MicroReview

Extracellular reduction of solid electron acceptors by Shewanella oneidensis

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Summary

Shewanella oneidensis is the best understood model organism for the study of dissimilatory iron reduction. This review focuses on the current state of our knowledge regarding this extracellular respiratory process and highlights its physiologic, regulatory and biochemical requirements. It seems that we have widely understood how respiratory electrons can reach the cell surface and what the minimal set of electron transport proteins to the cell surface is. Nevertheless, even after decades of work in different research groups around the globe there are still several important questions that were not answered yet. In particular, the physiology of this organism, the possible evolutionary benefit of some responses to anoxic conditions, as well as the exact mechanism of electron transfer onto solid electron acceptors are yet to be addressed. The elucidation of these questions will be a great challenge for future work and important for the application of extracellular respiration in biotechnological processes.

Introduction

Exactly 30 years ago in 1988 Ken Nealson and Charles Myers published a report in which they describe bacterial manganese reduction and growth with manganese as the sole electron acceptor. Their model organism was a bacterium at this point named Alteromonas putrefaciens MR-1 (Nealson and Myers, 1988). Since then, the genus of this strain has been renamed Shewanella to honour the Scottish microbiologist James M. Shewan (MacDonell and Colwell, 1985) and the species is called oneidensis, since the organism was isolated from lake Oneida in Upstate New York (Venkateswaran et al., 1999). The genus Shewanella shows a very high respiratory versatility. Most of its representatives can reduce a variety of inorganic and organic electron acceptors that can be soluble (e.g. dimethylsulfoxide (DMSO), fumarate, nitrate, nitrite, trimethylamine-N-oxide (TMAO), oxygen, humic acids) or in a solid state (ferrihydrite, hematite, birnessite, electrodes). Their niches seem to be redox stratified environments in which the electron donor is not the limiting factor (Nealson and Scott, 2006; Fredrickson et al., 2008). To date, Shewanella oneidensis MR-1 is the best understood model to study extracellular electron transfer processes. Its strategy is to use c-type cytochromes as electron transfer proteins and flavins to facilitate the electron transfer process. In fact, the use of c-type cytochromes is a widespread solution to transfer electrons to the cell surface within various bacterial genera and a high number of c-type cytochrome encoding genes is characteristic for many dissimilatory metal reducers (Heidelberg et al., 2002). S. oneidensis is a Gram-negative organism and respiratory electrons will have to pass two membranes and the periplasm in order to get into contact with the solid electron acceptor at the cell surface. The final electron transfer step for the reduction of insoluble electron
acceptors in \textit{S. oneidensis} seems to be rather unspecific. Consequently, outer membrane cytochromes can reduce a wide range of substrates ranging from different insoluble minerals and electrodes to soluble compounds like humic acids or metal complexes (Richter et al., 2012; Table 1). The reduction of toxic metals that become insoluble upon reduction like uranium or chromium and the reduction of electrodes attracted the interest of applied microbiologists and engineers to the physiology of the model organism \textit{S. oneidensis}. Moreover, its ability to reduce insoluble iron minerals is of high importance from an environmental science perspective. Iron is the fourth most abundant element in soil. Hence, its reduction has widespread implications for biogeochemical cycling. The reductively dissolved ferrous iron is an important trace nutrient and can initiate a number of environmentally relevant abiotic redox transformations as for instance the reduction of nitroaromatic compounds and azo dyes (Rügge et al., 2004).

In this review, we will follow the path of the electrons from the cytoplasm to the cell surface (illustrated in Fig. 1). We will summarize the achievements of many groups working with \textit{S. oneidensis} and will highlight novel results and research directions. Even after 30 years of research, \textit{S. oneidensis} has still secrets that are not understood and we will formulate several open research questions at the end of the review.

\textbf{The central carbon metabolism in \textit{S. oneidensis}}

\textit{Shewanella oneidensis} can use only a limited number of carbon sources under anoxic conditions. Here, growth was so far reported with lactate, pyruvate, N-acetyl-glucosamine and DNA (Lovley et al., 1989; Pinchuk et al., 2008; Hunt et al., 2010; Brutinel and Gralnick, 2012a). Still, under oxic conditions growth is possible with a wider variety of substrates including different dipeptides, amino acids and short organic acids (Table 2). While some \textit{Shewanella} strains are able to grow on glucose, \textit{S. oneidensis} lacks the ability to import and phosphorylate glucose to glucose-6-phosphate. Nevertheless, Howard and colleagues could show a rapid adaptation of \textit{S. oneidensis} to aerobic growth on glucose (Howard et al., 2012). This ability is due to a deletion in a genomic region that includes \textit{nagR}, the gene for the regulator of the N-acetyl-glucosamine catabolism. This leads to the constitutive expression of the N-acetyl-glucosamine permease and kinase genes. The corresponding enzymes both have a promiscuous activity toward glucose (Chubiz and Marx, 2017). Other researchers achieved growth on glucose also under anoxic conditions by the heterologous expression of a glucose facilitator and a glucokinase and established a glucosedependent current production in a bioelectrochemical system (Choi et al., 2014; Nakagawa et al., 2015). An adaptation strategy similar to the experiments conducted by Howard lead recently to the development of an \textit{S. oneidensis} strain that can use xylose as carbon and electron source under oxic and anoxic conditions (Sekar et al., 2016). Now, with the design of xylose and glucoseconsuming strains, lignocellulose hydrolysates could be used as a sustainable carbon source for biotechnological conversions catalysed by \textit{S. oneidensis}.

\textit{Shewanella oneidensis} uses the EntnerDoudoroff (ED) pathway for sugar (N-acetyl-glucosamine) catabolism (Scott and Nealson, 1994; Serres and Riley, 2006; Scott and Nealson, 2014; Nakagawa et al., 2015). The corresponding enzymes both have a promiscuous activity toward glucose (Chubiz and Marx, 2017). Other researchers achieved growth on glucose also under anoxic conditions by the heterologous expression of a glucose facilitator and a glucokinase and established a glucosedependent current production in a bioelectrochemical system (Choi et al., 2014; Nakagawa et al., 2015). An adaptation strategy similar to the experiments conducted by Howard lead recently to the development of an \textit{S. oneidensis} strain that can use xylose as carbon and electron source under oxic and anoxic conditions (Sekar et al., 2016). Now, with the design of xylose and glucoseconsuming strains, lignocellulose hydrolysates could be used as a sustainable carbon source for biotechnological conversions catalysed by \textit{S. oneidensis}.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Extracellular electron acceptor & Main terminal reductase & \textbf{Main terminal reductase} \\
\hline
DMSO & DmsA/B & Grainick et al. (2006) \\
Fe(III) minerals & MtrC & Coursolle and Grainick (2010) \\
Soluble Fe(III) complexes & MtrC & Coursolle and Grainick (2010) \\
Mn(IV) minerals & MtrC, OmcA, MtrF & Bretschger et al. (2007); Gao, Barua et al. (2010) \\
Soluble Co(III) complexes (in presence of MgSO$_4$) & MtrC, OmcA & Hau et al. (2008) \\
Soluble heavy metals & MtrC, OmcA & Marshall et al. (2006) \\
& OmcA, MtrC & Gao, Barua et al. (2010); Belchik et al. (2011) \\
U$^{V+}$ & MtrC, OmcA & Myers et al. (2004) \\
C$^{V+}$ & MtrC, OmcA, HyaB & Marshall et al. (2007) \\
V$^{II}$ & MtrC, OmcA & Kim et al. (2012) \\
Tc$^{VII+}$ & MtrC, OmcA & Lies et al. (2005) \\
Tellurite & MtrC & Marsili et al. (2008); Coursolle et al. (2010) \\
AQDS & MtrC & Coursolle et al. (2010); Jiao et al. (2011) \\
Electrodes & MtrC, OmcA & Bretschger et al. (2007); Coursolle et al. (2010); Jiao et al. (2011) \\
Graphite, glassy carbon, graphene oxide & MtrC, OmcA & \\
\hline
\end{tabular}
\caption{Extracellular electron acceptors of \textit{Shewanella oneidensis} MR-1 with known electron transport pathways elucidated by mutant studies. Of note, the table lists only the most important terminal reductase for the individual electron transfer pathways.}
\end{table}
Yang et al., 2006). Contrary to what was believed for a long time, the ED pathway is widespread within aerobic or facultative anaerobic heterotrophic organisms (Chen et al., 2016). It has a lower ATP yield compared to glycolysis but it is thermodynamically more favourable, which results in considerably less costs for the amount of enzymes required to sustain the flux through the pathway (Flamholz et al., 2013). In other words, the ED pathway could be advantageous under conditions where ATP is not the limiting factor, which might be the case in respiratory organisms. Still, under anaerobic respiratory growth conditions ATP from substrate level phosphorylation is the only available energy pool, while electron transfer to the terminal electron acceptor is not accompanied with an oxidative phosphorylation-based ATP production. Consequently, a deletion mutant lacking all genes of the ATP synthase showed almost no phenotype under anoxic growth conditions with fumarate as electron acceptor, while it was highly affected under oxic conditions (Hunt et al., 2010).

In line with the apparent anaerobic substrate level phosphorylation based ATP production is a common downregulation of the citric acid cycle under anoxic conditions, which mainly serves for the production of precursor molecules for biomass growth. Uncommon is that the organism uses a longer oxidative branch to produce succinyl-CoA instead of a reductive branch leading from oxaloacetate to succinyl-CoA (Brutinel and Gralnick, 2012b). Also uncommon is the apparent use of the 2-methylcitrate synthase under anoxic conditions instead of the canonical citrate synthase for the conversion of oxaloacetate and acetyl-CoA into citrate (Brutinel and Gralnick, 2012b). Respiratory electrons enter the quinone-pool under anoxic conditions via NADH oxidation mainly by Nqr1 or the oxidation of carbon compounds by D- or L-lactate dehydrogenases or formate dehydrogenases (Myers and Myers, 1993; Saffarini et al., 2002; Myers et al., 2004; Pinchuk et al., 2010, 2009; Duhl et al., 2018). Recent results indicate that the redox potential of the terminal electron acceptor influences the percentage to which the NADH- and formate-dehydrogenases contribute to the reduction of the quinone pool. Using a bioelectrochemical system and a working electrode poised to either +0.5 V, +0.2 V or 0 V vs. SHE (Standard Hydrogen Electrode), Hirose and colleagues could elucidate that a deletion mutant in all four NADH dehydrogenase encoding gene clusters is almost
completely unable to produce current at +0.5 V (Hirose et al., 2018). In contrary, the difference in current production was not significant compared to the wild type at the lower potentials tested. This observation, that higher redox potentials of anoxic electron acceptors lead to a higher percentage of electron flux via NADH dehydrogenases could also be verified using fumarate \((E^0 = –0.03 \text{ V})\), nitrate \((E^0 = –0.43 \text{ V})\) and \(\text{MnO}_2 \) \((E^0 = –0.53 \text{ V})\). The NADH dehydrogenase mutant showed a decreased ability to use nitrate and \(\text{MnO}_2\) but showed a similar growth rate compared to the wild type with fumarate. The regulation of the contribution of these two ways to reduce the intracellular quinone pool is advantageous to the cell, as NADH dehydrogenase-dependent quinone reduction leads to a higher production of proton motive force per electron transferred compared to formate dehydrogenase. Not only the contribution of membrane bound dehydrogenase but also that of inner membrane quinols change with the potential of the electron acceptor. Low potentials lead predominantly to a reduction of the cellular menaquinone pool while higher potentials also trigger the use of ubiquinone-8 as electron carrier (Hirose et al., 2018). Still, the dominant quinone under anoxic conditions seems to be menaquinone-7 \((\text{MQ}-7, \text{ Venkateswaran et al.}, 1999)\), which has a dual function for the transfer of respiratory electrons onto the cell surface, as it is also a specific cofactor for the menaquinol oxidase and tetraheme \(c\)-type cytochrome CymA \((\text{cytoplasmic membrane protein A})\) located on the outer leaflet of the cytoplasmic membrane (McMillan et al., 2012). Interestingly, the dominant catalytic mode of CymA under \textit{in vitro} conditions is the reduction of MQ-7 and hence the reverse of its natural role (McMillan et al., 2013). Nevertheless, it was also shown \textit{in vivo} that it is possible to use CymA for the import of electrons into the quinone pool if the organism is supplied with a cathode poised to a suitable potential as electron donor and either fumarate or oxygen as electron acceptor (Ross et al., 2011; Rowe et al., 2018).

CymA distributes electrons to a variety of electron transfer pathways, with terminal electron acceptors that are reduced either within the periplasm (nitrate, nitrite, fumarate, hydrogen peroxide) or at the cell surface (e.g. metal oxides, quinone analogues, dimethyl sulfoxide (DMSO)) (Myers and Myers, 2000; Schwabla et al., 2002; Table 2. Carbon sources sustaining the metabolism of \textit{Shewanella oneidensis} MR-1. Of note, the study by Rodrigues et al. used the Biolog system for analysis of substrate conversion (Rodrigues et al., 2011). The table lists only compounds that were metabolized similarly to lactate.

| Oxic          | Anoxic                        |
|---------------|-------------------------------|
| 1C & 2C       |                               |
| Acetate       | Nealon and Myers (1990)       |
| Propionate    | Scott and Nealon (1994)       |
| Lactate       | Nealon and Myers (1990)       |
| Pyruvate      | Nealon and Myers (1990)       |
| Glycerate     | Rodionov et al. (2010)        |
| Succinate     | Venkateswaran et al. (1999)   |
| Fumarate      | Venkateswaran et al. (1999)   |
| 3C            |                               |
| Galactose     | Venkateswaran et al. (1999)   |
| Inosine       | Driscoll et al. (2007)        |
| N-Acetyl-D-Glucosamine | Hunt et al. (2010)      |
| Casamino acids| Driscoll et al. (2007)        |
| DNA           | Pinchuk et al. (2008)         |
| N-Acetyl-D-Glucosamine | Hunt et al. (2010)      |
| DNA           | Pinchuk et al. (2008)         |
| Biochemical data |                                  |
| Adenosine     | Rodrigues et al. (2011)       |
| Inosine       | Rodrigues et al. (2011)       |
| Uridine       | Rodrigues et al. (2011)       |
| Methylypyruvate| Rodrigues et al. (2011)       |
| D-Lactic Acid Methyl Ester | Rodrigues et al. (2011)   |
| Gly-Glu       | Rodrigues et al. (2011)       |
| 2’-Deoxyadenosine| Rodrigues et al. (2011)   |
| Gly-Asp       | Rodrigues et al. (2011)       |
| Gelatin       | Rodrigues et al. (2011)       |
| 4C            |                               |
| Galactose     | Venkateswaran et al. (1999)   |
| Inosine       | Driscoll et al. (2007)        |
| N-Acetyl-D-Glucosamine | Hunt et al. (2010)      |
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| Gly-Asp       | Rodrigues et al. (2011)       |
| Gelatin       | Rodrigues et al. (2011)       |
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| Gly-Glu       | Rodrigues et al. (2011)       |
| 2’-Deoxyadenosine| Rodrigues et al. (2011)   |
| Gly-Asp       | Rodrigues et al. (2011)       |
| Gelatin       | Rodrigues et al. (2011)       |
| N-Acetyl-D-Glucosamine | Hunt et al. (2010)      |
| DNA           | Pinchuk et al. (2008)         |
| Biochemical data |                                  |

\(^{a}\)Mixed results, see also Serres and Riley, (2006).
\(^{b}\)contrary to Biolog data by Rodrigues et al. (2011).
2003; Gralnick et al., 2006; Schuetz et al., 2011). Since CymA is also necessary for nitrate and MnO$_2$ reduction and Hirose and colleagues observed also the use of ubiquinone-8 as electron carrier under anoxic conditions, it must be possible to oxidize with this enzyme menaquinol and ubiquinol. The redox potential window of CymA is between −0.3 and 0 V (Hirose et al., 2018). Hence, the oxidation of ubiquinol-8 would most probably necessitate the completely oxidized state of CymA to render this reaction thermodynamically feasible.

**Electron transfer in the periplasmic space of Shewanella**

The periplasm of *S. oneidensis* spans approximately 235 Å and for this reason was proposed to be too wide for direct electron transfer between CymA and the decaheme metal reducing protein A (MtrA) bound to the outer membrane complex MtrCAB (Dohnalova et al., 2011; Fonseca et al., 2013; Edwards et al., 2018). Multiheme cytochromes predicted to be localized to the periplasm of *S. oneidensis* and present in high amounts during anaerobic growth may receive electrons from CymA and transfer them to the inner face of the outer membrane, including to MtrA (Richardson et al., 2012). The most abundant periplasmic proteins found in anaerobi- cally grown *Shewanella* cells are the monoheme cytochrome ScyA, the fumarate reductase FccA and the small tetraheme cytochrome STC (Tsapin et al., 2001; Meyer et al., 2004). Although the genes of these cytochromes are up-regulated during extracellular respiration (Rosenbaum et al., 2012), individual gene deletion mutants suggested that none of these proteins per se play a critical role in metal reduction (Schuetz et al., 2009; Coursolle and Gralnick, 2010; Gao, Barua et al., 2010). Recent studies have shown that this protein is involved in several anaerobic respiratory processes (Fonseca et al., 2013; Alves et al., 2015) including the reduction of solid electron acceptors at the cell surface. STC functions as an electron transfer hub, receiving electrons from CymA and distributing them to a number of terminal oxidoreductases (Alves et al., 2015). It is able to interact with MtrA for the reduction of metal compounds (Fonseca et al., 2013), DmsE for the reduction of DMSO, octaheme tetrathionate reductase (OTR) for the reduction of nitrogen compounds, but not with c-type cytochrome Nir for nitrite reduction (Alves et al., 2015). This study, together with Fonseca et al. (2009), also revealed that STC does not operate as a molecular wire, and that it functions like a *cul-de-sac* that forces electrons to enter and leave the protein by the same heme (Fonseca et al., 2009; Alves et al., 2015). This enables *Shewanella* to transfer electrons within the periplasm in a controlled and efficient manner, preventing the risk of diverting electrons to side redox pathways or production of radical species that would damage the cell.

A double deletion mutant of *S. oneidensis* in both *fccA* and *cctA* (the gene encoding STC) has shown that these proteins share a functional redundancy (Sturm et al., 2015), being both involved in extracellular respiration. These proteins have an overlapping activity and at least one is necessary for coupling respiratory oxidation of...
CymA to efficient electron transfer to ferric citrate, DMSO and nitrate (Sturm et al., 2015). Interestingly, in vitro studies have shown that STC and FccA do not interact with each other (Fonseca et al., 2013), which suggests the coexistence of two non-mixing redox pathways to transfer electrons across the periplasmic gap to terminal reductases, one involving FccA and the other involving STC (Fonseca et al., 2013).

Electron flux across the outer membrane

In *S. oneidensis* the electron flux across the outer membrane can occur via one of four porin cytochrome conduits; the MtrCAB complex, (Richardson et al., 2012); the MtrFED complex (McLean et al., 2008) the DmsEFA DMSO reductase system (Gralnick et al., 2006) and the SO_4359SO_4360 system (Schicklberger et al., 2013). Of these the MtrCAB complex, which is constitutively expressed, is the best characterized and has a clear role in dissimilatory metal reduction. The MtrFED and SO4359-60 complexes have no clear phenotype, but allow extracellular respiration on soluble Fe(III)chelates when exogenously expressed in mtrCAB deletion strains (Coursolle and Gralnick, 2010; Schicklberger et al., 2013). Both MtrCAB and MtrFED porin cytochrome complexes contain a transmembrane barrel that forms a putative channel in the outer membrane. Two multiheme cytochromes enter the channel from opposite sides of the membrane and bind close enough so that electrons are capable of hopping between hemes of adjacent cytochromes, forming a functional electron conduit across the outer membrane (Hartshorne et al., 2009). This electron conduit can be isolated as a stable complex with a length of approximately 170 Å and inserted into proteoliposome models to show bidirectional electron transfer across the lipid bilayer, as well as rapid reduction of different types of insoluble iron oxide (White et al., 2013; Edwards et al., 2018).

The genes mtrC, mtrA and mtrB are expressed within the same operon and the synthesized peptides are transported through the Sec pathway to the periplasm in an unfolded state (Shi et al., 2008). Both MtrC and MtrA are folded by the *S. oneidensis* cytochrome c maturation pathway (ccm) in the periplasm and MtrC is transported to the cell surface by the Type II secretion pathway while MtrA remains in the periplasm (DiChristina et al., 2002). MtrB is transported through the cytoplasmic membrane and across the periplasm before assembling as a porin-cytochrome complex in the outer membrane with MtrA (Schicklberger et al., 2011). Extracellular MtrC, which is anchored to the outer membrane by a covalently attached lipid, binds to the MtrAB complex on the surface of the cell to generate the fully functional MtrCAB complex (Hartshorne et al., 2009; Edwards et al., 2018).

The interactions between the three proteins indicate that MtrA interacts more tightly with MtrB, and is essential for correct folding of MtrB. Different knockout studies have shown that correct MtrB and folding in the outer membrane requires the presence of MtrA (Hartshorne et al., 2009; Schicklberger et al., 2011) and it is possible that MtrA also functions as a chaperone for MtrB, by acting as a scaffold for MtrB assembly.

The molecular structure of MtrB is not yet known, but topology predictions suggest that MtrB is a transmembrane β-barrel with 28 antiparallel β-strands (Beliaev and Saffarini, 1998). Consistent with the structure of other outer membrane cytochromes, the loops connecting the β-strands on one side of the predicted β-barrel are longer than the other. These extended loops are typically exposed on the cell surface, allowing them to interact with extracellular ligands. They also play an important role in the maturation of transmembrane barrels, as they fold inside the barrel during membrane insertion and then interact with the charged groups on the membrane exterior. Surprisingly, the extended loops of MtrB are not on the surface but appear to interact with MtrA, rather than MtrC, suggesting that the folding mechanism for incorporation of MtrB into the outer membrane of *Shewanella* is different to that of other transmembrane β-barrels (White et al., 2013).

The soluble N-terminal domain of MtrB is approximately 16 amino acids long, and contains a CXXC motif that is present within the *Shewanella* MtrB family, but not within paralogs of MtrB in other strains. The first of the two cysteines (cysteine-42 in *S. oneidensis*) was shown to be essential for Fe(III) reduction in *S. oneidensis* and it has been suggested that these cysteines may be involved in MtrB transport across the periplasm and correct insertion into the outer membrane (Wee et al., 2014).

The locus containing the mtrCAB operon also contains omcA, which encodes for a second outer membrane cytochrome that is expressed independently of the mtrCAB operon. In contrast to the essential mtrA and mtrB genes, deletion of either mtrC or omcA only causes a partial loss of Fe(III) reduction (Coursolle and Gralnick, 2010). Deletion of both mtrC and omcA had a cumulative effect resulting in a near complete loss of Fe(III) reduction, suggesting that both OmcA and MtrC are capable of accepting electrons from the MtrAB transmembrane electron conduit. This is surprising as the only interactions shown *in vitro* have been between MtrC, MtrA and MtrB (Ross et al., 2007). *In vivo* crosslinking studies are still the only evidence for an MtrCABOmcA interaction, and this might have been the trapping of a transitional interaction rather than a stable complex (Myers et al., 2004; Shi et al., 2006).

The X-ray crystal structures of both MtrC and OmcA have been solved to atomic resolution and show significant
structural similarity (Edwards et al., 2014; 2015). Both structures contain hemes arranged in two chains that intersect, forming a ‘staggered-cross’ pattern with hemes II, V, VII and X at the termini of each chain. Hemes V and X are exposed at opposite ends of the structure, while hemes II and VII are close to two β-barrel domains that flank the heme-containing domains. The hemes in both MtrC and OmcA are superposable with the exception of heme V, which is displaced and in a different orientation in each structure (Edwards et al., 2015). The cross-like arrangement of hemes gives four possible electron ingress/egress routes, with hemes V and X suggested as potential sites for direct metal oxide reduction and hemes II and VII as responsible for flavin reduction. Heme VII has been shown to be most likely involved in flavin interaction through both molecular dynamic simulations on MtrC and mutagenesis studies on axial ligands of OmcA hemes (Babanova et al., 2017; Neto et al., 2017).

Recent results indicate that the outer membrane of S. oneidensis undergoes dynamic structural rearrangements that are triggered by an electron acceptor limitation. These structures were first reported in 2010 and were called nanowires according to conductive extracellular structures that were observed in G. sulfurreducens first (Gorby et al., 2006). Meanwhile we know that the structures are not pili as they are in G. sulfurreducens but chains of outer membrane vesicles that are filled with periplasmic proteins (Pirbadian et al., 2014; Subramanian et al., 2018). It is therefore still under debate whether these structures can be called nanowires. Although they are not composed of pili-subunits, they can serve for the same purpose, which is the transfer of respiratory electrons beyond the dimensions of an individual cell (El-Naggar et al., 2010). It seems as if they can catalyse electron transfer along their length by electron hopping between outer membrane cytochromes and diffusion of cytochromes along the surface of the vesicles (Subramanian et al., 2018). The formation of these structures might be of special importance for growth of S. oneidensis in biofilms in which only certain layers of the cells can be in direct contact with the electron acceptor and other cells might depend on electron transfer over micrometre distances to reach the terminal electron acceptor.

The role of flavins

Two studies reported independently the excretion of flavins by S. oneidensis cells grown under batch conditions (Canstein et al., 2008; Marsili et al., 2008). FAD is transported through the cytoplasmic membrane via the bacterial FAD exporter Bfe (Kotloski and Gralnick, 2013). Thereafter the 5’-nucleosidase UshA processes FAD to AMP and FMN which is the major flavin molecule in the culture supernatant (Canstein et al., 2008; Covington et al., 2010). Experiments with a mutant in the FAD transporter revealed that electron transport was accelerated 4-fold in the presence of extracellular flavin (Kotloski and Gralnick, 2013). Moreover, a potential flavin binding site was observed in the structure of the MtrC analogue MtrF (Clarke et al., 2011) and the Mtr-pathway was additionally revealed to be necessary for the reduction of flavins (Coursolle et al., 2010). There are still two hypotheses regarding the role of flavin molecules. The first hypothesis is, that these molecules act as freely diffusible shuttle molecules (Canstein et al., 2008; Marsili et al., 2008) while the other opinion is that these flavins are in fact cofactors of outer membrane cytochromes facilitating one electron transport via the formation of semiquinones (Okamoto et al., 2013; 2014; Xu et al., 2016). Recent results from differential pulse voltammetry conducted in different groups strongly support the second hypothesis and emphasize the importance of outer membrane cytochrome bound semiquinones for extracellular electron transfer in S. oneidensis (Okamoto et al., 2013; 2014; Xu et al., 2016). Moreover, keeping the flavins in close proximity to the cell surface in the form of cofactors would also minimize the risk that these compounds could be lost via diffusion or be used as growth-supporting substrate by other microorganisms of the respective ecosystem. Of note, Oram and Jeuken proposed recently, that flavin-independent electron transfer by S. oneidensis is in fact not direct but mediated by soluble iron (Oram and Jeuken, 2016). This soluble iron could be released via partial cell lysis and is necessary for electron transfer in the high potential range between 0 and 0.2 V. Evidence for this model stems from experiments with the iron siderophore and chelator deferoxamine. The addition of this substance almost completely disabled electron transfer at the high potential range in the chosen setup.

Secreted flavins will have an additional role as shuttling molecules especially under laboratory batch conditions that allow the accumulation of flavins in the medium. This role is accentuated for instance by the study of Jiang et al. in which the removal of spent medium in a bioelectrochemical system was shown to lead to a drastic decrease in current while the re-addition of this medium to the system increased the current to 80% of the original level. Interestingly, it was almost irrelevant if the cells could directly contact the electrode or if the electrode was masked by a nonconductive material with nanoholes that hamper direct microbe-electrode-interaction but allow for flavin diffusion (Jiang et al., 2010). Furthermore, an overproduction of flavins can be used as a tool to enhance current production in bioelectrochemical systems. This increased flavin production can either be achieved by the synthetic overproduction of flavin molecules or by adding limiting amounts of oxygen as co-electron acceptor to the working electrode. Addition of oxygen increases cell growth and flavin production (Teravest et al., 2014; Yang et...
Regulation of extracellular respiration

Compared to model organisms like *E. coli*, *S. oneidensis* uses a differing way to establish its anaerobic physiology. *E. coli* uses cAMP and the cAMP receptor protein (CRP) for the hierarchical usage of different carbon sources. The two different master regulators ArcAB (aerobic respiration control protein) and FNR (fumarate and nitrate reduction regulatory protein) are used to sense the redox potential and to adapt to anoxic conditions (Postma et al., 1993; Escalante et al., 2012; Förster and Gescher, 2014). In contrast, *S. oneidensis* mainly deduces the availability of oxygen from cAMP levels and uses CRP as the master regulator to switch on the expression of proteins involved in anaerobic energy generation. Consequently, *crp* mutants are unable to thrive with Fe$^{3+}$, Mn$^{4+}$, nitrate, fumarate or DMSO (Saffarini et al., 2014). In contrast, *S. oneidensis* mutants are unable to thrive under anoxic conditions (Postma et al., 1993; Escalante et al., 2012; Förster and Gescher, 2014). In contrast, *S. oneidensis* mainly deduces the availability of oxygen from cAMP levels and uses CRP as the master regulator to switch on the expression of proteins involved in anaerobic energy generation. Consequently, *crp* mutants are unable to thrive with Fe$^{3+}$, Mn$^{4+}$, nitrate, fumarate or DMSO (Saffarini et al., 2003). In contrary, neither deletion of the *arcA* nor the *fnr* analogue *etrA* resulted in mutants with a growth difference compared to the wild type with ferric iron or MnO$_2$ as electron acceptor (Beliaev et al., 2002; Gao, Wang, et al., 2010; Cruz-Garcia et al., 2011). The deletion of *arcA* leads to a decreased expression of cytochromes linked to anaerobic respiration like cymA, omcA and dmsAB. Nevertheless, an impact on anaerobic growth with ferric iron or manganese oxide was not detectable, while a growth defect could be observed for DMSO as electron acceptor. (Gralnick et al., 2005; Gao et al., 2008). Recent results indicate that the Arc-system might be involved in sensing the extracellular redox status and that it uses this as a trigger to regulate the expression of several proteins in the cytoplasm and the cytoplasmic membrane. Therefore, it was speculated that the Arc-system might be involved in the observed metabolic shift (see above) from anoxic conditions with a low redox potential electron acceptor to a high redox potential electron acceptor (Hirose et al., 2018). Still, as growth with nitrate and MnO$_2$ does not seem to be affected by an *arcA* deletion, it is not clear how important the Arc-system is for a redox potential-based metabolic shift under anoxic conditions (Gao et al., 2008; Hirose et al., 2018).

The production of cAMP is catalyzed by an adenylate cyclase. *S. oneidensis* contains three genes for putative adenylate cyclase (Charania et al., 2009). Nevertheless, only a double deletion of the gene for the cytoplasmic membrane bound enzyme CyaC and the predicted soluble enzyme CyaA lead to a phenotype that was similar to the *crp* deletion strain (Charania et al., 2009). Expression of *cyaC* alone from a plasmid was sufficient to complement a triple mutant in all adenylate cyclase genes, while *cyaA* expression lead to an incomplete suppression of the phenotype. CRP is directly involved in the regulation of key genes for extracellular respiration as CRP binding sites were detected upstream of omcA, mtrC, mtrA, cymA and cta (Gao, Wang, et al., 2010; Kasai et al., 2015; Barchinger et al., 2016). Moreover, CRP is involved in D-lactate oxidation by activating the expression of the *lldP-dld* operon encoding the genes for a lactate permease and a novel membrane bound D-lactate oxidase. Consequently, the Δ*crp* mutant can only grow on D,L- or L-lactate (Kasai et al., 2017). Interestingly, a CRP binding site could not be detected upstream of the *fcaA* gene. Hence, the effect of the *crp* deletion on fumarate reduction seems to be indirect, possibly since CRP also positively regulates heme synthesis and c-type cytochrome maturation (Charania et al., 2009; Barchinger et al., 2016). Of note, the concentration of cAMP influences the expression of many more genes than CRP alone, since a *cyaC* deletion mutant showed a defect in the up- or downregulation of 1255 genes, compared to only 359 genes for the *crp* strain (Barchinger et al., 2016). So far, it is not clear how the adenylate cyclases are regulated and what the phosphate donating factor for cAMP production might be.

Another regulatory factor that was recently revealed to play a role is the extracytoplasmic function sigma factor RpoE. So far it was known that RpoE is essential for growth under suboptimal conditions (high or low temperatures, high salinity, oxidative stress (Dai et al., 2015) but a corresponding mutant is also negatively affected in growth on minimal medium as it reaches less than 50% of the final optical density compared to the wild type. Targets for RpoE are genes involved in outer membrane lipoprotein transport and folding, lipopolysaccharide production and periplasmic proteases (Dai et al., 2015). Moreover, RpoE binding sites were detected upstream of the genes for the outer membrane cytochromes OmcA and MtrC as well as genes involved in c-type cytochrome maturation and heme biosynthesis (Barchinger et al., 2016). Expression of the *rpoE* gene itself is upregulated twofold in the absence of oxygen (Barchinger et al., 2016) and the presence of metals and thiosulfate (Beliaev et al., 2005). Further analysis will have to reveal what the overall impact of RpoE on extracellular respiration is. Still, it will be difficult to distinguish this direct function on extracellular respiration from its general involvement in stress response.

The production of c-type cytochromes is also affected by the availability of iron. Iron depletion leads to a downregulation of *mtr*-gene expression. This process relies to a major extent on the ferric uptake regulator (Fur) that binds
ferrous iron and is important for iron homeostasis (Yang et al., 2008). The impact of iron-sensing by Fur on the expression of some c-type cytochrome encoding genes seems to be mediated by the small RNA RyhB. Iron depletion leads to an upregulation of this sRNA and its interaction with certain mRNAs causes an accelerated degradation of these target mRNAs. RyhB targets are for instance cymA, cctA as well as mtrABC (Meibom et al., 2018).

Open questions
Extensive efforts have been made by numerous groups across the world to unravel the mechanism of extracellular electron transport by S. oneidensis. The results have already clarified tremendously our understanding of the process. Still, there are several aspects that remain unclear and that are relevant for our fundamental understanding of extracellular electron transfer and also for the implementation of knowledge based strategies to harness this interesting metabolism for biotechnological applications:

Why c-type cytochromes? The vast majority of the organisms known to perform extracellular electron transfer contain c-type cytochromes and these play a central role in the process (Koch and Harnisch, 2016). However, it is so far not understood what prevents other redox proteins, for example containing iron sulfur clusters, which cover an even broader range of potentials (Liu et al., 2014) from fulfilling this role. Model organisms are what they are also because they are easy to grow under laboratory conditions. Hence, we may be in a situation like the story of ‘looking where the light is better’ and find more and more c-type cytochrome using organisms because we are blind for other solutions that evolved in the environment. It might just not be easy to find these different solutions since bioinformatic approaches do not help without model organisms and since the organisms that use these solutions might not be easy to cultivate so far.

Why so many c-type cytochromes? Again, the research shows that the contribution of cytochromes does not rely on a single cytochrome, but that at least dozens to more than (in the case of Geobacter sulfurreducens) cytochromes are encoded in the genomes of the organisms and that multiple of them are simultaneously expressed. The question why so many, even though genome surveys show that very few are conserved across species (Gao, Barua, et al., 2010; Liu et al., 2014) is so far not answered although we begin to understand that some of the cytochromes evolved to fulfil their role only under certain redox potential conditions.

Why multiheme proteins? It is still unclear what advantage is accrued from the process being mediated by cytochromes with multiple hemes. While on one hand, none of these multiheme cytochromes are long enough to span the width of the periplasm and therefore establish a fixed electrically conducting wire, on the other hand, the arrangement of the multiple cytochromes associated with the outer membrane porins is clearly excessive if the objective was simply to span the thickness of the outer membrane to deliver electrons to the cell surface.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.