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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.**

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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 *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 *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*., 1998; Elsner *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 *S. oneidensis* and will highlight novel results and research directions. Even after 30 years of research, *S. oneidensis* has still secrets that are not understood and we will formulate several open research questions at the end of the review.

The central carbon metabolism in S. oneidensis

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 *Shewanella* strains are able to grow on glucose, *S. oneidensis* lacks the ability to import and phosphorylate glucose to glucose-6-phosphate. Nevertheless, Howard and colleagues could show a rapid adaptation of *S. oneidensis* to aerobic growth on glucose (Howard *et al*., 2012). This ability is due to a deletion in a genomic region that includes *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 *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 *S. oneidensis*.

Shewanella oneidensis uses the EntnerDoudoroff (ED) pathway for sugar (N-acetyl-glucosamine) catabolism (Scott and Nealson, 1994; Serres and Riley, 2006;

Table 1. Extracellular electron acceptors of *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.

Extracellular electron acceptor	Main terminal reductase	
DMSO	DmsA/B	Gralnick *et al*. (2006)
Fe(III) minerals	MtrC	Coursolle and Gralnick (2010)
Soluble Fe(III) complexes	MtrC	Coursolle and Gralnick (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		
U^{VI+}	MtrC, OmcA	Marshall *et al*. (2006)
Cr^{VI+}	OmcA, MtrC	Gao, Barua *et al*. (2010); Belchik *et al*. (2011)
V^{V+}	MtrC, OmcA	Myers *et al*. (2004)
Tc^{VII+}	MtrC, OmcA, HyaB	Marshall *et al*. (2007)
Tellurite	MtrC, OmcA	Kim *et al*. (2012)
AQDS	MtrC	Lies *et al*. (2005)
Flavins	MtrC	Marsili *et al*. (2008); Coursolle *et al*. (2010)
Electrodes		
Graphite, glassy carbon, graphene oxide	MtrC, OmcA	Bretschger *et al*. (2007); Coursolle *et al*. (2010); Jiao *et al*. (2011)

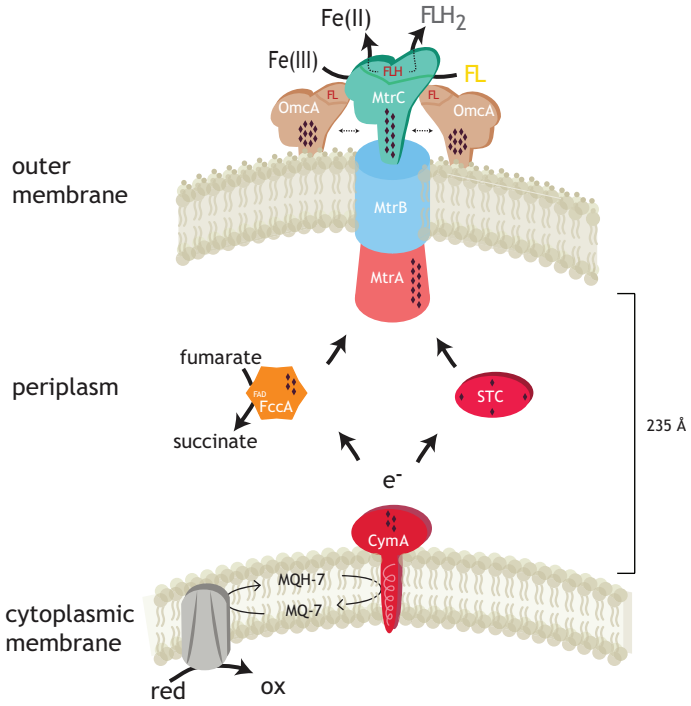

Fig. 1. Illustration of the respiratory electron transport in *Shewanella oneidensis* MR-1. Cytoplasmic substrates are oxidized and the resulting electrons are transferred via membrane bound oxidoreductases to a pool of quinones in the cytoplasmic membrane. Menaquinone 7 (MQ-7) is dominant under anoxic conditions. It interacts with CymA, a tetraheme cytochrome, in two modes: A. as an electron shuttle and B. as a specific cofactor. CymA is regarded as an electron hub, distributing electrons to a broad range of periplasmic *c*-type cytochromes. In case of extracellular respiration FccA and STC seem to have the largest share among a mostly redundant network of cytochromes. The outer membrane is bridged by the MtrCAB complex, capable of reducing soluble and insoluble terminal electron acceptors at the cell surface. The complex consists of two decaheme cytochromes, the MtrA and MtrC, each located on one side of the membrane, and the β-barrel protein MtrB in between. MtrC was found in a 1:2 complex with OmcA, facilitating the reduction process. FL: flavin fully oxidized, FLH: flavin semiquinone, FLH_2: flavin fully reduced.

Yang *et al*., 2006). Contrary to what was believed for a long time, the ED pathway is wide spread 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 seems to be the only or at least major available energy pool, while electron transfer to the terminal electron acceptors 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 form 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 (**S**tandard **H**ydrogen **E**lectrode), Hirose and colleagues could elucidate that a deletion mutant in all four NADH dehydrogenase encoding gene clusters is almost

Table 2. Carbon sources sustaining the metabolism of *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	Nealson and Myers (1990)		
3C			
Propionate	Scott and Nealson (1994)[a,b]	Lactate	Nealson and Myers (1988); Lovley *et al.* (1989)
Lactate	Nealson and Myers (1990)	Pyruvate	Lovley *et al.* (1989)
Pyruvate	Nealson and Myers (1990)		
Glycerate	Rodionov *et al.* (2010)		
4C			
Succinate	Venkateswaran *et al.* (1999)[b]		
Fumarate	Venkateswaran *et al.* (1999)[b]		
>4C			
Galactose	Venkateswaran *et al.* (1999)	N-Acetyl-D-Glucosamine	Hunt *et al.* (2010)
Inosine	Driscoll *et al.* (2007)	DNA	Pinchuk *et al.* (2008)
N-Acetyl-D-Glucosamine	Hunt *et al.* (2010)		
Casamino acids	Driscoll *et al.* (2007)		
DNA	Pinchuk *et al.* (2008)		
Biochemical data			
Adenosine	Rodrigues *et al.* (2011)	Formate	Scott and Nealson (1994)[a]
Inosine	Rodrigues *et al.* (2011)		
Uridine	Rodrigues *et al.* (2011)		
Methylpyruvate	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)		

[a]Mixed results, see also Serres and Riley, (2006).
[b]contrary to Biolog data by Rodrigues *et al.* (2011).

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 ($E^{0\prime} = -0.03$ V), nitrate ($E^{0\prime} = -0.43$ V) and MnO_2 ($E^{0\prime} = -0.53$ V). The NADH dehydrogenase mutant showed a decreased ability to use nitrate and 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 dehydrogenasede-pendent quinone reduction leads to a higher production of proton motif 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 (MQ-7, (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 (**cy**toplasmic **m**embrane protein **A**) located on the outer leaflet of the cytoplasmic membrane (McMillan *et al.*, 2012). Interestingly, the dominant catalytic mode of CymA under *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 *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; Schwalb *et al.*, 2002;

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 **m**et**a**l **r**educing protein A (MtrA) bound to the outer membrane complex MtrCAB (Dohnalkova *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 anaerobically 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). While ScyA was identified as a mediator of electron transfer between CymA and the diheme *c*-type cytochrome peroxidase CcpA (Schuetz *et al*., 2011), FccA was shown to be the only respiratory fumarate reductase in *S. oneidensis* MR-1 (Myers and Myers, 1997). FccA is a soluble 64 kDa unidirectional fumarate reductase (Pealing *et al*., 1992) that is composed of an N-terminal domain with four *c*-type hemes which are homologous to STC, a C-terminal flavoprotein domain with a non-covalently bound FAD group and a flexible clamp domain that may control the access of the substrate to the active site (Taylor *et al*., 1999). The four hemes in FccA are arranged in a quasi-linear architecture that allows an efficient conduction of electrons across the length of the N-terminal domain to the active site of the protein (Taylor *et al*., 1999). Thermodynamic and kinetic studies have shown that the reduction of the heme domain occurs through hemes I and II of FccA, that receive the electrons from its physiological partner (Fonseca *et al*., 2013) and distribute them by intramolecular electron transfer to the other hemes according to their reduction potential (Pessanha *et al*., 2009; Paquete *et al*., 2014). Since electron exchange among the hemes is faster (> 10^5 s^{-1}) than electron transfer to the FAD group (~100 s^{-1}) (Jeuken *et al*., 2002; Pessanha *et al*., 2009), two electrons are always available for the reduction of fumarate. Besides fumarate reduction, FccA also functions as an electron transfer shuttle between CymA and MtrA for the reduction of extracellular substrates (Schuetz *et al*., 2009). It was demonstrated that the degree of reduction of FccA controls the activity of this moonlighting protein (Paquete *et al*., 2014). At low electron flux from the cell metabolism, FccA receives the electrons from CymA and transfers them to the outer-membrane complexes for the reduction of insoluble electron acceptors (Schuetz *et al*., 2009; Fonseca *et al*., 2013). As the electron flux increases, FccA will become fully reduced, which enhances the catalytic efficiency of fumarate reduction, offering another option for discharging the electrons to prevent metabolic arrest. This switching mechanism allows *Shewanella* to quickly alternate between reduction of soluble and insoluble electron acceptors, without the production of new enzymes (Paquete *et al*., 2014).

The physiological function of the 12 kDa tetraheme cytochrome STC was for several years unclear, mainly due to a lack of a phenotype under conditions of metal or DMSO reduction (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 proteolipsome 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 **b**acterial **F**AD **e**xporter 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*

al., 2015). Moreover, an increased flavin production was also observed, when DMSO was added to ferric iron reducing cultures. Although the effect of adding a second electron acceptor might have several points of actions and not only flavin production, it becomes clear that it can be advantageous although the initial thought would be that the breakup of electron transfer routes might decrease the efficiency of ferric iron or anode reduction (Cheng *et al*., 2013).

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 **c**AMP **r**eceptor **p**rotein (CRP) for the hierarchical usage of different carbon sources. The two different master regulators ArcAB (**a**erobic **r**espiration **c**ontrol protein) and FNR (**f**umarate and **n**itrate reduction **r**egulatory 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*., 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-García *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 potentialbased 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 *cctA* (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 *fccA* 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 extend on the **f**erric **u**ptake **r**egulator (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 ironsulfur 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.