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Microbial nanowires for bioenergy applications

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Microbial nanowires are electrically conductive filaments that facilitate long-range extracellular electron transfer. The model for electron transport along *Shewanella oneidensis* nanowires is electron hopping/tunneling between cytochromes adorning the filaments. *Geobacter sulfurreducens* nanowires are comprised of pili that have metal-like conductivity attributed to overlapping pi–pi orbitals of aromatic amino acids. The nanowires of *Geobacter* species have been implicated in direct interspecies electron transfer (DIET), which may be an important mode of syntrophy in the conversion of organic wastes to methane. Nanowire networks confer conductivity to *Geobacter* biofilms converting organic compounds to electricity in microbial fuel cells (MFCs) and increasing nanowire production is the only genetic manipulation shown to yield strains with improved current-producing capabilities. Introducing nanowires, or nanowire mimetics, might improve other bioenergy strategies that rely on extracellular electron exchange, such as microbial electrosynthesis. Similarities between microbial nanowires and synthetic conducting polymers suggest additional energy-related applications.

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Introduction

Several bioenergy strategies require that microorganisms exchange electrons with their external environment (Figure 1). For example, effective metabolism of alcohols and fatty acids, which is essential for the anaerobic digestion of organic wastes to methane, requires an electron exchange between the microorganisms oxidizing these substrates and the methanogens that accept the electrons for the reduction of carbon dioxide to methane [1••]. The conversion of organic wastes to electricity in microbial fuel cells (MFCs) requires that microorganisms transfer electrons to electrodes [2,3]. Microbe-electrode exchange is also required for processes such as electromethanogenesis and microbial electrosynthesis in which microorganisms use electrons derived from electrodes for the reduction of carbon dioxide to methane or multi-carbon fuels [4,5].

One mechanism for external electron exchange is for microorganisms to reduce an electron acceptor to generate an electron carrier molecule that can serve as an electron donor for the electron-accepting microbe or electrode (Figure 1). Interspecies H_2 transfer, in which the electron-donating organism reduces protons to H_2 and the electron-accepting organism oxidizes the H_2 with the reduction of an electron acceptor, is an important example of this form of electron exchange [1••]. Organic electron shuttles, either produced by cells (i.e., flavins, phenazines, cysteine) or commonly found in the extracellular environment (i.e., humic substances) can facilitate electron transfer to electrodes and interspecies electron transfer [2,6,7••]. These electron shuttles, as well as terminal electron acceptors such as metals or electrodes, may be reduced by outer-surface redox-active molecules, such as *c*-type cytochromes [8–11]. Conductive materials like magnetite and granular activated carbon (GAC) can also promote interspecies electron exchange [6,12,13].

Another, more controversial, mechanism for extracellular electron exchange is via electrically conductive filaments, collectively referred to as microbial nanowires [2,14]. The purpose of this review is to summarize the recent literature on microbial nanowires and their potential role in bioenergy applications.

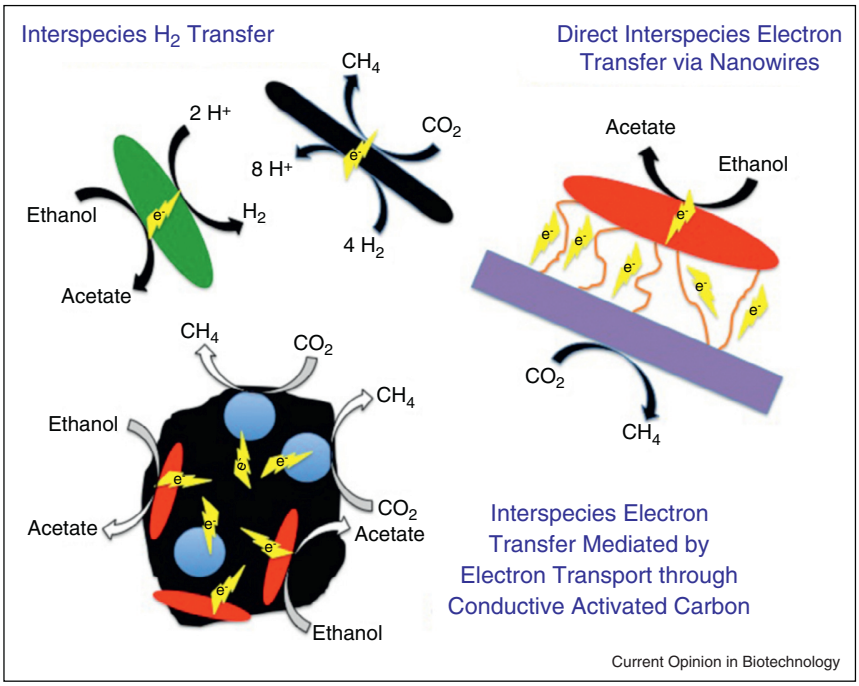
Nanowire diversity in microbes and conduction mechanism

Microbial nanowires have been implicated in extracellular electron transfer in many organisms, but have only been studied in detail in two microorganisms, *Shewanella oneidensis* [15] and *Geobacter sulfurreducens* [16]. Studies to date have suggested that the nanowire function in these two organisms is remarkably different (Figure 2).

Shewanella oneidensis nanowires

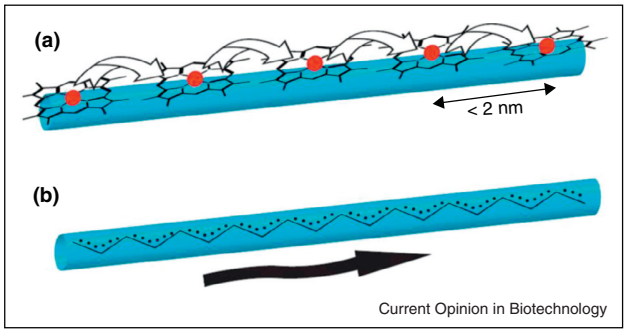
The current model for electron conduction along *S. oneidensis* nanowires is electron hopping (Box 1) between cytochromes (Figure 2a) adorning a filament of as yet unspecified composition [15,17,18,19••,20,21]. The initial evidence suggesting an electron hopping mechanism was the finding that a strain that lacked genes for the outer surface cytochromes MtrC and OmcA produced non-conductive filaments [15,18]. A theoretical model indicated that multistep hopping model along chains of cytochromes could account for conductivity of *S. oneidensis* pili, assuming that the cytochromes are aligned along

Figure 1



Mechanisms for extracellular cell-to-cell electron exchange in methanogenic digesters. Intercellular electron carriers can be reduced and oxidized within cells and diffuse through the extracellular environment between electron-donating and electron-accepting microbes, such as in interspecies H_2 transfer. Cells can forge biological electrical connections, with components such as conductive pili as shown here. Alternatively, cells can attach to conductive materials, such as granular activated carbon, which serves as a conduit for electron transfer between electron-donating and electron-accepting cells. Similar extracellular electron transfer mechanisms are possible in which an electrode is substituted for either the electron-donating or electron-accepting microorganism. A diversity of compounds can potentially substitute for H_2 as the electron shuttle, some of which are extracellularly reduced/oxidized. Various redox-active proteins, such as *c*-type cytochromes, may facilitate electron exchange between pili and electron acceptors/donors or may directly serve as biological electrical contacts.

Figure 2



Two contrasting models of electron flow along microbial nanowires. The electron hopping model for filaments of *Shewanella oneidensis* (a) and the metallic-like conduction for pili of *Geobacter sulfurreducens* (b). In the electron hopping mechanism, charges hop from cytochrome to cytochrome (shown in red). In metallic-like conduction, delocalized charges (shown as black dots) are spread across the entire filament.

the filament with spacing within 0.7 nm, which would enable sufficient electronic coupling [19••]. However, association of cytochromes with the *S. oneidensis* filaments, especially at a density high enough to permit cytochrome-to-cytochrome electron hopping/tunneling, has yet to be demonstrated [22••]. Theoretical considerations suggest that, even if cytochromes could be closely packed (<1 nm separation) along the filaments, the high conductivities reported [18] are likely to have been an artifact of sample treatment [22••]. This is an important consideration given the suggestion that the filaments that have been observed are in fact comprised of extracellular polymeric material that collapses into filaments during sample preparation [23•]. Thus, determining the actual composition of *S. oneidensis* nanowires and documenting the presence of cytochromes and their spacing along the filaments should be high priorities in future research.

Geobacter sulfurreducens nanowires

The nanowires of *G. sulfurreducens* are type IV pili [16]. The available evidence suggests that the pili have metal-like conductivity (Box 1), which can be attributed to

Box 1 Mechanisms of conductivity

There are three primary modes for electron flow in materials [79]. These are: tunneling, hopping, and delocalization (i.e., metal-like conductivity). In both tunneling and hopping electrons are associated with discrete electron carriers and the electrons can traverse one or more sites to migrate over large distances (Figure 2a). The main difference between tunneling and hopping is the involvement of the nuclear motion. Tunneling is a quantum mechanical phenomenon in which an electron overcomes the energy barrier at the conductor/insulator interface due to the wave nature of the electrons, allowing the electron to penetrate the barrier [79]. Tunneling can be a single step event or it can involve a series of multiple consecutive steps. In hopping the electron transport involves a physical displacement of the redox molecules due to diffusion and electrons hop from one reduced molecule to an adjacent oxidized molecule. In hopping, there is no electron transfer until the thermal motion of nuclei yields a favorable molecular geometry which permits electron motion *over* the barrier by rearrangement of the molecule. In contrast, in tunneling electrons move *through* the barrier due to the finite probability of finding the electron on the other side of barrier, without requiring nuclear motion [80]. Metallic conductivity is substantially different than hopping or tunneling. Instead of electrons being localized in individual molecules, electrons are delocalized along a chain of molecules and electrons are free to move throughout the material [81] (Figure 2b). Metallic conductivity arises due to a process of electron delocalization or spreading of electron wave functions [79].

electron delocalization (Figure 2b) due to overlapping pi–pi orbitals of aromatic amino acids. Although metal-like conductivity is a well-known phenomenon in synthetic organic conducting polymers [24], *G. sulfurreducens* pili are the first biological protein filaments found to have this property [14,25••,26].

Multiple lines of evidence have ruled out the alternative hypothesis [27–29] that the conductivity of *G. sulfurreducens* pili can be attributed to electron hopping between pili-associated cytochromes, in a manner similar to that proposed for *S. oniedensis* nanowires. Cytochromes are associated with *G. sulfurreducens* nanowires [30], but atomic force microscopy [31] has demonstrated that their spacing (100–200 nm) is greater than the ca. 1–2 nm spacing that is required for cytochrome-to-cytochrome electron hopping (Box 1). The distance between the cytochromes is also greater than that required for a proposed 'stepping stone mechanism' in which cytochromes would serve to bridge electron transfer between aromatic-rich regions of the pili [28]. Furthermore, denaturing the cytochromes associated with the pili does not diminish their conductivity [25••]. Scanning tunneling microscopy [32] and electrostatic force microscopy (N.S. Malvankar *et al.* 'Visualization of charge propagation along individual pili proteins using ambient electrostatic force microscopy', manuscript submitted) have also suggested that the conductivity of *G. sulfurreducens* nanowires cannot be attributed to cytochromes.

G. sulfurreducens nanowires are comprised of PilA, a protein with homology to the PilA of other gram-negative bacteria that produce type IV pili from the assembly of the PilA monomer [33]. The N-terminus of the PilA sequence of *G. sulfurreducens* and other organisms is highly conserved. However, the PilA sequence of *G. sulfurreducens* differs from that of other microorganisms outside the genus *Geobacter* in that the carboxyl terminus is highly truncated. The pili of *Pseudomonas aeruginosa*, which has the longer carboxyl PilA sequence, have low conductivity [16], and expressing the *P. aeruginosa* PilA in *G. sulfurreducens* yielded poorly conductive pili, even though *G. sulfurreducens* properly localized cytochromes on the *P. aeruginosa*-PilA pili [34].

Specific aromatic amino acids in the carboxyl terminus of the PilA sequence of *G. sulfurreducens* appear to confer conductivity [35•]. Substituting an alanine for each of the five most distal aromatic amino acids in the carboxyl terminus of PilA led to the expression of pili with properly localized cytochromes, but with greatly diminished conductivity [35•]. The strain producing these modified pili was no longer able to effectively carry out extracellular electron transfer functions, such as current production and Fe(III) oxide reduction [35•].

The necessity for aromatic amino acids for conductivity, and the truncated carboxy terminus that is likely to allow these aromatic amino acids from separate PilA monomers to establish close contact, is consistent with the concept [14,25••] that overlapping pi–pi orbitals in the pili structure enable metal-like conductivity. Changes in conductivity in response to temperature and proton doping followed patterns expected for a material with metal-like conductivity and were inconsistent with electron hopping/tunneling [25••].

Attempts to elucidate the conductivity mechanism for *G. sulfurreducens* pili from modeling based on the PilA structure [36,37] have also suggested that aromatic amino acids are likely to be important in electron transfer along the pili, but have not yielded a predicted filament structure in which electrons would be transported via metal-like conductivity. Structural studies on assembled filaments are needed to further evaluate the metal-like conductivity hypothesis [38].

Bioenergy strategies to which microbial nanowires may contribute

Anaerobic digestion

Anaerobic digestion is a well-proven strategy for extracting energy from organic wastes in the form of methane [39]. Microorganisms closely related to known *Geobacter* species are abundant in some anaerobic digesters [40–42] in which they are likely to be functioning as syntrophs [43]. The capacity for syntrophic growth has been demonstrated in *G. metallireducens*, which like *G. sulfurreducens* requires pili for long-range extracellular electron transfer [44]. Adaptive evolution, transcriptomic, and genetic

approaches demonstrated in proof-of-concept studies that *G. metallireducens* and *G. sulfurreducens* exchange electrons via pili in electrically conductive aggregates during syntrophic growth [45–47].

A similar direct interspecies electron transfer (DIET) appeared to be taking place within aggregates in anaerobic digesters in which *Geobacter* species were the predominant bacteria and *Methanosaeta* species were the most abundant methanogens [41]. The aggregates had a metal-like conductivity, consistent with conductivity via *Geobacter* pili [41]. *G. metallireducens* donated electrons to the digester isolate *Methanosaeta harundinacea* in defined co-culture aggregates, but a strain of *G. metallireducens* in which the gene for PilA had been deleted could not function in this manner [48••]. Although it was previously thought that *Methanosaeta* species specialized in the conversion of acetate to methane, *M. harundinacea* used electrons derived from DIET to reduce carbon dioxide to methane, and metatrascriptomic analysis suggested that *Methanosaeta* species within an anaerobic digester were also actively reducing carbon dioxide [48••]. These previously unrecognized capabilities of *Methanosaeta* species have implications beyond anaerobic digestion because *Methanosaeata* is considered to be the most prodigious methane producer on earth [48••].

The diversity of microorganisms capable of participating in DIET and its prevalence in methanogenic environments is as yet unknown. *Pelobacter carbinolicus*, which is in the same phylogenetic family as *G. metallireducens*, was incapable of DIET, instead relying on H_2 or formate as the interspecies electron carrier [49]. Other intensively studied syntrophic organisms also appear to have evolved to specialize in interspecies H_2 or formate transfer [1••] as have some methanogens [48••]. The methanogen *Methanasarcina barkeri* can grow via DIET or H_2 transfer (A.-E. Rotaru *et al.*, unpublished data).

The finding that methanogens such as *Methanosaeata* and *Methanosarcina* species have evolved the capacity to accept electrons via DIET suggests that DIET offers a competitive advantage in some methanogenic environments and there is circumstantial evidence that DIET may enhance the function of anaerobic digesters. For example, GAC is often added to anaerobic digesters to improve anaerobic digestion and GAC promotes DIET [6]. GAC is much more electrically conductive than pili and can serve as an electrical conduit for syntrophic growth, even alleviating the need for pili for DIET [6]. Conductive iron minerals may serve a similar function [12]. Carbon cloth stimulated anaerobic digestion [50] and it seems likely that this response might be attributed to syntrophs and methanogens plugging into the conductive cloth matrix. Further research into strategies for favoring DIET in anaerobic digesters seems warranted.

Microbial fuel cells

MFCs are a potential alternative to anaerobic digestion for harvesting energy from organic wastes [3]. MFCs already have applications as sensors, powering electronic monitoring devices, and bioremediation [51••,52]. However, at present, the current densities of MFCs are too low to permit this technology to be competitive with anaerobic digestion [39].

The only proven strategy for creating microbial strains with the capacity for increased current densities is to promote nanowire production [53–55]. *G. sulfurreducens* strains with more pili produce more conductive biofilms, and more conductive anode biofilms generate higher current densities [54]. Biofilm conductivity permits cells at distances of up to 400 μm from the anode surface to remain metabolically active and contribute to current production [56••,57,58]. For example, *G. sulfurreducens* strain KN400, which was a rare variant [59] in a culture of *G. sulfurreducens* strain DL-1, was selected for its superior current-producing capability [53]. KN400 produces more pili than DL-1 [53], as proven by a threefold greater abundance of PilA protein [25••], and its biofilms are fivefold more conductive [25••]. These findings are consistent with the concept that pili confer conductivity to *G. sulfurreducens* biofilms, which have a similar metal-like conductivity [14,25••]. Genetically altering a regulatory switch for pili production also enhanced biofilm conductivity and current production [55]. As previously reviewed [14], *G. sulfurreducens* naturally strives to produce more pili when using an electrode as an electron acceptor by greatly upregulating its PilA gene expression.

In searching for strategies to further increase current output it is important to consider alternatives other than manipulating pili density. For example, it has been proposed that electron transport through current-producing biofilms is the result of electron hopping/tunneling between *c*-type cytochromes dispersed throughout the biofilm matrix [27,29,60–63]. If so, it might be expected that increasing the density of extracellular cytochromes in biofilms would increase conductivity and current production. However, just the opposite has been observed. In fact, the best strategy for generating new strains of *G. sulfurreducens* with increased capacity for current production has been to delete genes encoding outer-surface *c*-type cytochromes [25••,54].

The likely reason that the cytochrome model lacks practical utility for enhancing current output is that its development was based on a flawed experimental approach. The model is based on an inference of conductivity from the oxidation and reduction of cytochromes within the biofilm rather than direct conductivity measurements. This approach neglects the fact that most of the *G. sulfurreducens* cytochromes are localized within the cell and that cytochromes both within and outside the cell are

oxidized or reduced in response to electrochemical manipulations regardless of the route of electron conduction through the biofilm [31]. In fact, the formal potential of *G. sulfurreducens* biofilms corresponds with that of the periplasmic cytochrome PpcA, the most abundant cytochrome in *G. sulfurreducens* [31]. The apparent 'diffusive behavior' of electrons through *G. sulfurreducens* biofilms (see [64] for a review) primarily reflects the kinetics of oxidation/reduction of cytochromes within cells, rather than electron transport through the biofilm.

Thus, at the practical level the debate over mechanisms for long-range electron transport through *G. sulfurreducens* biofilms has been resolved. The approach that has generated strains of *G. sulfurreducens* with enhanced capacity for current production has been to reduce expression of outer surface *c*-type cytochromes and increase expression of pili [25••,31,53,55]. However, *G. sulfurreducens* requires the outer surface cytochrome OmcZ [65] to facilitate electron exchange at the biofilm/anode interface [11]. It has yet to be determined whether this final electron transfer to anodes can be improved through genetic manipulation.

A wide diversity of organisms other than *Geobacter* species are capable of oxidizing organic compounds with electron transfer to electrodes [51••]. However, *Thermincola ferriacetica* is the only other organism that has been shown to produce the thick, conductive biofilms that are necessary for high current densities [66••]. For example, *S. oneidensis* relies on flavin electron shuttles rather than nanowires as its primary mode of electron transport to electrodes [2] and the slow diffusive flux of shuttles limit current densities [57].

Microbial electrosynthesis — the electric economy meets synthetic biology

Another potential bioenergy application of microbe-electrode exchange is microbial electrosynthesis, a process in which microorganisms accept electrons from an electrode for the reduction of carbon dioxide to multi-carbon fuels or other organic commodities [4,67,68]. When solar energy is the source of electrical power microbial electrosynthesis is an artificial form of photosynthesis:

$$CO_2 + H_2O \xrightarrow{\text{Solar energy}} \text{Organic products} + O_2$$

that has a number of potential advantages as a sustainable practice over strategies that rely on the conversion of biomass to fuels [4,69]. Solar panels harvest solar energy much more efficiently than plants; the solar energy collected is channeled directly to the production of desired products rather than biomass, reducing water usage and waste production; arable land that could be devoted to food production is not required for electrosynthesis; and the environmental degradation associated with intensive biomass production is avoided.

Although proof-of-concept studies have demonstrated the feasibility of microbial electrosynthesis with acetogenic bacteria as the catalyst [67,68,70], volumetric rates of product formation are low. This can be attributed to sparse biofilm formation on cathodes. Some improvements in biofilm density were achieved with modifications of cathode materials [71], and membrane-spanning compounds may aid electrode-to-microbe electron transfer [72], but thick conductive biofilms, comparable to the conductive anode biofilms of *G. sulfurreducens* [56••] or natural communities [73], have yet to be achieved.

A potential solution is to employ microorganisms that produce conductive filaments as the electrosynthesis catalysts. However, *Geobacter* species lack the Wood–Ljungdahl pathway, which is likely to be the most effective and efficient of the known microbial pathways for generating extracellularly released organic products from carbon dioxide reduction [4]. *Clostridium ljungdahlii*, which possesses the Wood–Ljungdahl pathway is an attractive chassis for electrosynthesis because it is genetically tractable [74]. Although *C. ljungdahlii* appears to directly accept electrons from cathodes [67], it only forms monolayer biofilms. This is similar to another gram-positive microbe, *Thermincola potens*, which is capable of direct electron transfer to electrodes via extracellular *c*-type cytochromes, but only cells in contact with the anode appear to be active [75•,76]. However, the closely related *Thermincola ferriacetica* can form thick biofilms that are potentially conductive via nanowires [66••], demonstrating that the capacity for long-range electron transport can be found in gram-positive microbes. This is encouraging because it may be easier to introduce the capacity to express nanowires into a chassis, such as *C. ljungdahlii* that already possesses the Wood–Ljungdahl pathway, than to introduce the Wood–Ljungdahlii pathway and associated energy conservation mechanisms into a microbe, such as *G. sulfurreducens*, that has the ability to produce conductive biofilms.

Conclusions

The limited numbers of studies on microbial nanowires to date have demonstrated that long-range electron conduction via proteinaceous microbial filaments is possible and that this form of extracellular electron transport is important in several anaerobic bioenergy processes. Synthetic conducting polymers, which also rely on pi–pi stacking for electron conduction, are being increasing employed in energy-related applications such as electronic circuits, light-emitting displays, and solar cells [24]. Microbial nanowires provide a model for how similar materials might be sustainably produced from inexpensive, renewable feedstocks. The ability of conductive pili of *G. sulfurreducens* to wire cells together to form biofilms with supercapacitor [77] and transistor properties [25••] has already been demonstrated. The increase in the power

output of MFCs with a simple genetic manipulation that increased nanowire production [55] demonstrates the possibility of enhancing processes not only with more nanowires, but also potentially with nanowires genetically engineered for higher conductivity or with artificial materials that function as nanowire mimetics.

Such practical applications will benefit from additional basic research. The study of *Shewanella* and *Geobacter* nanowires has already suggested that different species produce nanowires that have different mechanisms for electron conduction. Therefore, it is important to further explore the diversity of the microbial world for other nanowires. Many microbial communities might exchange electrons via nanowires not only for anaerobic respiration, but also potentially as an electrical signal that is more specific and direct than alternatives, such as quorum sensing molecules. Likely places to find such direct electrical connections include the wide diversity of consortia that establish contact to syntrophically oxidize organic substrates; communities anaerobically oxidizing methane may be a good place to start [78]. The potential of gut microorganisms or symbionts within protozoa electrically interacting with their eukaryotic hosts via nanowires is another intriguing possibility. These surveys of nanowires in the natural world should be coupled with further structural and biophysical investigations of nanowires in order to better understand the mechanisms of this amazing biological phenomenon.

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