model of electron transfer. However, at high oxidative potentials, conformational change, unfolding, and irreversible process could alter the catalytic abilities of enzymes, decreasing the anodic (oxidation) current (27, 52). In all experiments described, the potential step used was 0.24 V versus SHE. This potential was chosen based on preliminary CV experiments, which showed that the oxidation current from cells was independent of applied potential in the range of 0.1 to 0.3 V versus SHE.

After inoculation, the potential step was applied, and a rapidly increasing anodic (oxidation) current was detected (Fig. 2). As this rate of increase was many times higher than what could be explained by known growth rates of *G. sulfurreducens*, it was likely due to cell attachment to the electrode. The attachment phase was most rapid when fumarate-limited cells (compared to mid-log-phase cells) were used as the inoculum, consistent with reports linking acceptor limitation to the expression of enzymes important to metal reduction (21, 23). This was followed by a growth phase, characterized by an exponential increase in current, which doubled at a rate typically observed for *G. sulfurreducens* reducing Fe(III)-citrate or fumarate (doubling time, ∼7 h).

Within 72 h, electrodes reached a saturation phase characterized by a stable maximum electron transfer rate with a standard deviation between biological replicates of less than 3% ($n = 6$). Confocal imaging of electrodes always revealed uniform thin films of ∼15 μm with few structural features such as pillars (see Fig. S1 in the supplemental material). During the attachment and growth phases, electrodes were tested for the effect of voltammetry analyses on the rate of growth or current plateau. Repeated CV and DPV (see Materials and Methods) did not alter key midpoint potentials, colonization rates, or final current densities (Fig. 2).

In order to minimize contribution of other electron transfer agents, we used a medium lacking redox-active species, such as vitamins (i.e., riboflavin), redox indicators (resazurin), and chemical reductants (cysteine and dithiothreitol) (47, 64). In our recent study (47), we showed that flavin concentrations in the mM range can be detected under the experimental conditions applied in the present work. When the medium in reactors was replaced, removing planktonic cells and any potential microbially produced soluble electron transfer agent, the electron transfer rate to electrodes remained unchanged. In addition, removal of nitrolotriacetic acid-chelated minerals from the medium (once electrode colonization had occurred) did not affect current production. These findings created stringent conditions for collection of baseline data in the absence of most common soluble redox-active compounds. A systematic examination of the effects of commonly present redox species...
on electron transfer by *Geobacter* spp. will be the focus of a future report.

The effect of microscale surface roughness on bacterial attachment and direct electron transfer has not been reported. Figure 3 shows the surface features of carbon electrodes polished with different sandpapers. When *G. sulfurreducens* biofilms were grown on electrodes polished with increasing grit size, the maximum current density increased over 100% for the same geometric area (Fig. 3). Polishing with particles smaller than ~5 μm did not significantly reduce the maximum current. Therefore, even micrometer scale features have to be controlled to allow reproducibility and comparisons between cultures and electrodes in this and future studies.

**CV**. CV is the principal diagnostic method in protein film voltammetry, which studies immobilized enzymes on rotating and stationary carbon electrodes. When in the presence of a suitable substrate and slowly cycled through a range of applied potentials at low scan rates, enzymes have time to undergo multiple turnovers at each sampled potential (5–7, 33). As scan rates increase, slow reactions may not have time to occur before the potential changes to the next step, and concentration gradients representative of steady-state conditions may not have time to establish. Thus, as the scan rate increases, the kinetics of interfacial electron transfer between redox protein(s) and electrodes affects the voltammetric response more strongly and can hide the kinetics of continuous enzymatic turnover. As the purpose of this study was to establish methods for measuring catalytic (turnover) kinetics in viable bacterial biofilms, in the absence of prior knowledge regarding interfacial electron transfer rates, low scan rates represented the most conservative experimental conditions. Parameters for the study of nonturnover voltammetry at high scan rates will be discussed in subsequent reports.

Application of CV to living bacteria required limiting the potential range to prevent harmful oxidizing or reducing conditions, as well as selection of informative scan rates. While it is known that application of potentials can disrupt biofilms either by producing hydrogen (28) or by inducing unfolding/oxidation of adsorbed proteins at oxidizing potentials (52), *G. sulfurreducens* biofilms were not harmed by exposure to potentials between −0.55 and 0.24 V versus SHE. For example, routine CV did not slow or impede biofilm development, as the anodic current after such analyses was 100.1% ± 3.4% (n = 6) of the current recorded before analyses. This was not unexpected, as *Geobacter* spp. are commonly exposed to metals in this potential range. Future work with stricter anaerobes, or organisms adapted to higher-potential environments, should independently verify the potential range which is not harmful to electron transfer.

Stable catalytic features with a high signal-to-noise ratio were observed when CV was performed at low scan rates (1 mV/s). Under these conditions, the rate of electron transfer rose rapidly at a potential higher than ~0.3 V, reaching a limiting current above a potential of −0.1 V (Fig. 4). This response, commonly called a “reversible catalytic wave” (7), indicated continuous regeneration of the entire series of proteins catalyzing electron transfer from the substrate to the electrode. In preliminary investigations, each increase in the scan rate significantly increased the ohmic current and shifted the location of peaks in voltammetric waves, as expected from kinetic effects (see Fig. S2 in the supplemental material). However, increasing the scan rate by fivefold did not significantly alter the limiting current, demonstrating that at low scan rates, conditions were within a timescale sufficient to establish sustained catalysis by cells at each applied potential. Since the most informative scan rate could vary for each experimental setup, scan rates used in this report should be taken only as an aid in choosing conditions for new organism or electrode surface studies.

The inflection point(s) in such catalytic waves was visualized as a peak using first-derivative analysis of voltammetry data (using software described in Materials and Methods) (2, 16). Such analysis of four independent biofilms is shown in Fig. 4. One dominant inflection point could be detected by this analysis, centered at about −0.15 V, which increased in height with the age of electrode-attached culture, while smaller intensity peaks, centered at −0.220 V and −0.020 V versus SHE, could also be detected. The dominant peak revealed in derivative analyses was consistent with the presence of a single oxidation/reduction event in electron transfer that was rate limiting (1, 9, 15). As shown in Fig. 4, these values were highly repeatable between individual biological replicates.

Once a biocatalyst is adsorbed, it is also possible to determine its response to increasing concentrations of substrate (24). To test this approach, the electron donor was first re-

![FIG. 3. SEMs of glassy carbon polished with P400 versus P4000 grit treatment (top). Bar graph (below) shows maximum current measured (n = 2 for each roughness) during CA after 72 h of growth. Also indicated (■, right y axis) is the average grit size of each polishing treatment.](http://aem.asm.org/)
moved from the medium, and electrodes were incubated at 0.24 V for 36 h until no residual anodic (oxidation) current was observed. At this point, the effect of lowering substrate concentrations could be determined. The anodic-limiting current at 0.1 V was determined from CV and was found to increase linearly with the acetate concentration when acetate was 200 M. At acetate concentrations higher than 3 mM, the anodic-limiting current did not change (Fig. 5). The midpoint potential of catalytic waves remained centered at the same position for all concentrations, suggesting a similar electron transfer mechanism and rate-controlling reaction, even at low current densities. This dose-response test detected acetate at concentrations as low as 3.5 M (signal-to-noise ratio, >3).

**DPV.** Although CV is the most informative method used to investigate catalytic substrate oxidation by adsorbed enzymes, it has a low detection limit, and subtraction of the ohmic (capacitive) current is necessary to reveal small features (9). Furthermore, when electron transfer between adsorbed enzymes and electrodes is slow, as is expected for complex electron transfer chains studied in stationary electrodes, CV requires substantial time, and proper derivative analysis requires postprocessing of data (40). In comparison, pulse methods have the potential to reveal characteristic peaks while canceling out capacitive current, even at higher scan rates, and are often used as complementary techniques to CV (20, 44, 65).

Preliminary experiments with *G. sulfurreducens* biofilms indicated that DPV could also be used to monitor biofilms non-destructively, across a range of scan rates (up to 50 mV/s) and pulse heights (up to 100 mV). The parameters chosen (see Materials and Methods) represent a compromise between the time of analysis and sensitivity. When performed on mature biofilms, DPV always revealed a broad peak, which increased in height with the age of the biofilm. These voltammograms were highly reproducible, with the peak centered at 0.105 ± 0.005 V versus SHE (Fig. 6).

Additional peaks at lower and higher potential with respect to the main peak were also detected. The results were consistent with multiple redox centers exposed on the surfaces of *G. sulfurreducens* cells that spanned a range of potentials (from approximately −0.2 to +0.1 V). In recent studies with *Shewanella* biofilms grown on similar electrodes, we observed strikingly different behavior, as DPV revealed a large, less convoluted peak at −0.21 V. These *Shewanella* cells were found to secrete redox-active flavins that mediated electron transfer (47), and changes in the DPV peak height could be used to monitor both the accumulation of this redox-active mediator at the electrode and its loss when the medium was changed to remove soluble compounds. As the DPV peak height was unaffected by medium changes in experiments with *Geobacter* biofilms, this represented additional support for accumulation of an attached electron transfer agent and lack of soluble mediators within *G. sulfurreducens* biofilms. These results demonstrate the utility of combining sweep and pulse methods in the study of living systems.

**EIS.** While originally applied to investigation of solid-solid interfaces, EIS is today the most common alternating current method applied to the characterization of aqueous biointer-

**FIG. 4.** (A) Low scan rate CV of *G. sulfurreducens* biofilms after inoculation (0 h) and at maximum current production (72 h). (B) First derivatives of cyclic voltammograms of biofilms (P400 grit polishing treatment) (n = 4) showing the midpoint potential detectable in catalytic waves of mature biofilms.

**FIG. 5.** Low scan rate CV (1 mV/s) of *G. sulfurreducens* at different concentrations of electron donor. The biofilm was poised at oxidizing potential without an electron donor until no anodic current was observed. Then electron donor was added, and the limiting current was recorded (inset). The midpoint potential of catalytic curves was constant with increasing concentration of the electron donor.

![Graph](https://example.com/graph.png)
faces (10, 13). In most EIS methods, a small sinusoidal potential perturbation is applied to the sample. The frequency of this perturbation is changed in the range between few mHz and 10^5 Hz. The resulting sinusoidal current is analyzed via fast Fourier transform techniques to calculate the impedance (Z) of the interface in the frequency domain to estimate charge transfer resistance (32), diffusion at surfaces covered by protein mono- layers (20), charge transfer time constants (59), and mechanisms of electron transfer (8).

Applications of EIS are numerous in microbially influenced corrosion, in which the sample is studied at the open circuit potential (42, 51). However, we have shown that electron transfer processes by *G. sulfurreducens* are voltage dependent (example shown in Fig. 3). Thus, while EIS is typically performed at open-circuit potentials, a second, slower time constant was observed in the 0.01 to 0.1 Hz range. The addition of a second time constant to the model did not significantly improve the dispersion of residuals, likely due to the low frequency of the second time constant.

The importance of potentiostatic EIS was illustrated by the significant influence that the imposed potential had on the charge transfer resistance and time constants for *G. sulfurreducens* biofilms (Fig. 8; Table 1). When the imposed potential was similar to the midpoint potential of the catalytic wave observed in CV, the lowest resistances and time constants were measured. At potentials outside of this range, differences in charge transfer were much smaller (in agreement with CV results), resulting in higher apparent values for the charge transfer resistance and time constant. Across the potential range explored, the solution resistance (R = 17.2 ± 0.4 Ω), double-layer capacitance (Cp = 3.0 ± 0.9 mF), and nonideality coefficient (α = 0.72 ± 0.06) did not change appreciably. These results again highlighted how data from one method (CV) could be used to select parameters in a complementary method (EIS). They also demonstrated the importance of investigating the proper analysis parameters for each bacterial system, as another strain with a different midpoint potential for its catalytic process would need to be studied at a different imposed voltage to obtain the fastest time constant.

**Implications.** Many studies providing voltammetry data from bacterium-electrode interactions have now been published. While reports differ in their pretreatment of bacteria, biofilm age, growth medium, environmental conditions, electrode materials, and electrochemical parameters, a general trend of catalytic wave-like behavior is clear (14, 31, 53, 68), showing that many bacteria and communities behave in a man-
ner similar to the early stage Geobacter biofilms grown on geometrically simple electrodes shown in this work. As a result, we can confirm what early observations suggested, that Geobacter bacteria behave as attached biocatalysts, stimulated by changes across a narrow potential range. For example, in a Geobacter we can confirm what early observations suggested, that geometrically simple electrodes shown in this work. As a result, Geobacter similar to the early stage 

In particular, this study shows how the response of an intact 

The high reproducibility between biological replicates and the ability to perform experiments with a range of electrodes provided a robust measurement of G. sulfurreducens current densities, which approached 2 A/m² for electrodes polished with 35-μm-grit-size particles. The catalytic wave consistently observed for G. sulfurreducens is an independent demonstration that interior oxidative processes of this organism are linked via a continuous pathway to surfaces and that the entire collection of attached organisms (i.e., the biofilm) behaves as an adsorbed catalyst. The midpoint potential of the catalytic wave at −0.15 V supports a model with a dominant rate-limiting electron transfer reaction and shows that G. sulfurreducens respiration rate does not increase when cells are provided with an electron acceptor with a potential greater than 0 V. The latter result implies that the final step of electron transfer (e.g., between a terminal external protein and the electrode) is not rate limiting, as this process can always be accelerated by additional applied potential (as described by the standard rate law $k_f = k_0 e^{(\Delta F^\parallel)}$ (9). The midpoint and the limiting current potential found in this study are consistent with G. sulfurreducens being adapted for the reduction of iron oxides with a potential between −0.2 and 0 V versus SHE in the environment and suggest that cells do not derive any additional energetic benefit from higher-potential electron acceptors.

While the study of an intact electron transport chain in G. sulfurreducens represents perhaps a complex example of this electrochemical approach, similar methods have been used to characterize extracts from bacterial cells adsorbed onto, or

### TABLE 1. EIS parameters for biofilms at 72 h

<table>
<thead>
<tr>
<th>Potential vs SHE (V)</th>
<th>Charge transfer resistance (Ω)</th>
<th>Characteristic frequency (Hz)</th>
<th>Time constant (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+0.042</td>
<td>23,500</td>
<td>&lt;0.01</td>
<td>&gt;100</td>
</tr>
<tr>
<td>−0.058</td>
<td>1,050</td>
<td>0.032</td>
<td>32</td>
</tr>
<tr>
<td>−0.158</td>
<td>204</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>−0.258</td>
<td>2,440</td>
<td>0.04</td>
<td>25</td>
</tr>
</tbody>
</table>

* Data were fitted with the single time constant model described in Materials and Methods. Values are averages of data from two separate experiments.
incorporated into electrode materials (26, 45, 46, 61). In addition, researchers have washed and concentrated whole cells for brief voltammetry analyses and detected possible redox-active agents on the external surfaces of cells (39). The benefits of using living cells in the work reported here include the lack of a protein purification step and the fact that cells are allowed to assemble the actual biofilm structure responsible for this unique extracellular electron transfer process.

The multiple peaks visible in both CV and DPV analyses also confirm the complexity of the *G. sulfurreducens* surface. Multiple cytochromes and redox proteins have been previously implicated in outer membrane-based electron transfer in proteomic and labeling studies (23, 36, 41). As most proteins implicated in electron transport by *G. sulfurreducens* contain multiple hemes or redox centers, the detected redox centers could reflect individual hemes, domains that act as a single center, or individual proteins. Recent work with the multiheme cytochrome MtRC (30) showed that multiheme proteins do not demonstrate classic, individual redox behavior for each heme but rather act as a cluster with a broader midpoint. In another study (67), the same protein was observed to behave as two pentaheme domains with broad midpoint potentials. Future work with specific mutants lacking key redox proteins in *G. sulfurreducens* will aid in identifying the origin of these peaks.

Based on the results reported here, voltammetric methods previously developed to characterize electron transfer phenomena by enzymes adsorbed at carbon electrodes can be extended to the characterization of viable biofilms. By choosing the appropriate conditions, these methods are not destructive and allow in vivo determination of electron transfer from whole cells to electrodes under conditions that are comparable to those encountered in natural environments. Both thermodynamic and kinetic parameters can be determined and used to define the phenotype of an organism for comparison with other strains or mutants. The proposed methods can be applied to well-defined pure cultures, as well as to complex microbial communities, and could allow for quantitative comparisons in the development of better microbial catalysts based on direct electron transfer between bacteria and electrodes.

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