A long way to the electrode: how do *Geobacter* cells transport their electrons?

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Abstract

The mechanism of electron transport in *Geobacter sulfurreducens* biofilms is a topic under intense study and debate. Although some proteins were found to be essential for current production, the specific role that each one plays in electron transport to the electrode remains to be elucidated and a consensus on the mechanism of electron transport has not been reached. In the present paper, to understand the state of the art in the topic, electron transport from inside of the cell to the electrode in *Geobacter sulfurreducens* biofilms is analysed, reviewing genetic studies, biofilm conductivity assays and electrochemical and spectro-electrochemical experiments. Furthermore, crucial data still required to achieve a deeper understanding are highlighted.

Introduction

The mechanism of extracellular electron transport in *Geobacter sulfurreducens* biofilms is still a matter of controversy and has raised fruitful discussion in recent work [1,2]. Although it is widely accepted that both pili and outer membrane *c*-type cytochromes are important for high current production [3–6], the specific role that each one plays in the electron transport to the electrode remains to be elucidated [5].

Two different mechanisms involving cytochromes and pili are being proposed for the transport of electrons from cells to the electrode: the metallic-like conduction model and the electron-hopping (superexchange) model (Figure 1). The former states that electron transport occurs through conductive proteinaceous filaments (modified pili, called 'nanowires') extending from the cell membrane into the extracellular environment [6–9], with outer-membrane cytochromes gating the electron transference to the electrode [4,6,10]. Pili were found on the extracellular space of cells using soluble and solid electron acceptors [7,11,12] and were proposed to confer conductive [9] and structural properties [13] to *Geobacter* biofilms. On the other hand, the electron-hopping (superexchange) model considers that electrons are transferred from the cells to the electrode through a sequence of redox reactions between cytochromes located in the extracellular matrix [14] that connect each biofilm cell with the electrode. In this model, pili play a structural role, providing the support to order cytochromes in the biofilm matrix and thus improving the electron-transfer process [5,14–16].

Although several outer-membrane cytochromes were found to be important for current generation, the only one described to date as indispensable for both sustaining current production at a high rate and forming well-developed conductive biofilms is the outer membrane *c*-type cytochrome Z (OmcZ) [17–19], an octahaem cytochrome with a wide potential range [20] located in the extracellular space [21].

The two proposed mechanisms have much different structural requirements and predict distinct responses to physical/chemical perturbations [1]. The metallic-like conduction model states that electrons are delocalized, forming a 'sea of electrons' moving without the need for thermal activation [1]. Conductivity is hypothetically conferred by the π-stacking of aromatic residues in pilin, the protein conforming pili [10], which may allow electron delocalization. If confirmed, metallic-like conduction will represent a paradigm shift in biology, since in all known mechanisms for biological electron transfer, electrons move from molecule to molecule via tunnelling or hopping over short distances [4]. The superexchange model [14,15], on the other hand, is based on mechanisms for electron transport in redox hydrogels [22] and states that electrons are transported through sequential redox reactions between aligned redox cofactors [5,14]. According to this model, the driving force for the transport of electrons is the potential gradient produced by the simultaneous reduction of redox mediators by cells and their oxidation at the anode surface [5,14].

A lot of work has been carried out in order to determine which of these mechanisms prevails in the electron-transport process, but the evidence is not yet conclusive; whereas conductivity measurements and the metallic-like dependence of this conductivity with pH and temperature [9] supports the proposal of the pili-based model, cyclic voltammetry [17,23–25], spectro-electrochemical evidence [24] and the presence of different cytochromes found on the external matrix [20,21,24] (either on pili [12] or on polysaccharides in the matrix [26]), represents increasing evidence supporting the electron-hopping hypothesis. Analyses of mutant strains are also insufficient to solve the controversy, mainly due to uncertainties in the interpretation of phenotypes, as deletion

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Abbreviations used: OCP, open circuit potential.
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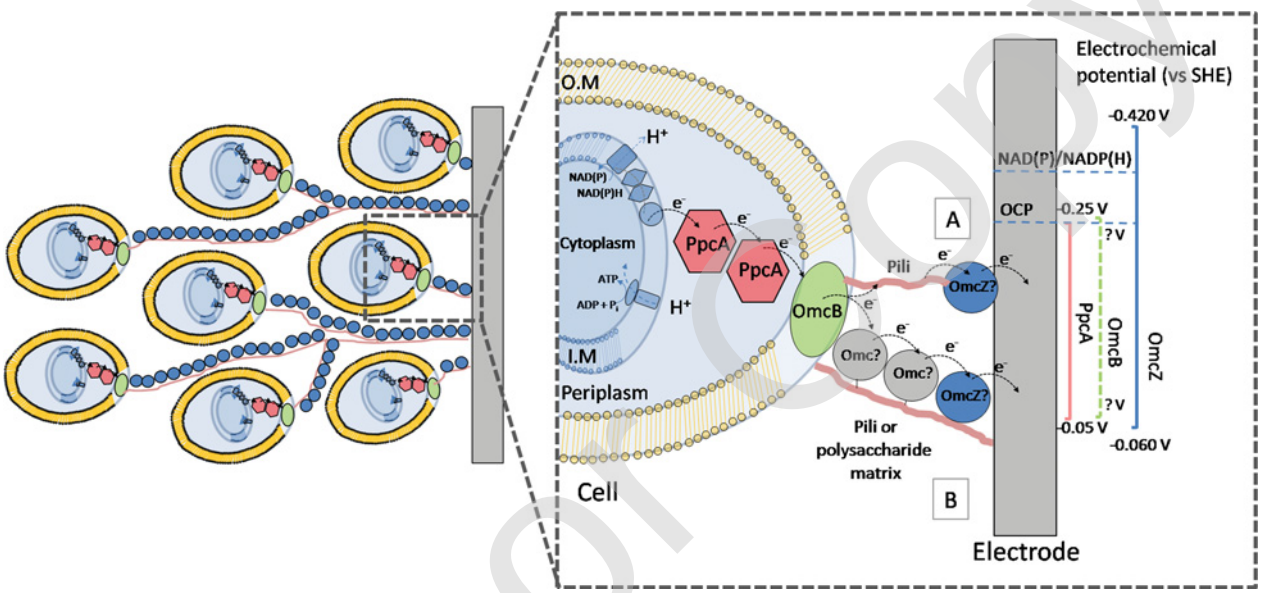
Figure 1 | Schematic representation of the electron-transport process from the inner membrane to the electrode in *G. sulfurreducens* biofilms

Beyond the respiratory chain, electrons are transferred to PpcA in the periplasm, which is proposed to interact with the OmcB in the outer membrane. For extracellular electron transport, two mechanisms, explained in detail in the text, are proposed: the metallic-like conduction model (A) and the electron superexchange model (B). Electrochemical potential windows of the involved proteins, the measured open circuit potential and the redox potential of the NAD(P)/NAD(P)H couple are shown on the right as a reference (not to scale). I.M, inner membrane; O.M, outer membrane; SHE, standard hydrogen electrode.



of certain cytochromes leads to changes in expression of other cytochromes and proteins, often replacing the function of the deleted ones [17,19,27–29]. Meanwhile, important structural information is still lacking [2,30]; although the primary structure of the *G. sulfurreducens* pili is known [31], its tertiary structure under physiological conditions has not yet been determined [2], mainly owing to difficulties in crystallization of the protein [9].

Unfortunately, on the inner side of the cell (transport to the external membrane through the cell periplasm), the picture is not much clearer. Proteins that play an important role in intracellular electron transport have been identified and characterized [12,24,32–34], but their arrangement in the intracellular network and the process-controlling steps are not yet clearly defined.

In the following sections, electron transport from inside the cell to the electrode is analysed, reviewing results of studies with genetic mutants, conductivity assays and electrochemical and spectro-electrochemical experiments. Hopefully, this work will help us to understand the state of the art of the electron-transport mechanism and to recognize which crucial data are still required to achieve a deeper understanding of the topic.

Evidence from genetic studies

Analysis of the current produced by *G. sulfurreducens* mutant strains lacking pili or specific cytochromes and the regulation of genes in these mutants reveals interesting features that may help to identify molecules involved in electron transport from the respiratory chain to the electrode. Nevertheless, it should be kept in mind that deletion of certain genes encoding cytochromes typically leads to changes in the expression pattern of other cytochromes, thus conditioning the interpretation of results [17,19,27–29]. Indeed, conditions used for culture maintenance, such as the number of successive transfers on soluble electron acceptors, have been identified as factors affecting the attachment and final current density of *G. sulfurreducens* biofilms (D.R. Bond, personal communication). In spite of these considerations, some general conclusions can be taken from genetic analysis.

Type IV pili, as well as OmcB [27], OmcE and OmcZ [20], were found to be more abundant and genetically up-regulated in current-harvesting biofilms compared with biofilms grown on fumarate as the electron acceptor [19], suggesting a role for these proteins in extracellular electron transport. More specific evidence indicates that mutants lacking *pilA* (the gene encoding pili protein) or *omcZ* not only produce less current [17,19], but also fail to adapt to current production even after incubations of more than 50 days [19]. These results indicate that both PilA and OmcZ are essential for efficient electron transport to the electrode and, more importantly, that they are needed simultaneously.

In addition to current production, electrochemical data from mutants may provide useful information. Voltammetry

results obtained from the Δ*omcZ* mutant appear to be crucial for the discussion of the electron-transport mechanism, as, under oxidizing conditions, current shows a pronounced linear dependence with the applied potential [17]. This behaviour has been explained considering the existence of a resistive pathway for cells far from the electrode [17], which, besides highlighting the significant role of OmcZ in electron transport through the biofilm, may be direct evidence of pili playing a conductive role when OmcZ is not present.

Biofilms of the Δ*pilA* mutant (which, interestingly, also has a lower amount of external cytochromes [35,35a]) produce a lower current per electrode unit area compared with biofilms of the wild-type strain [8,17]. Nevertheless, current density production of the mutant was the same [8] or even higher [17] when normalized by the biofilm thickness. Voltammetry of biofilms of this mutant revealed that they are still conductive, rapidly exchanging electrons with the electrode, and limited for current production by the cellular rate of acetate oxidation [17], suggesting that pili may not play a central role in electron transport, at least for cells located near the electrode [8]. On the basis of this evidence, the superexchange model proposes that the role of pili is mainly structural, which is supported by the participation of pili in cell–cell aggregation [13] and in optimal biofilm development. In this context, pili contribution to efficient electron transport would be the promotion of highly ordered biofilms [24], by providing a structural network for cytochromes arrangement. This might reduce the potential decay in the external matrix, allowing the respiration of cells at large distances from the electrode and, consequently, the development of thicker biofilms [5,36].

Polysaccharides located outside the cells are also involved in surface attachment and cell–cell interactions [26,37]. It was reported recently that *Geobacter* biofilms produce an exopolysaccharide matrix rich in cytochromes that may interconnect cells for electrode respiration [26]. Notably, in spite of bearing pili with attached cytochromes and retaining the ability to transfer electrons to external soluble acceptors [26], the mutant lacking the gene encoding exopolysaccharide matrix production fails to develop electrogenic biofilms on electrodes [26]. These results strongly suggest a structuring role for the external polysaccharide matrix similar to that proposed for the pili, favouring both biofilm growth and electron transport [26].

Evidence from biofilm conductivity measurements

In favour of the metallic-like conduction model, it was demonstrated that biofilms with higher pili and lower cytochrome content exhibit higher conductivity [9]. In return, it was argued that the higher pili content could improve the spatial organization of cytochromes, consequently improving the electron transfer in between them, thus giving an alternative explanation to the increased conductivity [2].

An interesting aspect of the conductivity is its dependence on temperature and pH. Whereas the activity of outer membrane cytochromes in physiological conditions decreases with decreasing temperature [38], conductivity of pili and biofilms *ex situ* preparations increases as in a disordered metal, giving support to the metallic-like conduction mechanism [9]. Conductivity of these preparations also increases with decreasing pH [9], which is consistent with protons acting as dopants to the pili structure and functioning as a source of carriers, as expected for a metallic-like conduction mechanism [9]. This last piece of evidence was accounted by the superexchange model by considering that protons act as charge-compensating ions that decrease the reorganizational energy of redox cofactors, thus accelerating the electron-transport rate [2].

Conductivity measurements of mutant strains are also quite controversial. Biofilms lacking OmcB, OmcE and OmcS/OmcT showed higher conductivity than the wild-type DL-1 strain [9], indicating that these cytochromes are not essential for the production of conductive biofilms and implicitly suggesting that conduction is made by pili. Unfortunately, the effect of deleting OmcZ, the only outer-surface cytochrome found to be necessary for high current density production [19], on conductivity remains to be determined.

Besides the above-mentioned studies, additional lines of evidence need to be considered for identifying the most relevant mechanisms behind biofilm conductivity. Of particular importance, structural information such as the verification of π-stacking of aromatic residues present in pili *in vivo* or *in situ*, to give support to the metallic-like conduction model, or the direct measurement of intermolecular haem spacing, for demonstrating the feasibility of the superexchange model, is needed. In this regard, calculations made on a multihaem cytochrome from *Shewanella oneidensis* [39] demonstrated that distances between haem groups in this protein may support conductivity in the range measured on biofilms [40]. Unfortunately, this study does not provide any information about the structural support for conduction between molecules, which has only been determined for adjacent molecules of the periplasmic cytochrome PpcA [41]. Clearly, similar calculations are needed for cytochromes found in the external matrix of *G. sulfurreducens* biofilms.

Voltammetry, OCP (open circuit potential) and spectro-electrochemical data

In several studies, peaks in the voltammetric profile of biofilms in non-turnover conditions (no electron donor) scale in magnitude with the square root of scan rate [17,23–25,42]. This behaviour is usual for systems in which the interfacial heterogeneous transfer is fast compared with the rate at which electrons are transported through redox species within a film [24,36] and indicates that a diffusive process governs the electron transport [2,5,43]. In the particular case of biofilms, the effect can be caused either by slow migration of charge-compensating ions [17] or by the limit imposed to charge transport by the availability of oxidized mediators [5,14,25], as proposed by the superexchange model

[14,36]. Spectro-electrochemical data give support to the occurrence of diffusional kinetics, revealing that spectral changes with voltage (caused by the variation of the redox state of cytochromes) show an increasing lag at scan rates exceeding 1 $mV \cdot s^{-1}$ [24]. Nevertheless, it has to be noted that pili are thought to transport electrons from cytochromes in the cell interior to those at the biofilm–electrode interface [6] (Figure 1), a configuration that may also justify the observed diffusive behaviour.

Spatial localization of proteins limiting (i.e. gating) electron flux from inside the cell to the electrode is crucial for a proper electrochemical analysis. In the light of very recent information showing that interfacial and matrix redox processes are orders of magnitude faster than cell-associated ones [15], controlling proteins might be located at the cell level, either on the external membrane or in the periplasm.

OmcB was proposed to gate electron transport to the cell exterior, playing a role similar to that recognized for decahaem outer membrane cytochrome MtrF in *Shewanella* (for which the structure has recently been resolved [44]), owing to its location at the cell outer membrane [27]. In fact, voltammetry of the Δ*omcB* mutant shows a shift in the half-wave potential to positive values (+0.09 V) [17], indicating that cells have to use an alternative high-potential pathway that controls the flow of electrons when this cytochrome is not present.

PpcA, a trihaem cytochrome located at the periplasm of cells [32], has also been proposed to 'gate' electron transport to the electrode [24]. It has been cleverly noted that this cytochrome has the highest redox potential in a putative pathway including OmcB, OmcZ and OmcS, which hypothetically warrants an oxidized external network always able to act as an electron acceptor for the periplasmic pool [24]. The proposal is supported further by the observed correlation between the redox potential window of PpcA (−250 to −50mV [45–47]) and the current produced by biofilms at each potential [24]. Unfortunately, the redox potential window of OmcB is still unknown and only electrochemical data obtained from the isolated molecule are available, showing a half-wave potential of approximately −190 mV [48].

The evolution of the OCP of biofilm electrodes reveals some interesting points in this regard. When polarization applied to a biofilm electrode is interrupted, electrons leaving the cells accumulate at the interface, progressively driving the OCP to more negative values [16]. The lower value of the OCP is thus considered a direct indication of the limit potential at which electrons can be released by the cells [16]. Notably, the OCP of wild-type cells [15–16,49] is close to the lower potential end of PpcA (−250 mV), indicating that not only OmcB, but also all molecules participating in the electron-transport pathway, must have a lower potential bound at least as negative as this value (Figure 1).

An important aspect of this discussion is that the redox potential window of involved proteins is not the only factor determining which protein limits the electron-transport process. The maximum amount of charge that each protein can transfer per unit of time (which ultimately limits the current produced by the biofilm) might also be determined by the abundance of the given protein and the effectiveness of its interaction with surrounding proteins. In this sense, the relative abundance of PpcA and OmcB, or other membrane electron conduits, remain to be explored.

Conclusions

Evidence collected to date is insufficiently conclusive on either the exclusive role of cytochromes or pili in the extracellular electron transfer, or the protein(s) that gate the electron flux to the electrode.

From the data available, external cytochromes (in particular OmcZ) appear to be fundamental for electron conduction, but a role for pili cannot be discarded. Direct evidence for the presence of pili in electrode-respiring biofilms and its association or not with cytochromes in the biofilm matrix are mandatory. Additional information on the structure of pili in their functional form is also needed to support the metallic-like conduction, and demonstrating the occurrence of the required intermolecular spacing between cytochromes for enabling electron hopping is crucial to support the superexchange model.

Finally, in order to determine which protein is gating the electron flux to the electrode, some important parameters such as the relative abundance of each protein involved in the electron-transport pathway have to be explored.

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