Review

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Electron transfer process in microbial electrochemical technologies: the role of cell-surface exposed conductive proteins

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Abstract

Electroactive microorganisms have attracted significant interest for the development of novel biotechnological systems of low ecological footprint. These can be used for the sustainable production of energy, bioremediation of metal-contaminated environments and production of added-value products. Currently, almost 100 microorganisms from the Bacterial and Archaeal domains are considered electroactive, given their ability to efficiently interact with electrodes in microbial electrochemical technologies. Cell-surface exposed conductive proteins are key players in the electron transfer between cells and electrodes. Interestingly, it seems that among the electroactive organisms identified so far, these cell-surface proteins fall into one of four groups. In this review, the different types of cell-surface conductive proteins found in electroactive organisms will be overviewed, focusing on their structural and functional properties.

Keywords: extracellular electron transfer; cell-surface exposed conductive proteins; multiheme *c*-type cytochromes; electroactive bacteria; microbial electrochemical technologies

1. Introduction

Microbial electrochemical technologies (METs) have become the focus of intense fundamental and applied research to harness the vast metabolic versatility of microorganisms towards sustainable industrial processes (Logan and Rabaey, 2012). The versatility of the concept has spawned a veritable zoo of device designs among which microbial fuel cells (MFC) and microbial electrosynthesis (MES) are the most prominent examples (Wang and Ren, 2013). Microorganisms in MFCs are used as biocatalysts to produce bioenergy from organic matter, while in MES electroactive microorganisms collect electrons from the electrode to generate useful chemical compounds. In both cases, the microorganisms have to perform extracellular electron transfer (EET), i.e. transfer electrons across the cell envelope and establish an electrical contact with the electrode, or vice-versa. The study of EET has been mainly focused on two model organisms, *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA, both Gram-negative mesophilic bacteria that are able to transfer electrons to extracellular substrates during respiration. However, electroactivity is not limited to organisms with an outer-membrane since the ability to transfer electrons to electrode surfaces was also observed for some Gram-positive bacteria, Archaea, microalgae and even fungi (Koch and Harnisch, 2016; Logan, 2009; Salar-García et al., 2016). Indeed, recently it was shown that the hyperthermophile iron-reducing archaea *Ferroglobus placidus* and *Geoglobus ahangari* present electroactive behavior in single-chamber microbial electrolysis cells (Yilmazel et al., 2018). Moreover, thermophilic Gram-positive bacteria that belong to *Thermincola* genus were identified as the dominant organisms in the anode of an operating microbial fuel cell operating at high temperature (Mathis et al., 2008; Wrighton et al., 2008).

From the extensive studies on *S. oneidensis* MR-1 and *G. sulfurreducens* PCA, it is now established that the general mechanism for EET involves multiheme *c*-type cytochromes (MHC) that are responsible for linking the cellular metabolism to the electrode, or vice-versa (Breuer et al., 2015b; Santos et al., 2015; Shi et al., 2012).

Two general mechanisms for EET are now recognized: *direct electron transfer* (DET) and *indirect electron transfer* (IET) (Gralnick and Newman, 2007). In DET microorganisms establish direct contact with the insoluble electron acceptor (e.g. electrode or insoluble substrates) via proteins that decorate the cell surface (David J. Richardson et al., 2012), or through cellular appendages such as pili or nanowires (Gorby et al., 2006). In several organisms these appendages are also covered with *c*-type cytochromes (Leang et al., 2010; Pirbadian et al., 2014). On the other hand, IET relies on the ability of microorganisms to use soluble redox active compounds as electron shuttles to mediate the electron transfer between cell-surface exposed conductive proteins and insoluble electron acceptors, such as electrodes in METs (Brutinel and Gralnick, 2012).

Cell-surface exposed cytochromes are among the most prevalent proteins in EET pathways of electroactive organisms being responsible for both DET and IET (Beliaev et al., 2001; Coursolle et al., 2010; Leang et al., 2010; Liu et al., 2014; Myers and Myers, 1997; Richter and Ludwig, 2009). Due to their cellular position and functional role, these proteins form one of the most important classes of proteins to be targeted for genetic manipulation (Teravest and Ajo-Franklin, 2015). This review focusses on the insights gained over the years on the role of surface exposed proteins, mainly MHC in mediating EET in METs (Table 1).

2. Cell-surface exposed conductive proteins of electroactive organisms

Besides the cytoplasmic membrane, which is the primary barrier to the external environment, the microbial cell envelope often includes other structural features, such as the peptidoglycan, the outer-membrane and the S-layer. Microorganisms have evolved specialized cellular components to overcome this physical barrier for EET reactions. So far, four types of proteins have been identified to be responsible for the transfer of electrons across the cell-surface of electroactive bacteria (Scheme 1):

(i) *Porin-cytochrome complexes*, typically composed by one porin protein and one or more redox proteins, mainly MHC;

(ii) *Cell-surface exposed cytochromes*, which can be lipoproteins or cytochromes loosely bound to the cell surface;

(iii) *Conductive nanowires*, typically pili composed by protein filaments anchored to the cell;

(iv) *Other redox proteins*, including copper and iron-sulfur proteins.

2.1. Porin-cytochrome complexes

Up to date, porin-cytochrome complexes were only found in Gram-negative bacteria (Shi et al., 2014), where a β-barrel protein is long and wide enough (White et al., 2013) to cross the outer-membrane and incorporate redox proteins, most commonly MHC. These complexes are responsible for conducting electrons across the outer-membrane for the reduction of extracellular electron acceptors outside of the cell (David J. Richardson et al., 2012). One of the most intensely studied, and also the most understood porin-cytochrome complex is MtrCAB from *S. oneidensis* MR-1 (Hartshorne et al., 2009; Ross et al., 2007). This complex is composed of the outer-membrane decaheme cytochrome MtrC, the periplasmic decaheme cytochrome MtrA, and the β-barrel MtrB where the cytochromes are embedded (Hartshorne et al., 2009;

Ross et al., 2007). The arrangement of these proteins in the complex MtrCAB that spans the ~40 Å of the outer-membrane of *Shewanella*, enables the transfer of electrons from one side of the lipid bilayer to the other through a chain of 20 hemes that is formed between the two cytochromes (David J. Richardson et al., 2012). *S. oneidensis* MR-1 contains a series of paralogs of these porin-cytochrome complexes: the MtrDEF, highly homologous to the MtrCAB complex; the DmsEFABGH responsible for DMSO reduction (Gralnick et al., 2006) and the SO4362-SO4357 cluster that was shown to function as a terminal reductase at the cell surface (Schicklberger et al., 2012) (Figure 1).

Comparative analysis of *Shewanella* spp. revealed that MtrCAB are well conserved among all analyzed species capable of mineral reduction (Fredrickson et al., 2008), and that *mtrAB* homologs can be found in numerous other organisms (Shi et al., 2012). Indeed, MtrAB has become a model system for electron transfer to occur across the bacterial outer-membrane (Hartshorne et al., 2009; Shi et al., 2012). This type of complex was found in numerous other electroactive organisms, such as in the iron-reducing organisms *G. sulfurreducens* PCA, *Aeromonas hydrophi*la, *Rhodoferax ferrireducens* and *Desulfuromonas acetoxidans* (Alves et al., 2011; Liu et al., 2014; David J. Richardson et al., 2012; Risso et al., 2009; Shi et al., 2014, 2012), and in the iron-oxidizing bacteria *Rhodopseudomonas palustris* TIE-1, *Sideroxydans lithotrophicus* ES-1, and *Acidithiobacillus ferrooxidans* (Jiao and Newman, 2007; Shi et al., 2012; Yarzábal et al., 2002) (Figure 1 and Table 1).

Geobacter sulfurreducens PCA contains two highly similar porin-cytochrome complexes: the OmaB/OmbB/OmcB and the OmaC/OmbC/OmcC, that are composed by the β-porin proteins OmbB(C), the outer-membrane dodecaheme cytochromes OmcB(C) and the periplasmic octaheme cytochromes OmaB(C) (Liu et al., 2014; Shi et

al., 2014). Although the observed similarities between these porin-cytochrome complexes and MtrCAB from *S. oneidensis* MR-1 suggest a common mechanism for EET (Liu et al., 2014; Shi et al., 2014), they are phylogenetically unrelated (Liu et al., 2014). This has led to the proposal that they must have evolved independently to provide a similar function (Shi et al., 2014).

The porin-cytochrome complexes in iron-reducing organisms are mainly formed by three or more proteins, while in iron-oxidizing organisms, such as *S. lithotrophicus* and *R. palustris* only two proteins are required to mediate electron transfer from the cell-surface (Figure 1 and Table 1). These include the β-porin protein and a MHC that is embedded in the pore (see Scheme 1A). The genome of *S. lithotrophicus* ES-1 contains the *mtoA* gene that encodes for the decaheme cytochrome MtoA and the *mtoB* gene that is predicted to encode a porin protein. These two proteins form a porin-cytochrome complex in the membrane of *S. lithotrophicus* ES-1, that together with MtoD and CymA form a conductive pathway that couples the extracellular oxidation of iron to the reduction of quinone to quinol in the cytoplasmic membrane (Beckwith et al., 2015; Shi et al., 2012). Homologous MtoAB clusters were also identified in the genomes of the iron-oxidizing organisms *Dechloromonas aromatica* RCB and *Gallionella capsiferriformans* ES-2 (Shi et al., 2012).

The pioABC operon found in the genome of *R. palustris* TIE-1 encodes for the periplasmic decaheme *c*-type cytochrome PioA, the outer membrane porin PioB, and the periplasmic high-potential iron protein Pio C (Jiao and Newman, 2007). This phototrophic iron-oxidizing bacterium is able to use light as an energy source and iron as electron source to fix CO_2 (Jiao et al., 2005). While PioAB form the porin-cytochrome complex responsible to receive electrons from insoluble iron complexes and transfers them across the outer-membrane, PioC was proposed to transfer these

electrons to the photoreaction center in a light-dependent way (Bird et al., 2014; Saraiva et al., 2012).

In contrast to the other porin-cytochrome complexes, Cyc2 from *A ferrooxidans* is a single protein that belongs to a fused porin-cytochrome family, where the N-terminus contains the heme domain and the C-terminus is a porin domain of 18 β-strands (Yarzábal et al., 2002). This protein is the first electron carrier in the iron oxidation respiratory pathway of this organism, being co-transcribed with three other proteins: a aa_3 cytochrome c oxidase, a rustacyanin and a c_4-type cytochrome. It is proposed that the porin domain wraps around the heme domain with a single CXXCH *c*-type heme-binding motif, forming a fused porin-cytochrome in the outer-membrane of *A. ferrooxidans* (Yarzábal et al., 2002). This protein is responsible for transferring electrons from the oxidation of iron to rustacyanin in the periplasmic space (Valdés et al., 2008).

2.2. Cell-surface exposed cytochromes

Besides porin-cytochrome complexes, electroactive organisms contain a large number of cell-surface exposed cytochromes that are important for EET to electrodes (Santos et al., 2015; Shi et al., 2012) (Table 1). One example is the outer-membrane decaheme cytochrome OmcA from *S. oneidensis* MR-1 that is associated with the porin-cytochrome complex MtrCAB (Hartshorne et al., 2009; Shi et al., 2006). OmcA, like MtrC, is considered to be the terminal extracellular reductase in *Shewanella*, being responsible for both DET and IET to insoluble electron acceptors or electrodes (Ross et al., 2009). Analysis of *Shewanella* spp. genomes showed that OmcA is highly conserved among species, and that in some strains the *omcA* gene is replaced by *undA*

that codes for an undecaheme cytochrome highly homologous to OmcA (Fredrickson et al., 2008; Shi et al., 2012).

The genome of *G. sulfurreducens* PCA contains genetic information for at least 30 outer-membrane cytochromes. Besides OmcB and OmcC, which are part of porin-cytochrome complexes, five were shown to play important roles in iron and electrode reduction: the monoheme cytochrome OmcF, the hexaheme cytochromes OmcT and OmcS, the tetraheme cytochrome OmcE and the octaheme cytochrome OmcZ (Mehta et al., 2005). While OmcE is loosely attached to the outer-membrane of *G. sulfurreducens* PCA, OmcS was proposed to be localized along pili (Holmes et al., 2006; Lovley, 2008). Both proteins were shown to facilitate electron transfer to iron oxides and electrodes (Holmes et al., 2006; Leang et al., 2010). OmcZ is crucial for current production in MFCs (Richter and Ludwig, 2009), and it was shown to accumulate at the biofilm-electrode interface in METs (Inoue et al., 2011). This protein is particularly important for long-distance electron transfer in biofilms, facilitating electron transfer between the biofilm and the anode (Inoue et al., 2010). Although the *omcF* deficient mutant of *G. sulfurreducens* PCA was impaired in iron reduction and electricity production, it was found that this monoheme protein is required for the appropriate transcription of outer-membrane cytochromes involved in EET (Kim et al., 2005, 2008).

Gram-positive bacteria were found to generate more current than the representative electroactive mesophilic organisms *S. oneidensis* MR-1 and *G. sulfurreducens* PCA in the same type of MET (Wrighton et al., 2011, 2008). Unlike Gram-negative bacteria, Gram-positive bacteria lack the outer-membrane and contain a thick cell wall (10 to 80 nm) and a glycoprotein S layer. Although these structural features were previously considered to prevent Gram-positive bacteria to perform DET to electrodes (Wrighton et al., 2011), genome sequencing of *Thermincola potens* JR revealed the presence of

genes that code for MHC (Byrne-Bailey et al., 2010) that were shown to be involved in EET (Carlson et al., 2012). From these, the nonaheme cytochrome TherJR_2595 was predicted to be associated with the cell-surface, and together with the hexaheme cytochrome TherJR_1122 that is embedded in the cell wall, mediate the electron transfer from the cellular metabolism to insoluble electron acceptors outside of the cell (Carlson et al., 2012).

Hyperthermophilic Archaea are capable to couple anaerobic acetate or benzoate oxidation with the reduction of insoluble electron acceptors, including iron (Holmes et al., 2011; Lovley, 2011; Manzella et al., 2013) and electrodes in MFCs (Yilmazel et al., 2018). Although a mechanistic understanding of microbial iron reduction performed by these organisms is still lacking, genome analysis has identified several MHC as promising candidates for EET (Table 1) (Kletzin et al., 2015; Smith et al., 2015). Interestingly, *F. placidus* contains more MHC than any other hyperthermophilic archaeon, with several of them being highly expressed during EET (Smith et al., 2015).

2.3. Conductive nanowires

Bacterial pili have been found as electrically conductive nanowires during iron and electrode reduction in *G. sulfurreducens* PCA (Reguera et al., 2006, 2005). After this discovery, bacterial nanowires were observed in numerous other electroactive organisms, including in *S. oneiden*sis MR-1 (Gorby et al., 2006). While in *Geobacter* sp. nanowires are composed by type IV pilin proteins (Reguera et al., 2005) mainly comprised by one subunit protein, the pilA, nanowires from *Shewanella* are essentially membrane extensions that include the porin-cytochrome complexes and outer-membrane MHC responsible for EET in this organism (Pirbadian et al., 2014).

Geobacter spp. nanowires are essential for iron reduction (Malvankar et al., 2011), for electricity production in MFCs (Reguera et al., 2006) and for direct interspecies electron transfer (Rotaru et al., 2014a). Indeed, it was shown that PilA is essential for *G. metallireducens* to participate in direct interspecies electron transfer (Rotaru et al., 2014b). It was proposed that the aromatic amino acids of PilA are organized in the pili in close proximity to allow the transfer of electrons by a metallic-like electron transfer mechanism (Malvankar et al., 2011). An alternative model suggests that electron transfer along pili in *Geobacter* spp. may occur through a multistep hopping mechanism (Feliciano et al., 2015; Lampa-Pastirk et al., 2016). It has also been hypothesized that OmcS may facilitate the nanowire electron transfer, since OmcS was shown to be distributed along the pili and to facilitate electron transfer from pili to iron oxides (Leang et al., 2010). The significance of OmcS in electroactive biofilms is still questionable by the fact that the separation between these outer-membrane cytochromes in the nanowire is too long for DET between two proteins to occur (Leang et al., 2010), and because the deletion of *omcS* gene had no effect in biofilm growth and current production (Richter and Ludwig, 2009).

2.4. Other redox proteins

Besides MHC, electroactive bacteria may employ other redox proteins to transfer electrons to extracellular electron acceptors (Gralnick et al., 2006; Schicklberger et al., 2012), including electrodes in METs (Deutzmann et al., 2015; Mehta et al., 2006).

G. sulfurreducens PCA mutant in the pseudopilin *oxpG* gene involved in the type II secretion pathway has led to the identification of the multicopper protein OmpB as an outer-membrane protein involved in EET (Mehta et al., 2006). It was suggested that

OmpB may be involved in the association of the bacterial cell with insoluble electron acceptors.

Genetic and biochemical studies revealed that the electromethanogenic archaeon *Methanococcus maripaludis* produces surface-associated redox enzymes, such as hydrogenases and formate dehydrogenases to mediate direct electron uptake during electrosynthesis in METs (Deutzmann et al., 2015). This organism releases these proteins during growth, being responsible to catalyze the formation of H_2 or formate.

3. Understanding the role of cell-surface exposed conductive proteins through *in vivo* experiments

3.1. Potential pitfalls with in vivo experiments

Transcriptomic and genomic studies have demonstrated that electroactive organisms contain many more genes for *c*-type cytochromes than would be necessary to transport electrons from the cytoplasmic membrane to the cell surface (Bretschger et al., 2007). Indeed, the model organisms *S. oneidensis* MR-1 and *G. sulfurreducens* PCA contain 41 and 111 *c*-type cytochrome encoding genes, respectively (Methé et al., 2003; Romine et al., 2008). However, many of these genes are (i) simultaneously expressed, (ii) present in the same cellular location and (iii) seem to have overlapping functions (Beliaev et al., 2005). Hence, many deletion mutants do not show clear phenotypes and the genomic plasticity of the organisms can lead to suppressor mutants that hide the phenotype of the targeted mutation (Bücking et al., 2012; Cordova et al., 2011; Schicklberger et al., 2012). This is the reason why the gold standard for the physiological analysis of the function of *c*-type cytochromes seems to be the development of mutants lacking all genes that could encode for *c*-type cytochromes with a similar function. In *G. sulfurreducens* PCA, seven outer-membrane cytochrome encoding genes

(Δ*omcBCESTVW*) had to be deleted to lead to a strain that is almost completely unable to reduce ferric citrate (Ueki et al., 2017). In another study, five *omc* genes were deleted to produce a strain that could not reduce the humic acid analogue AQDS (Δ*omcBCSTEZ*) (Voordeckers et al., 2010). A similar *S. oneidensis* MR-1 mutant is also available. In this organism, all five putative outer-membrane cytochrome encoding genes (*mtrC*, *omcA*, *mtrF*, SO_1659, SO_2931) were deleted (Bücking et al., 2010). Another factor that has to be considered in all experiments with outer-membrane cytochrome mutants is the potential for polar effects. For instance, deletion of the membrane associated cytochrome MacA in *G. sulfurreducens* PCA lead to a strain with highly reduced capacity to reduce ferric citrate. Still, later experiments revealed a polar effect of the *macA* mutation that resulted in drastically reduced production of the outer-membrane cytochrome OmcB (Bücking et al., 2012; Kim and Lovley, 2008).

3.2. S. oneidensis MR-1 cell-surface exposed protein mutants affected in electrode reduction rates

A *S. oneidensis MR-1* mutant devoid of all outer-membrane cytochromes (MtrC, OmcA, MtrF, SO_1659, SO_2931) is almost unable to reduce ferric citrate or birnessite (a manganese oxide mineral) but still retains 25% of the current production rate of the wild-type when using graphite electrodes (Bücking et al., 2012, 2010). A similar observation was made with mutants that are only *mtrC* and *omcA* negative (Bretschger et al., 2007). Mutants in *mtrA* or *mtrB*, in which periplasmic electron transfer is totally separated from the extracellular site of the outer-membrane, are more affected with regard to current production than a *mtrC*/*omcA* double mutant. Nevertheless, the impact of outer-membrane cytochrome deletion is more severe regarding flavin reduction than current production.

Expression of either SO_1659 or SO_2931 in the quintuple mutant did not lead to a detectable extracellular respiration activity (Bücking et al., 2010). OmcA production in this mutant had only an effect on manganese oxide but not on ferric iron reduction rates. Expression of *mtrC* in these mutants from a plasmid rescued the phenotype of the mutation almost completely, while expression of MtrF could only partially restore the wild type current production rates (Bücking et al., 2010). This was surprising, as its expression sustained similar or even superior ferric iron reduction rates compared to MtrC. Hence, although MtrC and MtrF are highly similar, and MtrF might be the more effective ferric citrate reductase, it seems to be less suited for microbe-electrode electron transfer. A variation of the production rate of either MtrC or MtrF in the quintuple mutant affected over a wide range of inducer concentration the rate of ferric iron reduction. Moreover, insertion of *mtrF* in an operon consisting also of *mtrA* and *mtrB* that is under control of an arabinose inducible promoter could be used as a biosensor as the current production rate of this strain linearly correlated with the arabinose concentration in the medium (Golitsch et al., 2013). Using a similar strategy, Webster *et al.* constructed a *Shewanella* based biosensor for arsenic by placing the *mtrB* gene under control of an inducible promoter (Webster et al., 2014).

3.3. G. sulfurreducens PCA cell-surface exposed conductive protein mutants affected in electrode reduction rates

The transcriptomic comparison of *G. sulfurreducens* PCA cells grown on an anode surface compared with cells grown on the same biofilm substratum but with fumarate as electron acceptor revealed that only two outer-membrane cytochromes were upregulated if respiratory electrons had to be transported to the electrode surface. These two cytochromes were OmcB and OmcZ (Nevin et al., 2009). Interestingly, deletion of the

omcB gene had no impact on electrode reduction, while *omcZ* deletion lead to 90% reduced current production at graphite electrodes (Richter et al., 2009). It was demonstrated that *omcZ*-deficient mutants form thinner biofilms, but that the pili production is not affected (Nevin et al., 2009). It seems that OmcZ is the terminal electrode reductase, although, besides this cytochrome, two other electron transfer systems to the electrode exist that operate at higher anode potentials (Peng and Zhang, 2017). This is in agreement with the study of Peng and Zhang, in which the authors observed that, unlike to the study by Richter et al., the Δ*omcZ* phenotype is less severe when higher anode potentials are applied (Peng and Zhang, 2017). OmcZ seems to be ubiquitously distributed in *G. sulfurreducens* PCA biofilms on anodes (Franks et al., 2012). Interestingly, its expression or localization seems to be affected by the composition of the extracellular polymeric matrix of *G. sulfurreducens* PCA (Rollefson et al., 2011). Indeed, deletion of a gene in a cluster for polysaccharide production lead to cells that were severely affected in biofilm production, but that also contained a reduced amount of OmcZ at the cell surface. It might be that polysaccharides arrange OmcZ at the cell surface in a way that allows for efficient hopping of extracellular electrons from the biofilm towards the electrode (Rollefson et al., 2011). In contrast to the above mentioned, an earlier study by Nevin and colleagues, revealed that the gene with the greatest transcription increase during the initial growth on electrodes compared to growth with ferric citrate is *omcS* (Holmes et al., 2006). It was proposed that this cytochrome together with OmcE might play a crucial role in the initial phase of current production on graphite electrodes (Holmes et al., 2006). OmcS is associated with nanowires and it facilitates electron transfer from pili to iron oxides (Leang et al., 2010). Nevertheless, the phenotype of *omcS* or *omcE* deletion mutants was almost undetectable

in the later study by Nevin *et al*. Hence, it seems possible that these mutations can be complemented by a redundant activity of OmcZ.

Deletion mutants on PilA prevented the production of the abundant type IV pili, and inhibited electron transfer to iron oxides and electrodes in METs (Reguera et al., 2006, 2005). It was shown that deficient mutants on PilA form thinner biofilms on electrodes, and that the amount of dead cells increases with the distance to the electrode (Nevin et al., 2009; Reguera et al., 2006), indicating that pili are required for long-range electron transfer across the multilayer biofilms on anodes. It is however not understood by which underlying mechanisms this occurs, being this an area of open investigation.

The higher conductivity observed for *G. sulfurreducens* KN400 biofilms were shown to be associated with a higher abundance of electrically conductive pili and a lower outer-surface cytochrome content (Malvankar et al., 2012, 2011). In order to alter biofilms properties, a deletion mutant on the gene GSU1240 predicted to encode PilZ domain, has led to a strain with a highly cohesive biofilm that were six times more conductive that wild-type biofilms, and produced higher current densities than the wild-type strain (Leang et al., 2013).

Interestingly, although deletion of several cell-surface exposed proteins in *G. sulfurreducens* decreases electron transfer to electrodes, it has an opposite effect on electron transfer from electrodes. Indeed, it was shown that individual deletion mutants on PilA, OmcZ, OmcB and OmcS show increased current consumption in a dual chamber electrode system (Strycharz et al., 2011).

4. Structural studies of cell-surface exposed conductive proteins

In contrast to the large number of genes expressing putative cell-surface exposed conductive proteins, the number of structurally resolved proteins remains limited. This

is due to the challenges involved either in obtaining sufficient amount of proteins for their structural characterisation or in obtaining structural data on membrane associated proteins.

4.1. Structure of S. oneidensis MR-1 cell-surface exposed conductive proteins

The proteins used by *S. oneidensis* MR-1 to transfer electrons across the outer-membrane and into extracellular acceptors are the best understood of all the extracellular electron transport systems (Richardson et al., 2012b). Phylogenetic analysis of the outer-membrane cytochromes reveals that they can be differentiated into four clades: the MtrC, OmcA, MtrF and UndA clades (Edwards et al., 2012a; Edwards et al., 2015).

Crystal structures of representatives for each of these clades have been obtained: MtrF, OmcA and MtrC from *S. oneidensis* MR-1, and UndA from *Shewanella* sp. HRCR-6 (Clarke et al., 2011; Edwards et al., 2014; Edwards et al., 2012b; Edwards et al., 2015). The structures are well conserved between the members of the clades, despite poor amino acid sequence homology. Each cell-surface exposed cytochrome is comprised of 4 domains (domains I-IV). The N-terminal domain is a 7-strand β-barrel structure (domain I) that is connected to the pentaheme domain (domain II). An α-helix links domain II to a second 7 strand β-barrel (domain III), which is linked to the C-terminal multiheme domain (domain IV). This last domain contains 5 *c*-type hemes in MtrC, OmcA and MtrF, and 6 hemes in UndA. The domains are arranged such that both heme domains II and IV associate at the center of the structure, bringing hemes 1 and 6 within 6 Å of each other.

The two β-barrel domains that are characteristic of the *Shewanella* outer-membrane cytochromes share a common core structure. The β-strands are folded barrels with 7 β-

strands arranged in an antiparallel Greek-key fold, that is a common topology of soluble β-barrel proteins. A flexible N-terminal membrane anchor is connected to the first β-barrel that, when removed, causes the soluble proteins to dissociate from the cell-surface (Edwards et al., 2012b). Comparative alignment of the amino acid sequences revealed a completely conserved $CX_{8-15}C$ motif in the second β-barrel (domain IV). A second motif $CX_{2-8}C$ sequence was conserved in the N-terminal region of all sequenced OmcA, MtrF, UndA and approximately half of all known MtrC. The currently available structures of OmcA, UndA and MtrC show that the two cysteines of these motifs form disulfide bonds between adjacent β-strands. In MtrF the 3.2 Å resolution precluded defining the CX_XC of the N-terminal β-barrel domains, however a recent molecular dynamics simulation generated an MtrF structure with both CX_xC motifs forming disulphide bonding (Watanabe et al., 2017).

The hemes of domains II and IV are arranged in a cross-like configuration in the structure of every outer-membrane cytochrome. The cross consists of two intersecting chains of 8 and 4-5 hemes with distances of 4-6 Å between adjacent hemes, allowing for rapid electron exchange. The eight heme chain spans the entire length of the protein with terminal hemes 5 and 10 (11 for UndA) exposed at the edges of the protein. The second chain consists of four hemes arranged edge to edge across a distance of 45 Å in MtrC, OmcA and MtrF. The terminal hemes are not exposed, but are positioned at the interface of the β-barrel domains. In UndA the chain is five hemes across due to an extra heme in domain IV. After co-crystallisation with soluble iron nitrilotriacetic acid and iron citrate, soluble iron chelates were identified bound close to the extra UndA heme, indicating a possible substrate binding site. Indeed, kinetic studies on UndA

show that it is faster at reducing soluble iron chelates than other *Shewanella* cytochromes (Shi et al., 2011).

Superposition of the hemes from all four structures shows that the overall heme positions are heavily conserved, with the biggest differences in the structures being the extra heme in UndA and the position of heme 5 in domain I. In OmcA, heme 5 is displaced compared to MtrC and MtrF, while in UndA heme 5 is both displaced and rotated. The variation observed in heme 5 position would significantly affect the properties around the area of the heme, and could be responsible for the observed differences in respiration of manganese and iron oxides or soluble iron chelates (Shi et al., 2011). The position of heme 5 is conserved between MtrC and MtrF, which are proposed to form stable porin-cytochrome complexes with MtrAB and MtrDE respectively (Richardson et al., 2012b). This structural conservation would be consistent with a conserved protein-protein interface within the porin-cytochrome complex. A hematite binding motif close to heme 10 of MtrC and OmcA was previously identified through peptide phage display technology (Lower et al., 2008). This S/T-hyd/aro-S/T-P-S/T motif was found to bind hematite strongly and was observed in MtrC and OmcA but not in UndA or MtrF.

4.2. Structure of G. sulfurreducens PCA cell-surface exposed proteins

There is very little structural information about the cell surface cytochromes of *G. sulfurreducens* PCA. Only the structure of a solubilised form of OmcF has been reported, which has been solved by both X-ray crystallography and solution state NMR (Dantas et al., 2017; Pokkuluri et al., 2009). This monoheme cytochrome is anchored to the membrane via an N-terminal lipid anchor and has significant similarity to cytochrome c_6, the electron transfer partner within photosystem I of plants and

cyanobacteria. OmcF contains a *c*-type heme cofactor where the hexacoordinate iron atom ligands are histidine and methionine.

The nanowires from *G. sulfurreducens* PCA are polymeric assemblies of proteins that belong to the type IV family of pilin proteins (Reardon and Mueller, 2013). The structure of these proteins was determined by NMR spectroscopy, that showed that PilA consists of a N-terminal α-helix composed by 52 amino acid residues (~75 Å long) combined with a short and flexible C-terminal region. It was demonstrated that the aromatic residues present in this protein are highly conserved among species that utilize similar EET mechanisms (Vargas et al., 2013). It is the clustering of these amino acid residues that plays an important role in electron transfer along the nanowire (Malvankar et al., 2011; Reardon and Mueller, 2013). Indeed, substitution of these aromatic amino acids with alanine, makes the nanowires non-conductive, impairing the reduction of iron oxides and production of current in MFCs (Vargas et al., 2013).

5. Functional properties of cell-surface exposed conductive proteins

5.1. Redox properties of cell-surface exposed conductive proteins

The redox characterization of cell-surface exposed proteins is not a straightforward process due to the high number of cofactors present in most of these proteins (Paquete and Louro, 2014). While the redox properties of a protein with only one redox center can be determined directly from the application of the Nernst equation, in multicenter redox proteins this requires the discrimination of the reduction potential of each individual center (Paquete and Louro, 2014). This usually depends on experimental techniques that allow to monitor the titration of each redox center individually (Fonseca et al., 2009).

For the monoheme cytochrome OmcF from *G. sulfurreducens* PCA, the fit of the redox titrations followed by visible spectroscopy with a Nernst equation with n=1 allowed to determine the values of reduction potentials of the soluble form of OmcF of +180 mV and +127 mV (vs standard hydrogen electrode, SHE) at pH 7 and 8, respectively (Pokkuluri et al., 2009). The midpoint potential is significantly higher than other surface exposed cytochromes, making it unlikely to be directly involved in extracellular electron transfer, as has been demonstrated (Kim et al., 2008).

The complexity of the other cell-surface exposed proteins, with a high number of hemes, and the absence of data that discriminates their individual hemes, has precluded the determination of the detailed characterization of their redox properties. Up to date, only a macroscopic characterization of their redox properties has been achieved. Potentiometric titrations monitored by visible spectroscopy and electron paramagnetic resonance spectroscopy, revealed that the hemes in MtrC from *S. oneidensis* MR-1 titrate over a broad potential range that spans between ~+100 mV and ~-400 mV (vs SHE) (Hartshorne et al., 2007). This potential window was also observed by protein film voltammetry, that also demonstrated that MtrC presents redox-Bohr effect (Firer-sherwood et al., 2008; Hartshorne et al., 2007).

The titration envelope observed for OmcA is very similar to the one observed for MtrC (Firer-sherwood et al., 2008). Furthermore, it was also demonstrated that this decaheme cytochrome also exhibits redox-Bohr effect within physiological pH (Firer-sherwood et al., 2008). Modelling of titration curves of OmcA from *S. frigidimarina* NCIMB400 led to the identification of two sets of near iso-potential components centered at -243 mV and -324 mV (vs SHE) with contributions of 30% and 70 %, respectively (Field et al., 2000), while modelling of titration curves of OmcA from *S. oneidensis* MR-1 with the sum of two Nernst curves with equal contribution led to two

groups of hemes with -73 mV and -243 mV (vs SHE) (Bodemer and Antholine, 2010). In the case of OmcA from *S. oneidensis* MR-1, the reduction potential of two hemes appear to shift in the presence of detergents, suggesting that two hemes are more exposed to the solvent than the remaining hemes, that are more buried in the protein and therefore less affected by changes in the external environment (Bodemer and Antholine, 2010). The X-ray structure suggests that these are most likely hemes 5 and 10 that are at opposite ends of the eight heme chain across the OmcA structure.

The characterization of MtrF revealed that this cell-surface exposed protein behaves similarly to MtrC, although the titration envelope is shifted to more positive values (David J. Richardson et al., 2012). Computational methods were used to determine the redox potentials of the ten hemes of MtrF in aqueous solutions (Breuer et al., 2012). The free energy landscape obtained for this protein revealed that there is no significant potential bias along the protein, suggesting that under aqueous conditions this protein works as a reversible two-dimensional conductor (Breuer et al., 2012). Recently, Watanabe and co-workers determined the reduction potential for the ten hemes in MtrF by solving the linear Poisson-Boltzmann equation and taking in consideration the protonation states of all titratable residues and heme propionic groups (Watanabe et al., 2017). This study revealed that the electron transfer pathway may proceed from domain IV to II, but when MtrF is reduced, the direction of the electron transfer pathway switches and FMN, in domain I and III, becomes the terminal electron acceptor (Watanabe et al., 2017).

The redox behavior of OmcZ from *G. sulfurreducens* PCA revealed that it titrates in a broad range from -60 mV to -420 mV (vs SHE). This large potential range was attributed to the wide range of the redox potentials of the eight hemes in the molecule (Inoue et al., 2010). The potential range of OmcZ covers the lowest anode potential

observed in MFC of this organism, suggesting that it has a low enough potential to directly transfer electrons to the anode (Inoue et al., 2010).

The apparent midpoint reduction potential of OmcS from *G. sulfurreducens* PCA at pH 7 was -212 mV (vs SHE), determined by electrochemical redox titrations (Qian et al., 2011). The low apparent redox potential of this hexaheme cytochrome suggested that it could react with a wide range of electron acceptors, including soluble and insoluble compounds (Qian et al., 2011).

5.2. Kinetic studies of cell-surface exposed conductive proteins

Electron transfer between cell-surface exposed proteins and electrodes can be determined through the measurement of interfacial electron transfer kinetics (Léger et al., 2006). This was performed for MtrC giving an estimated rate of 200-300 s^{-1} (Firer-sherwood et al., 2008; Hartshorne et al., 2009). The value is very similar to that observed for the MtrCAB complex (Hartshorne et al., 2009), suggesting that MtrC is the cytochrome that interacts primarily with the electrode surface.

Kinetic studies showed that OmcA and MtrC are kinetically competent to catalyze the reduction of soluble iron in whole cells of *Shewanella*, but are not responsible for direct electron transfer to insoluble iron-containing minerals (Ross et al., 2009). It was shown that addition of flavins increased the reaction rates of these proteins, demonstrating the importance of electron shuttles in the reduction of solid iron compounds (Ross et al., 2009).

For MtrF, computational simulations were used to explore the microscopic kinetics of each heme pair (Breuer et al., 2014). These studies showed that electron flow through solvated MtrF can occur at 10^4-10^5 s^{-1}, which is consistent with the measured rate for the related multiheme protein complex MtrCAB (White et al., 2013).

Despite the similar three-dimensional structure of the outer-membrane cytochromes, kinetic studies have shown that the undecaheme outer-membrane cytochrome UndA reduces soluble iron chelates faster than its homologues, MtrC and OmcA from *S. oneidensis* MR-1. It was proposed that this cytochrome serves as an extracellular metal reductase, being involved in iron and uranium reduction in *Shewanella* sp. strain HRCR-6 (Shi et al., 2011).

5.3. Interactions studies with physiological partners (electron shuttles and other proteins)

The availability of the structure of the outer-membrane cytochromes from *S. oneidensis* MR-1 prompted the study of the molecular details of their interaction with electron shuttles, including flavins (Paquete et al., 2014). NMR spectroscopy revealed that despite the common architecture of the heme core of these outer-membrane cytochromes, there are substantial differences in their interactions with soluble redox shuttles. Whereas OmcA, MtrC and UndA bind AQDS in the vicinity of hemes, this does not occur in the case of MtrF (Paquete et al., 2014). For the case of FMN, the monitoring of its phosphorous atom revealed that it does not bind to MtrF nor UndA, but binds to OmcA and MtrC with dissociation constants of 29 and 256 uM, respectively (Paquete et al., 2014). Interestingly, the binding stoichiometry to these two outer-membrane cytochromes matches the number of redox-active disulfide bonds found in these proteins (Edwards et al., 2015, 2014). These disulfide bonds are proposed to be redox active under physiological conditions leading to conformational changes in the outer-membrane cytochromes upon reduction and enhanced affinity for redox shuttles such as flavins (Edwards et al., 2015). Molecular docking simulations using Autodock 4 showed that the lowest energy solutions for AQDS binding to UndA are

found near hemes 2 and 7. The same procedure revealed that in the case of OmcA the polypeptide appears to direct different shuttles to different hemes according to their electrostatic nature. FMN and AQDS that are negatively charged dock near heme 2, with the conserved calcium atom that is not derived from the crystallization buffer playing an important role. Docking of riboflavin, which is neutral, occurs near hemes 9 and 10, and docking of phenazine methosulphate which is positively charged occurs near the heme 10. These computational results are corroborated by NMR spectroscopy data that show different heme signals being disturbed upon binding of the various shuttles to OmcA (Paquete et al., 2014). Two independent sets of molecular docking simulations of riboflavin binding to OmcA revealed a preference for binding near hemes 5 and 7, with heme 7 being considered the most likely of the two possibilities due to the putative role of heme 5 in protein-protein interaction (Babanova et al., 2017; Hong and Pachter, 2016). Docking of FMN to OmcA revealed solutions similar to those obtained for riboflavin (Babanova et al., 2017) The NMR spectroscopy data showing different heme signals being disturbed upon binding of FMN or riboflavin reveals that despite the similarity of the calculated docking solutions there is a clear discrimination between the binding sites for the two shuttles under the experimental conditions (Paquete et al., 2014).

The availability of the crystal structure of MtrC prompted also the investigation of the molecular details of shuttle binding to this protein. Binding of FMN was found to take place close to heme 2 with a calculated dissociation constant of 490 uM (Breuer et al., 2015a). Molecular dynamics simulations revealed that the reductive cleavage of the disulfide bond in this protein can lead to conformational changes in the structure. These changes reveal a new binding pocket near to heme 4 with a poise that is compatible with electron transfer but does not lead to an increase in the affinity for FMN (Breuer et al.,

2015a). Four possible docking positions for FMN were identified near hemes 1, 4, 7 and 9 using the crystal structure of MtrC with the shortest distance for heme 4. By contrast, riboflavin does not show a solution near heme 4 and docking near heme 7 shows the shortest distance (Babanova et al., 2017).

In contrast to spectroscopic and computational results indicating weak binding of shuttles to outer-membrane cytochromes, voltammetric signals of electrode grown *Shewanella* assigned to presumed flavocytochromes were interpreted as indicating strong flavin binding to outer-membrane cytochromes (Okamoto et al., 2014a). This different result may have two causes: first a strong increase of the affinity of flavins for outer-membrane cytochromes with increasing ionic strength was deduced from voltametric data (Okamoto et al., 2014a); second, the outer-membrane cytochromes in living cells are not in the fully oxidized state for which crystal structures were obtained and the disulfide bridges may be reduced leading to the proposed increase in flavin affinity (Edwards et al., 2015).

The other model electroactive Gram-negative bacterium *G. sulfurreducens* PCA is generally accepted to perform DET because: i) it is unable to reduce iron that is entrapped in alginate beads with pore size too small for direct cell contact with the medium, and ii) current production of electrode grown culture is not affected by medium exchange (Bond and Lovley, 2003; Nevin and Lovley, 2000). This does not mean that outer-membrane cytochromes from *G. sulfurreducens* PCA are not able to opportunistically use flavins that may be available in the medium to enhance EET (Okamoto et al., 2014b).

Conclusions

The increasing interest in METs as promising devices for sustainable industrial processes has stimulated the investigation of EET mechanisms performed by electroactive organisms. This has relied on the identification and characterization of cell-surface exposed conductive proteins responsible for exchanging electrons with electrodes. These fall into one of four types of proteins: porin-cytochrome complexes, cell-surface exposed cytochromes, conductive nanowires and other redox proteins. To broaden our understanding of the diversity of the portfolio of proteins that sustain EET it is necessary to characterize cell-surface exposed conductive proteins from other electroactive organisms, including those that do not contain MHC.

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Figure Captions

Figure 1. Alignment of genes identified to encode for proteins belonging to porin-cytochrome complexes. (A) in *S. oneidensis* MR-1; (B) in other representative electroactive organisms. The genes are colored for putative function in EET (yellow are porin proteins, red are *c*-type cytochromes, green are iron-sulfur proteins and purple molybdopterin containing proteins).

Scheme Captions

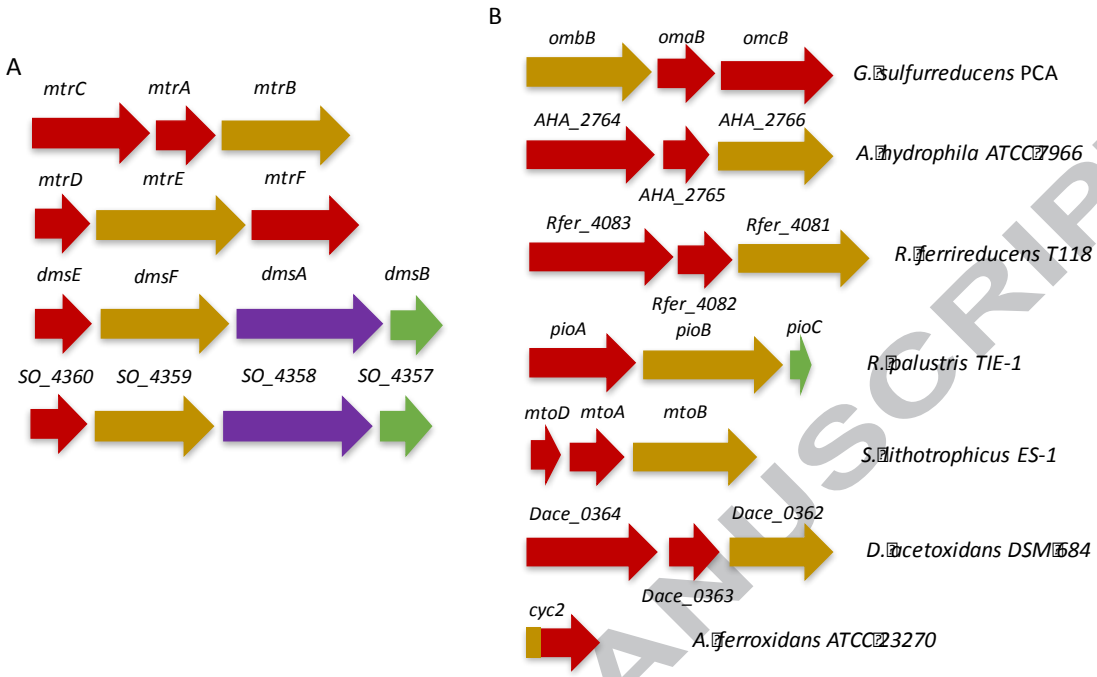
Scheme 1. Representation of the four types of cell-surface exposed conductive proteins from electroactive organisms: (A) porin-cytochrome protein complexes; (B) cell-surface exposed cytochromes, which can be lipoproteins or cytochromes loosely bound to the cell surface; (C) conductive nanowires and (D) other redox proteins. The proteins are colored for type of protein: red are *c*-type cytochromes, yellow are porins, grey are pilin, blue and green are iron-sulfur and copper proteins, respectively.

Table 1. Cell-surface exposed cytochromes identified in electroactive organisms.

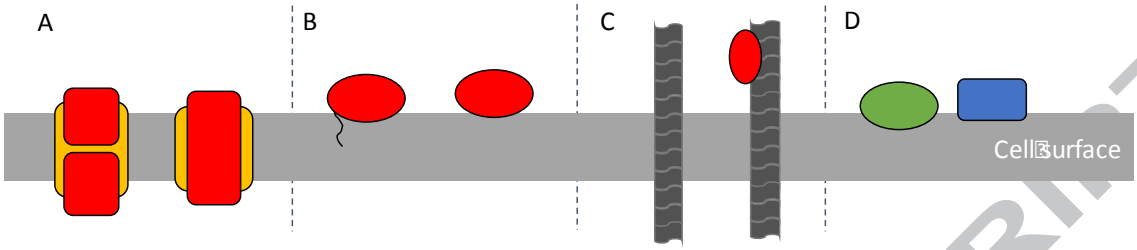
Microorganism	Locus tag	Protein name	Heme bind. motif [a]	Number of aminoacids	Reference
Acidithiobacillus ferrooxidans ATCC 23270	AFE_3153	Cyc2	1	485	(Yarzábal et al., 2002)
Aeromonas hydrophila ATCC 7966	AHA_2764		10	743	(Shi et al., 2012)
Desulfuromonas acetoxidans DSM 684	Dace_0364	OmcB	13	762	(Alves et al., 2011)
Geobacter sulfurreducens PCA	GSU2737	OmcB	12	744	(Kim et al., 2005; Leang et al., 2010; Liu et al., 2014; Richter et al., 2009)
	GSU2731	OmcC	12	768	
	GSU0618	OmcE	4	232	
	GSU2432	OmcF	1	104	
	GSU2076	OmcS	6	432	
	GSU2432	OmcZ	8	473	
Ferroglobus placidus	Ferp_0670		35	1732	(Smith et al., 2015)
	Ferp_0672		31	1639	
Rhodoferax ferrireducens T118	Rfer_0244		4	261	(Liu et al., 2014; Risso et al., 2009)
	Rfer_4079		10	885	
	Rfer_4080		10	936	
	Rfer_4083	MtrC	10	826	
Rhodopseudomonas palustris TIE-1	Rpal_0817	PioA	10	540	(Jiao and Newman, 2007)
Shewanella oneidensis MR1	SO_1778	MtrC	10	671	(Beliaev et al., 2001; Hartshorne et al., 2007; Myers and Myers, 1997)
	SO_1780	MtrF	10	639	
	SO_1779	OmcA	10	735	
Sideroxydans lithotrophicus ES-1	Slit_2497	MtoA	10	355	(Shi et al., 2012)
Thermincola potens JR	TherJR_2595		9	525	(Carlson et al., 2012)

[a] The heme binding motif is identified by the sequence CXXCH, where X is any aminoacid.

Figure 1



Scheme 1



**Electron transfer process in microbial electrochemical technologies: the
role of cell-surface exposed conductive proteins**

Highlights

- Electroactive organisms in MET perform extracellular electron transfer processes
- Extracellular electron transfer relies on cell-surface exposed conductive proteins
- There are 4 groups of cell-surface conductive proteins in electroactive organisms
- Most electroactive organisms support their activity with multiheme cytochromes