Bacterial Power: An Alternative Energy Source

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Abstract

The demand for energy and the limited supply of fossil fuels and their impact in the environment have required the development of alternative energy sources. Among the next generation of energy sources, microbial fuel cells (MFCs) have emerged as a promising technology due to their ability to recover energy from wastewaters in the form of electricity using electroactive microorganisms as catalysts. Among the various factors that affect power generation performance in MFCs, the efficiency of extracellular electron transfer (EET) is one of the most important. Several enzymes, specifically multiheme cytochromes, have been implicated in this process although the electron transfer chain organization remains to be fully understood. In this chapter, we review in detail the mechanisms that support EET from electroactive microorganisms to the anode in MFCs. We focus on the model organism *Shewanella oneidensis* MR-1, due to the existence of an extensive molecular characterization of its EET processes. The recent developments in the characterization of the multiheme cytochromes involved in these mechanisms will also be reviewed.

Keywords

Alternative energy · Microbial fuel cell · Electroactive microorganism · *Shewanella oneidensis* · Extracellular electron transfer · Enzyme · Multiheme cytochrome

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1 Introduction

The constant demand for energy and the limited supply of fossil fuels and their impact in the environment have required the development of alternative energy sources. Among the next generation of energy sources, microbial fuel cells (MFCs) continue to attract wide attention due to their ability to recover energy in the form of electricity using microorganisms as catalysts. This technology is among the most studied bioelectrochemical systems (BES), with these devices also being used for other eco-friendly purposes such as the production of biofuels and chemicals, biosensors, bioremediation, wastewater treatment and desalination [1–5].

MFCs employ microorganisms to produce electrical current while metabolizing nutrients available in the medium [6, 7]. The capacity of using organic waste (e.g., wastewater) as substrate has opened the possibility of producing electricity in a way that is close to carbon neutral [8, 9]. These cells consist of an anode that is kept under anoxic conditions and receives electrons from the bioenergetic metabolism of the microorganisms growing on its surface. MFCs also contain a cathode that transfers electrons to the terminal electron acceptor. It is the electron flow from the anode to the cathode through an external circuit that allows the production of electrical current. Nowadays, there are a wide variety of designs, where the anode and the cathode may coexist in a single compartment (single-chamber) or can be separated by a physical barrier that is permeable to ions (dual-chamber) (Fig. 1) [10].

In BES, electron transfer efficiency depends on several parameters, with the electron transfer processes performed by the microorganisms among the core factors that affect power generation performance [11, 12]. Microorganisms that oxidize organic compounds and transfer electrons to the anodes of BES are called electroactive but are also known under several other names in the literature, such as, electricigens, exoelectrogenic, anode-respiring or anodophilic microorganisms [13]. These electroactive microorganisms are united in their ability to perform extracellular electron transfer (EET), directly and/or mediated, to the electrode.

The concept of electric current generation by microorganisms is not new and was reported over 100 years ago [14], with research on MFCs making several advances in the last decade [7, 15]. These include different MFC architectures and construction materials for the anode and cathode, diverse microbial communities and knowledge on the biochemical characteristics of the EET performed by the microorganisms [7, 16]. Nevertheless, the commercialization of MFCs is still limited due to low performance, expensive core parts and materials, and bottlenecks in scale-up [15, 17]. Therefore, many challenges and room for improvement remain in BES, including the identification of new electroactive microorganisms with high electrochemical activities and the characterization of the electron transfer process between cells and electrodes. This has been a crucial aspect in the enhancement of MFC performance and paramount in promoting their future applications [18–20].

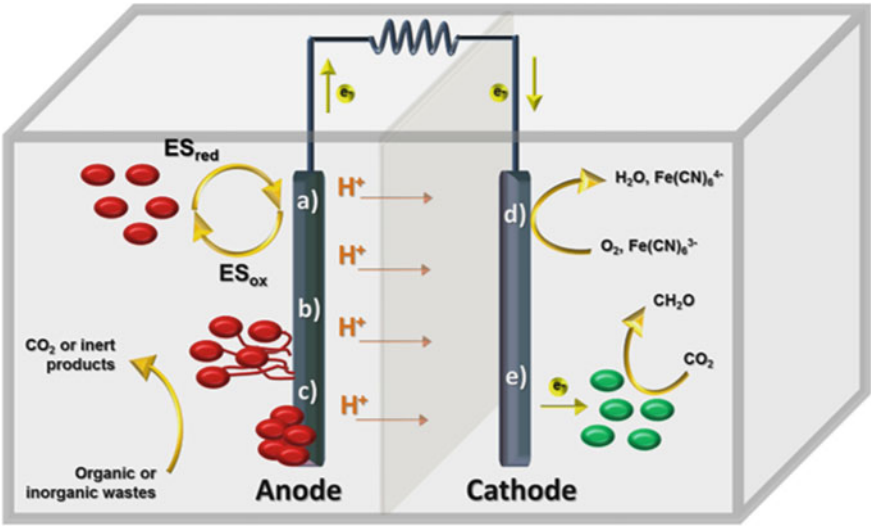


Fig. 1 Schematic representation of a MFC, where the difference between a single and double chamber design is the presence or absence of a permeable barrier that is often an ion-exchange membrane separating the anode from the cathode. Bacteria at the anode chamber (circles) feed on organic or inorganic wastes and transfer electrons to the anode through: **a** electron shuttles (ES), **b** nanowires or conductive pili, or **c** directly through cell surface redox active proteins. The protons produced flow through the selectively permeable membrane to the cathode chamber and the electrons flow through an electrical circuit to the cathode. The electrons are then transferred to the final electron acceptor. This can be **d** abiotic or **e** biotic

In this chapter, we review in detail the mechanisms that support EET from electroactive microorganisms to the anode in BES. We focus on the model organism *Shewanella oneidensis* MR-1, due to the existence of an extensive molecular characterization of its EET processes. The recent developments in the characterization of the enzymes involved in these mechanisms will also be reviewed.

2 Extracellular Electron Transfer Mechanisms

Extracellular electron transfer is defined as a metabolic process that enables electron transfer between cells and extracellular solid materials and is based on one of the oldest types of microbial respiration, the dissimilatory reduction of iron [21]. The EET process between electroactive microorganisms and electrodes is the footstone for developing MFCs and other BES, which connect the intracellular bioenergetic pathways of microorganisms with the electrochemical reactions of electrodes [22, 23].

The molecular mechanisms of electron transfer to, or from, extracellular substrates can be divided in direct and mediated EET [24] (Fig. 2). EET is a very complex phenomenon and in vivo an absolute separation between direct EET and mediated EET is often difficult since both type of EET can occur simultaneously within one single organism [23, 25]. The study and elucidation of these mechanisms have led to a better understanding on how EET occurs and provides guidance for the optimization of MFCs.

2.1 Direct EET

In direct EET, microorganisms attach to solid surfaces, to or from which they directly transfer electrons without involvement of any diffusible redox compounds. Although early studies with *S. oneidensis* MR-1 supported a mechanism of physical contact for growth on insoluble manganese oxide [26], it was only latter that the first experimental evidence for this mechanism was revealed [27]. Atomic force microscopy experiments showed that *Shewanella* cells grown anaerobically bind preferentially metal oxides in contrast to aerobically grown cells.

Direct EET is achieved through physical contact of the cells to solid surfaces, with the efficiency of this electron transfer mechanism limited by the maximum cell density in the bacterial monolayer [28]. This physical contact occurs via redox active proteins present on the outer membrane or cell envelope [29]. A high number of multiheme *c*-type cytochromes (MHCs) have been found in organisms capable of performing EET, with several of them directly implicated in direct EET [29–32]. These proteins are characterized by multiple heme cofactors that are covalently attached to the polypeptide chain and can switch between oxidized Fe(III) and reduced Fe(II) states. Their distances are typically less than 14 between closest neighbors enabling fast long-range electron transfer via electron hopping [33, 34].

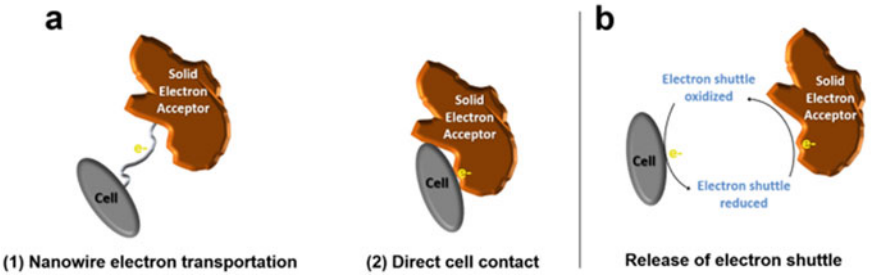


Fig. 2 Strategies employed by microorganisms for electron transfer to insoluble extracellular electron acceptors. Extracellular electron transfer can occur by direct EET. **a** through cell appendages of diverse nature called pili or nanowires (1) or through direct cell contact via cell surface redox active proteins (2); or indirect EET, **b** mediated by electron shuttling compounds

Some microorganisms can establish a thick multilayer electrochemically active biofilm, and through long-range conductive filamentous appendages, such as nanowires or pili, achieve higher electron transfer rates and densities per surface area when compared to cell monolayers [35]. Scanning tunneling microscopy images show thin filaments with about 8 nm in diameter and 10 μm in length [35]. These conductive filamentous appendages allow the bacteria to conduct electrons over a large distance within the multiple layers of a biofilm. In terms of morphology, *Shewanella* nanowires are partially composed of *c*-type cytochromes [28, 36–39]. This was demonstrated by deleting the genes coding for the outer-membrane cytochromes, MtrC and OmcA, resulting in non-conductive nanowires [38]. Also, deleting the *gspG* gene which is involved in the type II secretion pathway, that is required for the proper export of the outer-membrane cytochromes MtrC and OmcA to the cell exterior [40, 41], resulted in non-conductive nanowires. Later studies using fluorescence microscopy revealed that *Shewanella* nanowires are, in fact, extensions of the outer-membrane and periplasm [42].

Other examples of conductive filaments are the pili [43] and the more recent multiheme cytochrome OmcS filaments [44, 45] from the *Geobacter* genus. The hypothesis that the pili might function as conductive filamentous appendages resulted from the observation that pili were specifically expressed during growth on insoluble electron acceptors [46]. Studies showed that type IV pilus monomer PilA deletion mutant of *G. sulfurreducens* could not reduce Fe(III) oxide and displayed a much lower current production in MFCs [28, 47]. The NMR structure of the PilA monomer of *G. sulfurreducens* shows that it is shorter than the PilA from other microorganisms [48]. Indeed, the truncation of PilA in *G. sulfurreducens* was proposed to be essential for iron respiration, suggesting that an adaptive evolution of this organism to dissimilatory iron reduction in natural environments has been achieved with the truncation of this protein [49]. Using cryo-electron microscopy, the structure of a different conductive filament was solved, with particle reconstructions showing that only the outer surface MHC OmcS monomers alone could produce a perfect fit [44, 45]. This argues for conductive filaments in *Geobacter* to be composed entirely of OmcS and that no arrangement with PilA monomers is present, as previously proposed [50]. These observations provide a context for the fact that, whereas the mechanism by which electrons are transferred along the PilA filaments is still fiercely debated [51], the OmcS polymer provides a continuous chain of hemes at close distance for efficient conduction along the length of the whole filament [44].

2.2 Mediated EET

Besides direct EET, some bacteria can also reduce extracellular substrates through mediated electron transfer, using small organic electron shuttles. These serve as the terminal electron acceptors, and once reduced, can themselves transfer electrons to iron oxides or anodes in MFCs.

Electron shuttles are available in the media (e.g., humic acids) or can be endogenously produced (*e.g.* flavins) by microorganisms. The possible involvement of endogenous electron shuttles in reduction of poorly soluble metal minerals by *Shewanella* was first proposed by Newman and Kolter [52]. Later, Lies et al. demonstrated that iron oxide entrapped within nanoporous glass beads could be reduced by *S. oneidensis* MR-1, confirming the participation of electron shuttles in the dissimilatory iron respiration of this bacterium [53]. The ability of flavins to enhance iron reduction was first examined by Myers and Myers [54], showing that addition of flavins to the growth medium increased ferric reductase activity in *S. oneidensis* MR-1. Since then it was confirmed by numerous researchers that members of the *Shewanella* genus are capable of secreting flavins, such as riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [55–57]. Also, *S. oneidensis* MR-1 can accumulate these flavins to high concentrations in solution (250–500 nM) to be used as electron shuttles for EET to the electrodes [55].These high levels of flavins enhance the electron transfer efficiency by several fold and therefore are cost effective since the ATP used on flavin production and secretion is negligible when compared with the resulting energetic advantage.

Although, electron shuttling seems to be the primary mechanism of EET, outer membrane MHCs still play a key role in mediated EET and are responsible for at least 95% of the reduction of extracellular flavins at physiological relevant rates [58]. Indeed, the *Shewanella* Mtr complex plays an essential role in flavins' reduction, with the outer-membrane MHC MtrC accounting for approximately 50% of the activity observed [59]. Kinetic results showed that direct contact between the outer-multiheme cytochromes (*e.g.* OmcA and MtrC) and insoluble iron substrates or MFC anodes could not account for the rates of electron transfer observed when using whole cells assays [60, 61], with this gap in electron transfer rates resolved with the addition of flavins. This demonstrated that outer-membrane cytochromes are not the only elements responsible for the EET at relevant kinetic rates and that direct and mediated electron transfer occur in tandem in *S. oneidensis* MR-1 [60]. Indeed, it has been shown that mediated EET, and not direct EET, is the primary mechanism of EET employed by *S. oneidensis*, accounting for approximately 75% of its EET capacity [57].

With reduction potentials of −219 mV (FMN and FAD) and −208 mV (riboflavin) [30], flavins have the capability to act as efficient extracellular redox mediators for the reduction of metal oxides at neutral pH (redox couple ferrihydrite/Fe(II) has a reduction potential ranging from −100 to +100 mV [62]). Thus, *Shewanella* species that can secrete and utilize flavins as electron shuttles have an advantage in environments that contain poorly soluble metal oxides but lack exogenous redox mediators, such as humic acids. Another advantage of flavin secretion by *Shewanella* is their potential application in the construction of MFCs without addition of costly exogenous redox mediators [63]. Furthermore, flavin secretion by *Shewanella* may also support mediated EET by other microbial species present in the BES and thereby increase the efficiency of current generation in mixed cultures [56].

3 Electroactive Microorganisms

In Nature, there is a great diversity of microorganisms that can be used in MFCs, with more than 100 different electroactive species presently identified [16, 64]. Most of these electroactive microorganisms are Gram negative bacteria, with more than half belonging to the Proteobacteria phylum. Also, the majority of these electroactive species are: (i) mesophilic; (ii) have low tolerance to high salinity; (iii) possess motility; (iv) have biofilm formation capabilities; and (v) exhibit anodic EET activity, with most species performing EET via mediated electron transfer and only a small percentage capable of performing direct EET [64]. As MFC research advances, we expect that significantly more electroactive microorganisms will be discovered, especially those that can exist and thrive in more extreme environments [65].

Presently, it is well known that mixed microbial cultures colonizing anodes in MFCs produce greater current densities with higher columbic efficiency, compared with experiments using pure cultures [66]. Presently, the highest current densities obtained are from microbial mixed cultures that are dominated by δ-Proteobacteria of the *Geobacter* genus [16]. The reason for this is the fact that mixed cultures have a higher flexibility towards external factors due to symbiotic effects. This allows a greater diversity regarding metabolic pathways, as well as the combination of different electron transfer mechanisms that permit a complete oxidation of the organic substrates existent in the MFC reactor [67, 68].

Though mixed microbial cultures produce more current density, MFCs operating with pure cultures of electroactive bacteria are preferred for the detailed investigation of EET mechanisms as they allow a better characterization of the pathways than in mixed cultures. Understanding the processes by which electroactive organisms transfer electrons to an electrode, as well as microbial-electrode interactions will allow the enhancement of EET and ultimately benefit operational performance of the MFCs and enable their future practical applications. The Gram negative proteobacterium *S. oneidensis* MR-1 has been used as a model organism to understand EET [23, 32]. The ability to grow *Shewanella* robustly under oxygen conditions, the large quantity of sequenced genomes, and their easy genetic manipulation makes these bacteria ideal to work with, both in the laboratory and in BES applications.

3.1 *Shewanella*: A Model Organism

Organisms currently assigned to the genus *Shewanella* have been recognized for nearly 90 years, having first been isolated from the surface of rotten butter in 1931 [69]. Over the subsequent decades, these bacteria received little attention, with the exception of the name, that was frequently changed. In 1985, based on 5S rRNA sequence data a reclassification was proposed [70]. At this time the new genus *Shewanella* was created, to honor Dr. James Shewan for his contributions in the

study of these microorganisms. Although, most newly discovered *Shewanella* strains were initially classified as *S. putrefaciens*, DNA:DNA hybridization and 16S rRNA sequences resulted in the identification of more than 60 species within this genus [71].

It was only in 1988, with the discovery of members of the *Shewanella* genus with the capacity to perform EET, that these microorganisms started to find a prominent position within the scientific community [26]. These findings strongly suggested that this genus could play important roles in the biogeochemical cycles of the elements and in biotechnological applications, such as in BES [23, 32].

Members of the genus *Shewanella* are facultative anaerobic Gram negative γ-Proteobacteria. They generally possess a single polar flagellum and a rod shape with 2–3 μm in length and 0.5–0.6 μm in diameter [72]. The vast majority of the *Shewanella* isolates were obtained from marine environments, where they are sometimes found as fish pathogens with important impact in the aquaculture industry [73, 74]. *Shewanella* are also found in other habitats such as the freshwater *S. oneidensis* MR-1 [72].

Numerous *Shewanella* species are capable of growing at low temperatures (<5 °C) even though their optimal growth temperature is above 16 °C [75]. By contrast, those species found to be opportunistic human pathogens such as *S. algae* can grow at the relatively high temperature of 42 °C [76]. Another major aspect of the versatility of *Shewanella* is their ability to utilize a broad variety of organic and inorganic compounds as a final electron acceptor [77]. This allows them to thrive in a wide range of aquatic habitats, both marine and freshwater, and play a significant role in several biogeochemical redox cycles, including those of iron and manganese [26]. Since many of these organic and inorganic compounds are toxic or highly insoluble, they do not enter the bacteria and are extracellularly reduced by terminal reductases localized on the surface of the cell [31].

In 2001, the genome of *S. oneidensis MR-1* was sequenced, primarily due to its position as a model organism for dissimilatory metal reduction and its potential role in several biotechnological applications [78]. Since then, approximately 40 other *Shewanella* genomes have been sequenced [79]. Analysis of the *S. oneidensis* MR-1 genome revealed that the chromosome encodes for 41 putative *c*-type cytochromes (9 in the cytoplasmic membrane, 27 in the periplasm, and 5 in the outer membrane) [80, 81]. The capability to transfer electrons to a vast range of electron acceptors and perform EET is linked to this large number of *c*-type cytochromes, which spans from the cytoplasmic membrane to the outer membrane [82, 83]. Using a variety of genetic (e.g., knock-out studies) and biochemical techniques (e.g., protein characterization), some of the components involved have been identified and characterized in detail (Fig. 3). The so called “minimal setup” of redox proteins which are assigned to the EET process will be discussed below.

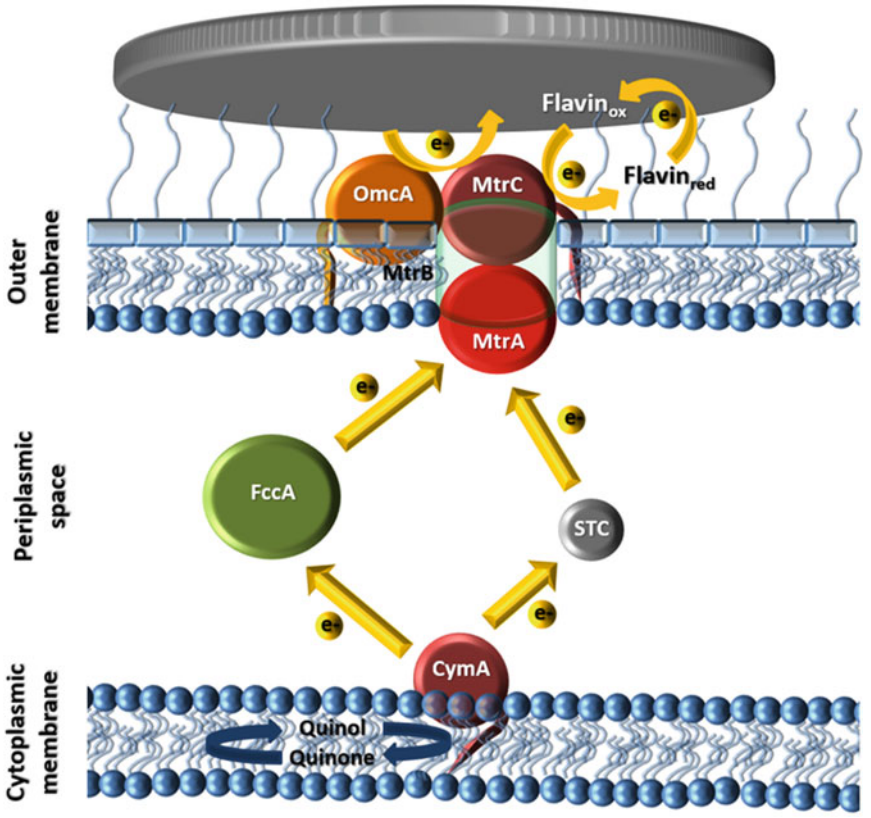


Fig. 3 Scheme of the so called "minimal setup" of redox proteins involved in the EET process of *S. oneidensis* MR-1. Arrows represent electron transfer processes

4 Extracellular Electron Transfer (EET) Pathway

MHCs play an important role in EET pathways, being critical elements for extracellular respiration and current output in electroactive microorganisms. In *S. oneidensis* MR-1, the MtrCAB pathway is the major EET pathway and is composed by several *c*-type cytochromes, that allow electrons generated from substrate oxidation to be transferred from the inner membrane, through the periplasmic space, to the outer-membrane for the reduction of terminal extracellular electron acceptors (e.g., metal oxides in the natural environment or electrodes in BES) (Fig. 3).

4.1 Cytoplasmic Membrane

Electron transfer at the cytoplasmic membrane involves the linkage of dehydrogenases responsible for oxidation of carbon sources in the cytoplasm (*e.g.* formate dehydrogenase), through a lipid soluble quinone pool, to electron transfer proteins (*e.g.* cytochromes) bound to the cytoplasmic membrane [84]. This mechanism generates a proton-electrochemical gradient that is used to produce ATP via the ATP synthase [85]. In parallel, the electron flow through the quinone pool towards extracellular electron acceptors appears to serve also as a pathway to discharge electrons without coupling to the generation of transmembrane electrochemical potential [86].

S. oneidensis MR-1 is known to produce three quinones (menaquinone, methylmenaquinone and ubiquinone) [87]. The deletion of *menD* and *menB* genes involved in the biosynthesis of menaquinone produced a phenotype incapable of iron respiration, revealing that menaquinone but not ubiquinone plays a role in metal respiration [88, 89]. This is in line with the difference in the reduction potentials of these two quinones with the ubiquinone potential more aligned to participate in aerobic respiratory chains.

4.1.1 CymA

Presently, it is well established that the linkage between the membrane quinone pool and EET chain is provided by a tetraheme *c*-type cytochrome called CymA that is attached to the periplasmic surface of the cytoplasmic membrane by a α-helical anchor [88, 90–93]. CymA from *Shewanella* has 21 kDa and is a member of the NapC/NirT protein family. It is able to bind quinol (Kd = 0.1–1 μM) [94], functioning as a quinol oxidase [88, 92, 95]. Deletion of the *cymA* gene severely hindered the reduction of a variety of substrates including Fe(III)/Mn(IV) oxides, fumarate, nitrate, nitrate, and DMSO [82, 90, 91]. This supported the proposal that CymA is one of the major hubs for electron transfer to the periplasm, being essential for EET. CymA's ability to interact with multiple periplasmic cytochrome partners has been amply demonstrated and explored [92, 96, 97]. Also, overexpression of this gene is enough to enhance electricity generation by *S. oneidensis* MR-1 in an MFC [98]. CymA's role in EET was further confirmed by cloning the gene in *E. coli* and observing that the heterologous expression of CymA is enough to make this bacterium capable of EET [99].

Although no structural characterization is presently available for CymA, it contains three low spin hemes with bis–histidine axial ligation and one high-spin heme with a histidine–water axial ligation [88, 100]. This high-spin heme forms an intrinsic part of the quinol oxidation site. Also, site-directed mutagenesis experiments revealed that the amino acid Lysine-91 is essential for quinol interaction with CymA from *Shewanella sp.* strain ANA-3 [94]. Redox properties were determined for CymA from *S. oneidensis* MR-1, with macroscopic midpoint potentials at pH 7.0 of approximately −110, −190 and −265 mV for the three low-spin hemes and −240 mV for the high-spin heme [88]. These potentials are below that of the menaquinol/menaquinone couple ($E^{o'} \approx -80$ mV), and thus electron transfer only

becomes spontaneous when the menaquinol/menaquinone balance is shifted towards menaquinol. Once the electrons enter the heme network, electron flow to the metal oxides becomes thermodynamically favorable due to progressively less negative redox potentials of the electron transfer proteins that are downstream of CymA [101].

Despite the importance of CymA for EET, the quinol dehydrogenase complex SirCD is capable of partially replacing it, restoring the capability of *Shewanella* Δ*cymA* strains to use Fe(III), fumarate or DMSO as terminal electrons acceptors [102]. *Shewanella* contains another tetraheme cytochrome attached to the inner-membrane denominated TorC [103]. Like CymA, TorC is a quinol dehydrogenase and is involved in the reduction of the terminal electron acceptor, Trimethylamine N-oxide (TMAO) to trimethylamine (TMA). This capability is the origin of the designation *putrefaciens* for the smell of rotten fish [104].

4.2 Periplasmic Space

In *S. oneidensis* MR-1, the periplasmic space has a width of approximately 235 Å [105] and contains an abundance of soluble electron transfer proteins (*e.g.* MHCs), in extremely high concentration, estimated to reach the mM range [106]. These proteins can be terminal reductases of soluble electron acceptors, or proteins that mediate electron transfer to the outer-membrane proteins for the reduction of insoluble electron acceptors. The two most abundant periplasmic cytochromes are the tetraheme flavocytochrome *c* FccA [97, 107, 108] and the small tetraheme cytochrome *c* STC [97, 107, 109].

4.2.1 FccA

Fc*c*A, a 64 kDa tetraheme *c*-type flavocytochrome, is a unidirectional fumarate reductase with a FAD cofactor in the active site [110]. This enzyme is unique in comparison to other fumarate reductases since it is a monomeric and soluble periplasmic protein. X-ray crystal structures of Fc*c*A from *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1 are available [111, 112], showing that these proteins fold into three domains: a N-terminal cytochrome domain with four bis–histidine low-spin *c*-type hemes, a C-terminal flavoprotein domain with a non-covalently bound FAD group and a clamp domain that was proposed to control the access to the active site of the enzyme. The hemes found in the N-terminal domain of Fc*c*A are arranged in a quasi-linear architecture that allows an efficient conduction of the electrons across the length of the protein to the FAD catalytic center [111–113]. Electron transfer to the active site is performed by heme IV, which is in close proximity (≈5 Å) to the FAD cofactor.

A microscopic redox characterization was obtained for Fc*c*A from *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1, revealing that despite their similar structure the details of the redox properties of the hemes are different [113, 114]. However, the differences are compatible with a common theme of internal control of the electron transfer flow that appears to direct electrons to the flavin catalytic

site only when the protein is reaching full reduction. From these observations a molecular mechanism for regulating the contribution of FccA in the EET vs fumarate reduction in *Shewanella* was proposed [114].

Fc*c*A can also transfer electrons to MtrA, the outer-membrane associated deca-heme cytochrome implicated in EET [97, 108, 115]. Binding studies performed in vitro demonstrated that Fc*c*A interacts with its redox partners, CymA and MtrA, through a single heme (heme II), avoiding the establishment of stable redox complex capable of spanning the periplasmic space [97]. Gene knock-out experiments of Fc*c*A showed defective phenotypes in several anaerobic cell growth conditions involving extracellular electron acceptors [106], which corroborates the previous studies implicating FccA as a periplasmic electron shuttle involved in the EET pathway of *Shewanella* [97, 108, 115]. Furthermore, in vitro and in vivo studies have also shown the occurrence of electron transfer between CymA and Fc*c*A [92, 97, 108].The high abundance in the periplasm, the interactions with CymA and MtrA, and the phenotypes of the deletion mutants make Fc*c*A a major player in electron shuttling in the periplasm during EET.

4.2.2 STC

STC is a highly abundant small tetraheme cytochrome *c* from the periplasm of *Shewanella* with a molecular weight of 12 kDa [116]. Based on gene knock-out experiments, STC is recognized as a key component in the EET pathway of *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1 [106, 109, 117, 118]. This was rationalized by studies with double STC and FccA knock-out experiments showing that at least one of these two cytochromes must be present in the periplasm to allow reduction of DMSO, ferric citrate or nitrate [106]. Overexpression of STC showed that it is a major component in the EET pathway of *Shewanella* [119].

High-resolution crystal structures of STC from *S. oneidensis* MR-1 and *S. algae* are available [120, 121] and a nuclear magnetic resonance (NMR) solution structure exists for STC from *S. frigidimarina* NCIMB400 [122]. Comparison of the structures from these three proteins showed that the general fold is very similar, and the relative positions of the heme groups are well conserved [121]. All four hemes are low-spin and have a bis–histidine axial ligation to the polypeptide chain [120–122]. The arrangement of the pairs of hemes in perpendicular and parallel geometries allows a short distance between the cofactors that enables a rapid intramolecular transfer of the electrons [123].

Microscopic redox properties measured for STC from *S. frigidimarina* NCIMB400, *S. oneidensis* MR-1 and *S. algae* DSM 9167 revealed similarities between these three ortholog proteins [121, 124, 125]. The microscopic reduction potentials for the four hemes of all three STCs cover similar, although not entirely overlapping reduction potential ranges: −190 to −229 mV; −171 to −243 mV; and −153 to −207 mV for *S. frigidimarina* NCIMB400, *S. oneidensis* MR-1 and *S. algae* DSM 9167, respectively. The redox potentials are in the range expected for bis-histidinyl-ligated heme groups with substantial exposure to the solvent [126]. Also, the results showed that electrostatic effects dominate the heme-heme interactions (covering a range of 8–56 mV for *S. frigidimarina*; 11–72 mV for

S. oneidensis; and 6–61 mV for *S. algae* [121, 124, 125], in agreement with the modest redox-linked structural modifications that occur in all three STCs. Furthermore, protonation has a considerable influence (redox–Bohr effect) on the redox properties of the hemes (covering a range of −4 to −36 mV for *S. frigidimarina* NCIMB400; −9 to −56 mV for *S. oneidensis* MR-1; and −1 to −51 mV for *S. algae* DSM 9167 [121, 124, 125], with heme III having in all three STCs the strongest redox-Bohr interaction, with a value similar to those reported for protonation of heme propionates [127]. In comparison, all three studied STC differ in their relative order of oxidation of the hemes due to changes that have occurred over time in their amino acid composition and/or structural arrangement. However, these three STC still possess a common feature that is heme III always presenting the highest reduction potential, and therefore is always the last heme to be oxidised [121, 124, 125].

In vitro binding studies showed that STC could interact with both inner membrane cytochrome CymA and outer-membrane protein MtrA from the MtrCAB complex [97]. These studies also showed that STC interacts with its redox partners, CymA and MtrA, through a single heme (heme IV), which forces detachment from the donor before attaching to the acceptor, preventing the formation of stable redox complexes that can span the periplasmic space of *Shewanella* [97]. Interestingly, although STC and FccA coexist and are highly abundant in the periplasmic space of *Shewanella*, they do not exchange electrons among themselves. This ensures that electron transport across the periplasmic space via these two proteins is segregated [97].

4.3 Outer Membrane

In order to reduce insoluble electron acceptors, electrons must cross the outer membrane and reach the cell exterior. Several redox proteins from *Shewanella* have been shown to be associated or bound to the outer membrane. Of these, the MtrCAB-OmcA protein complex is required to achieve maximal extracellular iron reduction rates [31, 128–130]. The genes encoding for this complex are clustered in an operon organized in the order: *omcA-mtrC-mtrA-mtrB*, where MtrC and OmcA are decaheme cytochromes present at the cell surface, MtrA is a decaheme periplasmic cytochrome and MtrB is a porin in which MtrA and MtrC are embedded on [131, 132].

4.3.1 MtrB

The outer-membrane *ß*-barrel protein MtrB is a 78 kDa protein with no cofactors but essential for EET [128]. Its pore size is estimated to be approximately 70 × 55 × 45 Å and embed MtrA and MtrC [132, 133]. The role of MtrB in metal reduction was first demonstrated by showing that a MtrB knock-out mutant strain lost its ability to reduce Fe(III) and Mn(IV) oxides [128]. MtrB knock-out mutants in *S. oneidensis* MR-1 showed mis-localization of both outer-membrane cytochromes MtrC and OmcA [134]. Furthermore, it was also demonstrated that the

decaheme cytochrome MtrA is only associated with the outer membrane when MtrB is expressed [135]. Using knock-out mutations and subsequent monitoring of complex assembly, revealed the existence of a synergetic relationship between MtrA and MtrB [136]. The assembly of the MtrAB subcomplex stabilizes MtrB, while subcomplex MtrBC does not assemble in the absence of MtrA. Three other stable modules similar to MtrAB have been identified in *S. oneidensis* MR-1. The MtrDE is proposed to be alternative to MtrAB, the DmsEF is part of porin cytochrome complex specific for DMSO reduction and SO4359-60 forms a secondary alternative iron reducing pathway to MtrAB [118, 130, 137, 138]. Moreover, gene clusters encoding for homologous MtrAB modules are phylogenetically distributed among organisms capable of electron exchange with the extracellular environment [135, 139]. Both metal-reducing (e.g., *Shewanella* and *Geobacter*) and metal-oxidizing (e.g., *Rhodopseudomonas* and *Sideroxydans*) bacteria have homologous MtrAB modules (Fig. 4) [30, 31, 135, 140–142]. This strengthens the hypothesis that the MtrAB module is essential for both outwards and inwards EET [131, 143].

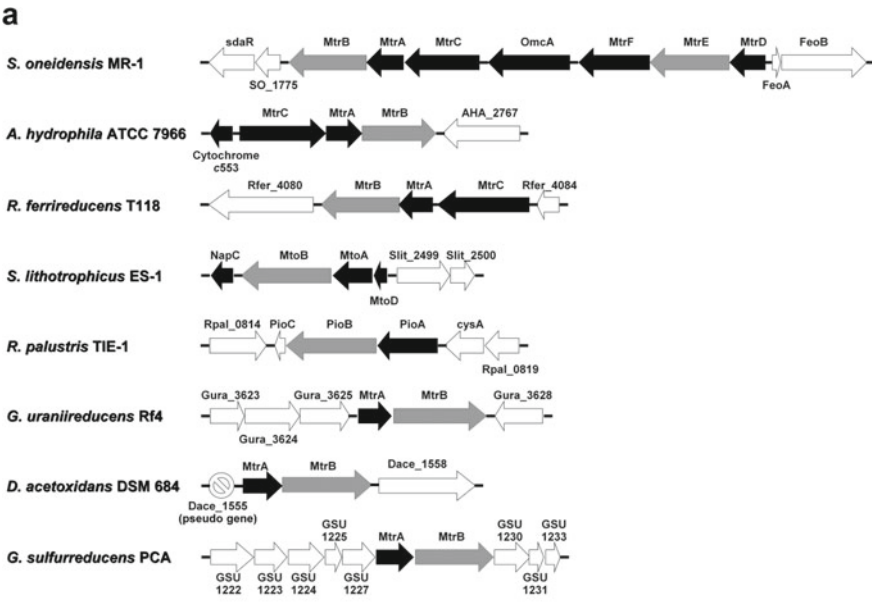
4.3.2 MtrA

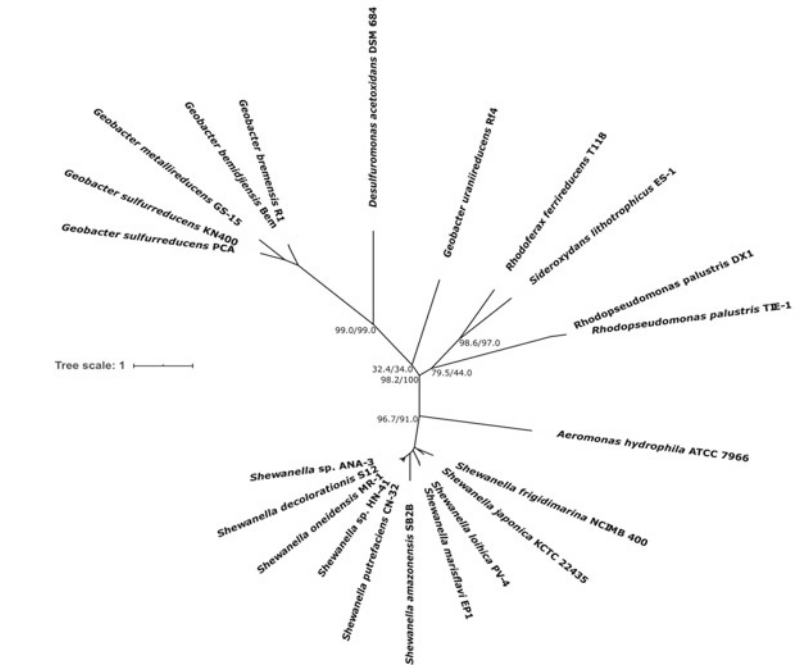
Decaheme cytochrome MtrA is a 37 kDa periplasmic cytochrome with 10 bis-histidine low-spin *c*-type hemes that is associated with the outer membrane via the integral membrane protein complex MtrCAB [30, 31, 135, 144, 145]. In vivo cross-linking assays showed that MtrA interacts on the periplasmic side with the outer membrane *ß*-barrel protein MtrB [144]. Also, it was shown in vitro that MtrA forms a stable protein complex with a dissociation constant stronger than 0.1 μM with its outer membrane partners, MtrB and MtrC [135]. However, under different experimental conditions MtrA was found to be present in the soluble periplasmic fraction hinting to weaker affinity for MtrB [108, 145]. In vitro studies showed that MtrA can interact and receive electrons from the periplasmic cytochrome Fc*c*A [97, 108] and also interact with the periplasmic cytochrome STC [97]. It was also revealed that MtrA can be directly reduced by CymA [96, 108]. Potentiometric redox titrations showed that MtrA is active over a potential range from −100 to −400 mV at pH 7.5 [145].

A high-resolution structure of MtrA has not been reported yet but its aminoacid sequence shows that it is likely to be evolutionarily related to the structurally characterized pentaheme cytochrome NrfB [146]. Small-angle X-ray scattering showed that MtrA is shaped like an extended molecular "wire" with overall dimensions 104 Å × 20 Å × 50 Å [133]. Given that the thickness of the

▶

Fig. 4 **a** MtrAB gene cluster in different electroactive microorganisms and their context in the genome. Cytochromes and β-barrel membrane proteins (MtrB/PioB homologues) are represented in black and gray, respectively. **b** Phylogenetic distribution of MtrAB modules in organisms known to be electroactive [16, 64]. Maximum-likelihood phylogeny with ModelFinder method (best model: LG + I + G4) of concatenated sequences of MtrA and MtrB. Bootstrap and SH-Like Test (-alrt) confidence values (from 1000 replicates each) are shown near each node of the major splits


a
S. oneidensis MR-1
sdaR
MtrB
MtrA
MtrC
OmcA
MtrF
MtrE
MtrD
FeoB
SO_1775
FeoA
A. hydrophila ATCC 7966
MtrC
MtrA
MtrB
AHA_2767
Cytochrome c553
R. ferrireducens T118
Rfer_4080
MtrB
MtrA
MtrC
Rfer_4084
S. lithotrophicus ES-1
NapC
MtoB
MtoA
Slit_2499
Slit_2500
MtoD
R. palustris TIE-1
Rpal_0814
PioC
PioB
PioA
cysA
Rpal_0819
G. uraniireducens Rf4
Gura_3623
Gura_3625
MtrA
MtrB
Gura_3628
Gura_3624
D. acetoxidans DSM 684
MtrA
MtrB
Dace_1558
Dace_1555 (pseudo gene)
G. sulfurreducens PCA
GSU 1225
MtrA
MtrB
GSU 1230
GSU 1233
GSU 1222
GSU 1223
GSU 1224
GSU 1227
GSU 1231


b
Desulfuromonas acetoxidans DSM 684
Geobacter bremensis R1
Geobacter bemidjiensis Bem
Geobacter metallireducens GS-15
Geobacter sulfurreducens KN400
Geobacter sulfurreducens PCA
Geobacter uraniireducens Rf4
Rhodoferax ferrireducens T118
Sideroxydans lithotrophicus ES-1
Rhodopseudomonas palustris DX1
Rhodopseudomonas palustris TIE-1
Aeromonas hydrophila ATCC 7966
Shewanella sp. ANA-3
Shewanella decolorationis S12
Shewanella oneidensis MR-1
Shewanella sp. HN-41
Shewanella putrefaciens CN-32
Shewanella amazonensis SB2B
Shewanella marisflavi EP1
Shewanella loihica PV-4
Shewanella japonica KCTC 22435
Shewanella frigidimarina NCIMB 400
99.0/99.0
98.6/97.0
32.4/34.0
98.2/100
79.5/44.0
96.7/91.0
Tree scale: 1

Gram-negative outer membrane is ≈70 Å [147], the estimated length of MtrA would be sufficient for transferring electrons heme-to-heme across the outer membrane even though the Small Angle Neutron Scattering model of the whole MtrCAB complex suggests that MtrA is only partially inserted in MtrB [143]. Indeed, the 3D-structure determination of an MtrCAB complex revealed that MtrA has very little secondary structure [132], which confer greater flexibility to the protein, allowing it to accept electrons from its various physiological partners [97, 108].

4.3.3 Decaheme Cytochromes MtrC and OmcA

Decaheme cytochromes MtrC and OmcA are two cytochromes with 75 and 85 kDa, respectively, anchored to the outer membrane via a lipidated cysteine [129, 148, 149]. Treatment by proteinase K significantly degraded MtrC and OmcA by 31 and 71%, respectively [148]. This indicates that both proteins are exposed on the outer surface of the cells and that MtrC is not as exposed to the extracellular environment as OmcA, which is coherent with the proposal that MtrC becomes partly buried upon association with the β-barrel protein MtrB [143]. The Small Angle Neutron Scattering structural data agree with previous in vivo cross-linking studies that revealed an interaction of MtrC with the β-barrel protein MtrB forming in combination with the decaheme cytochrome MtrA an outer membrane protein complex MtrCAB, with a 1:1:1 stoichiometry [144]. Cross-linking assays demonstrated that MtrC and OmcA physically interact with each other on the bacterial cells [144, 150], and in vitro studies reported a dissociation constant smaller than 0.5 μM for the MtrC-OmcA complex [151].

Several studies showed that MtrC and OmcA are highly expressed by *S. oneidensis* MR-1 under ferric iron reducing conditions [129, 130, 152] and are capable of direct electron transfer to iron oxides [153–155]. Both MtrC and OmcA polypeptides were shown to contain a putative hematite-binding motif (Ser/Thr-Pro-Ser/Thr) [156] and the physical interaction between MtrC and OmcA synergistically boosts the metal reductase activity of these outer-membrane cytochromes [151]. Disruption of the *mtrC* or *omcA* genes did not affect the growth of *S. oneidensis* MR-1 on soluble terminal electron acceptors, such as fumarate, nitrate and DMSO [149]. In contrast, reduction of insoluble iron oxides and electron transfer to MFC anodes was severely diminished [58, 82, 118, 152, 157, 158]. A series of knock-out mutations of all the outer-membrane cytochromes and subsequent expression of each one individually, showed that MtrC is critical for EET and that mutants containing only the OmcA cytochrome were not capable of transferring electrons to iron [130]. This fact suggests that while OmcA is an iron terminal reductase [60, 149], its contact with the periplasmic redox chain is mediated by MtrC [130]. Additionally, it has been shown that MtrC is responsible for most of the electron transfer to carbon electrodes, while OmcA is mainly involved in cellular attachment to solid surfaces, playing a smaller role in electron transfer [58]. This is coherent with data obtained by antibody functionalized atomic force microscopy (AFM) tips that showed OmcA in the interface between the cell and insoluble substrate, while MtrC displays a more uniform distribution across the

cell surface [158]. Purified MtrC and OmcA were reported to reduce iron oxides at much slower rates compared to measurements with intact *Shewanella* cells and that with the addition of flavins, rates were increased to values comparable to those measured with intact cells [60]. These results demonstrate a role of electron shuttle for flavins during MtrC and OmcA mediated reduction of ferric iron oxides.

Both cytochromes have 10 low-spin *c*-type hemes [95, 148, 157, 158]. Potentiometric titrations revealed that both MtrC and OmcA titrate over a broad range of redox potential from +100 mV to −500 mV and −20 mV to −320 mV, respectively [101, 151, 157]. Crystal structures of these proteins have revealed that the proteins are formed by 4 domains, two multiheme domains that are flanked by two β-barrels with β-strands arranged in Greek key motifs [159, 160]. Both MtrC and OmcA contain a conserved decaheme staggered cross cofactor arrangement, where an octaheme chain formed by hemes V, IV, III, I, VI, VIII, IX, X is crossed by a tetraheme chain consisting of hemes II, I, VI, VII. All the hemes display bis-histidine axial ligand coordination to the heme iron, with each heme within 7 Å of its nearest neighbor, ensuring rapid intra-molecular electron transfer. In vitro experiments revealed that OmcA and MtrC (as well as its homologues MtrF and UndA) are capable of transferring electrons to chemically varied soluble electron acceptors typically found in the oxic-anoxic interface habitats where *Shewanella* is found, with clear differences in the rates for different acceptors [161]. NMR and computational docking studies revealed that whereas for negatively charged FMN and AQDS binding occurs near heme II, neutral riboflavin binds near hemes IX and X and positively charged phenazine methosulphate binds near heme X in a different position. For OmcA, which plays a more important role in surface attachment than MtrC, it was observed that iron oxide particles and graphene oxides do not come into close proximity to the hemes, in agreement with experimentally observed slow electron transfer [162]. Altogether these studies reveal that the structure of these proteins appears to be designed such as the staggered cross provides different exit points for electron through different exposed hemes [161].

EET Enhancement in *Shewanella*

To increase EET in MFCs, over-expression of the MHC involved in EET of *S. oneidensis* MR-1 has been used to enhance current output. For example, it was observed that overexpression of *mtrC* in *S. oneidensis* MR-1 could generate 35% more current in MFCs than that of wild-type organism [82]. Furthermore, the co-expression of the metal-reducing biosynthesis gene cluster *mtrC-mtrA-mtrB* also exhibited an increase in maximum current density of approximately 87% [163]. More recently, the genetic manipulation of *S. oneidensis* MR-1 where the proteins that may compete with STC for EET processes in the periplasmic space were replaced by STC, led to the creation of a mutant that presented 23% higher current generation when compared with the wild-type strain [119]. These studies highlight the importance of genetic engineering to design and tailor MHC towards enhanced electron transfer processes to push forward the practical implementation of electroactive organisms in BES [18].

Genetic engineering was also shown to be crucial to increase the metabolic capacity of *S. oneidensis* MR-1. The heterologous incorporation of metabolic pathways allowed *S. oneidensis* MR-1 to use glucose, xylose or glycerol as the sole carbon and energy source for electricity production in MFC [164–166]. Furthermore, the heterologous expression of proteorhodopsin, a light-dependent proton pump, led *Shewanella* to consume lactate at an increased rate when it is illuminated which was reflected by the increase in current generation when compared with wild-type organism [167]. Recently, genetic manipulation of *S. oneidensis* MR-1 allowed the modification of this organism to use electrons from a cathode to drive reduction of acetoin to 2,3-butanediol, demonstrating the capacity to genetically engineer a microbial electrosynthesis pathway [168].

Another approach used to enhance the rate of EET in *S. oneidensis* MR-1 was the increase of the intracellular electron pool, by engineering and driving the metabolic flux toward the enhancement of intracellular NADH regeneration [169]. In this work three different modules (the de novo pathway, the salvage pathway and the universal biosynthesis pathway) were over-expressed, and the capacity for electricity production of mutated *S. oneidensis* MR-1 was evaluated. The increase in electricity generation and Coulombic efficiency showed that an increase in the NAD(H+) pool results in the transfer of more electrons from increased oxidation of the electron donor to the EET pathway, enhancing intracellular electron flux and EET rate [169].

Mediated electron transfer has been demonstrated to be one of the most important mechanism for *S. oneidensis* MR-1 in performing EET. Promoting redox shuttle biosynthesis in this organism also enhances EET efficiency in BES [163, 170]. The heterologous expression of the flavin biosynthesis pathway from *Bacillus subtilis* enhanced EET rate of *S. oneidensis* MR-1, with an increase of 13,2 times of the maximum power output when compared with wild-type strain [170]. Likewise, the homologous expression of the flavin biosynthesis gene cluster *ribA-E* in *S. oneidensis* MR-1 increased the maximum current density by approximate 110% [163]. Furthermore, overexpression of the gene *ydeH* from *E. coli* in *S. oneidensis* MR-1, responsible for the biosynthesis of c-di-GMP enhanced biofilm formation and bioelectricity generation [171]. The high levels of intracellular c-di-GMP promote the expression of adhesive matrix components, which facilitates bacterial biofilm formation. The maximum power density obtained with the engineered strain was $\sim$2.8 times higher than that achieved by the wild-type strain [171].

The recent work on the CRISPR/Cas9 approach to manipulate *S. oneidensis* MR-1 enables the precise site-directed mutagenesis of the bacterial chromosome [172]. This allows the modification of several different genes, and the introduction of various types of mutations, including individual base changes and net gene deletion in this model strain [172]. This approach will simplify the genetic manipulation of this electroactive organism facilitating the implementation of high-throughput genomic engineering technologies, contributing to the improvement of this type of organisms towards the practical implementation of BES.

5 Conclusion

MFCs have been intensively investigated over the past decades with tremendous advances. Despite all the improvements made to increment MFC power output, the low EET rate from electroactive microorganisms to the electrode surfaces remains a bottleneck that prevents the practical application of BES [173–175]. At this time, means to improve electroactivity are being explored, on the biochemical, genetic and technological fronts [121, 176–178].

EET has been observed and studied in phylogenetically diverse microorganisms [16], indicating that this microbial trait is widespread in nature. Despite the microbial diversity, only a few species have emerged as model organisms for the study of EET (e.g., *Geobacter sulfurreducens, Shewanella oneidensis* and *Thermincola spp.*). Of all the EET model organisms, *S. oneidensis* MR-1 is the most extensively studied and presently has the best characterized molecular mechanism of EET [23, 32, 179]. The extensive study of *Shewanella versus* other electroactive bacteria has to do with a number of combined factors such as robust growth under oxygen conditions, existence of sequenced genome, straightforward genetic manipulation, and robust strategies for overexpression of the relevant multiheme cytochromes which makes these bacteria ideal to work with. It is well known that *Geobacter* species are the dominant members in acetate fed BES electrode biofilms and that *Thermincola spp.* are able to grow at higher temperature, and both produce higher current densities compared to *Shewanella* [16, 176, 180–182], making them more attractive candidates for BES applications. Despite these positive aspects, several difficulties mainly involving growth and genetic manipulation have rendered these bacteria more challenging to study and fully characterize their EET pathways, with numerous gaps in the understanding of their molecular mechanisms of EET.

Using as model organism bacteria such as *S. oneidensis* MR-1, our understanding on how EET occurs has increased greatly. Here, MHCs continuously revealed themselves as key players, creating an efficient redox network that stretches from the cytoplasmic membrane, across the periplasmic space and through the outer membrane, transferring electrons directly or indirectly to their insoluble acceptors [29, 183]. The detailed functional characterization of the MHCs from microorganisms capable of EET will ultimately lead to a more rational design and optimized biotechnological applications which use these organisms. This optimization can be biological or technological, using different approaches such as molecular biology to tune the reduction potentials of hemes found in the MHC involved in the electron transfer pathway [121, 162, 184, 185], manipulation of electron mediator synthesis pathways [186] reprogramming gene regulatory circuits to enhance electron transfer pathways [187] or even surface enhancement of electrodes for improved cellular contact [188]. Either way, all stand to benefit from the full characterization of these complex electron transfer pathways.

In this chapter, the key *c*-type cytochromes for EET in *S. oneidensis* MR-1 were reviewed in order to shed light on how electrons are delivered to the cell surface during EET and possible mechanisms that could be applied to enhance the function of MFCs and other BES, bringing them closer to commercial applications.

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