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## The relay network of *Geobacter* biofilms

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While actual models explaining electron conduction in electricity producing biofilms have evolved separately to apparent irreconcilable conceptual positions, finding cytochrome complexes in the external matrix of *Geobacter* biofilms supports the proposal of a new functional model that takes fundamental elements from confronting theories. In this model electrons expelled by cells are conducted to the collecting electrode along a network of supramolecular cytochrome arrangements interconnected by semiconducting pilus fibres that provide equipotential conditions within physically distant points. This arrangement resembles, from our point of view, a relay network for *Geobacter* biofilm, which allows a concerted physiological response of the entire population to any local redox change.

During recent years there has been considerable debate regarding the mechanisms of electron conduction across biofilms of the electro-active bacteria *Geobacter sulfurreducens*, the most effective electricity producer described up to present days. In these biofilms every cell is interconnected to its neighbour in such a way that electrons obtained from the oxidation of the carbon source are channelled to a collector electrode. To reach the electrode, electrons pass along a conductive exocellular matrix, where electron transport occurs across distances of tens of micrometres. This exocellular matrix has been shown to be composed of polysaccharides,<sup>1</sup> as typically found in biofilms of other bacteria, but also contains a high abundance of cytochromes, as well as pili proteins that give the structural support to electron conduction mechanisms described up to now.

Two main mechanisms have been proposed for explaining long distance electron transport: the first one based on conduction along pili, recognized as the

“metallic like” conduction mechanism<sup>2</sup> and the second one based on conduction by electron hopping between cytochromes, identified as the “superexchange” conduction model.<sup>3</sup>

Aiming at conciliating critical experimental evidence in a single model, in a perspective article published in PCCP during 2013,<sup>4</sup> we proposed a third alternative mechanism that we denoted as the “stepping stones” mechanism, according to which cytochrome units may act as relay stations at conduction gaps in the pili structure, while acting as the contact between different pili as well. Several publications have appeared since that moment adding evidences that support one or the other proposal, but unfortunately, accordance seems not possible in depicting a concerted conduction model.

From our point of view and in spite of recent arguments in favour or opposing each model, there are facts that cannot be disregarded, independently of which model one is intending to defend. The first one we highlight is that evidence confirms that electrons can be conducted along pili at a level that suffices the requirements for biofilm cells activity,<sup>5</sup> strongly contending against hopping, which has intrinsically a lower efficiency.<sup>2, 6, 7</sup> The second fact is the limitation

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found in electricity production as biofilms approaches a critical thickness of about 50  $\mu\text{m}$  that clearly points to a limitation in electron conduction, not compatible with high conduction levels measured on isolated pili nets.<sup>3, 8-10</sup>

In this opinion article we add new evidence that we think can change the way in which the structural basis for electron conduction in *Geobacter* biofilms is seen. Around this evidence we elaborate a hypothetic explanation that rewords the stepping stones model to a new version in which roles are inverted; because now conduction is centred on cytochromes with pilus acting as connecting elements that bridge gaps, where supramolecular cytochrome complexes are not ideally organized. This complex structure acts as a continuum in terms of electrochemical potential and responds to a potential difference with a diffusional limitation. Indeed, it accommodates well the evidence showing that electron conduction has not a predefined direction, allowing electrons to flow towards any point in the network, provided it has the most oxidative potential. In order to reflect all these facts we parallel this hypothetic structure with a relay network that allows response of every cell in the biofilm according to local redox or respiratory variations to reach the electrode no matter how distant they may be.

### Organization of external cytochromes in supramolecular complexes

To this date six outer membrane C-type cytochromes named OmcZ, S, T, E, C and B are known to be involved in external Direct Electron Transfer (DET) in *G. sulfurreducens*.<sup>11, 12</sup> Highly relevant to this process,<sup>13</sup> OmcZ and OmcS are molecules exhibiting a wide redox potential window adding respiratory versatility to this strain.<sup>14</sup> While OmcZ localizes preferentially (but not only) close to the electrode surface,<sup>15</sup> OmcS seems to be uniformly distributed along biofilm layers.<sup>16, 17</sup> Functionally redundant OmcB and OmcC on the other hand, are known to play a role in transporting electrons through the outer membrane to the cell exterior,<sup>18</sup> with OmcB found in higher concentration at layers further away from the electrode.<sup>19</sup>

Assays combining electrochemical and spectro-electrochemical tools have implicated C-type cytochromes as the redox cofactor involved in long distance electron transfer based on "superexchange".<sup>8, 20-22</sup> The main argument opposing this proposal is that these redox cofactors can hardly be organized along the biofilm exocellular matrix in a way to warrant electron conduction at high rate, a point of view that was initially supported by the observation of gaps between gold dots in immunogold detection of OmcS associated to pili.<sup>16, 17</sup> The immediate reply by superexchange defenders claimed that those gaps may be filled by other cytochromes or redox proteins not detected by the specific antibody<sup>17</sup>, but more direct observation by AFM, confirmed the occurrence of proteinaceous globules associated with pili like structures that are separated by gaps as large as 100 nm.<sup>23</sup> It should be mentioned here that AFM images were collected from planktonic cell samples grown at 25 °C with fumarate as final electron acceptor, but has never been taken on anode biofilm samples.

In spite of the overwhelming number of work reporting characteristics of superexchange in *Geobacter* biofilms, one main aspect for validating this conduction model remains unexplored. No direct structural evidence of domains of closely spaced cytochromes within actively respiring *G. sulfurreducens* biofilm anodes exists.<sup>3</sup> Following the clue of supramolecular globules and aiming at shedding some light over structural organization of redox cofactors supposedly relevant to bacterial electroactivity, we performed native gradient gel electrophoresis of the extracellular protein fraction of mature electro-active biofilms of *G. Sulfurreducens*. Using this simple approach we observed that external c-type cytochromes are in fact organized into different protein complexes (relative molecular weights of 300, 150, 140 and 80 kDa) named here as OCA, OCB, OCB' and OCC (for Outer Complex A, B, B' and C, respectively)(Fig1). These complexes, when solved in semi-denaturing SDS-electrophoresis second dimension gels (Fig 1), showed overlapping components for OCA, OCB and OCB' including at least two of five different C-type cytochromes of 76, 50, 45, 30 and 28 kDa relative

molecular weight, while OCC does not share components with other complexes. Mass spectrometry confirmed that included in these arrangements are dodecaheme cytochrome OmcC (MW 80kDa), plus OmcS (45kDa) and OmcE (24kDa) with 6 and 4 heme-binding sites each (data not shown). Even though information on the identity of 50 and 30kDa bands is presently under research, they most likely correspond to OmcZ large and small forms, respectively.<sup>12, 24</sup>

comprised of one subunit protein, PilA<sup>26</sup> and synthesized by combination of two isoforms (short and long). Notably the short one was found essential for the correct localization of OmcZ.<sup>27</sup> As UREA (8M) treatments must be applied to achieve the disassembly of pili into their subunits<sup>25</sup> and considering the results of pull down experiments showing pilin co-precipitation with cytochromes,<sup>28</sup> we are prompted to consider OCP (Fig. 1) as a supramolecular assembly including OmcS, OmcZ and PilA.

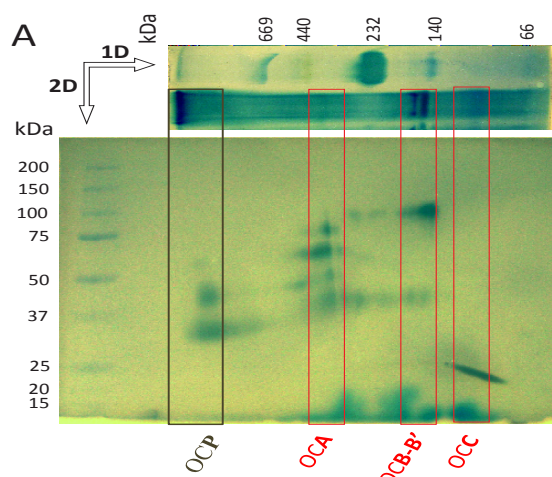


Fig 1: **Blue native gel electrophoresis of external soluble proteins from *G. sulfurreducens* electro-active biofilms.** Extracellular *Geobacter* protein complexes from biofilms grown to stable current over graphite electrodes polarized at 0,2 V (Ag/AgCl) were solved under native conditions using a 4-15% acrylamide gradient gels, first dimension (1D), and non-reducing 12% polyacrylamide SDS gels, second dimension (2D), for the separation of proteins component of each complex. Native (1D) or pre-stained (2D) Molecular weight markers (kDa) are observed at the upper side in 1D gels and to the left in 2D gels. **A** TMBZ staining and c type cytochrome detection. Complex containing cytochromes interacting or not with protein polymeric fibres are mark in green and red boxes respectively.

Given the same cytochromes were observed in more than one complex, we are inclined to think that bands detected may correspond to different oligomerization states of essentially one or two major respiratory complexes. This interpretation is supported by detection in the sample of a single high molecular fraction during gel filtration chromatography, which split into several aggregates upon analysis in hydroxyapatite chromatography under different buffering conditions, each one exhibiting a different combination of the same five cytochromes mentioned above (data not shown).

During analysis of native gels we also observed a larger complex we named OCP (MR higher than 669kDa) composed of two c-type cytochromes of about 45 and 30 kDa and a very high molecular weight non-redox protein (Fig 1 upper left region). In *Geobacter* biofilm, exocellular polymeric protein assemblies typically belong to type IVa family of pili proteins,<sup>25</sup> mainly

### Structural characteristics of electron transfer in *Geobacter*

In order to achieve inter or/and intra-molecular electron transfer between Fe or Fe-S centres, non-prohibited tunnelling distances lower to 14 Å need to exist.<sup>29</sup> Generally, tunnelling distances between redox centres are achieved by a more or less dynamic complex formation of redox cofactors involved in respiratory chains.<sup>30</sup> Recently, Liu and co-worker<sup>31</sup> published the characterization of the first supramolecular respiratory complex of outer membrane localization in *G. sulfurreducens*. The components of this complex included external cytochromes OmcB and OmcC interacting with porin-like proteins OmbB/OmbC and periplasmic cytochromes OmaB/OmaC. The authors hypothesized that this complex is responsible for conducting respiratory electrons to the extracellular space. Similarly, we show here several external multiheme c-type cytochromes organized in supramolecular

complexes that we believe are responsible for effective electron transport to solid electron acceptors, in this case the collector electrode. Importantly, the participation of OmcC in both, trans-outer membrane<sup>31</sup> and outermost respiratory complexes (Fig. 1) is compatible with the existence of an integral supramolecular arrangement connecting periplasmic redox cofactors with its counterpart at the external conductive matrix, composing a route with increasing potential that can warrant the contact to virtually any external solid acceptor. According to our results in this arrangement both, OmcS and OmcE are found interacting with each other as well as with OmcC and probably OmcZ, with PilA also involved (Fig.1).

Recent reports have shown that *Geobacter* may develop different respiratory pathways in response to external redox potential above or below -0,1V vs SHE.<sup>32, 33</sup> Two inner membrane heme-containing proteins named ImcH and CbcL were described as key components of each one.<sup>32-35</sup> In this context, previously described PAS sensor redox molecules of periplasmic localization<sup>36, 37</sup> would be functional to respond directly to the environmental redox potential cells may indeed detect. Complementing this machinery, the broad operating potential range of OmcS and OmcZ,<sup>14</sup> may provide outer complexes the flexibility to bridge virtually any potential signal for triggering the internal cell physiology response.<sup>8, 38</sup> Taken together, these findings provide a regulatory context, not fully understood yet, for cells respiration at different biofilm layers, which most likely compensate the fast changing redox environment.<sup>8, 9, 21</sup>

### The relay network concept

In our view, dynamic protein-protein interaction as described here may take place during “selfexchanging” electron pathways within *Geobacter* biofilms, in which a diffusive controlled process enables anode electrodes to collect metabolic electrons produced close or far away into the biofilm. In other words, oxidized cytochromes at any point of the biofilm matrix may be reduced by electrons produced near or far from its original environment. For this to happen, cytochromes

should either form a continuous chain between cells surrounding them and the anode, which today we know not to happen, or this redox cofactor may rely on a connecting conductive structure like pilus fibres.

Great amount of experimental and modelling results support electron conduction through certain aromatic residues of *Geobacter* pilA fibres,<sup>2, 6</sup> with conductivity ranging from 5  $\mu$ S/cm to 4 S/cm when measured in a complex pili mixture<sup>2</sup> to isolated individual pili in cytochromes-free regions.<sup>5, 39</sup> It becomes evident from these values the need for considering the pilus-to-pilus contact resistance<sup>5</sup>, which we think may play a role also in explaining low conductivities measured or calculated in biofilms.<sup>40, 41</sup> From the structural point of view and far from arguing against pili conduction, we instead highlight the low probability for every cell in the biofilm of having a direct pilus connection to the electrode and propose a new scenario in which contrary to experimental situation of pili fibres extended over electrode probes,<sup>5, 6</sup> they are expected to arrange extracellularly in an intricate net that allows electric contact between cells, protein globules and the electrode, providing equipotential conditions between physically distant points. At the molecular level, semi-conducting polymer pili may act as a contact system allowing long distance electron transport through sequential redox chains formed within distant redox complexes.<sup>8</sup> For this to occur: a) respiratory complexes need to accommodate along pilus fibres, as already demonstrated for the complex component OmcS<sup>16</sup> (Fig. 1); b) electric connection between redox complexes and pili need to be ensured, as already demonstrated in a previous work where redox proteinaceous globular structures were charged through pilus fibres<sup>6</sup> and c) less specific interactions may exist so that additional complex-pili and complex-complex connections are achieved as a way to improve network function, as those inferred from bioinformatics predictions and structural information showing the occurrence of regularly distributed heme-binding sites along pili structure.<sup>4, 42</sup>

Based on all elements discussed, we are prompted to put under consideration a combined conductivity

model for *Geobacter* biofilms during which a recognizable electron pathway is formed in the direction given by the most favourable redox gradient, along a structure that connects individual cells, influencing their activity in response to the network (redox) state. In this model, electrons reaching the extracellular space will be transferred from one reduced external complex to a more oxidized one by passing through aromatic amino acids residues of pilus fibres connecting both redox complexes and thus providing equipotential conditions. The reaction will then proceed to other complex or pili in the electric pathway along the biofilm thickness to finally reach the electron acceptor. This arrangement is from our point of view a kind of *relay network* for *Geobacter* biofilm, which combines external respiratory complexes and proteinaceous conductive fibres to allow a concerted physiological response of the entire population to any local redox change.

This proposal is compatible with the existence of a redox gradient around a more oxidized network point.<sup>8, 9, 43</sup> As mentioned, cells are expected to adapt its internal respiratory machinery in response to this gradient, where at least two different inner membrane cytochromes would act depending on the potential the cell is sensing.<sup>32-35</sup> If both pathways can transfer their electrons to slightly different external redox complexes exhibiting slightly different oligomerization states remains to be confirmed, but seems to be possible in view of the differential distribution of OmcB as well as OmcZ along biofilm layers.<sup>15, 19, 35</sup>

Bacteria have developed several strategies in order to efficiently adapt to electronic donor and final acceptor presence in their environment. Anaerobic respiration of insoluble acceptors demands molecular mechanisms to expel electrons produced within the cells limits to the external medium. The *relay network* conductivity model that we put under consideration incorporates a new strategy developed by these bacteria in order to achieve efficient high rates long distance electron transfer.

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## Notes and references

### Glossary:

- Relay network: is a broad class of network topology commonly used in wireless networks, where the source and destination are interconnected by means of some nodes that "relay" the information to different devices. In such a network the source and destination cannot communicate to each other directly because the distance in between them is greater than their transmission range. Often the relay network is complex and branches off in multiple directions to connect many intermediate points in order to have access to the most efficient path.

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**Something in the way *they* move.** When biology reaches its limits is when it's ready to overcome them. This seems to be the case of electro-active bacteria able to communicate electrically to a final acceptor located tens of microns away. In this article we aim at organizing previous relevant evidence and new biochemical data, to produce a model that gives structural and functional support to that electrical communication. In our model, external cytochrome are organized in complexes interconnected by semiconducting pilus fibres that providing equipotential conditions within physically distant points. The structure works essentially as a *relay network* that helps explaining redox gradients and electron transport limitations observed in electro-active biofilms, relevant in improving bioelectrochemical systems and related technological applications.