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On Electron Transport through *Geobacter* Biofilms

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Geobacter spp. can form a biofilm that is more than 20 μm thick on an anode surface by utilizing the anode as a terminal respiratory electron acceptor. Just how microbes transport electrons through a thick biofilm and across the biofilm/anode interface, and what determines the upper limit to biofilm thickness and catalytic activity (i.e., current, the rate at which electrons are transferred to the anode), are fundamental questions attracting substantial attention. A significant body of experimental evidence suggests that electrons are transferred from individual cells through a network of cytochromes associated with cell outer membranes, within extracellular polymeric substances, and along pili. Here, we describe what is known about this extracellular electron transfer process, referred to as electron superexchange, and its proposed role in biofilm anode respiration. Superexchange is able to account for many different types of experimental results, as well as for the upper limit to biofilm thickness and catalytic activity that *Geobacter* biofilm anodes can achieve.

Introduction

*Geobacter spp. (*e.g., *Geobacter sulfurreducens* strain DL1) can acquire energy by coupling the intracellular oxidation of organic matter, such as acetate, with extracellular electron transfer to an anode (an electrode maintained at a sufficiently positive electrochemical potential to act as an electron drain) resulting in an electric current (Figure 1).[1] The anode-respiring ability of *Geobacter* derives from their ability to reduce insoluble oxidants, such as Fe^{III} oxides, observed in natural environments.

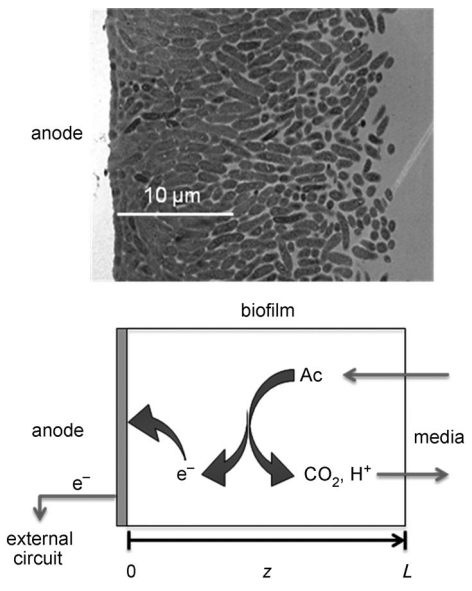

Figure 1. Top: TEM image of cross section of *Geobacter sulfurreducens* strain DL-1 biofilm anode grown to the point where growth becomes limited (image courtesy of E. V. LaBelle). Bottom: Schematic depiction of the overall process of *Geobacter* biofilm anode respiration. Here, acetate is the electron donor, *L* is the biofilm thickness (typically > 20 μm), and *z* is the distance from the anode surface inside the biofilm.

Although other species are known to catalyze anodic reactions,[2] *Geobacter* are distinct for the high rate at which they can directly transfer electrons to an anode surface, comparable to the rate at which they can respire soluble oxidants such as Fe^{III} citrate, while not relying on soluble electron transfer mediators such as flavins.[3] Furthermore, on anodes that are inexhaustible electron acceptors, *Geobacter* can form multi-microbe thick, persistent biofilms not observed when respiring insoluble oxidants, in which microbes residing more than 20-cell lengths away utilize the anode as their terminal electron acceptor. The combination of robust direct electron transfer and high cell surface density (microbes across the entire biofilm generating electrons, which are collected by the underlying anode) enables *Geobacter* biofilms to achieve higher anodic current densities than any other species.[4] For this reason, *Geobacter* may play an important role in emerging technologies based on microbe-catalyzed anode processes for which abiotic catalysts do not exist, such as wastewater treatment and energy generation from biomass oxidation.[5]

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Evidence that superexchange controls biofilm anode electron transport

How cells and cellular components coordinate in the transport of electrons through a thick biofilm and across the biofilm/anode interface and what limits the rate and distance that electrons can be transported through an anode respiring biofilm are unresolved. Cyclic voltammetry,[3,6] conductivity measurements,[7] and spectroelectrochemical measurements[8] performed on living, actively respiring biofilms all indicate that *Geobacter* biofilm anodes contain discrete, biofilm-bound redox cofactors that are reversibly oxidized and reduced in response to the anode potential. The key observation from all three types of experiments is that the transport of electrons through a *Geobacter* biofilm anode exhibits diffusive behavior characterized by Fick's laws of diffusion,[6a,d,7,9] consistent with extracellular electron transport involving sequential electron-transfer self-exchange reactions (i.e., electron hopping) between discrete redox cofactors in a manner resembling an electron "bucket brigade".

We refer to this form of extracellular electron transport in *Geobacter* biofilm anodes as electron superexchange, in deference to the established use of this term to describe this form of electron transport within redox hydrogels including wired enzyme electrodes.[10] Based on superexchange, the driving force for extracellular electron transport toward the anode surface during anode respiration is the effective concentration gradient of electrons across the biofilm (i.e., a redox gradient), arising from the reduction of oxidized cofactors by microbes within the biofilm, and oxidation of reduced cofactors at the anode surface.[6d,7,9b] Accordingly, the local rate of electron transport at any given location inside the biofilm, including at the biofilm/anode interface, resulting in current is expected to be proportional to the local concentration gradient of reduced cofactor (Fick's 1st law of diffusion).

In the case of cyclic voltammetry, a set of transient anodic and cathodic voltammetric current peaks are observed for living, actively respiring *Geobacter* biofilms grown on anodes at sufficiently fast scan rates (typically $>0.02\ V\,s^{-1}$).[6c,9b] These peaks result from the change in oxidation state of redox cofactors within the biofilm in response to the changing anode potential, and the potentials at which these peaks occur [at approximately -0.2 V vs. standard hydrogen electrode (SHE)] are similar to the formal potentials of known *c*-type cytochromes purified from *G. sulfurreducens*.[11] In electrochemistry, the term pseudocapacitance is used to describe the charge (the integral of current over time) associated with such voltammetric peaks; a more thorough description of which is available.[6d] In all published studies, these voltammetric peaks scale in magnitude with the square root of the voltammetric scan rate. This observation indicates that a diffusive process (referred to as semi-infinite diffusion in a confined film[6d]) consistent with superexchange, governs the transport of electrons between biofilm redox cofactors and the anode surface as the oxidation state of the cofactors changes in response to the changing anode potential. Moreover, the formal potentials of these voltammetric peaks are centered on the midpoint potential of the sigmoid-shaped catalytic current–electrode potential dependency (i.e., catalytic voltammetry) observed at slower scan rates (typically $<0.02\ V\,s^{-1}$). This is consistent with a catalytic current limited by the rate at which cells deliver electrons to redox cofactors in the biofilm that subsequently transport electrons to the anode surface via superexchange, as determined by modeling *Geobacter* biofilm anodes as enzyme-functionalized electrodes (in the absence of pH considerations, vide infra).[9b] Voltammetric peaks observed in the absence of acetate (non-turnover condition) are also centered at the same approximate formal potential as when acetate is present, indicating that the same electron transport process observed by voltammetry of resting cells is involved in anode respiration.[9b]

In the case of spectroelectrochemistry,[8b,c] diffusive electron-transport behavior consistent with superexchange is also exhibited by actively respiring *Geobacter* biofilm anodes. Here, a lag in the change of oxidation state of biofilm redox cofactors, specifically *c*-type cytochromes (vide infra), is observed spectroscopically while the electrode potential is changed during voltammetry. This lag increases in duration with increasing voltammetric scan rate and increasing biofilm thickness. As above, this observation indicates that a diffusive electron transport process governs the transport of electrons between biofilm redox cofactors and the anode surface, where the time required for electrons to radiate via superexchange across the biofilm becomes more apparent the faster the electrode potential is changed and the thicker the biofilm becomes.

In the case of conductivity measurements,[7] diffusive electron transport behavior consistent with superexchange is also exhibited by actively respiring *Geobacter* biofilm anodes. Here, a sigmoid-shaped dependency (rather than a linear dependency) is observed for a current conducted through a *Geobacter* biofilm connecting two separated electrodes as voltage is applied to the electrodes.[7]

These independent measurements (electrochemical, spectral, and conductance) performed on actively respiring *Geobacter* biofilm anodes all yield results highly characteristic of systems for which long-range electron transport results from electron transfer reactions between discrete redox cofactors. This results in diffusive-like electron transport behavior consistent with known systems for which electron transport occurs via superexchange.[10]

Evidence for the role of *c*-type cytochromes

G. sulfurreducens cells possess an abundance of multiheme *c*-type cytochromes on their outer membrane, in extracellular polymeric substances, and along pili.[11a,b,12] *C*-type cytochromes are ubiquitous redox proteins involved in biological electron-transport processes, but are best known for systems in which single or diheme cytochromes shuttle electrons between larger electron transfer proteins.[13] In contrast, multiheme *c*-type cytochromes can act as immobilized electron conduits spanning large distances, as has been demonstrated for the *Shewanella* CymA-MtrA-MtrC system,[14] where three multiheme cytochromes transfer electrons from the inner membrane to the outer surface in a sequence of 24 interacting hemes.

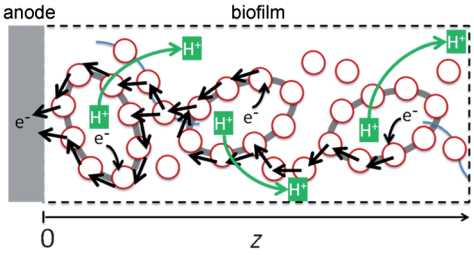
Spectroelectrochemistry specifically implicates cytochromes as the dominant redox cofactors involved in *Geobacter* biofilm extracellular electron transfer. Four different laboratories have used UV-Vis absorption spectroscopy,[8] surface-enhanced infrared absorption spectroscopy,[15] and surface-enhanced Raman spectroscopy[16] combined with electrochemistry to investigate *Geobacter* biofilm anodes, demonstrating characteristic spectra of *c*-type cytochromes that undergo changes in oxidation state with changes in the anode potential. Furthermore, Liu and Bond[8c] found a linear increase in catalytic current with the amount of *c*-type cytochrome in a *Geobacter* biofilm anode during biofilm growth, consistent with electrochemical data,[6d] demonstrating a linear increase in the amount of redox cofactor in a *Geobacter* biofilm anode with catalytic current during biofilm growth. Although they may be hidden by the large signal produced by *c*-type cytochromes, no significant spectroscopic signals indicative of other potential redox cofactors with formal potentials in this range (−0.2 to −0.5 vs. SHE), such as quinones and flavins, have been detected.

Analysis of surface-enhanced Raman spectra directly implicates *c*-type cytochromes in electron-transfer across the biofilm/anode interface.[16] This evidence is further supported by the observation of multiple sets of voltammetric peaks in cyclic voltammograms recorded under non-turnover conditions (where cells have no acetate to oxidize, e.g., Figure 9B, Strycharz et al.[9b]) comparable to that observed for isolated multiheme *c*-type cytochromes involved in electron transport of *Shewanella oneidensis* strain MR-1.[14b,c] Such peaks are consistent with the presence of a multi-electron accepting redox cofactor at the biofilm/anode interface, whose formal potential is influenced by the number of transient electrons residing in the cofactor due to repulsive electron–electron interactions (i.e., a Coulomb blockade).[9a] Prior modeling indicates that the formal potential of redox cofactors involved in electron transfer across the biofilm/anode interface cannot be more negative than the midpoint potential of the catalytic voltammetry (e.g., Figure 9A in Ref. [9b]), suggesting that during anode respiration, only one transient electron at a time may reside in multiheme *c*-type cytochromes transferring electrons to the anode.

Genetic data is less definitive with respect to implicating cytochromes as redox cofactors involved in biofilm anode electron transfer, as deletion of certain *G. sulfurreducens* cytochromes leads to multiple changes in expression of other cytochromes and proteins.[17] Perhaps the most compelling evidence involves the octoheme cytochrome OmcZ. This protein is secreted outside the cell, where it is loosely tethered to material extending far beyond the outer membrane.[12f,18] Consistent with its possible role as an extracellular electron transfer agent, OmcZ tends to aggregate to itself, is highly stable, and has a reduction potential of approximately −0.22 V versus SHE, consistent with thermodynamics of anode respiration (vide infra). Further, deletion of OmcZ eliminates the ability of *G. sulfurreducens* to utilize anodes as electron acceptors. Many other cytochromes, such as those shown to be organized along pili (OmcS), loosely attached to the outer surface (OmcE), or attached to the outer membrane (OmcB), also produce phenotypes in anode-grown biofilms,[6c] but these mutants tend to select for suppressor mutants able to express other cytochromes, which complicates the study of their specific roles.[17,19]

Scheme of biofilm-anode catalytic activity

A schematic depiction of the proposed catalytic process, including the superexchange extracellular electron-transport process that incorporates the results described above, is shown in Scheme 1. Details of independent components of this Scheme



Scheme 1. Schematic depiction of *Geobacter* biofilm anode respiration at the microbe layer. Here, biofilm-bound redox cofactors (*c*-type cytochromes) associated with cell surfaces, along pili, and within extracellular polymeric substances act as extracellular terminal electron acceptors for cells metabolizing acetate, as well as mediators facilitating electron-transport through the biofilm by self-exchange among adjacent cofactors, and ultimately as the electron donors for electron transfer to the anode surface resulting in electrical current. Concomitantly generated protons diffuse out of the biofilm, in the opposite direction of electron flow, toward adjacent media.

have been described extensively elsewhere.[4,7,9b,20] Here, for a given microbe in the biofilm, electrons resulting from intracellular acetate oxidation are transported from the cytoplasm to outer membrane cytochromes associated with the microbe. Once in the extracellular environment, electrons are transferred between cytochromes, either on cell outer membranes, aligned along pili,[12c] or in the extracellular polymeric substances,[12f] until they reach cytochromes associated with microbes at the biofilm/anode interface, where they are transferred to the anode (referred to as a heterogeneous electron transfer reaction as it involves an electrode). Concomitantly generated protons diffuse complexed with bases toward the media.

The following are key aspects of this scheme.

Electron transport must be very efficient

Cyclic voltammetry of *Geobacter* biofilms grown utilizing acetate as the electron donor has repeatedly demonstrated a thermodynamic threshold below which *Geobacter* is unable to donate electrons to an anode; this value is approximately −0.22 V versus SHE.[3,6a,c,9b,21] Taking into account the reduction potential of the CO_2/acetate half-reaction (−0.29 V vs. SHE), this indicates that less than 0.07 V (6.7 kJ per electron) is used during cellular respiration to generate proton motive force. Thus, for the complete eight-electron oxidation of acetate under standard conditions, only 53.6 kJ per mol acetate is potentially available for all adenosine triphosphate (ATP) generation needs, a value that will be even lower as acetate concentrations are reduced. These electrochemical observations are consistent with chemostat and modeling studies concluding

that *Geobacter* obtains much less than one ATP per acetate oxidized when metals are the electron acceptor.[22] The low energy gain from anode respiration may reflect the fact that iron(III) oxyhydroxides in the environment also typically have low redox potentials, and that, when acetate and Fe^{III} are at low concentrations, conserving any additional energy would be uncompetitive.

The second important observation derived from voltammetry is that *Geobacter* only requires anodes to be poised at potentials 0.1 V higher than this thermodynamic threshold to reach its maximum rates of anode respiration. In other words, when anodes are poised at −0.10 V versus SHE, cells respire just as fast as when the anode is raised to potentials 0.5 V higher.[3a,6c,7] This observation reveals two facts: *Geobacter* does not take advantage of excess available potential energy when anodes are poised at higher potentials, and only about 0.1 V is lost while driving electron transport across multiple insulating membranes, through multiple microns of extracellular space, and across the biofilm/anode interface. Such a low energy loss is only possible if the intrinsic rate of electron self-exchange between proteins, and with the anode surface, is very fast (i.e., activation energy barriers are very low).

A calculation investigating this low energy exchange hypothesis has been performed to model electron transport between two microelectrodes spanning an extracellular fiber isolated from *Shewanella oneidensis* strain MR-1.[23] Electron transport for this organism has also been observed with very low (≈0.1–0.2 V) driving forces, which was modeled by electron superexchange among redox cofactors proposed to be associated with the fiber.[24] Electron transfer along such fibers at observed rates[23] was possible via a superexchange mechanism as long as the redox cofactors were closely spaced and the calculated activation energy for electron transfer among adjacent cofactors was among the lowest exhibited for biological electron transfer reactions (on the order of 0.6 mV).[25]

pH gradient

Proton transport is crucial within a *Geobacter* biofilm due to high stoichiometric production of protons during anode respiration:

$$CH_3COO^- + 3\,H_2O \rightarrow CO_2 + HCO_3^- + 8\,H^+ + 8\,e^- \quad (1)$$

In fact, no other microbial metabolic pathway, respiratory or fermentative, produces as many protons per substrate consumed as anode respiration because in the case of anode respiration, negative charge (electrons) is removed at the base of the biofilm. As electrons are produced and transported to the anode, concomitantly produced protons must be transported out of the biofilm and into adjacent media.[20a] Proton transport is thought to occur by diffusion[26] of protons complexed with buffers (such as carbonate and phosphate ions)[20a,26b] owing to low proton concentration at near-neutral pH values favored by *Geobacter*.

The generation of protons by cells within a *Geobacter* biofilm anode, and their diffusion out of the biofilm, is predicted to result in the formation of a proton concentration gradient across the biofilm (Figure 2). In this gradient, a drop in the pH value is expected to occur close to the biofilm/anode interface. This drop becomes more pronounced as the biofilm grows thicker. Imaging by using pH-sensitive dyes confirms that a pH gradient does occur, in which a pH value as low as 6.1 can occur close to the biofilm/anode interface of a full grown *Geo-*

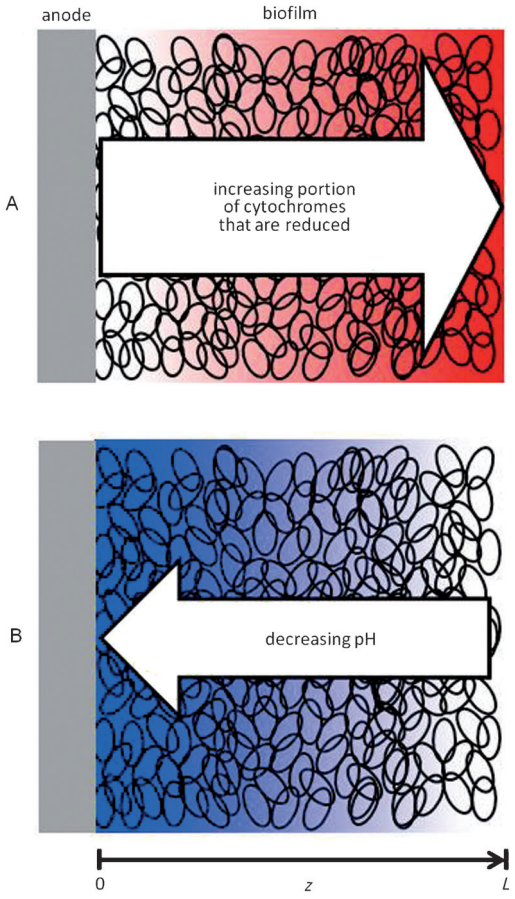


Figure 2. A) The proposed use for cytochromes in the biofilm to both accept electrons from cells they are associated with, as well as electrons transported from cells residing further from the anode surface, is predicted to result in generation of a redox gradient across the biofilm, represented by the arrow pointing away from the electrode surface. With increasing distance from the anode surface, the relative abundance of cytochromes in the oxidized state decreases while the relative abundance of cytochromes in the reduced state increases, represented here by a shift in biofilm color from white to red with increasing distance from the anode surface (*z*). In the absence of any other limitations, the biofilm will grow in thickness until the relative abundance of cytochromes in the oxidized state at the outer edge (*L*) is too low to act as electron acceptors for cells, and thus cannot support any additional biofilm growth. B) In addition, the generation of protons inside the biofilm due to anode respiration, and their transport out of the biofilm by diffusion, is expected to result in generation of the depicted pH gradient across the biofilm indicated by the arrow pointing toward the electrode surface. As the biofilm grows thicker, the pH drops further in the biofilm represented here by a shift in biofilm color from white to purple with decreasing distance from the anode surface (*z*). In the absence of any other nutritional limitations, the biofilm will grow in thickness until the rate of electron transport through the biofilm region closest to the anode surface is inhibited either by direct effects of low pH values on cytochromes, on the metabolism of cells in this low pH region, and/or on the availability of oxidized cytochromes distant from the electrode.

bacter biofilm anode,[27] a pH value known to decrease metabolic activity.[21] Such a pH gradient is expected to disproportionally affect metabolic activity of microbes closest to the biofilm/anode interface and is, therefore, expected to diminish the net contribution to current by additional cells as the biofilm grows thicker. The effect of a low pH value may be even more pronounced, creating a negative feedback loop that limits biofilm thickness, if the rate of electron transfer between adjacent cyctochromes and/or across the biofilm/anode interface itself is negatively affected by low pH values. Although little is known about the pH optima of cytochromes involved in these reactions, the distorted sigmoid-shaped current–voltage dependencies of *G. sulfurreducens* biofilm anodes recorded under conditions that exacerbate the effects of pH (e.g., Figure 6 in Torres et al.[20a]) are consistent with the hypothesis that as the pH value decreases, the rate of electron transfer across the biofilm/anode interface decreases (e.g., Figure 8 in Ref. [9b]). Decreasing the pH value has also been shown to shift the midpoint potential of cytochromes within the extracellular matrix positively by approximately 50 mV for each pH unit (close to the theoretical Nernstian value of 0.059 V per pH unit), which would reduce the driving force for transferring electrons to anodes.[21,28] Thus, while multiple experiments have shown that biofilm anodes are severely affected by the pH value,[20a,21,26b,c,27] more information related to both the physiological impact (effects on metabolic activity of cells), along with the biochemical and electrochemical effects (changes in cytochrome electron-transfer kinetics), is needed to elucidate the importance of proton export on electron transfer through biofilms to anodes. Independent of the exact mechanism, diffusive proton transport is predicted to result in less active microbes near the biofilm/anode interface that may limit current density and thus the extent of biofilm growth.

Oxidized cytochrome concentration gradient

Within a *Geobacter* biofilm, an oxidized extracellular cytochrome may both accept electrons directly from the microbe it is associated with and from neighboring reduced cytochromes originating from microbes residing farther from the anode surface (Scheme 1). This competition for use of cytochromes is expected to generate a concentration gradient of oxidized cytochromes (and due to mass balance, reduced cytochromes) across the biofilm (Figure 2). In this gradient, cells closer to the anode surface experience a higher concentration of oxidized cytochromes able to accept their electrons, whereas cells farther from the anode surface experience a lower local concentration of oxidized cytochromes able to accept their electrons. As a result, the activity of cells is expected to decrease with increasing distance from the anode surface, opposite the effect expected from the pH gradient described above. In the limiting case, the local concentration of oxidized cytochromes at the outer edge of the biofilm will be too low to support additional biofilm growth. Under this condition, modeling indicates that the biofilm is still predicted to exhibit an undistorted sigmoid-shaped catalytic current–voltage dependency, as has been observed experimentally in many laboratories.[3a,6a,9b,20b]

Three lines of experimental evidence indicate that such a concentration gradient occurs within a *Geobacter* biofilm anode. We (Glaven and Tender) recently obtained direct evidence for such a gradient, in which the electrochemical potential inside a *G. sulfurreducens* biofilm measured 10 μm from the anode surface is approximately −0.2 V versus SHE (Snider, et al., manuscript in preparation). At this potential, cytochromes are over 80% reduced, even though a highly favorable electron acceptor (an anode poised at +0.1 V vs. SHE) is only 10 μm away. Secondly, spectral evidence is provided by Liu and Bond,[8c] in which the proportion of reduced cytochromes within a respiring *Geobacter* biofilm anode increases with increasing biofilm thickness even though cells are again only microns away from an anode poised at a potential sufficiently positive to oxidize all *c*-type cytochromes. Lastly, transcriptional evidence obtained by slicing biofilms and comparing gene expression at the top versus near the electrode[29] revealed down-regulation of acetate oxidation and ribosomal genes, indicating respiration rates slowing with increased distance from the anode.

Role of nanowires

Proteinaceous filaments extending from the cell membrane into the extracellular environment have been observed for many electrode-reducing bacteria.[24b,30] Filamentous structures from two anode-respiring organisms, *G. sulfurreducens*[30,31] and *S. oneidensis*,[23–25,32] have been investigated in depth. Specifically, conductivity across the diameter of sheared *G. sulfurreducens* fibers[30] could be measured by means of conducting atomic force microscopy (AFM), which could not be detected in Type IV pili mutants. Conductivity could also be measured across the diameter and along the length of individual *S. oneidensis* fibers,[23,24b] but aside from mutants in the general Type II secretion pathway responsible for excreting a wide range of proteins to the *Shewanella* outer surface, no data is available on the identity or composition of *Shewanella* fibers. In the case of the lengthwise conductivity measurements, Polizzi et al.[25] calculated that this conductivity could arise from electron superexchange involving redox cofactors associated with the fiber rather than from metallic conductivity.[31,33] Although there are clear differences between these two organisms, the apparent conductivity of both *Geobacter* and *Shewanella* fibers has led to the general term of "nanowires" for these extracellular structures contributing to extracellular electron transport.

The *Geobacter* structures exhibiting this conductivity are believed to be primarily comprised of the Type IV pili structural protein PilA,[30] although other filamentous appendages are present in deletion mutants lacking PilA.[34] All *Geobacter* species, as well as close relatives, which are not capable of electron transfer to electrodes, possess a complete suite of Type IV pili genes typically studied for their ability to retract and pull cells closer to surfaces. The PilA protein of *Geobacter* is also significantly shorter than most commonly studied bacterial Type IV pilins, including those found in *Shewanella*, which has

also been interpreted as evidence that these structures perform additional roles.[35]

Deletion of the entire *pilA* gene significantly inhibits attachment of cells to each other[36] and affects formation of biofilms on glass slides even when electron transfer is not a consideration, further indicating that *Geobacter* pili contribute to adhesion and attachment. PilA mutants also attach poorly to anodes and secrete fewer essential cytochromes such as OmcZ,[35,37] offering a possible explanation for why PilA is required for maximum catalytic current generation.[6c,33] Deletion of the same pilin, however, does not affect electron transfer reactions in which a cathode serves as the electron donor.[38] Further complicating elucidation of the specific role of pili in electron-transport of actively respiring anode grown *Geobacter* biofilms is the fact that the *c*-type cytochrome OmcS has been shown to localize along pili during metal reduction[12c,35] and during interspecies electron transfer between cell aggregates undergoing syntrophic respiration.[39] Moreover, it has been shown that *pilA* contains two different translational start sites, resulting in a long and short isoform of the protein.[40] Expression of only the long form of PilA results in cells unable to produce pili, but rescues much of the cytochrome secretion defects and restores 75% of catalytic current production to *G. sulfurreducens pilA* mutants when grown on anodes.[40]

Despite the confounding effects of pili on attachment, adhesion, retraction, and anchoring of cytochromes, which could all lead to shorter electron transfer distances and increased biofilm growth, a decrease in current generation by *Geobacter pilA* mutants has been attributed to inherent conductive properties of these structures.[31] Temperature-dependency measurements of dried crude pili extracts, and on mutant biofilms that form uniquely tough layers able to be peeled from electrodes and placed across the gap of a split-gold electrode, suggest that direct conduction of electrons through these preparations occurs by a metallic-like process.[31] It is not clear at this time how this data relates to the mechanism controlling the electron transport through an intact, actively respiring wild-type biofilm, and the interpretation of these experiments has been debated elsewhere.[33] However, the hypothesis of metallic electron conduction by pili does not provide explanations for the finite thickness of biofilms, their diffusive redox characteristics, their conductive properties measured while active respiring, or the finding that cytochromes in actively respiring *Geobacter* biofilms are partially reduced.

Conclusions

We have described an evolving scheme of biofilm anode respiration that is ultimately controlled by superexchange among extracellular cytochromes. Although it is likely that other components are also involved, this model is able to account for many different types of experimental evidence reported for actively respiring *Geobacter* biofilm anodes and places specific physical limits on both the thickness and current densities obtainable by anode-reducing bacteria.

According to this concept, local pH values in the biofilm are expected to decrease with increasing biofilm thickness due to the finite rate of outward diffusion of protons generated inside the biofilm by respiration. Low pH values may inhibit the rate cytochromes operate at in superexchange directly by altering redox potentials as well as indirectly by slowing the metabolic activity of the cells they are associated with. In addition, the finite rate of electron exchange between cytochromes is expected to cause the local concentration of oxidized cytochromes in the biofilm to decrease with increasing distance from the anode surface. This phenomenon results from the dual use of oxidized cytochromes to both accept electrons emerging from local cells, and to accept electrons from cells residing farther from the anode surface.

Based on these outcomes, we propose that a biofilm anode will grow in thickness until either the pH value near the anode surface becomes sufficiently low that it inhibits cytochrome function of the innermost cells, thereby limiting the ability of all cells in the biofilm to transfer electrons to the anode, or until the local concentration of oxidized cytochromes experienced by the outermost cells becomes too low to support additional growth. As experiments have directly detected both of these inhibitory effects within living *G. sulfurreducens* biofilm anodes, they likely act synergistically to limit biofilm thickness and thus catalytic activity.

Key challenges that remain are experimental visualization of a network of cytochromes throughout the biofilm and the extracellular structures that ensure the close interactions required for rapid electron transfer. Extrapolating from an entirely different organism, to explain the conductivity measured along a single isolated *Shewanella* fiber,[24a] a superexchange-based mechanism would require cofactor spacing on the order of 1 nm, consistent with intra-heme spacing in multiheme *c*-type cytochromes.[7,23,25] With the identification of at least one multiheme cytochrome that aligns along *Geobacter* pili (OmcS), multiple cytochromes found associated with cell surfaces (e.g., OmcB and OmcE), cytochromes found associated with extracellular materials (OmcZ), and more than 50 genes encoding for additional multiheme cytochromes,[41] numerous candidates for interacting self-exchange pathways exist along cell surfaces and extracellular structures.

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