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Stepping stones in the electron transport from cells to electrodes in *Geobacter sulfurreducens* biofilms†

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Pablo Sebastián Bonanni,* Diego Massazza and Juan Pablo Busalmen

Geobacter sulfurreducens bacteria grow on biofilms and have the particular ability of using polarized electrodes as the final electron acceptor of their respiratory chain. In these biofilms, electrons are transported through distances of more than 50 μm before reaching the electrode. The way in which electrons are transported across the biofilm matrix through such large distances remains under intense discussion. None of the two mechanisms proposed for explaining the process, electron hopping through outer membrane cytochromes and metallic like conduction through conductive PilA filaments, can account for all the experimental evidence collected so far. Aiming at providing new elements for understanding the basis for electron transport, in this perspective article we present a modelled structure of *Geobacter* pilus. Its analysis in combination with already existing experimental evidence gives support to the proposal of the ''stepping stone'' mechanism, in which the combined action of pili and cytochromes allows long range electron transport through the biofilm.

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Bacteria obtain energy by transferring electrons from an organic electron donor to an electron acceptor and using the potential difference between these compounds to produce ATP. *Geobacter sulfurreducens* bacteria have the particular ability of growing on biofilms that use a polarized electrode as the electron acceptor[1,2] generating an electric current. This ability has opened a broad window of practical applications for these bacteria, including treatment of organic wastes with electric current production in microbial fuel cells,[3–5] microbial electrolysis cells for synthesis of high value products,[6,7] and microbial desalination cells,[8] among others.

The mechanism of electron transport from the cells to the electrode in *G. sulfurreducens* biofilms has raised intense discussion in recent works.[9–14] When respiring electrodes, *G. sulfurreducens* bacteria grow forming biofilms with an active thickness of about 70–80 μm.[15–18] These bacteria have developed an efficient exocellular electron transport mechanism that allows them to respire a polarized electrode located hundreds of cell layers below. Establishing a connection with an extracellular electron acceptor located at such large distances represents a challenge not faced by microorganisms that reduce soluble electron acceptors inside the cell.[19]

Laboratorio de Bioelectroquímica, Area de electroquímica y corrosión, INTEMA (Conicet), Mar del plata, Argentina. E-mail: sebastian.bonanni@fi.mdp.edu.ar; Tel: +54 223 816600 (248)

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A lot of experimental work, following physiological, biochemical and electrochemical approaches, has been carried out in order to determine how the electrons are transported from the cells to the electrode through such large distances, but a consensus has not been reached yet.

In contrast to other bacteria that also respire insoluble compounds, *Geobacter* does not excrete electron shuttles[20] and requires direct contact with the electron-accepting surface.[2] *Geobacter* cells are connected to the electrode by an extracellular matrix composed of pilA protein,[21] polysaccharides[22] and several c-type cytochromes.[22–25] All these proteins were found to be necessary for an efficient electron transport from the cells to the electrode, but their specific role in the process is not yet clear.

PilA protein forms a filament composed of predominantly helical subunits.[26] It has conductive properties,[21,27,28] proposed to be conferred on interchain stacking between aromatic residues,[28,29] and was found to be necessary for cell attachment to surfaces,[19,30] agglutination[19] and the development of thick biofilms when the electrode was the electron acceptor.[19,21,31] The pilA protein has two isoforms with different specific functions. The short isoform, bounded in the intracellular fraction, influences the secretion of several outer membrane c-type cytochromes to the extracellular space and stabilizes the long isoform.[30] The long isoform is required for secretion of PilA outside of the cell and is essential for biofilm formation on certain surfaces.[30] When the gene encoding for both isoforms is suppressed, yielding the ΔpilA mutant, the respiration of iron oxides[21] and the production of

current in *G. sulfurreducens* biofilms[28,31,32] are greatly inhibited. It has to be noted that the ΔpilA mutant, besides lacking PilA protein in the cell exterior,[30,32] shows a mislocalization of outer membrane cytochromes.[30,33] Interestingly, a mutant with unmodified distribution of outer membrane cytochromes and no PilA in the cell exterior yields thick biofilms that produce current densities lower but on the order of that of wild-type cells.[30] This indicates that PilA, while important, is not an absolute requirement for biofilm growth and current production, and also highlights the importance of an appropriate distribution of outer membrane cytochromes in the biofilm matrix for an efficient electron transport through the biofilm.[30]

The binding of cytochromes in the extracellular matrix may be a characteristic of microorganisms respiring insoluble compounds,[22] as cytochromes have also been detected beyond the cell[34] and specifically in the matrix[35] of *Shewanella oneidensis* MR-1 biofilms. In fact, in this species cytochromes were found to be necessary for the conduction of electrons along pili filaments.[36]

In *G. sulfurreducens*, outer membrane cytochromes were found to gate the electron transfer to the electrode[37–39] and to be bonded to pili and the polysaccharide matrix.[12,40] OmcZ is an octaheme cytochrome with a wide potential range[23] that was found to be highly expressed in high current density biofilms.[41] Although preliminary studies suggested that it is localized on the biofilm–electrode interface,[24] the gene encoding for its production was found to be expressed in cells through all the biofilms,[16] and it is thought to be anchored to the extracellular polysaccharide matrix.[22] This cytochrome is also essential for the electron transport from cells to the electrode and the production of high currents.[30,31,41] Interestingly, when the gene encoding for OmcZ is deleted, the production of current is substantially reduced, despite the cells possess pili.[41] What is more, cells lacking OmcZ form thin biofilms that produce very low current density.[31] Notably, the conductance of biofilms with lower abundance of OmcZ was higher than that of the wild type,[28] contrasting with the much higher resistance for electron transport measured in biofilms lacking OmcZ.[31] Unfortunately, the conductivity of a mutant lacking OmcZ was not reported yet.[14]

Two different mechanisms for explaining the electron transport

Based on the above-mentioned evidence, two different mechanisms were proposed to explain the electron transport in *Geobacter* biofilms; the conduction along pili[12,21,28,32,42] and the electron hopping through outer membrane cytochromes.[9,11,13] As it will be noted, neither can explain all the experimental evidence collected so far.

The conduction along pili states that electron transport occurs through the conductive pili filaments extending from the cell membrane into the extracellular environment.[21,28,32,42] A high metallic-like conductivity was measured on *Geobacter* biofilms.[28] Interestingly, this conductivity does not depend on the abundance of cytochromes in the biofilm.[12] The mechanism of conduction along pili proposes that the transport of electrons in the biofilm matrix is made solely through this filament. In this case, outer-membrane cytochromes serve only as intermediates between pili and the electrode, gating the heterogeneous electron transfer,[42] while those cytochromes bounded to pili are supposed to serve as temporary storage sites[43–45] in the absence of an electron acceptor.[42]

In contrast to this metallic-like conductivity, electrochemical analyses show diffusive behaviour of the electron transport through *Geobacter* biofilms.[46,47] In this context, the transport of electrons through the biofilm matrix is proposed to proceed *via* hopping through proteins in the biofilm network,[9,13] resembling the electron transport in abiotic redox polymers.[48,49] This implies a sequence of redox reactions between cytochromes located in the extracellular matrix that connect each biofilm cell with the electrode[9,13] and pili serving only as a structural support,[19] ordering cytochromes in the matrix and consequently improving the electron-transfer process.[9,13] Interestingly, a mutant lacking *pilA* growing with soluble fumarate as the electron acceptor can form biofilms as thick as the wild type.[41] This suggests that, in the absence of PilA, cells may also rely on the structural support of one or more of the non-PilA filaments they secrete[50] possibly explaining why biofilms lacking PilA protein in the matrix can produce current densities comparable to those of the wild-type.[30]

For biofilms of up to 60 μm the increase in current was found to be proportional to biomass accumulation[32] and metabolic activity was demonstrated to be high even in cells located farther from the electrode.[16,17] This evidence led to the suggestion that an efficient electron transport through such large distances could only be possible thanks to the high conductivity of pili filaments.[32,42,51] Nevertheless, recent modelling work predicted that electron transport by hopping between redox proteins can support metabolic activity at distances of 60 μm from the electrode.[18] According to the model, limitations due to electron hopping arise in thicker biofilms, as the existence of a redox gradient lowers the cell respiration rate in the upper layers of the biofilm. Such a gradient has been experimentally demonstrated in very recent works[15,52] and is considered an indication of electron transport through the biofilm being not as efficient as expected when considering the high conductivity measured on biofilms.[15] In the same line, UV-visible spectroscopic results have also shown an accumulation of reduced compounds in the biofilm.[46] Also, it was shown that current becomes independent of biofilm thickness for biofilms thicker than 60–70 μm,[15] further supporting the existence of differences between the respiration rate of cells at different distances from the electrode.[18] Modelling work has shown that all this experimental evidence can be explained by the electron hopping mechanism considering the diffusivity of electrons measured using cyclic voltammetry.[18] On the other hand, those results could not be explained when considering the high conductivities measured on the biofilms and proposed to be conferred by pili.[18]

The high conductivity of biofilms might thus not be representative of the pathway used by cells to transport electrons through the external matrix.[18] This would explain controversial evidence as the lack of correlation between biofilm conductivity and OmcZ abundance in the matrix,[12] despite this cytochrome

was found to be essential for current production,[41,53] and the mismatch between the variation of biofilm conductivity with applied potential[28] and recent experimental results.[52]

Diffusivity of electrons measured using cyclic voltammetry besides representing a much lower conductivity than that directly measured on biofilms is, from our point of view, a more appropriate parameter to explain the electron transport process. The lower equivalent conductivity does not exclude pili from the electron transport mechanism, but suggests that the whole process may not be as efficient as a metallic-like conduction and may proceed through hopping steps.

Anyway, it has to be noted that some experimental evidence is not in agreement with the electrons being transported solely by hopping through outer membrane cytochromes.[12] For instance, the spacing between cytochromes associated with pili was shown to be too large to sustain electron hopping between outer membrane cytochromes.[12,40] Biological electron transfer reactions often occur between separated redox partners, but the electron transfer reaction is feasible only when there is an overlap between donor and acceptor orbitals that allows electron tunnelling or super-exchange between them.[54] While the practical upper limit for reasonably fast transfer through proteins was set between 14[55] and 20[56] Angstroms (Å),[54] AFM microscopy shows a spacing between globules on the pili one order of magnitude higher than that distance.[12]

X-ray diffraction patterns of purified pili suggest π overlap and charge delocalization, indicating that the electrons might be transported along the protein by resonance of its aromatic residues supposed to be separated by approximately 3.5 Å.[28] Although the crystallographic structure of geopilin remains unresolved, aromatic residues were shown to be essential for pili conductivity[29] and bio-informatic simulations suggest that charge transfer between pilin subunits may be possible.[26] Unfortunately, no information regarding the distance between those subunits in the pilin oligomer is available.

Modelled pilus structure

In order to gain preliminary insight into the structure of the pilus filament of *G. sulfurreducens* and the possible binding sites with cytochromes, we built a hypothetical assembly by using the structure of the *Neisseria gonorrhoeae* GC pilus (PDB entry 2HIL) as a template. The model allows assessing the distribution of aromatic amino acids within the oligomeric structure and, thus, the prediction of the most likely pathway for electron transport through the filament. The modelled protein is available in pdb format in the ESI.†

Type IV pili are grouped on the basis of characteristic features on the amino acid sequence of pilins, such as a *N*-methylated N-terminus, a conserved hydrophobic N-terminal residue and a carboxy-terminal disulphide bond. These pilins are grouped into type IVa and IVb subclasses on the basis of amino acid sequence and length. Type IVa pilins have leader sequences of 5–6 amino acids and a mature sequence of ~150 amino acids.[57] Besides their sequences, structure of type IVa pilins is also conserved. They are organized as an α-helix across the 53 amino acids of the N-terminal (α1 domain) and a globular domain containing an anti-parallel β-sheet region and two conserved cysteines, forming a disulfide bond between the β-sheet and the C-terminal segment of the helix.[58] While most pilins have an average length of 150 amino acids, *G. sulfurreducens* pilin is only 61 amino acids long which, according to phylogenetic analyses, seems to be a general feature within *Geobacteraceae*.[21] The architecture of this shorter protein still contains the α1 domain of the type IVa pili but the globular head has been replaced by a small random-coiled segment at the C-terminal end (Fig. 1A). This is consistent with a divergent amino acid sequence that could be related to the peptide's specialized function in electron transfer.[26] All mature type IV pilin proteins studied to date have been assembled into filaments,[30] but information about the structure of these oligomers is still limited.

The impact of the deletion of the polar head in geopilin on the stabilization of the putative oligomeric structure is unknown. However, it is interesting to note that the prediction of the secondary structure of the hypothetical protein of unknown function GSU1497, located adjacent to the gene that encodes for the structural subunit of the pilin protein (pilA: GSU1496),[41] resembles the globular head of type IVa complete pilins, giving rise to the speculation that GSU1496 and GSU1497 together would make a complete protein complex comparable to the PilA of other bacteria in both, size and secondary structure.[59]

To our knowledge, the only report describing a pilin oligomer is the one by Craig *et al.* (2006),[60] who presented a structural prediction of the *Neisseria gonorrhoeae* GC pilus structure (PDB entry 2HIL) based on crystallographic and electron microscopy information. Considering the above information, the modelled *G. sulfurreducens* pilus filament structure was generated by superimposing a single GSU1496 pili subunit[26] onto each subunit of the *N. gonorrhoeae* GC pilus structure, using the *N. gonorrhoeae* GC pili conserved core (*i.e.* lacking the αβ-loop and the D-region). The fit between the N-terminal helices was optimized using the model building program in COOT. Although the proposed model is mechanistic, based on structural overlapping of different proteins, it nevertheless might stimulate the analysis of structural constraints for mechanisms of electron transfer in the biofilm matrix.

The modelled filament (Fig. 1B) has an outer diameter of about 50 Å that agrees with the diameter of *Geobacter* pili measured using scanning tunnelling microscopy.[27] As in *N. gonorrhoeae* pilus,[60] the filament shows a narrow central channel that expands along the filament and may account for the flexibility of the structure (Fig. 1C). Monomers are tightly packed and the aromatic residues appear to be helically distributed along the filament (Fig. 1B).

Recent predictions suggest that resonance is enhanced in the middle of the α-helix monomer, thus favouring electron transfer between the aromatic residues.[26] In agreement with this, in our modelled structure the smallest distances between aromatic residues in the filament are found in the middle of one single monomer (Fig. 1D), while some aromatic amino

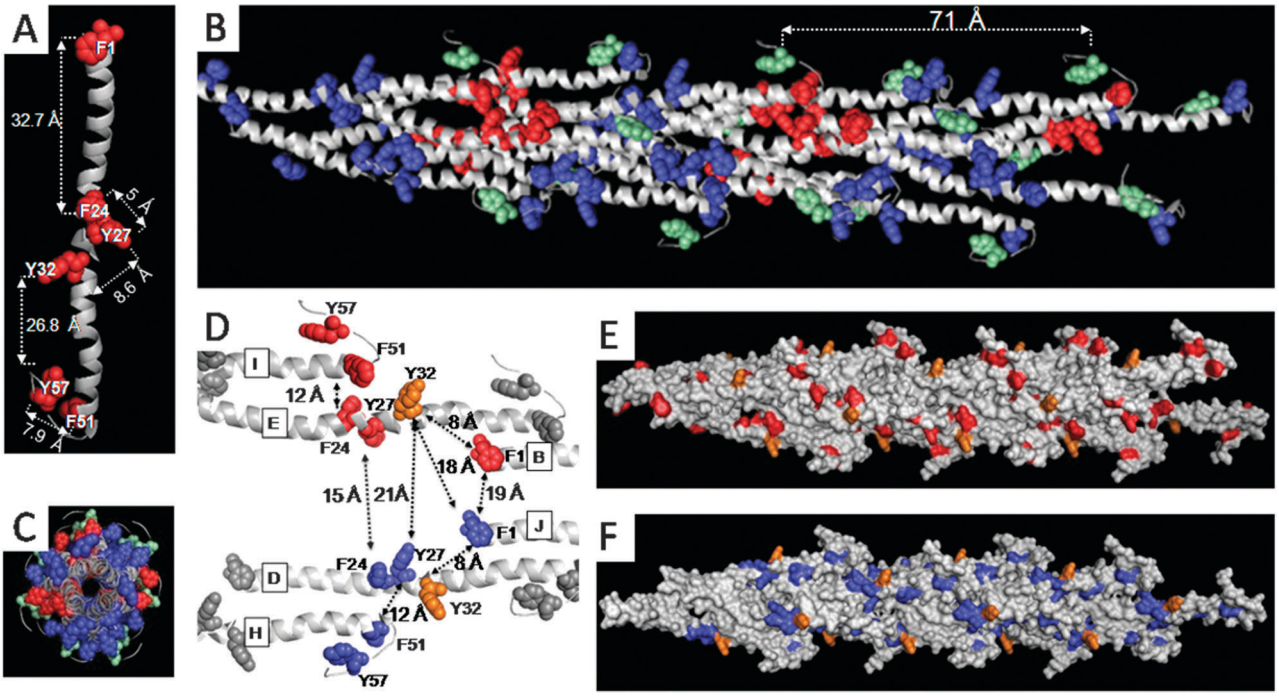

Fig. 1 (A) Structure of the GS pilin, modelled based on the structure of the PAK pilin.[26] The hydrophobic residues within the monomer are marked in red. The distances between Cα–Cα are indicated with dashed lines. (B) The GS pilus protein complex viewed along its longitudinal axis. The hydrophobic residues within the α-helices are shown in red or blue, while hydrophobic residues located at random coiled structure are shown in light green. The distance between Cα–Cα of hydrophobic residues located at the same longitudinal is denoted with a dashed line. (C) Cross section image of the GS pilus structure. Hydrophobic residues are colored as in B panel. (D) Distances between aromatic residues in the modelled filament. (E) Surface topography of the modelled pilus. Tyrosine 32 is colored in orange and all the other aromatic aminoacids are colored in red. (F) Surface topography of the modelled pilus showing the predicted heme binding sites in blue with tyrosine 32 colored in orange. The images were obtained using PYMOL software.

acids are packed in groups showing periodic distribution along the oligomer (Fig. 1B). Interestingly, the predicted distances between aromatic residues of amino acids within each group are very small, possibly allowing π orbital stacking and fast electron transfer rates between them.

The surface topography of the modelled pilus evidences that some of the groups of aromatic residues are exposed to the surface (Fig. 1E). In spite of being distributed all over the molecule, a periodic distribution of the aminoacids is found along any longitudinal transect of the filament. Interestingly, the longitudinal distance between surface exposed amino acids in the modelled structure agrees with the separation of high conductivity spots on pili determined using STM.[27]

It is important to note, on the other hand, that the predicted separation between groups of aromatic residues along the pilus is in the order of 15–21 Å (Fig. 1D). Such large distances not only impede orbital stacking and electron delocalization but also hamper an efficient electron hopping between aminoacids.[54,61] In this context, the electron transport solely through pili would not be as efficient as required for the transport of electrons through large distances as those in *G. sulfurreducens* biofilms.

External cytochromes are bonded to pili,[40] store electrons arising from cell's metabolism in the lack of an electron acceptor[44,62] and also link cells to the electrode.[37,38] Thus, there has to be a pathway for direct electron exchange between pili and cytochromes. Unfortunately, little is known about the nature of electron transfer and binding between these proteins.

It seems to be reasonable to think that electron exchange between cytochromes and pili could involve heme groups exposed at the cytochrome surface. Based on the structure of the pilin monomer, we have performed the prediction of the heme binding sites (plausible electron exchange sites) on the pilus surface using the Heme NET server.[63] The analysis yielded a regular distribution of heme binding motifs (score > 0.90) on the ridges of the α-helix core of the structure (Fig. 1F), composed of nine amino acids (S25, R28, V29, K30, A31, S37, R41, L47, A50).

Tyrosine 32 (Tyr32) is a non-conserved residue located near the C-terminus of the mature protein and was found to be subjected to a posttranslational modification with glycerolphosphate[59] that increases the affinity of the residue for Fe^{3+}.[64] Although affinity measurements were performed on soluble Fe ions, the interaction of Tyr32 with iron ions partially coordinated, as those in the core of heme groups of cytochromes, seems to be possible. In fact, aromatic residues such as tyrosine and tryptophan were found to mediate the electron transfer to cytochrome hemes in several biological systems.[56,65,66] In the modelled pilus structure, Tyr 32 is exposed at the C-terminus of the closely packed groups of aromatic amino acids (Fig. 1D), representing a possible electron exchange point on the conductive segment of the pilus structure.

In several protein complexes, the binding of the cytochrome is made by the interaction between the ring of positively charged lysine residues that surround the heme[67,68] and the negative residues (commonly aspartate and glutamate) exposed on the surface of the interacting protein.[65,67–70] Abundant aspartate and glutamete residues are exposed on the surface of the modelled pilus structure (D39, E48, D53 and D54, data not shown), which suggests that the same protein–protein interaction may govern the binding between outer membrane cytochromes and pili. Interestingly, those residues are separated from the proposed electron exchange sites (see above) by distances smaller than the typical diameter of a cytochrome (15 Å) which further supports the idea of tyrosine 32 and surrounding aromatic aminoacids serving as sites for electron transfer from pili to cytochromes.

Stepping stone mechanism

The alignment of metal-containing redox-cofactors, especially Fe–S-clusters and hemes, allows efficient electron transfer in proteins through otherwise prohibitive distances of more than 14 Å.[71] Besides, the side chains of aromatic amino acids, formed by cofactors with low and tuneable oxidation potentials, were found to serve as relay stations, ''stepping-stones'', for the electron transport reactions[61] leading to a 20- to 30-fold increase in the ET rate.[72]

In the particular case discussed in this work, based on the structural evidence collected from the predicted structure of geopilus (Fig. 1) and discussed in the previous section, we propose that outer membrane cytochromes bonded to pili might serve as intermediate ''stepping stones'' to overcome limitations in electron transfer introduced by the large distance that separates groups of aromatic amino acids in the pilus structure (Fig. 2).

The diameter of outer membrane cytochromes is about 8.5 nm,[73,74] which agrees with the structural periodicity within the pilin oligomer (~7 nm) (Fig. 1). In this scenario, the multiplicity of hemes in *Geobacter* external cytochromes suggests that they can act as nodes in a pili network, connecting aromatic residues either of single or neighbour filaments. Besides, cytochromes could also reduce the reorganization energy[75] of the pilin protein, accelerating the internal electron transfer process.

In the proposed mechanism electrons are transported along the pilus through groups of closely packed aromatic residues, to latterly hop to stepping stone cytochromes that bridge the gap to the next aromatic group. Owing to the relative rates of involved processes, hopping of electrons between cytochromes and between cytochromes and pili may be the limiting step of the mechanism, providing an explanation to the diffusive behaviour of the electron transport[9,13,18] and the sharp redox gradient found in the biofilms.[15]

External cytochromes of *G. sulfurreducens* often appear to be regularly distributed on the pili of single cells, separated by distances[12] that largely exceed the periodicity of the binding sites found in the pili structure (Fig. 1F). This indicates that some binding sites for cytochromes may not be fully occupied and, as a consequence, that in some regions of the pili aromatic residues may be still separated by distances that do not allow electron conduction. Anyhow, the absence of the stepping stones in those regions of the pili does not necessarily impede the electron transport through the biofilm. It has to be noted that the interactions between pili and cytochromes might produce conformational changes in the filament, possibly leading to smaller distances between aromatic residues than those on the isolated pilin. In addition, the pilus is likely to be folded in more complex structures than a simple extended filament, which would possibly bring the redox cofactors closer. Besides, electron transport from the cells to the electrode does not necessarily have to proceed through a single filament. Pili, cytochromes and polysaccharides surely form a complex conductive matrix with numerous pathways for the electron transport. Indeed, cytochromes associated with pili appear to be bumps of about 20–30 nm in size[12] which, taking into account the mean predicted diameter of already studied cytochromes,[73,74] suggests that protein complexes rather than single cytochrome units might be bonded to pili. In this context, the nature of those complexes, their interaction with pili and their spacing in actively respiring biofilms remain to be elucidated. Besides, although the arrangement of cofactor distances is the most important parameter in the redox chain protein complexes, for several protein structures electron transport rates cannot be explained only by electron donor to electron acceptor distances.[54] A deeper structural analysis of the peptide matrix, including the determination of the crystallographic structure of the involved proteins and its conformational changes during charge transfer, is necessary to understand how electrons are transported through the peptides.[56,66,76]

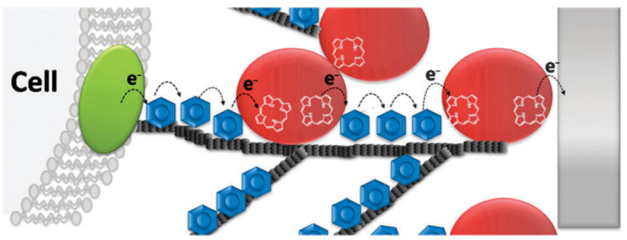

Fig. 2 Schematic representation of the electron transport from the cell to the electrode *via* aromatic residues in the pilA filament (blue hexagons) and using cytochromes (red circles) as stepping stones.

Concluding remarks

The transport of electrons along unusual large distances of more than 50 μm requires a very efficient transport mechanism that is unlikely to involve a single type of protein. The conductive properties of pili and the abundance of cytochromes in the biofilm matrix together with a putative electron exchanging role for the non-conserved Tyr32 located in the surface exposed binding sites for hemes of the modelled protein suggest a combined mechanism involving both cytochromes and pili.

The model presented in this work represents a first mechanistic approach that allows preliminary insight into the fundamentals of

the protein–protein interactions in the biofilm matrix. We hope that this work will help to stimulate specialists in fields such as molecular dynamics and protein structure and energetics to perform more detailed analyses on the proposed model.

We also believe that structural studies focused on the co-crystallization of pili and cytochromes would help to better understand the nature of the protein–protein interactions, including binding sites and electron transfer pathways. Anyway, as the crystallization of isolated pili and *Geobacter* matrix cytochromes has not been achieved, its co-crystallization seems to be improbable in the near future. Complementary experiments based on directed mutagenesis of those pili residues supposed to be involved in the interaction with cytochromes could be a possible way of determining which residues have an important effect on the protein–protein interactions and the electron transport process.

Additionally, the distribution of cytochrome complexes in the biofilm matrix and the nature of molecular and electronic interactions between them as well as with pili filaments and the polysaccharide matrix are important points that remain to be explored in depth and that will undoubtedly help to better understand the electron transport through the biofilm.

From our point of view, a consensus on the exocellular electron transport mechanism in *Geobacter sulfurreducens* biofilms will be achieved only after performing several independent studies aimed at solving the controversy rather than supporting a particular mechanism.

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