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Enhancing Microbial Electron Transfer Through Synthetic Biology and Biohybrid Approaches: Part I

Bioelectrochemistry for sustainable energy conversion

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Traditional microbial synthesis of chemicals and fuels often rely on energy-rich feedstocks such as glucose, raising ethical concerns as they are directly competing with the food supply. Therefore, it is imperative to develop novel processes that rely on cheap, sustainable and abundant resources whilst providing carbon circularity. Microbial electrochemical technologies (MET) offer unique opportunities to facilitate the conversion of chemicals to electrical energy or *vice versa*, by harnessing the metabolic processes of bacteria to valorise a range of waste products, including greenhouse gases (GHGs). However, the strict growth and nutrient requirements of industrially relevant bacteria, combined with low efficiencies of native extracellular electron transfer (EET) mechanisms, reduce the potential for industrial scalability. In this two-part work, we review the most significant advancements in techniques aimed at improving and modulating the efficiency of microbial EET, giving an objective and balanced view of current controversies surrounding the physiology of microbial electron transfer, alongside the methods used to wire microbial redox centres with the electrodes of bioelectrochemical systems *via* conductive nanomaterials.

Introduction

Bioelectrochemistry is concerned with electrical current generated through chemical reactions in biology and is intrinsic for life's processes. Electron flow provides biology with the free energy conversion mechanisms essential for survival, allowing the assimilation, storage and utilisation of energy from the environment *via* a set of oxidation and reduction reactions (1). The availability of oxygen in many ecosystems enables

aerobic respiration, employing gaseous oxygen as the terminal electron acceptor (TEA) of the respiratory transport chain. However, in oxygen-depleted environments, an alternate strategy must be employed. Instead of importing the TEA for intracellular respiratory processes, some microbial species have evolved systems to import or export electrons from their respiratory transport chain *via* a variety of EET mechanisms. By electrochemically communicating with their external surroundings, electroactive bacteria (EAB, a term disputed by the scientific community as most bacteria have some form of electron transfer mechanism) widen the pool of potential TEAs, enabling survival in environments where the optimal TEA, oxygen, is unavailable. First observed over 100 years ago (2), recent developments in the study of microbial EET mechanisms offer unique possibilities to several research areas including energy conversion and storage (3, 4), environmental remediation (5–7) and bioelectronics (8, 9).

A range of bacterial EET mechanisms have been identified, including redox-active 'mediator' molecules (10–13), membrane-bound or extracellularly-projected cytochromes (14–18) and conductive filamentous proteins termed 'nanowires' (19–21). In addition, there has also been reported hypothesis, and now some experimental evidence that electron transfer through the cell wall of Gram-positive bacteria and yeast can occur (22–24). The exact mechanisms for the latter are yet to be elucidated. A significant body of work has examined the use of EAB as chassis for a range of energy conversion processes such as microbial fuel cells (MFCs) and microbial electrosynthesis systems (MES) within the wider category of MET (25–30).

MFCs operate in an outward or anodic direction, generating electricity *via* bacterial liberation of electrons from reduced energy sources such as municipal wastewater or agricultural biomass (31, 32). MFCs offer opportunities to decarbonise society's electrical generation capacity, providing an alternate source of renewable electricity while reducing the energy expenditure of costly and environmentally damaging processes such as sewage treatment or environmental detoxification. Indeed, MFC reactors have been constructed running on energy sources as diverse as urine (33), car-wash wastewater (34) and lead-contaminated soil (35). They have also been studied as valuable mineral recovery processes, with a 2019 study achieving the recovery of cobalt from spent lithium ion batteries (36), an ongoing environmental issue that will only expand as electric vehicle production and renewable energy storage capacity increases.

MFCs also offer intriguing environmental and medical biosensing applications (37, 38) due to their long service life and self-powering nature. In one example, researchers constructed a MFC-biosensor capable of detecting sewage contamination of groundwater over a five month period, running autonomously (39). Although they promise such a wide array of environmentally beneficial applications, and research into MFCs has been conducted for almost 100 years (40), fundamental limitations have restricted their application to laboratory-scale, and occasionally pilot-scale, systems. This is due to low power generation, largely attributable to poor electron transfer efficiency at the interface of microbial cell and electrode.

The reverse of MFC, microbial electrosynthesis generates reduced chemical products from oxidised carbon in an inward or cathodic electron transfer direction, employing bacterial uptake of electrons to assimilate into valuable reduced chemical products such as biofuels or platform chemicals (31). These processes are suggested to provide solutions to a range of environmental issues, such as excess atmospheric carbon, and offer a route to the production of carbon neutral biofuels with no demand on arable land. Their attractiveness derives from the disparity between organic photosynthetic process and photovoltaic electricity generation: commercially available solar panels now exceed 20% solar conversion efficiencies, while the maximum theoretical efficiency for biological photosynthesis is approximately 12% and, realistically, agricultural crops only achieve 1–4% (41). However, renewable electricity generation creates issues surrounding intermittent production, to which MES may offer a solution by storing excess electrical energy in C–C bonds of reduced chemical fuels. In an recent example, researchers modified the metabolism of the photoautotrophic organism *Rhodopseudomonas palustris* TIE-1 to allow fixation of atmospheric carbon dioxide to produce the biofuel *n*-butanol directly from solar-panel generated electricity (42). Compared to nearly 100 years of MFC research (40), MES processes are in their infancy, with the term only coined in 2010 (43, 44). While MES processes offer significant promise in achieving a circular carbon economy, the outputs of chemical fuels are still below the limit for industrial feasibility and significant research and investment is required to realise the potential. While there are numerous

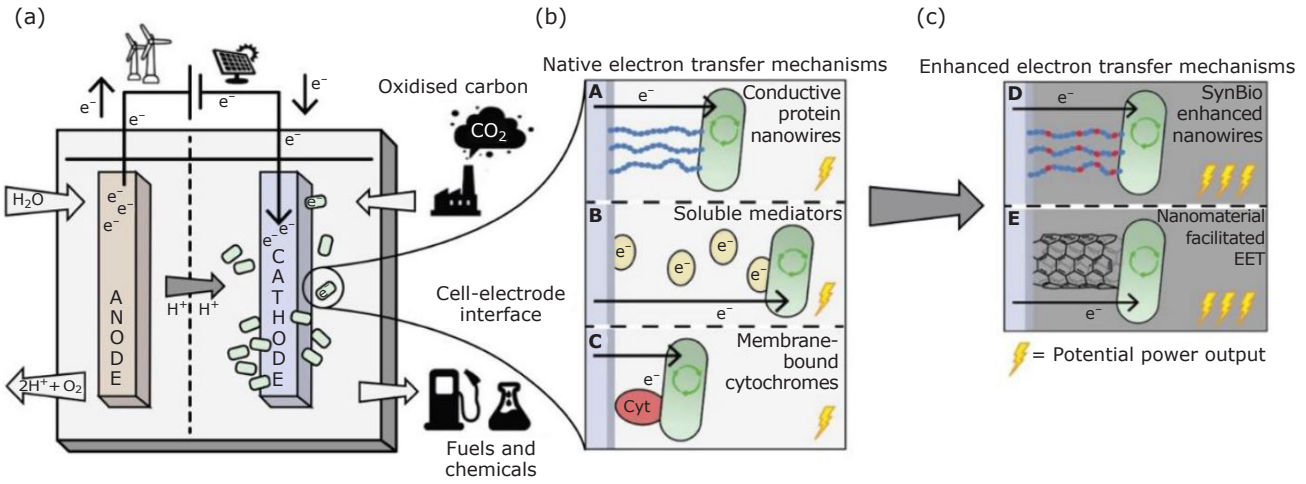

Fig. 1. (a) An example MES cell; (b) native EET pathways: A = conductive pili protein nanowires, B = secretion of soluble electron-carrying 'mediators' and C = outer membrane-bound cytochromes; (c) novel EET mechanisms with potential to increase the rate of microbial EET *via*: D = modification of aromatic amino acid (red dots) content and positioning on the pili protein sequence to increase conductivity; and E = wiring microbial redox centres to the electrodes of bioelectrochemical systems *via* conductive nanomaterials

bottlenecks affecting the low production rates, a fundamental cause of this is the low efficiency of biological EET mechanisms (45, 46), limiting the rate of cathodic electron uptake.

Current MFC reactors typically generate maximum power densities between 3800 mW m^{-2} to 4400 mW m^{-2} (47), with the maximum reported being >10,000 mW m^{-2} (48). For MES, the highest reported biofuel yield is 12.5 l_{CH_4} l^{-1} day^{-1}, representing a conversion efficiency of 30% (49). However, poor cost-effectiveness and low efficiency of native EET mechanisms have resulted in the limited commercial applications of MET (50–53). This is partly because exoelectrogenic bacteria often reside in specific environmental niches which are difficult to replicate in laboratory settings or within bioreactors (54) and limited metabolic optimisation means the biochemical processes underpinning MET are still reliant on the bacteria's cellular processes operating as they do in nature (55). Moreover, there is mismatch in abiotic/biotic material properties (56) which prevents seamless integration of the cells with the electrodes. This increases cell impedance, resulting in lower charge transfer. Furthermore, a fundamental limitation of current MET affecting both MFC and MES is the quantity of individual microbial cells that can physically contact with the electrode and participate in EET (57). Generating strategies to improve the EET rates of native mechanisms, as well as recombination of EET mechanisms in industrially important bacterial strains, are vital in advancing the application of MET research. While MET reactor setup and operational optimisation is a major route to improve the output efficiencies of both processes, this review aims to outline the approaches researchers have used to increase the underlying EET rates between cell and electrode, and increase the pool of potential EAB chassis available for use in MET system design. Two contrasting routes to modulate microbial EET, *via* synthetic biology and 'bio-hybridisation' with conductive nanostructured materials will be explored in subsequent sections of this review and are summarised in **Figure 1**. First, the current state-of-the-art of native EET mechanisms are summarised, followed in Part II (58) by a review of biological and biohybrid approaches used to modulate EET for MET applications.

Bacterial Extracellular Electron Transfer Pathways

Exoelectrogenic bacteria utilise one or a combination of several EET mechanisms to electrochemically communicate with their environment, either by direct or indirect electron transfer mechanisms (**Figure 2**). Direct EET mechanisms involve intimate physical contact between the organism and the electrode and our current understanding of this interaction is largely based on the study of two model systems: *Shewanella onedesis* MTR-1 (60, 61) and *Geobacter sulfurreducens* (53, 62, 63). *S. onedesis* MTR-1 is considered a model organism to study EET *via* outer-membrane-bound cytochromes (OMC), while *G. sulfurreducens* is

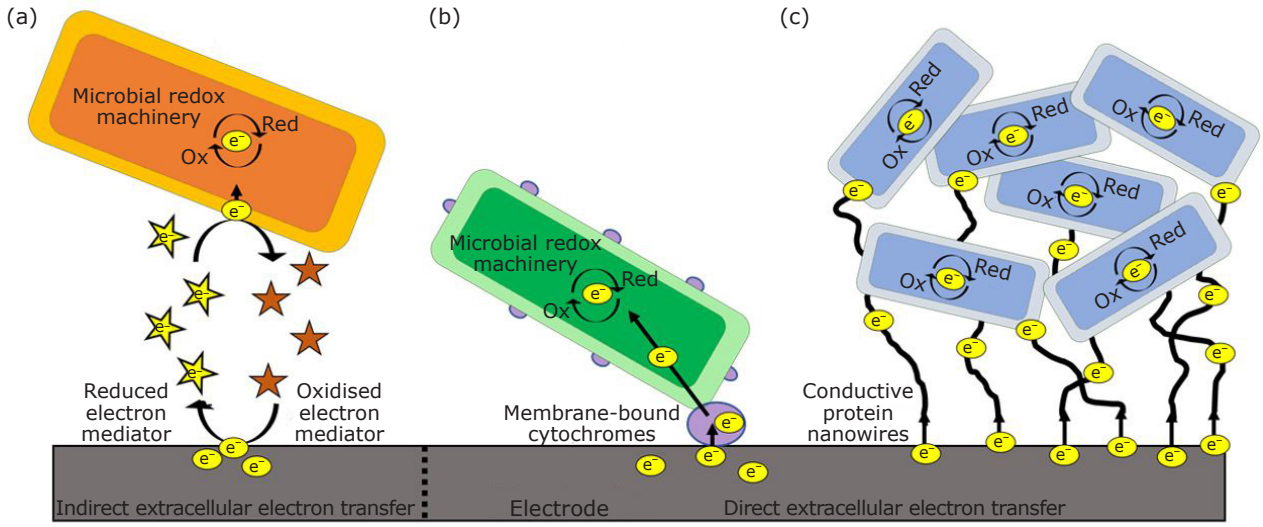

Fig. 2. Mechanisms of extracellular electron transfer: (a) indirect EET *via* the secretion of redox-active electron mediators; (b) short-range, direct EET *via* outer membrane-bound cytochromes as in *Shewanella oneidensis*; (c) long-range, direct EET *via* the production of an extracellular matrix comprised of conductive nanowires as in *Geobacter sulfurreducens*. Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature (59), Copyright (2009)

known for its conductive protein appendages, often referred to as 'nanowires'. While other *Shewanella* and *Geobacter* are capable of external charge transfer, and in some cases such as *Geobacter metallireducens* possibly exceed in its ability to transfer electrical charge when compared to the model organisms (64), the former strains were the first in which these mechanisms were described and to date, they form the most extensive knowledge base for EET. Direct EET can be further divided into long- and short-range mechanisms (65–67) (explained in more detail below) and organisms frequently express multiple EET pathways in response to environmental changes (68, 69).

Indirect EET mechanisms rely on soluble, redox-active 'mediator' compounds to transfer electrons when the organism is not in direct physical contact with the oxidising or reducing source (13, 70–73). Although exceptions exist, many bacterial cell membranes are comprised of non-conductive peptidoglycans and phospholipids, electrochemically insulating intracellular redox-active species with the external environment, and not yet shown to be capable of significant membrane faradaic electron transfer (74). To overcome this shortfall, a large number of bacterial strains utilise electron-carrying mediator compounds which allow the shuttling of electrons between metabolic machinery and external oxidation or reduction sources (75). Some of these compounds are synthesised by the organism, while other organisms rely on inorganic redox-active minerals. Additionally, some mediator compounds such as pyocyanin are membrane-diffusible, allowing microbial uptake and interaction with intracellular redox centres, while others are not and perform EET with membrane-bound redox machinery such as cytochromes.

Many electron mediators possess reversible redox states, enabling the cycling of oxidation and reduction reactions, meaning relatively low mediator concentrations can facilitate the indirect EET of a system (70). First studied in the 1930s (40), a large range of mediator compounds were identified throughout the 20th century including phenazines, phenoxazines, phenothiazines and quinones (1, 76). Furthermore, the addition of exogenous mediator compounds to MET was a technique used in early studies to artificially boost the rate of bacterial EET. However, they generate relatively low current densities compared to direct EET mechanisms (10–100 mA cm^{-2}) and introduce added cost and complexity to EET bioreactor design (77). For these reasons, modern MET research has largely moved on to engineering solutions for enhanced EET mechanisms (78).

Outer-Membrane-Bound Cytochromes

Shewanella spp. are Gram-negative, rod-shaped facultative anaerobes, of which many have found

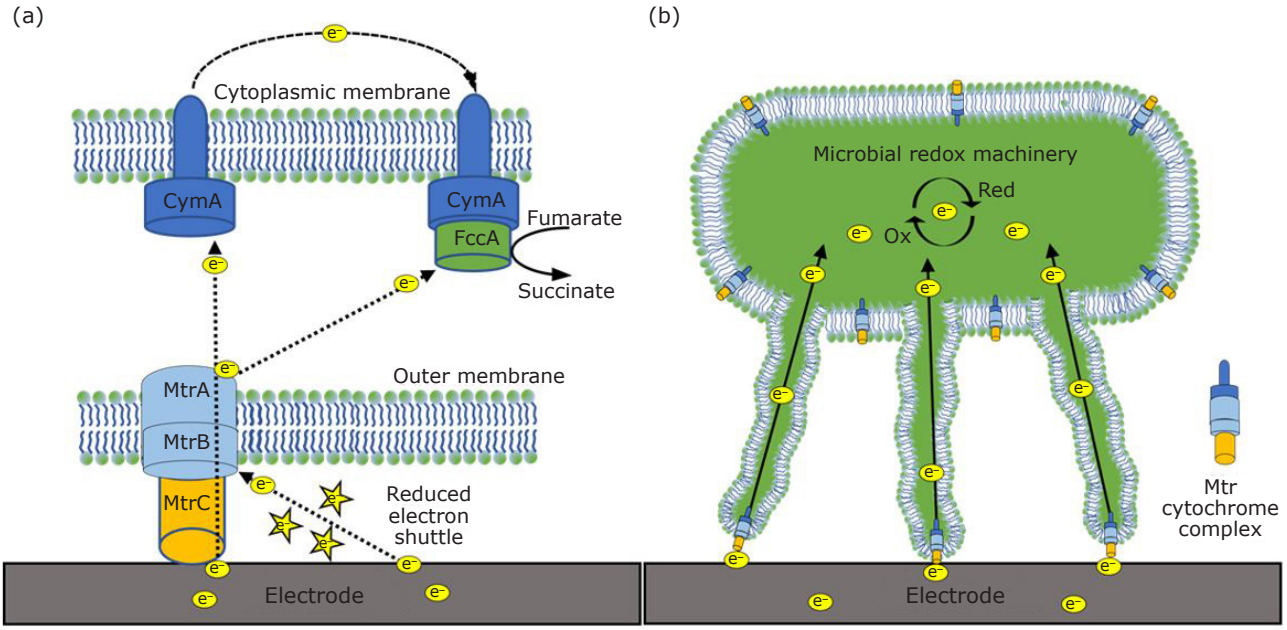

Fig. 3. Proposed reversible EET pathway of *S. onedesis* MTR-1: (a) MtrC is bound to the outer membrane, accepting electrons, and transferring to MtrB and MtrA where it crosses the periplasmic space *via* CymA, the menaquinone pool and CymA-FccA interaction. 15% of electron flux travels from MtrA directly to FccA; (b) proposed structure of *S. onedesis* MTR-1 membrane protrusions with embedded Mtr redox machinery, allowing EET to occur at extended distances. Adapted from (82) under a Creative Commons Attribution License

an ecological niche at redox interfaces (74). The *Shewanella* model of direct EET is based on a series of multi-enzyme complexes embedded in the bacterial cell membrane, forming a conductive bridge across the periplasmic space (79) with indirect methods also contributing substantially to electron flow. *S. onedesis MTR-1* is the model electroactive strain, from which much of the understanding of this genus is derived. In *S. onedesis MTR-1* the Mtr pathway (encoded by the *mtrCAB* operon) enables the electrons to move between living cells and inorganic materials. It is proposed that MtrC, a multiheme-containing cytochrome is bound to the outer membrane, accepting or donating electrons to the cell's surroundings with MtrB and MtrA spanning the periplasmic space (79–81) (**Figure 3(a)**).

The pathway is redox-reversible, making *S. onedesis* MTR-1 capable of both inward and outward EET (82, 83). However, this is dependent on the outermost enzyme, MtrC, directly contacting the anode or cathode. To allow this to occur at greater distances, *Shewanella* spp can form extensions of its outer membrane, with embedded MTR machinery in response to oxygen limitation (69, 79, 80, 84). By forming these cellular protrusions, *S. onedesis* MTR-1 has evolved a strategy to extend the spatial range of Mtr-facilitated EET (80), enabling long-range direct EET (**Figure 3(b)**).

However, the EET mechanism of these protrusions ('nanowires') in *S. onedesis* MTR-1 is less clearly understood. A seminal 2014 study used gene expression analysis, combined with directly labelling cellular components and immunofluorescence imaging to capture the structure of *S. onedesis* MTR-1 nanowires and suggested a series of outer membrane vesicles enabling EET over micrometre distances (80). This could explain the difficulties previous studies experienced in isolating *S. onedesis* MTR-1 nanowires, as they are indistinguishable from the rest of the cellular membrane, making the extraction and analysis of individual filaments a significant challenge. Building on this work, Subramanian *et al*. proposed that EET occurs *via* a combination of 'electron-hopping', and diffusion between redox-active proteins located on dynamic, outer-membrane-vesicles conjoined to form conductive 'nanowire' extensions (79).

While it has been suggested that *S. onedesis* MTR-1 is capable of lower current generation than other currently known exoelectrogenic strains (59, 85–87), it remains one of the most studied organisms in bioelectrochemical systems. The bidirectional EET nature of the Mtr pathway means

it can be used in both MFC and MES (82, 88–90), in contrast to other long-range direct EET mechanisms for which the ability to operate bidirectionally is unclear. Significant gains in expanding the synthetic biology toolkit of *S. onedesis* MTR-1 have been achieved, including the use of inducible expression and repression systems (91–93), and production of electrocompetent cells (94), important tools to study the underlying biology of EET and allowing precise, high-throughput genetic modification. Expression of the Mtr pathway in *Escherichia coli* has been undertaken by several research groups (17, 95–98), allowing the leverage of advanced genetic modification tools. Reactor setup is also highly influential on the rate of *S. onedesis* MTR-1 EET, with a highly cited study achieving 100% increases in power generation using oxygen-limiting conditions and high electrode surface area to reactor volume in a miniature 1.2 cm^3 MFC (99). These findings suggest further optimisation of reactor setup may allow higher current generation gains.

Conductive Protein Nanowires

Geobacter are obligately anaerobic, Gram-negative bacteria (100), commonly used as the biocatalyst in anodic MET processes. *G. sulfurreducens,* the most well-characterised strain, has generated the highest maximum current produced to date in MFC reactions (48). While the current scientific consensus is unclear whether the conductive nanowires of *Geobacter* spp are capable of bidirectional EET, *G. sulfurreducens* cathodic electron transfer has been demonstrated in MES (101–103). Although *Geobacter* spp are arguably the most well-studied genus of EAB, the complex array of EET mechanisms shown to contribute to its respiratory metabolism mean the precise nature of how *Geobacter* spp electrochemically communicates is still an enigma.

In contrast to *Shewanella* membrane extensions, *Geobacter* has been shown to produce conductive proteinaceous filaments. Originally, these filaments were thought to be a form of type-4 pili, termed 'nanowires', providing *Geobacte*r spp with a long range, direct electron transfer mechanism (104). The original model suggests *Geobacter* pilin nanowires are secreted by the type-4 apparatus shared with many Gram-negative, PilA-expressing organisms (21). They are thought to consist of repeating subunits of a single PilA monomer (105) with homology to other Gram-negative PilA proteins (**Figure 4**).

The PilA monomers are constructed into pili complexes by the T4aP assembly system, encoded by the pilMNOPQ genes. These genes are present in a wide range of Gram-negative organisms, including pathogens (106, 107) with typical functions including twitching motility and adherence to host cells, making type-4 pili an important virulence factor (108, 109). The PilA of *Geobacter spp* however is much shorter than other T4aP PilA monomers, with a highly truncated carboxyl terminal end (51). This is thought to prevent the neutralisation of electrons travelling along the PilA before moving the next subunit (110). Interestingly, electroactive pili are not exclusive to *Geobacter* spp. Recent analysis has suggested that *Flexistipes sinusarabici, Calditerrivibrio nitroreducens* and *Desulfurivibrio alkaliphilus* may express filaments of comparable conductivity to *G. sulfurreducens* (111), and electroactive filaments have been identified in archaea (112). This widespread diversity provides further evidence of the effectiveness of conductive nanowires as an EET option, as the mechanism may have evolved independently across phylogenetic kingdoms.

A substantial body of evidence has suggested a relationship between conductivity and aromatic amino acid content of PilA monomers (63, 110, 111, 113–115). The actual mechanism of how electrons transverse the filaments, however, is less clearly understood and is subject to much debate. Two contrasting models have been proposed, including metallic-like conductivity *via* aromatic amino acid residues along the pili monomer sequence, along with multistep electron-hopping *via* a network of type-c cytochromes distributed throughout the pili and biofilm (116, 117). Several studies have described pilus conductivity to be in a manner similar to metallic wires, suggesting a form of conductivity termed 'metallic-type' *via* π-π stacking of aromatic amino acid orbitals within the inner core of the pili structure (64, 113, 116, 118). However, others counter that the geometry of phenyl ring stacking is not sufficient to bring aromatic residues within the correct spatial distances to allow delocalised charge transfer and instead suggests an axial, multistep electron-hopping conductivity model (119). The PilA monomer of *G. sulfurreducens* contain higher proportions of aromatic amino acids than other type-4 pili (111) and are congregated at the c-terminal end (120). Experiments reducing the aromatic amino acid composition of *G. sulfurreducens* PilA have been shown to produce strains capable of under 10% electron transport rates compared to wild-type strain (113), indicating the correlation

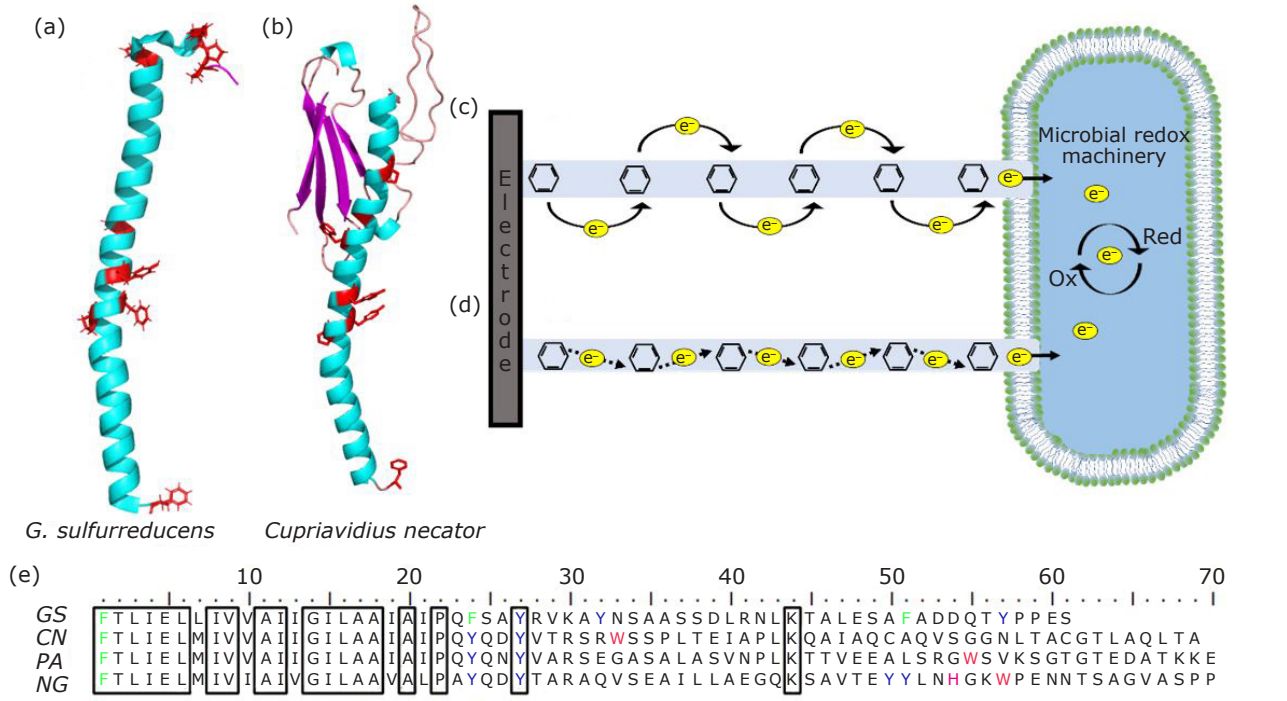

Fig. 4. *Geobacter* conductive protein nanowires: (a) predictive model of *G. sulfurreducens* PilA conductive nanowire; (b) the closely related type-4 pili of *Cupriavidius necator*, displaying differences in aromatic amino acid (red side chains) composition and secondary protein structure. Both models produced by Pymol. Contrasting models of electron flow: (c) electron-hopping/tunnelling model, where electrons move between aromatic amino acid residues along the PilA filament; and (d) metallic-like conduction, where delocalised charges are distributed throughout aromatic amino acid residues within the inner core of the filament. Reprinted from (51), Copyright (2014), with permission from Elsevier; (e) comparison of type-4 pili protein sequences from: *GS* (*G. sulfurreducens*), *CN* (*Cupriavidius necator*), *PA* (*Pseudomonas aeruginosa*), *NG* (*Neisseria gonorrhoeae*). Produced *via* BioEdit

between aromatic amino acid content and electron transport rates. Recent analysis has also suggested that microbial nanowires are in fact not PilA at all, but polymerised OmcS cytochromes with the stacking of heme rings within the inner core providing conductivity (14, 16). Further analysis then observed a second form of cytochrome-based nanowire, with 1000-fold conductivity increases over OmcS (121). However, direct atomic force microscopy analysis of *G. sulfurreducens* cells suggested that 90% of surface-expressed filaments were comprised of PilA proteins (122). This disparity has stimulated heated debate (21, 123) with others discussing at length the limitations of this electron transfer model. Recently, researchers suggested that the pili of *G. sulfurreducens* did not have a conductive function and were associated with structural roles of supporting biofilm growth on electrode surfaces (124). Contradicting this hypothesis and further blurring the scientific consensus, another 2022 study achieved a conductive pilus-based nanowire on the type-1 architecture of *E. coli* by modifying the aromatic amino acid content of the chassis' native monomer sequence (125), the first example of a conductive pilus not based on type-4 construction systems. Although these findings are contradictory regarding the structural identity of *G. sulfurreducens* conductive nanowires, they strongly indicate there may be multiple pathways contributing to EET for this organism (111). Given the myriad of EET mechanisms already shown to be present in *Geobacter* spp (100, 126–130), it is possible that both pili- and cytochrome-based conductive nanowires perform EET functions in *Geobacter* spp.

Limitations of Native EET for MET Applications

Despite the growing interest and literature on MET, their application beyond laboratory scale is still limited, mostly due to poor charge transfer efficiency at the interface of cell and electrode (50, 116, 131). In MFCs, the highest reported power densities are in the order of a few watts per square metre of electrode surface area, significantly lower than current

densities produced in chemical fuel cells (53). While the complexity of factors influences the poor power and current generation of MET, a key factor that this review aims to address is the degree of biofilm formation on the electrode. The number of cells in direct contact with the electrode limits the amount of EET that can occur, therefore restricting the amount of microbial respiration and subsequent current generation. Electroactive nanowires such as those expressed by *Geobacter* spp allow longer-range EET providing a connection between cell and electrode (21, 132, 133) and as such are a critical target to overcome this limitation, either by optimising the native mechanism, expressing in heterologous hosts or replicating using conductive nanomaterials.

To address the limitations posed by the native low EETs, engineering biology and biohybrid approaches have been explored as strategies to boost efficiency. Investigating these approaches both independently and in tandem could provide novel strategies in advancing MET past the laboratory stage and allowing society to benefit from their application. A wide range of biological approaches and nanomaterials have been studied to boost microbial EET mechanisms and will be outlined in Part II (58) of this review.

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