Long-range electron transport in Geobacter sulfurreducens biofilms is redox gradient-driven

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Geobacter spp. can acquire energy by coupling intracellular oxidation of organic matter with extracellular electron transfer to an anode (an electrode poised at a metabolically oxidizing potential), forming a biofilm extending many cell lengths away from the anode surface. It has been proposed that long-range electron transport in such biofilms occurs through a network of bound redox cofactors, thought to involve extracellular matrix c-type cytochromes, as occurs for polymers containing discrete redox moieties. Here, we report measurements of electron transport in actively respiring Geobacter sulfurreducens wild type biofilms using interdigitated microelectrode arrays. Measurements when one electrode is used as an anode and the other electrode is used to monitor redox status of the biofilm 15 μm away indicate the presence of an intrabiofilm redox gradient, in which the concentration of electrons residing within the proposed redox cofactor network is higher farther from the anode surface. The magnitude of the redox gradient seems to correlate with current, which is consistent with electron transport from cells in the biofilm to the anode, where electrons effectively diffuse from areas of high to low concentration, hopping between redox cofactors. Comparison with gate measurements, when one electrode is used as an electron source and the other electrode is used as an electron drain, suggests that there are multiple types of redox cofactors in Geobacter biofilms spanning a range in oxidation potential that can engage in electron transport. The majority of these redox cofactors, however, seem to have oxidation potentials too negative to be involved in electron transport when acetate is the electron source.

It is widely accepted that electron transport can occur over molecular-scale distances in biological systems by electron hopping among a small number of immobilized redox cofactors (1, 2). There is growing awareness, however, of the possibility of electron transport over length scales much longer than previously thought possible in biological systems by using immobilized redox cofactors organized into electron transport conduits. This is most evident by microorganisms, such as Shewanella spp. and Geobacter spp., that can use electron acceptors residing outside the cell for respiration (3). For example, in the case of Shewanella, it is proposed that the CymA-MtrA-MtrC complex comprised of 3 multiheme c-type cytochromes totaling 24 hemes acts as a multistep conduit that conducts respirred electrons originating in the cytoplasm through the periplasm and outer membrane to the cell outer surface (4–6). Outside the cell, both Shewanella and Geobacter secrete nanometer scale diameter, micrometer scale long proteinaceous filaments, referred to as pili and microbial nanowires (7), that extend from their outer surfaces into the extracellular matrix (ECM) thought to be involved in extracellular electron transport processes, including cell-to-cell electron transfer (8) and reduction of insoluble oxidents (9). Ex situ conductivity measurements of individual filaments of Shewanella oneidensis MR-1 confirm their lengthwise conductivity when isolated from cells under specific conditions (10, 11), and subsequent modeling of their current-voltage characteristics is consistent with multistep electron hopping involving redox cofactors proposed to be associated with these filaments (12–14). The ECM of both species, however, contain many proteins other than those comprising pili, including a number of c-type cytochromes, and their contributions to extracellular electron transport have not been determined under physiologically relevant conditions owing largely to the lack of information regarding their spatial organization. Recently, it has been proposed that microbes inhabiting marine sediments may use a network of such filaments and other extracellular proteins to couple oxidation of sulfate in anoxic sediment with reduction of oxygen in overlying oxic sediment, resulting in the transport of electrons over centimeter-scale distances (15).

Geobacter as a Model System for Studying Micrometer-Scale Biological Electron Transport

Geobacter spp. are microorganisms that inhabit subsurface environments, such as marine sediments (16), and they are widely studied for their distinct ability to acquire energy by coupling intracellular oxidation of organic matter, such as acetate, with extracellular electron transfer to insoluble (i.e., mineral) electron acceptors (17). Geobacter are also able to directly transfer electrons to noncorrosive anodes of electrochemical reactors, resulting in electrical current coupled to metabolic organic matter oxidation (18). When grown using an anode as their metabolic electron acceptor, Geobacter cells adhere to the anode surface, proliferate, and form a persistent multimicrofibe-thick biofilm in which all cells comprising the biofilm appear to contribute to current generation (19–21). Such biofilms are electrically conductive, inferred by the ability of cells not in direct contact with the anode surface to contribute to current generation (22), and confirmed by two-electrode conductivity measurements (20, 23). Geobacter cells possess an abundance of multiheme c-type cytochromes on their outer membrane, in the ECM, and along PilA-pili (24–33). Cyclic voltammetry (19, 34–37) and spectroelectrochemical measurements (38–43) of actively respiring Geobacter biofilms are consistent with long-range electron transport mediated by sequential electron transfer reactions through a network of bound redox cofactors comprised of ECM c-type cytochromes terminating with electron transfer to the anode surface. This proposed process, analogous to diffusive electron transport observed for redox polymers containing discrete redox moieties (20, 44–48), is an extension of the normal mode of electron transport in biological systems involving redox proteins (2) to longer length scales (up to 18 μm in the case of biofilms described here).

The ability of a Geobacter biofilm to use an electrode as its terminal metabolic electron acceptor allows precise measurement of the rate of extracellular electron transport to the anode as current and enables modulation of the rate and driving force for extracellular electron transport by external control of the electrode potential. This ability enables application of electrochemical techniques, such as cyclic voltammetry (19, 20) and
chronoamperometry (49–51), and combined with availability of its genome sequence and genetic system (52), it makes possible the investigation of the mechanism of extracellular electron transport to a degree not yet possible with non-electrode-using systems. Understanding the processes that govern long-range biofilm electron transport from cells comprising a biofilm to the underlying anode surface has important implications for optimization of microbe-catalyzed electrode reactions, such as anode-coupled oxidation of organic matter for wastewater treatment and energy transformation (53–57) and cathode-coupled reduction of carbon dioxide to liquid fuel precursors (58, 59) in microbial fuel cells and microbial electrolysis cells. It may also provide valuable insights into the mechanism of long-range biological electron transport in environmental processes not involving electrodes.

A Redox Gradient Is Expected to Accompany Multistep Electron Hopping

A predicted feature of long-range multistep electron hopping is the presence of a redox gradient, in which the local oxidation state of the biofilm decreases with increasing distance from the anode surface (14) (that is, the local concentration of electrons residing in biofilm cofactors is expected to be higher farther from the anode surface). This gradient is expected to result from re-duction of cofactors by cells distributed across the biofilm coupled to acetate oxidation at the anode surface, where the potential applied to the anode determines the local oxidation state of the biofilm in the vicinity of the anode surface. This gradient is also expected to provide the driving force for electron transport as occurs for redox polymers (44), in which electrons effectively diffuse from areas of high to low concentration (from cells to the anode surface) by electron transfer among cofactors.

Here, we provide experimental evidence for the existence of an intrabiofilm redox gradient within actively respiring G. sulfurreducens WT biofilms. Furthermore, the magnitude of the gradient seems to correlate with catalytic current coupled to acetate oxidation in response to changing potential applied to the anode in a manner consistent with multistep electron hopping. In addition, we provide additional experimental evidence indicating that electron transport through an actively respiring Geobacter biofilm between two electrodes acting as an electron source and drain results from generation of a redox gradient between the electrodes in response to the potentials applied to the electrodes. The magnitude of the gradient seems to depend on the electrode potentials in a manner also consistent with multistep electron hopping (20). In these source-drain measurements, electron transport is not coupled to acetate oxidation, but rather, the more negative electrode (the source) supplies the electrons that are conducted through the biofilm to the more positive electrode (the drain). Comparison of measurements of biofilm electron transport to an anode coupled to acetate oxidation with measurements of biofilm electron transport between a source and drain not coupled to acetate oxidation indicates that Geobacter biofilms seem to contain a number of cofactors able to participate in multistep electron hopping. The majority, however, do not seem able to participate in electron transport coupled to acetate oxidation, because the formal potentials are too negative to accept electrons originating from acetate.

Results

Biofilm-Modified Interdigitated Microelectrode Arrays. Fig. 1 depicts an SEM image of a G. sulfurreducens WT biofilm grown on a gold interdigitated microelectrode array (IDA). An electrode configuration developed to study conductive properties of polymer films (60). IDAs used here are comprised of 100 parallel 15-μm-wide × 48-cm-long gold microelectrode bands patterned onto a glass slide separated by 15-μm-wide gaps. Every other band is electrically connected at two opposite edges of the array, forming two interdigitated electrodes (electrode 1 and electrode 2), each comprised of 50 microelectrode bands, electrically separated by a single 15-μm-wide × 48-cm-long single serpentine-shaped gap (Fig. 1, Inset). The biofilm was grown by poising both electrodes as anodes at +0.300 V vs. Ag/AgCl [approximately +0.500 V vs. standard hydrogen electrode (SHE)] in media containing excess acetate (10 mM) until a self-determined limiting catalytic current of 50 μA (25 μA for each electrode) was achieved, corresponding to a biofilm thickness of 18 μm, on average, that was sufficiently thick to span the gap between adjacent interdigitated microelectrode bands (SI Materials and Methods). Two types of electrochemical measurements performed on actively respiring G. sulfurreducens WT biofilms grown on IDAs are presented here after the biofilms were first grown using both electrodes as anodes. In the first type (Fig. 2A), a potential is applied only to electrode 1, which continues to act as an anode, whereas electrode 2 is at open circuit and monitors the local oxidation state of the biofilm in the vicinity of electrode 2 while electron transport through the biofilm to electrode 1 coupled to acetate oxidation is occurring. In the second type (electrochemical gate measurements) (Fig. 2B), different potentials are applied to electrodes 1 and 2, which act as an electron drain and source while maintaining a constant potential difference between the electrodes inducing electron transport between the electrodes through the intervening biofilm that is not coupled to acetate oxidation.

Evidence for a Biofilm Redox Gradient During Catalytic Current Generation. Fig. 3A depicts catalytic (turnover) cyclic voltammetry (CV) recorded for electrode 1 while electrode 2 was at open circuit (Fig. 2A). Here, only the set of microelectrode bands comprising electrode 1 was used to collect electrons resulting from acetate oxidation. The CV exhibits the sigmoid-shape dependency of steady state catalytic current on applied potential reported for full grown G. sulfurreducens biofilms (19, 20, 36), consistent with an electrode catalytic (EC) reaction scheme (61), in which a reactant (in this case, acetate) that cannot be directly oxidized by an electrode owing to poor kinetics is coupled to reduction of a redox cofactor that can be reversibly oxidized by an electrode, such as a c-type cytochrome (41). The negative deviation in current observed during the anodic scan of the experimental CV has been previously noted, and it is attributed to possible inhibition of acetate oxidation and/or possible accumulation of electrons in cells occurring at the end of the cathodic scan and beginning of the anodic scan, when the potential applied to electrode 1 was fairly negative (20). Fig. 3A, Inset depicts nonturnover CV (recorded in the absence of acetate).
where current is attributed to changing the oxidation state of redox cofactors in the biofilm (i.e., pseudocapacitance) (19, 39, 40) and multiple voltammetric peaks indicate possible presence of multiple redox cofactors spanning a range in formal potentials. Overlaid on to the cathodic voltammetric scan are fits based on Eq. 1 derived by modeling catalytic current generation by a Geobacter biofilm as an EC reaction scheme (61), in which electron transport occurs by multistep electron hopping (20) (Eq. 1):

\[ i_{\text{cat}} = i_{z} (\Delta X_{\text{Ox}})_{z=0,1}. \]  

Here, steady catalytic current measured at electrode 1 coupled to biofilm acetate oxidation, \( i_{\text{cat}} \), scales in proportion to the local biofilm oxidation state in the vicinity of electrode 1, \( (\Delta X_{\text{Ox}})_{z=0,1} \) (portion of cofactors that directly exchange electrons with electrode 1 that are in the oxidized form where \( z \) is distance from the electrode surface), and the maximum catalytic current, \( i_{z} \) (Fig. 3A, 39.3 \( \mu \)A), which occurs when \( (\Delta X_{\text{Ox}})_{z=0,1} = 1 \). \( i_{z} \) is dependent on the IDA geometry, growth state of the biofilm (19), concentration of redox cofactors in the biofilm, and rate constant for electron exchange between adjacent cofactors (14). If electron exchange between electrode 1 and cofactors at the electrode surface is fast (20), then \( (\Delta X_{\text{Ox}})_{z=0,1} \) can be determined from the potential applied to electrode 1, \( E_{1} \), by a modified version of the Nernst Equation (Eq. 2):

\[ (\Delta X_{\text{Ox}})_{z=0,1} = \frac{F}{R T} \left( E_{1} - (E^{\circ})_{\text{avg}} \right) \left( 1 + \exp \left( \frac{n F}{R T} \left( E_{1} - (E^{\circ})_{\text{avg}} \right) / g \right) \right). \]

\( j = 1 \) indicates electrode 1, where \( (E^{\circ})_{\text{avg}} \) is the weighted average of formal potentials of cofactors that participate in multistep electron hopping, \( n \) is the number of electrons transferred at a time between a cofactor and electrode 1 (\( n = 1 \)) (36), and \( F \), \( R \), and \( T \) (303 K) have their standard meanings. \( g \) is an empirical factor invoked here to describe the degree of heterogeneity among cofactors with respect to formal potential. When \( g = 1 \), all of the cofactors have the same formal potential, and a smaller value of \( g \) indicates greater heterogeneity among cofactors, resulting in a wider potential range, over which the oxidation state of the biofilm in the vicinity of the electrode surface changes from being fully oxidized to fully reduced (14, 62).

In Fig. 3A, the best fit of the experimental CV to Eq. 1 based on matching the shape and midpoint potential (potential at which \( i_{\text{cat}} = i_{z}/2 \) for each voltammetric peak attributable to biofilm redox cofactors (same axes scales). (b) Potential measured for electrode 2 vs. potential applied to electrode 1. (b, left axis) Corresponding biofilm oxidation state in the vicinity of electrode 1, \( (\Delta X_{\text{Ox}})_{z=0,1} \), and electrode 2, \( (\Delta X_{\text{Ox}})_{z=0,2} \), vs. potential applied to electrode 1 calculated using Eq. 2.
calculated using Eq. 2 from $E_1$ (applied to electrode 1) and $E_2$ (measured at electrode 2) vs. $E_1$ using values for $(E^0)_{	ext{red}}$ and $g$ determined from Fig. 3A. Comparison of Fig. 3A with $B$ reveals that, for all instances in which catalytic current occurs, $(X_{Ox})_{z=0.1}$ is higher than $(X_{Ox})_{z=0.2}$, indicative of a redox gradient extending across the gap separating adjacent microelectrode bands (i.e., the effective concentration of electrons residing in cofactors in the vicinity of electrode 2 is higher than in the vicinity of electrode 1). Furthermore, increasing catalytic current coincides with increasing difference between $(X_{Ox})_{z=0.1}$ and $(X_{Ox})_{z=0.2}$, and the maximum catalytic current coincides with the maximum difference between these values, consistent with electron transport to electrode 1 during catalytic current generation that is redox gradient-driven. It is important to note the lack of a negative deviation in $E_2$ and therefore, $(X_{Ox})_{z=0.2}$ observed during the anodic voltammetric scan as observed for $i_{cat}$ indicating that the negative deviation observed for $i_{cat}$ appears not to be associated with extracellular electron transport.

Evidence for a Biofilm Redox Gradient Using Electrochemical Gate Measurements. Figs. 4 and 5 depict results of experiments in which potentials are applied to both electrodes that are changed in unison while maintaining a constant 0.1 V offset between them (i.e., electrochemical gate measurements) (60), where the different electrode potentials establish different values for $(X_{Ox})_{z=0.1}$ and $(X_{Ox})_{z=0.2}$. Fig. 4A depicts dependency of the resulting conducted current, $i_{con}$, recorded under turnover condition after acetate removal to the average of the potentials applied to the electrodes, $E_{avg} = (E_1 + E_2)/2$. Here, current was measured at both electrodes, where negative current indicates electron transfer from the more negative electrode (electrode 2, electron source) into the biofilm, whereas positive current, as in the case of an anode, indicates electron transfer out of the biofilm to the more positive electrode (electrode 1, electron drain). The magnitude of current measured at each electrode, $i_{con}$, is attributed to electron transport through the biofilm between adjacent microelectrode bands comprising electrodes 1 and 2 that is not coupled to acetate oxidation. Comparison of Fig. 4A with Fig. 3A (Inset) indicates that current due to pseudocapacitance is a negligible contribution to $i_{con}$. Depicted in Fig. 4A are fits to $i_{con}$ vs. $E_{avg}$ based on Eq. 3, $i_{con} = i_{M} \left[ (X_{Ox})_{z=0.1} - (X_{Ox})_{z=0.2} \right]$. 

![Fig. 4.](image-url)

![Fig. 5.](image-url)

![Fig. 6.](image-url)
that follows directly from equation 21 in the work by Strycharz-
Glaven et al. (14, 63), which is based on multistep electron
hopping. Here \( i_M \) is the maximum current that can be conducted
through the biofilm that is dependent on the same parameters as \( i_F \).
The condition at which \( i_M = i_F \) can occur is when \( \{X_{\text{con}}^c\} = 1 \) and \( \{X_{\text{con}}^f\} = 0 \), the condition at which the magnitude of the
redox gradient between adjacent microelectrode bands is maxi-
mized (44). The best fits based on peak potential and peak width
at one-half height are obtained for \( E_{\text{avg}} = -0.528 \pm 0.0025 \) V and
\( g = 0.47 \pm 0.01 \), indicating that redox mediators involved in gen-
eration of \( i_{\text{con}} \) by multistep electron hopping are considerably more
heterogeneous and have a considerably more negative average
oxidation potential than redox cofactors involved in generation of \( i_{\text{cat}} \).

The model (20) indicates that redox cofactors involved in generation of \( i_{\text{cat}} \) cannot have formal potentials that are more
negative than the midpoint potential of the catalytic CV (\( E_M =
-0.458 \pm 0.0005 \) V vs. Ag/AgCl) (Fig. 3A), which is ultimately
constrained by the oxidation potential of acetate (\(-0.49 \) V vs. Ag/
AgCl). As such, the majority of redox cofactors able to participate in
multistep electron hopping not coupled to acetate oxidation
resulting in \( i_{\text{con}} \) cannot participate in multistep electron hopping
that is coupled to acetate oxidation resulting in \( i_{\text{cat}} \), because the
supply of electrons during \( i_{\text{con}} \) generation is not constrained in
potential that is, the source electrode can supply electrons into the
biofilm with higher potential energy (at a more negative potential)
than those electrons originating from acetate oxidation.

Fig. 4B depicts the dependency of \( \{X_{\text{con}}^c\} = 0.1 \) and \( \{X_{\text{con}}^f\} = 0.2 \)
on \( E_{\text{avg}} \) determined from Eq. 2 using the best-fit parameters
from Fig. 4A. As expected for redox gradient-driven electron
transport, an appreciable \( i_{\text{con}} \) occurs only when there is an ap-
preciable difference between \( \{X_{\text{con}}^c\} = 0.1 \) and \( \{X_{\text{con}}^f\} = 0.2 \),
and the largest \( i_{\text{con}} \) coincides with the largest difference in these values
(4.6). Calculated values of \( \{X_{\text{con}}^c\} = 0.1 \) and \( \{X_{\text{con}}^f\} = 0.2 \) based on
Eq. 2, for which \( i_{\text{con}} \) is largest, are 0.32 and 0.74, respectively, the
largest difference between these values that can be achieved
given the 0.100 V offset maintained between the electrodes
(\( E_1 = -0.478 \) V, \( E_2 = -0.578 \) V) for \( g = 0.47 \).

Fig. 5A depicts results of measurements performed in an
identical manner as those measurements depicted in Fig. 4B but
under turnover condition. When acetate is present, \( i_{\text{con}} \) is super-
imposed onto \( i_{\text{cat}} \) (Fig. 5B), because each electrode also acts as an
anode. To a first approximation, \( i_{\text{cat}} \) can be background subtracted
using CV, in which the potentials of both electrodes are changed
in unison with no potential offset between them, resulting in only
\( i_{\text{cat}} \) at each of the electrodes (Fig. 5A, Inset). Fig. 5B depicts results of
this subtraction process, where the residual \( i_{\text{cat}} \) observed results
presumably from competition among cofactors in electron trans-
port associated with \( i_{\text{con}} \) and \( i_{\text{cat}} \). Regardless, Fig. 5B indicates that
\( i_{\text{con}} \) depends on the potentials applied to both electrodes in
nearly the same manner when acetate is present, suggesting that \( i_{\text{cat}} \)
may be localized to a relatively small portion of the biofilm directly
between adjacent microelectrode bands.

\section*{Discussion}

Two types of electrochemical measurements performed on G.
sulfurreducens WT biofilms grown on IDAs are presented here.
In the first type, a potential is applied only to electrode 1, which
continues to act as an anode. The applied potential directly
determines the local oxidation state of the biofilm in the vicinity
of electrode 1. In contrast, electrode 2 is at open circuit, such
that its potential reflects the local oxidation state of the biofilm
in the vicinity of electrode 2 that is indirectly influenced by the
potential applied to electrode 1. The difference in the electrode
potentials when catalytic current is generated at electrode 1 is
interpreted to reflect a difference in local oxidation state of the
biofilm in the vicinity of each of the electrodes, indicating

the presence of redox gradient that drives electron transport
through the biofilm to electrode 1 when such electron transport
is coupled to cellular acetate oxidation. In the second type
(electrochemical gate measurements), different potentials
are applied to electrodes 1 and 2, which act as an electron drain
and source, respectively, while maintaining a constant potential
difference between the electrodes. The applied potentials directly
determine the local oxidation state of the biofilm in the vicinity
of each of the electrodes. The resulting current conducted through
the biofilm between the electrodes is interpreted to reflect genera-
tion of redox gradient between the electrodes in response to the
applied potentials that is not coupled to acetate oxidation.

As shown here for a living, actively respiring G. sulfurreducens
WT biofilm, electrochemical gate measurements result in a com-
pletely reversible current peak spanning the formal potentials of
biofilm redox cofactors as observed for redox polymers known to
engage in multistep electron hopping (60). The peak in conducted
current lies more negative than expected based on the midpoint
potential of the catalytic current-potential dependency (Fig. 3A),
and it is broader than expected for a single type of redox cofactor,
suggesting that multiple types of redox cofactors possessing different
oxidation potentials can be accessed by electrons driven by the con-
ducting current, \( i_{\text{con}} \), that are not involved in catalytic current
generation, \( i_{\text{cat}} \) (39, 40). WT G. sulfurreducens contains genes encoding
for more than 50 multiheme cytochromes (64) in addition to those
cytochromes identified to be important in establishing biofilms grown
on anodes (36, 65); therefore, it is not surprising that some of them
can pass electrons but are not recruited for catalytic current.

\section*{Implications}

The results of our electrochemical gate measurements directly
contradict results recently reported in the work by Malvankar et al.
(23), which attributes long-range electron transport through Geo-
bacter biofilms not to multistep electron hopping but to an intrinsic
“metallic-like” conductivity of PilA-pili (23). According to their
model, however, the current–potential dependency depicted in
Figs. 4A and 5A should be sigmoid-shaped and not peaked-shaped
(63). The nature of Geobacter biofilm conductivity is contested
because of a lack of direct evidence for cytochrome spatial distri-
bution within the biofilm ECM or direct structural evidence for the
proposed conductive PilA-pili conclusively supporting either a
model for multistep electron hopping or metallic-like conduc-
tivity model (21, 66–67). Because of the role of PilA-pili in biofilm
formation (22) as well as cytochrome secretion (9, 52) and extra-
cellular localization (32), we subscribe to the supposition that PilA-
pili are important for Geobacter biofilm structure and cytochrome
organization (52), and like in other biological systems (2), redox
proteins are the primary charge carriers. The results also raise
questions regarding the thermodynamics of electron transport
through such biofilms.

\section*{Materials and Methods}

IDAs were fabricated in house using standard procedures. Two electrode
experiments were performed under anaerobic conditions (80:20 N
\textsubscript{2}:CO) using a biopotentiostat (AFCBP1; Pine Instruments), counterelectrode (graph-
ite rod), and reference electrode (Ag/AgCl, 3 M KCl; CH Instruments) in
freshwater medium. G. sulfurreducens strain DL1 (S1575; ATCC) was main-
tained in freshwater media containing 40 mM fumarate and 10 mM sodium
acetate as described previously (14, 20, 68, 69). Electrochemical cells containing
10 mM acetate were inoculated with log-phase G. sulfurreducens
drain DL1 (3% vol/vol inoculum, OD = 0–0.6; S1573; ATCC), while IDA electrodes 1 and
2 were maintained at +0.300 V vs. Ag/AgCl. Details of the experimental pro-
cedures are provided in SI Materials and Methods.

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