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PAPER

Lack of cytochrome involvement in long-range electron transport through conductive biofilms and nanowires of *Geobacter sulfurreducens*

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Two competing models for long-range electron transport through the conductive biofilms and nanowires of *Geobacter sulfurreducens* exist. In one model electrons are transported *via* pili that possess delocalized electronic states to function as protein wires with metallic-like conductivity. In the other model electrons are transported by more traditional electron transfer *via* electron hopping/tunneling between the *c*-type cytochromes in *G. sulfurreducens* biofilms and pili. The cytochrome hypothesis was further examined. Quantifying *c*-type cytochromes in *G. sulfurreducens* biofilms and pili indicated that there are insufficient cytochromes to account for electron transport through the bulk of the biofilm or pili and demonstrated that there is a negative correlation between cytochrome abundance and biofilm conductivity. Direct imaging using atomic force microscopy revealed that cytochromes were not packed close enough on pili to permit electron hopping/tunneling along the pili. Inactivating cytochromes had no impact on biofilm conductivity. The results of electrochemical gating studies were inconsistent with electron transport *via* cytochromes. Theoretical considerations suggest that a cytochrome model cannot explain the previously reported response of biofilm conductivity to temperature changes. These multiple lines of evidence, which rely on approaches with different sets of assumptions, demonstrate that the hypothesis that long-range electron transport through *G. sulfurreducens* biofilms and nanowires can be attributed to electron hopping/tunneling between *c*-type cytochromes is incorrect. In contrast, these multiple lines of evidence are consistent with long-range electron transport through the biofilms *via* networks of pili that possess metallic-like conductivity.

Introduction

Evidence for long-range (μm to cm) electron transport *via* metallic-like conductivity in the pili and biofilms of *G. sulfurreducens* suggests a new paradigm in biological electron transfer.[1–4] Metallic-like conductivity differs significantly from the more well-known short-range (<2 nm) electron hopping/tunneling between discrete redox-active molecules that typifies most

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Broader context

The discovery that *Geobacter* species form electrically conductive pili and that networks of these pili can confer conductivity on *Geobacter* biofilms has broad implications. These findings provide an important foundation for the emerging field of electro-microbiology and suggest novel applications in bioelectronics. Furthermore, long-range electron transport *via* pili is thought to play a central role in the reduction of Fe(III) by *Geobacter* species, an important process in the natural cycling of carbon and metals as well as for bioremediation of contaminated groundwater. The conductive pili of *Geobacter* species have also been shown to be important in the interspecies electron exchange that is necessary for the conversion of organic wastes and biomass to methane. Therefore, developing an understanding the mechanism for long-range electron transport along *Geobacter* pili and through conductive biofilms of *Geobacter* species is important for a number of environmental and bioenergy fields. Strong statements in the literature have suggested that this long-range electron transport can be explained *via* electron hopping/tunneling between discrete redox carriers such as *c*-type cytochromes. The studies reported here refute the cytochrome hypothesis and provide additional data which suggests that electron transfer is *via* a metallic-like conductivity.

biological electron transfer.[3,5] Long-range electron transport *via* metallic-like conductivity is likely to have significance for electron transfer to insoluble minerals such as Fe(III) oxides,[6] as well as interspecies electron transfer,[4,7–10] and microbe-electrode electron exchange.[4,11]

Several studies have suggested that traditional electron hopping/tunneling between *c*-type cytochromes confers conductivity to biofilms transferring electrons to electrodes.[12–17] However, biofilm conductivity was not measured in any of these studies. Instead, conductivity was inferred from measurements of electron transfer into or out of the biofilm with a single electrode using cyclic voltammetry. It is true that the *c*-type cytochromes that are often abundant in current-producing biofilms are oxidized and reduced as the electrode potential is changed in such studies.[18] However, this merely reflects the fact that, as expected, the cytochromes of the biofilm cells can respond to redox changes within the cells and their nearby environment. In fact, many of the most abundant *c*-type cytochromes in *G. sulfurreducens*, such as the periplasmic PpcA,[19] are not extracellular and though oxidized and reduced during current production, cannot participate in extracellular electron conduction. Not surprisingly, the formal potential of *G. sulfurreducens* DL-1 biofilm (−150 mV *vs.* SHE) is close to that of center macroscopic potential of PpcA (−146 mV).[13,18] Thus, cytochrome oxidation and reduction alone is not sufficient evidence that cytochromes are involved in long-range electron transport through the biofilms.

The physical meaning of electronic conductivity is that there are free electrons that can move under ordinary thermal conditions (~25 mV).[20] Catalytic current and electron transfer measurements cannot query conductivity in biofilms or pili because they involve a large potential range and the measured current results from capacitive charging as well as the faradic electron transfer reactions at the biofilm/electrode interface.[21] The most direct way to measure electronic conductivity is to apply a small potential difference between two well separated portions of biofilms or pili with ion-blocking electrodes and measure the resultant steady-state electronic current.[20,22,23] This approach enables measurement of long-range electron transport rather than short-range electron transfer process and facilitates the evaluation of conductivity.[24]

Such measurements revealed that current-producing biofilms of *G. sulfurreducens* were highly conductive (two-probe conductivity 0.5 mS cm^{-1}), in contrast to the poor conductivity (0.01 mS cm^{-1}) of biofilms of other organisms not capable of producing current.[1] *G. sulfurreducens* produces electrically conductive pili,[1,25] and biofilm conductivities of different *G. sulfurreducens* strains were directly correlated with the abundance of PilA, the structural pilin protein.[1] Furthermore, the temperature dependence of biofilm conductivity had a metallic-like response similar to that observed in networks of pili sheared from cells.[1] The conductivity of biofilms and pili increased upon cooling[1] whereas conductivity would decrease upon cooling if conductivity was the result of electrons hopping/tunneling between cytochromes.[15] The results of proton-doping, cytochrome-denaturing, and structural studies provided multiple lines of additional evidence consistent with metallic-like conductivity along pili and inconsistent with electron hoping/tunneling *via* discrete electron carriers such as cytochromes.[1,3,5] For example, the highest conductivity for pili was obtained at pH 2, a pH at which *c*-type cytochromes are denatured. In addition, cytochrome localization studies[26,27] and recent scanning tunneling microscopy studies[28] have also suggested that cytochromes are unlikely candidates to confer conductivity to either *G. sulfurreducens* biofilms or pili.

In spite of these results, the hypothesis that cytochromes are responsible for long-range conduction of electrons through biofilms and along the pili of *G. sulfurreducens* persists.[15,16,29,30] Here we describe studies designed to specifically evaluate the potential role of *c*-type cytochromes in long-range electron transport through biofilms of *G. sulfurreducens* with an approach that directly measures conductivity through the biofilms. The results demonstrate that *c*-type cytochromes do not contribute to conductivity through the bulk of the biofilms or pili. These results, coupled with multiple lines of evidence from our previous studies,[1,31–33] suggest that long-range electron transport *via* networks of pili with metallic-like conductivity is the most likely explanation for the high conductivity of *G. sulfurreducens* biofilms.

Results and discussion

Cytochromes are not abundant enough to promote conduction through the biofilm

If *c*-type cytochromes were involved in electron conduction through *G. sulfurreducens* biofilms, it would be expected that the conductivity, σ, would be described by the following Nernst–Einstein relation:[34]

$$\sigma = \frac{ne^2D}{k_BT} \quad (1)$$

where n is the number density of electron carriers, e is the electronic charge (coulombs); k_B is the Boltzmann constant (J K^{-1}); and T is the temperature (K). The diffusion coefficient of redox carriers, D (cm^2 s^{-1}), resulting from a combination of physical displacement and electron hopping is given by following Dahms–Ruff expression:[34,35]

$$D = D_{phys} + \frac{k_{ex}C_E\delta^2}{6} \quad (2)$$

where D_{phys} (cm^2 s^{-1}) is the diffusion coefficient for physical displacement of the redox molecules; k_{ex} (cm^3 mol^{-1} s^{-1}), is the bimolecular rate constant for electron self-exchange; C_E (M) is the total concentration of redox species; and δ (cm) is the contact distance between redox centers at the time of electron transfer.

These equations were applied to strain KN400, the *G. sulfurreducens* strain which produces the most conductive biofilms. We can compute the biofilm conductivity potentially available from *c*-type cytochromes with eqn (1) by substituting the reported diffusion coefficient for *c*-type cytochromes (~1×10^{-6} cm^2 s^{-1}),[36] and the measured number density (n) where $n = N/V$. The density of hemes in the KN400 biofilm can be obtained by measuring the heme concentration (nmoles ml^{-1}) spectrophotometrically (Fig. 1) and multiplying it by the total amount of the extracted biofilm (ml). For a biofilm of KN400 strain, the amount of cytochrome hemes, N = 17.69 nmoles × Avagadro number = 1.07×10^{16}, V = 0.0258 cm^3 for a 40 μm thick biofilm grown on 1″ × 1″ electrode, e is the electronic charge = 1.6×10^{-19} C, and k_BT at room temperature is 25 meV.

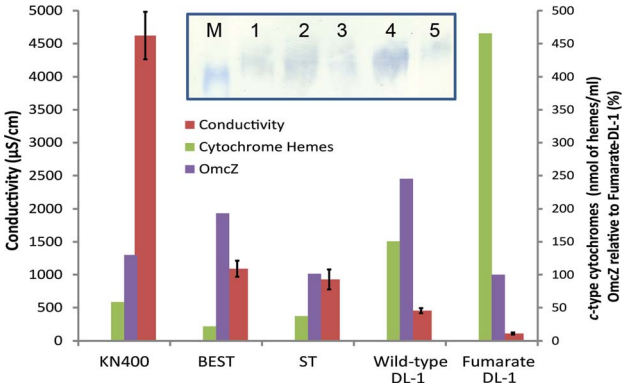

Fig. 1 Comparison of 4-probe conductivity of biofilms of various strains (red bars) and corresponding concentration of cytochromes hemes (green bars) and OmcZ (purple bars) in the biofilms. Error bars indicate standard deviation. Inset: Western immunoblot for OmcZ. M: Marker; Lane-1: KN400; Lane-2: BEST; Lane-3: ST; Lane-4: Wild-type DL-1; Lane-5: Fumarate DL-1. The results are representative of replicate measurements on multiple biofilms.

The computed conductivity for KN400 biofilms that could be due to *c*-type cytochromes is 2.65 μS cm^{-1}, 2000 times lower than the measured conductivity[1] of 5 mS cm^{-1}. This demonstrates that *c*-type cytochromes cannot account for the biofilm conductivity. In a similar manner, the predicted conductivities based on cytochrome abundance are *ca.* 1000, 500, and 100-fold too low for the current-producing biofilms of the BEST, ST, and DL-1 strains, respectively. Furthermore, these calculations substantially overestimate the conductivity that could actually be attributed to cytochromes because: (1) it was assumed that all the hemes measured would be available to participate in extracellular electron exchange, but many of the most abundant cytochromes cannot because they are localized in the periplasm or in membranes; and (2) known outer-surface cytochromes are multiheme and thus the number of cytochrome molecules available for electron exchange are in fact much lower than the number of hemes measured.

Recently, Strycharz-Glaven *et al.* have proposed a model for electron conduction in biofilms and nanowires based on superexchange by redox-active proteins such as *c*-type cytochromes.[15,37] However, the diffusion coefficients up to 10000 cm^2 s^{-1} are used.[37] This value is 11 orders of magnitude higher than the experimentally measured diffusion coefficient for *c*-type cytochromes[36] and therefore physically unrealistic.

Lack of correlation between biofilm conductivity and the concentration of *c*-type cytochromes

Eqn (1) and (2) predict that conductivity should be directly proportional to the total concentration of redox species,[35] but there was a negative correlation ($r = -0.93$) between biofilm conductivity and the concentration of *c*-type cytochrome hemes in different DL-1 strains of *G. sulfurreducens* (Fig. 1). For example, biofilms of strain DL-1 that were grown with fumarate as the electron acceptor had more *c*-type cytochrome hemes than biofilms grown with an electrode as the electron acceptor, but lower biofilm conductivity. A double mutant in which the genes for the two outer-surface *c*-type cytochromes OmcS and OmcT were deleted[38] had less cytochrome hemes than the wild-type DL-1, but higher conductivity. A quadruple mutant in which the genes for the cytochromes OmcB and OmcE, as well as OmcS, and OmcT, were deleted[39] had even fewer cytochrome hemes, but still higher conductivity. Strain KN400, which was selected for its enhanced current-producing capacity,[40] also had lower cytochrome heme levels than strain DL-1, but the highest biofilm conductivity. This negative correlation between cytochrome heme abundance and biofilm conductivity contrasts with the previously reported positive correlation ($r = +0.94$) for the abundance of PilA, the structural pilin protein, and biofilm conductivity.[1] On the other hand, cytochrome hemes show a very strong positive correlation ($r = +0.998$) with the biofilm capacitance,[41] suggesting that, overall, cytochromes contribute to electron storage rather than electron transport in biofilms.

Spacing of proteins associated with pili is too great for electron hopping

It has been suggested that *c*-type cytochromes specifically associated with pili might be responsible for long-range conduction along pili and through conductive biofilms.[15,30]

However, OmcS is the only cytochrome known to be associated with pili,[27] and deletion of OmcS was associated with an increase in biofilm conductivity,[1] and current density,[31] not the decrease, expected if OmcS was important in conferring conductivity required for long-range electron transport.

Furthermore, conduction *via* OmcS is physically impossible because OmcS molecules are spaced too far apart. This was initially suggested from studies with immunogold labeling which indicated average spacing between OmcS molecules of *ca.* 30 nm and spacing between OmcS clusters of 100–200 nm.[27] These values far exceed the distances of <2 nm required for electron hoping/tunneling[42,43] as the rate of electron exchange (k_{ex}) decreases exponentially with the distance (d) between the donor and acceptor molecule. The rate is given as:[42]

$$k_{ex} \propto \exp(-\beta d) \quad (3)$$

where β is the decay constant for direct or superexchange mediated tunneling. The parameter β sensitively depends on the electronic coupling between donor and acceptors across an insulating tunneling energy barrier. Recent theoretical studies demonstrated that cytochromes aligned along microbial nanowires need to packed less than 1 nanometer apart at all times to allow any electron flow.[43]

Because there was a possibility that inefficient immunogold labeling might lead to an overestimation of OmcS spacing on pili, or that redox-active proteins other OmcS might be aligned along pili, the spacing of proteins on intact filaments were directly examined with atomic force microscopy (AFM) (Fig. 2 and 3). *G. sulfurreducens* cells were grown similarly to that described previously for immunogold labeling studies.[27] In order to directly image native pili under physiologically relevant conditions, chemical fixatives were avoided and the samples were gently air dried to maintain a water layer on the sample.[44] AFM studies revealed several cytochrome-like globules aligned along the pili filaments (Fig. 2 and 3). The height of the pili and globules is consistent with previous reports of pili,[25,28] and *c*-type

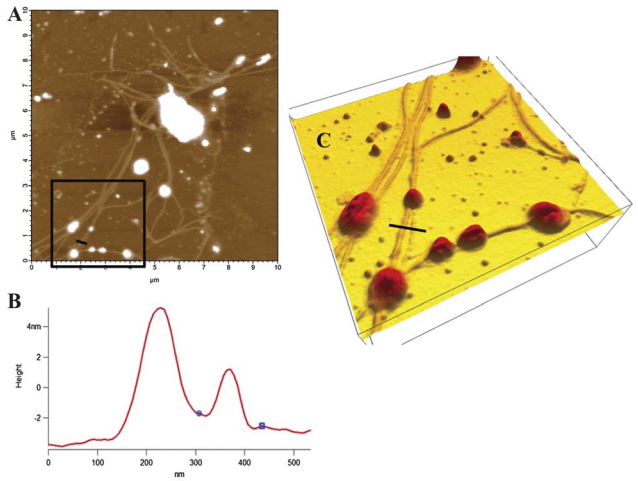

Fig. 2 (A) Atomic force micrograph of *c*-type cytochromes-like globules aligned along intact pili filaments of *G. sulfurreducens* strain KN400. (B) Height profile of pili at the region denoted by a black line. (C) Colorized 3-D image of the 4.5 μm^2 square section in image (A).

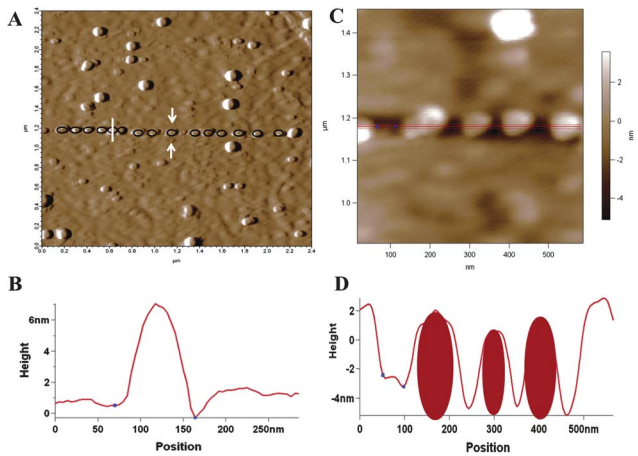

Fig. 3 (A and C) Atomic force micrograph of proteinaceous globules (black circles) aligned along pili filaments. (B) Height profile of globules along a white line shown in (A). Total globule height is *ca.* 5–7 nm, consistent with known size of *c*-type cytochromes. (D) Height profile of globules along a red line shown in (C). Typical spacing among globules is 100 nm.

cytochromes.[45] The apparent broadening of pili filaments and globules is due to electronic convolution of AFM tip.[44] The true height of pili is presented in Fig. 2B which is 3–6 nm, consistent with previous reports.[1,25] Typically the globules were separated by long distances (Fig. 2) but sometimes globules were closely spaced (Fig. 3). Notably, the spacing between these protein globules was greater than 100 nm, similar to that previously observed using immunogold labeling studies.[27] Thus, AFM studies independently confirm that proteins attached to pili filaments are spaced too far apart to account for conduction along pili and through biofilms.

Lack of correlation between OmcZ and conductivity

OmcZ[46] is the only outer-surface *c*-type cytochrome known be necessary for current production[32] and previous studies have suggested that OmcZ confers conductivity to biofilms of *G. sulfurreducens*.[13,14] However, subsequent studies demonstrated that OmcZ is specifically localized at the anode–biofilm interface,[26] suggesting that it cannot be responsible for conduction of electrons through the bulk of the biofilm.[26]

In order to further evaluate the role of OmcZ in conferring conductivity to biofilms, the amount of OmcZ in biofilms of the different strains was quantified (Fig. 1). There was no direct correlation ($r = 0.15$ for DL-1 strains) between OmcZ and biofilm conductivity (Fig. 1), further confirming that OmcZ does not account for the conductivity through the bulk of the biofilms.

Denaturing *c*-type cytochromes has no impact on biofilm conductivity

As an alternative approach to evaluate the role of *c*-type cytochromes in biofilm conductivity, biofilms were treated with acetyl methionine, which binds to *c*-type cytochromes making them nonfunctional.[47] Previous studies[41] demonstrated that addition of acetyl methionine prevented *c*-type cytochromes in *G. sulfurreducens* biofilms from accepting electrons and it also inhibited current production in *G. sulfurreducens* fuel cells (Fig. 4B). This was associated with a lack of redox peaks in cyclic voltammetry of the biofilms following the addition of acetyl methionine

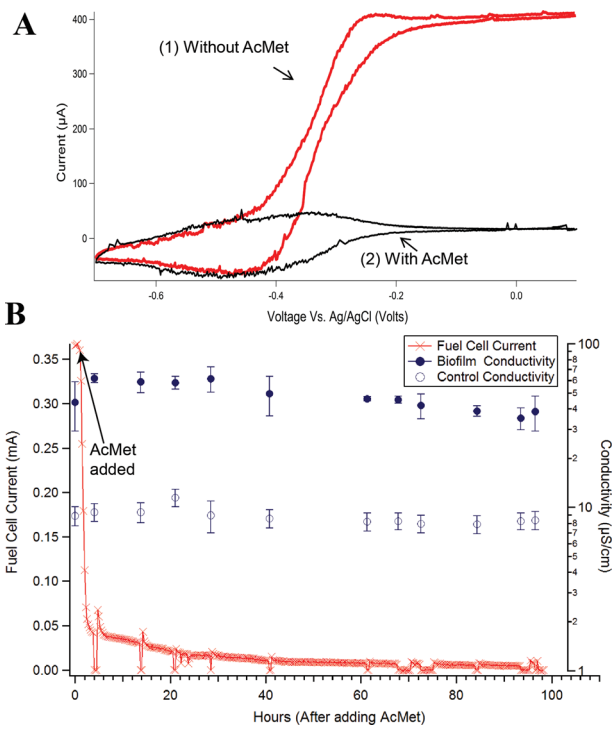

Fig. 4 (A) Effect of addition of acetyl methionine (AcMet) on redox activity of DL-1 biofilm monitored by cyclic voltammetry at scan rate 10 mV s^{-1}. Data shows voltammograms before (red trace 1) and after (black trace 2) adding AcMet (120 mM) into the anode chamber. Voltage indicated is against Ag/AgCl reference electrode. (B) Effect of addition of acetyl methionine (AcMet) on the microbial current generation and biofilm conductivity measured with the 2-probe method. Conductivity of the DL-1 biofilms (blue solid circles) and controls (blue open circles) that lacked biofilm over time are presented after adding acetyl methionine (AcMet). Red trace shows the microbial current at the time of conductivity measurements. Error bars indicate standard deviation.

(Fig. 4A) even though acetate was continuously supplied to the biofilm. However, there was no impact of acetyl methionine addition (shown in figure as time zero) on biofilm conductivity (Fig. 4B). These results are consistent with previous studies on pili which demonstrated that cytochrome denaturants did not negatively impact on conductivity in pili networks[1] and further suggest that *c*-type cytochromes do not contribute to long-range electron transport.

Electrochemical gating analysis

Electrochemical gating analysis to measure the conductivity as a function of redox potential[48] offers another approach to evaluate the possibility of conduction through *G. sulfurreducens* biofilms *via c*-type cytochromes. When conduction is *via* cytochromes or other redox carriers there should be a maximum when the surface potential is near the formal potential for the redox process.[22,23,49–51] This reflects the equal density of reduced and oxidized species at the formal potential, which allows maximum possibility for electron exchange. When the film is poised at a potential that is much more positive or negative than the formal potential, the conduction is poor due to an overwhelming majority of the reduced (electron-loaded) or oxidized (hole-loaded) redox carriers. For example, electrochemical gating on redox-active metalloproteins exhibited a conductance peak whereas non-redox controls did not show any peak in conductance.[52]

Electrochemical gating with an electrolyte-gated, field-effect transistor geometry revealed that the biofilm conductivity does not peak at the formal potential (Fig. 5), including at the formal potential of −0.350 V *vs.* Ag/AgCl for the biofilms of DL-1 strain observed in this (Fig. 4A) and previous studies.[13,41] There was no peak in conductivity for live biofilms of the DL-1 strain (Fig. 5A) as shown previously[1] or when acetyl methionine was added to denature cytochromes (Fig. 5B). This shows that the observed sigmoidal response in conductivity as a function of gate voltage, which is a characteristic of organic metals, (ref. 1 and references therein), cannot be attributed to cytochromes. The decrease in gating efficiency for the denatured biofilm is most likely due to the higher ionic strength of the electrolyte after addition of 120 mM acetyl methionine that screens the electric field[48] because the biofilm conductivity was unchanged after cytochrome denaturation (Fig. 4B).

The conductivity of biofilms of KN400 strain was also measured with four probes as a function of surface potential (Fig. 5C), which eliminated the possible contribution from the gate-voltage dependent contact resistance.[50] The formal potential for the biofilm of KN400 strain is −0.370 V *vs.* Ag/AgCl.[37,41]

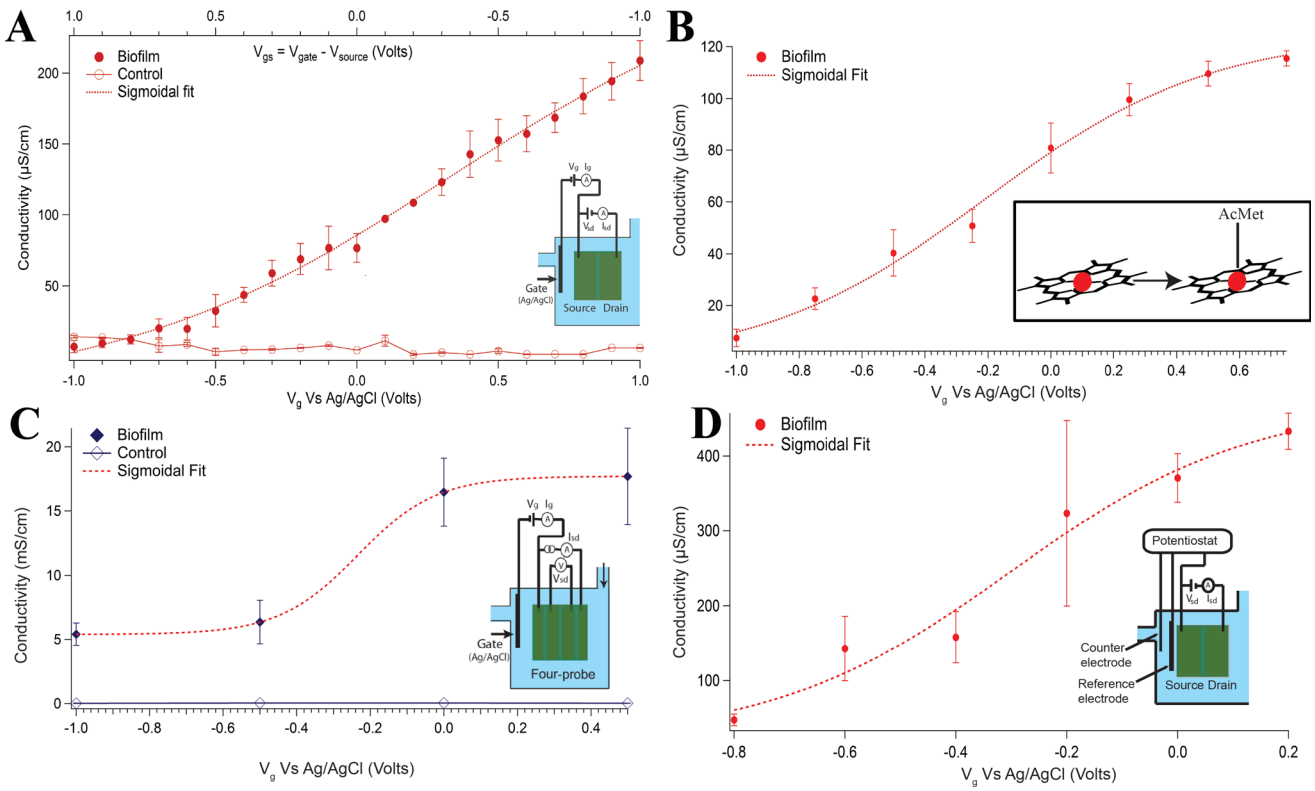

Fig. 5 Electrochemical gating of *G. sulfurreducens* biofilms showing sigmoidal response with no peak at the formal potential. Gate voltage indicated is against Ag/AgCl, 3 M KCl reference electrode. (A) Gating response on the biofilm of strain DL-1 when gating and conductivity measurements were performed in two electrode setup (inset). Figure adapted from ref. 1 with permission. (B) Gating response on the biofilm of strain DL-1 in which cytochrome activity was inhibited with acetyl methionine. Measurement setup is similar to that used in (A). Inset: ligand coordination reaction of AcMet to heme iron. (C) Four-probe conductivity of biofilm of KN400 strain (blue solid triangles) no biofilm control (blue open triangles) measured by electrochemical gating. Measurement setup is shown in the inset. (D) Conductivity of *G. sulfurreducens* biofilm strain ZMS123 measured by electrochemical gating using a three-electrode potentiostat configuration (inset). Error bars indicate standard deviation. Note that with the exception of panel A, all studies are reported here for the first time.

Similar to studies with DL-1 biofilms, there was no peak in 4-probe conductivity of KN400 biofilms at the formal potential. It has been argued that the current flowing through the reference electrode can cause artifacts in the response of electrochemical gating.[53] In order to evaluate any possible effect of current flowing through the reference electrode on the observed response, we used a potentiostat for electrochemical gating with independent counter and reference electrodes so that all the current was diverted to the counter electrode, with no current flowing through the reference electrode (Fig. 5D inset). Notably, the response of the biofilm was similar irrespective of the gating configuration with no peak at the formal potential (Fig. 5D). The differences in gating efficiency in four-probe and potentiostat gating experiments can be attributed to the differences in the electric field applied using differing geometries.[54,55]

At a highly oxidizing potential, the cytochrome-dependent conduction model predicts a large suppression in conductivity.[15] In contrast, there was an increase in conductivity, over two orders of magnitudes, at oxidizing potentials. Therefore, these studies further confirm that the observed conductivity is unlikely due to *c*-type cytochromes.

Temperature dependence of conductivity of biofilms, nanowires is inconsistent with cytochrome-dependent conduction

Redox conduction *via* cytochromes is a thermally activated process (eqn (1)). Conductivity *decreases* with a decrease in temperature since the diffusion coefficient that comprises the rate constant, k_{ex}, for the redox reactions is also temperature dependent (eqn (4)) and the rate decreases exponentially with a decrease in temperature[42] according to:

$$k_{ex} = A\exp\left(\frac{-\Delta G}{RT}\right) \quad (4)$$

where A is the equilibrium rate constant and ΔG is the change in free energy during the reaction.

However, conductivity of biofilms and pili *increased* when these materials were cooled from the room temperature, until a crossover temperature after which disorder dictates the response.[1] It was suggested that potential contact effects negated the relevance of these measurements,[53] but the studies were performed with a four-probe configuration that avoids such artifacts. Furthermore, the increase in conductivities were observed as the temperature was dropped through physiologically relevant temperatures of 27 °C to 0 °C and the lack of chemical fixatives and the presence of bound water further ensured physiological relevance.[3]

Conclusions

The results demonstrate from multiple approaches, with different inherent assumptions, that electron hopping/tunneling between *c*-type cytochromes cannot account for the conductivity of *Geobacter sulfurreducens* biofilms. This is consistent with the results of earlier studies which suggested that long-range electron transport through the biofilms could be attributed to networks of pili with metallic-like conductivity.[1]

Long-range electron transport through biofilms *via* *c*-type cytochromes is physically impossible because the density of cytochromes is too low and cytochromes are spaced too far apart for electron hopping/tunneling through the biofilm. There is a negative correlation between cytochrome abundance in biofilms and biofilm conductivity. The only *c*-type cytochrome that is required for effective current production, OmcZ, is concentrated at the anode–biofilm interface and thus could not be responsible for conducting electrons through the bulk of the biofilm. Inactivating *c*-type cytochromes does not eliminate conductivity. Multiple responses of the biofilm to perturbations such as changes in temperature, pH, or oxidation potential are inconsistent with conduction *via* cytochromes, but consistent with metallic-like conductivity.

The claim for *c*-type cytochromes contributing to conduction in biofilms primarily rests on the observed oxidation and reduction of cytochromes in biofilms when electrons are electrochemically injected or withdrawn from biofilms. However, this response does not mean that the cytochromes are responsible for long-range electron transport. Any mechanism that conducts electrons to cells as well as their associated cytochromes could make it possible to reduce and oxidize cytochromes in a biofilm. Modeling studies designed to more specifically address the mechanisms for electron transport have shown that physically unreasonable assumptions are required to model cytochrome-dependent conductivity. For example, cytochromes aligned along the pili have been assumed to be spaced apart less than 1 nm,[43] whereas in actuality for *G. sulfurreducens*, they are spaced more than 100 nm apart. The high conductivity of pili could not be achieved in the model when the distance between charge carriers was greater than 1 nm, even if the reorganization energy was set to zero.[43] Without a multiplicative factor of 1000, corresponding to the number of carriers in the individual pili coupled to the electrodes, a hopping model cannot yield reasonable conductivities.[43] The number of cytochromes observed on individual pili is much less than 1000.

These considerations, and the many experimental observations inconsistent with long-range electron conduction *via* *c*-type cytochromes, make electron hopping/tunneling *via* *c*-type cytochromes a highly unlikely mechanism for the conductivity of *G. sulfurreducens* biofilms and nanowires. Recent studies using scanning tunneling microscopy on *G. sulfurreducens* nanowires also conclude that conductivity is unlikely due to *c*-type cytochromes.[28]

It might be argued that electron transfer relays other than *c*-type cytochromes could conceivably contribute to conductivity *via* traditional electron hopping/tunneling. But the metallic-like conductivity of *G. sulfurreducens* biofilms and pili cannot result from electron tunneling or hopping.[42] Furthermore, electrochemical analyses failed to detect any redox-active proteins other than *c*-type cytochromes.

The sheer abundance of *c*-type cytochromes in *G. sulfurreducens* does suggest an important role for the cytochromes, such as for electron storage,[41] but it should also be considered that the ability of *Geobacter* species to produce high current densities on electrodes may be a fortuitous outcome of their evolution for other activities as the conditions on electrodes are not replicated in known natural environments.[56,57] In fact, selective pressure for enhanced current production yielded a strain of *G. sulfurreducens* that produced fewer cytochromes and more pili.[40] The only outer-surface *c*-type cytochrome that has been shown to be

required for high-density current production in *G. sulfurreducens* is OmcZ,[32] which is specifically localized at the anode–biofilm interface.[26] Further evaluation of the mechanisms by which OmcZ contributes to electron exchange with electrodes is warranted.

Materials and methods

Bacterial strains and culture conditions

Geobacter sulfurreducens strain DL-1 (ATCC 51573),[40] strain KN400,[40] strain BEST,[39] strain ST,[38] and strain ZMS123,[58] were obtained from our laboratory collection. The cultures were maintained under strictly anaerobic conditions in growth medium supplemented with fumarate (40 mM) as the electron acceptor with acetate (10 mM) as the electron donor as described previously.[59]

Biofilms were grown in a split-anode microbial fuel cell as described previously.[1] The two gold anodes separated by a 50 μm nonconducting gap were connected to a carbon-cloth cathode through a 560 ohms load resistor. The cathode was immersed in a 50 mM potassium ferricyanide solution. Control split-anodes were not connected to a cathode, prohibiting biofilm formation on control electrodes. All the results were confirmed by repeated measurements on multiple biofilms.

Electron transport (conductivity) measurements

Conductivity of biofilms was measured as described previously.[1] Voltage was applied across the electrodes in the range 0–0.05 V in steps of 0.025 V for a minimum of 100 seconds and current was measured at every second. After allowing exponential decay of the ionic current, steady state electronic current was used to create current–voltage characteristics and to extract conductance. For 4-probe measurements, a source meter (Keithley 2400) was used to apply a fixed current between outer of the four electrodes and to measure the potential drop between two inner electrodes,[54] by measuring the voltage for each current every second over a period of 100 seconds, after reaching the steady-state. An additional high-impedance voltmeter (Keithley 2000) was used to record the output voltage of the current source to calculate conductance.[54] Note that the output voltage of inner electrodes was used for all four-probe measurements which eliminate the contribution of contact resistance as the very high input impedance of the voltmeter prohibits any current flow through the inner circuit (Fig. 5C inset).

Biofilm conductivity (σ) was calculated from the measured conductance (G) using conformal mapping (the Schwarz–Christoffel transformation)[60] as described previously[1] using the following relation:

$$\sigma = G\frac{\pi}{L}\Big/\ln\left(\frac{8g}{\pi a}\right) \quad (5)$$

where L is the length of the electrodes ($L \approx 2.54$ cm); a is the half-spacing between the electrodes ($2a \approx 50$ μm) and g is the biofilm thickness measured using confocal microscopy. Above formula is valid for the limiting case $a < g \ll b$ where b is the half-width of the electrodes ($2b \approx 2.54$ cm).

Cytochrome measurements

The biofilms were removed from the gold electrodes using 600 μl isotonic wash buffer. Collected biomass was immediately frozen with liquid nitrogen and stored at −20 °C. After thawing, vortexing, sonicating, and centrifuging for 5 min at 9000 RPM, supernatants and pellets (cell debris) were collected. Supernatant protein concentration was measured by Quick Start Bradford Dye Reagent (BioRad, Hercules, CA, USA). Standards were prepared by using Bovine Serum Albumin (Sigma, St. Louis, Mo, USA). Pellets were suspended in 200 μl deionized water and boiled with 0.5% SDS for 10 minutes and protein concentration was determined by the bicinchoninic acid method with bovine serum albumin as a standard.[61]

Heme content was estimated by UV/visible scanning of disrupted cells using UV2401-PC spectrophotometer (Shimadzu, MD, USA) as described previously.[41] The oxidized cytochromes had a distinct peak at 410 nm and the obtained absorbance value of that peak was used to calculate heme content by comparing to a standard curve. A standard curve was made with oxidized bovine heart cytochrome *c* (Sigma, St Luis, USA). For heme staining, the cytochrome contents were analyzed by 12.5% Tris–Tricine denaturing polyacrylamide gel electrophoresis followed by staining with *N*,*N*,*N*′,*N*′-tetramethylbenzidine as described previously.[62,63]

In order to determine the amount of OmcZ protein in the biofilms, immunoblots were probed with the OmcZ-specific antiserum. Immunoreactive bands were visualized with One-Step Western Kit (GeneScript Co., NJ, USA) according to manufacturer's instructions. Each lane was loaded with 8 μg of cell protein. The intensity of OmcZ bands was quantified by densitometry using ImageJ software (NIH).

Atomic force microscopy imaging

Atomic force microscopy (AFM) was performed as described previously.[1] Cells of KN400 strain were placed on freshly cleaved Highly Oriented Pyrolytic Graphite (HOPG) surface and allowed to settle for 15 minutes. Excess buffer was removed using a micropipette. The tapping mode of AFM, which is known to be ideal for imaging soft biomolecules,[64] was used to image cells, and pili.[1]

Cytochrome inhibition experiments

Acetyl methionine (AcMet) was used to inactivate the cytochromes in biofilm as described previously.[41,47] When microbial current production became stable, growth medium in the anaerobic anode chamber was removed under sterile, anaerobic conditions. Chambers were refilled with a sterile, anaerobic growth medium with addition of 120 mM AcMet. The medium pH was adjusted to 7. Within a few hours, current dropped to zero. High performance liquid chromatography confirmed that acetate was no longer being consumed.

Cyclic voltammetry (CV)

CV was performed by sweeping the voltage between +0.1 V and −0.7 V *vs.* Ag/AgCl at scan rate 10 mV s^{-1} using a Solartron 1287 potentiostat. Both the working electrodes (source and

drain) were connected to each other and voltage was applied between working and Ag/AgCl, 3 M KCl reference electrode. Current was measured between working and counter electrode. The counter electrode was a carbon cloth located in the cathode chamber. The formal potential was determined by cyclic voltammetry agreed very well with the literature.[12,13,37,65]

Electrochemical gating

For two-probe measurements, Keithley 2400 sourcemeter was used to apply voltage (V_g) between gate (Ag/AgCl, 3 M KCl reference electrode) and the source–drain to create the electrolyte gate field effect (Fig. 5A inset).[1] As described earlier,[1] gate current (I_g) was continuously monitored over the entire experiment. No monotonic dependence of the gate current on the measured biofilm conductivity was observed, confirming that the largest part of this ion current does not flow through the biofilm across the gap but originates from the gold electrodes, which are partially covered by electrolyte.[66,67] A second Keithley 2400 sourcemeter was used to apply voltage between source and drain to measure conductance as described in the method section about conductivity measurements.

For electrochemical gating using four-probe, gate voltage was applied between reference electrode and one of the outer electrode[54] and conductance was measured as described in the method section about conductivity measurements (Fig. 5C inset).

For electrochemical gating using a potentiostat, Solartron 1287 was used. Platinum wire was used as a counter electrode (Fig. 5D inset).[35] IGOR Pro software (WaveMetrics Inc. OR, USA) was used for all data analysis.

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