

Review

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Microbial nanowires: an electrifying tale

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Electromicrobiology has gained momentum in the last 10 years with advances in microbial fuel cells and the discovery of microbial nanowires (MNWs). The list of MNW-producing micro-organisms is growing and providing intriguing insights into the presence of such micro-organisms in diverse environments and the potential roles MNWs can perform. This review discusses the MNWs produced by different micro-organisms, including their structure, composition and mechanism of electron transfer through MNWs. Two hypotheses, metallic-like conductivity and an electron hopping model, have been proposed for electron transfer and we present a current understanding of both these hypotheses. MNWs not only are poised to change the way we see micro-organisms but also may impact the fields of bioenergy, biogeochemistry and bioremediation; hence, their potential applications in these fields are highlighted here.

Introduction

Micro-organisms are known to produce sophisticated nanomachines, like bacterial flagellar nanomotors, that are made up of several proteins (Chalmeau *et al.*, 2009). Scientists are using peptides and proteins as building blocks for the construction of nanodevices including sensors and drug delivery vehicles (Petrov & Audette, 2012; Rosenman *et al.*, 2011; Scanlon & Aggeli, 2008). Several peptide nanotubes have been built and can be used as a casting module for synthesis of metal nanowires (Reches & Gazit, 2003; Scanlon & Aggeli, 2008).

One desired property of peptide/protein nanotubes is electrical conductivity. Such electrically conductive nanotubes or nanowires are an essential requirement in the field of nanoelectronics. Most proteins made of natural amino acids are insulating (Scanlon & Aggeli, 2008) and thus efforts were made to build electrically conductive protein nanotubes that in turn can act as nanowires (Creasey *et al.*, 2015; Scanlon & Aggeli, 2008). However, Reguera *et al.* (2005) discovered extracellular electrically conductive protein nanofilaments in *Geobacter sulfurreducens* and termed them microbial nanowires (MNWs). This discovery opened many new avenues of research in nanotechnology and microbiology. This review

deals with MNWs produced by diverse micro-organisms and discusses several important aspects of MNWs including their types, role, mechanism of electron transfer and potential applications.

Discovery of MNWs in different microbes

As a part of anaerobic respiration, some bacteria are capable of transferring electrons to extracellular electron acceptors in a process termed extracellular respiration (Lovley, 2008). Extracellular respiration is commonly found in metal-reducing bacteria like *G. sulfurreducens* and *Shewanella oneidensis*. There are three known strategies by which extracellular respiration is carried out by bacteria; first, bacteria transfer electrons directly to metals through proteins present on the cell surface (Fig. 1a); second, metal chelators (citrate and nitrilotriacetic acid) deliver metals to intracellular metal oxidoreductases (Fig. 1b) or finally small molecules (humic substances) act as a shuttle to transfer electrons between the cell and the substrate (Fig. 1b) (Gralnick & Newman, 2007; Richardson, 2000). An addition to this list is extracellular MNWs which act as a conduit of electrons between cell and distant substrates (Fig. 1c) (Reguera *et al.*, 2005). The conductivity of proteins has been studied earlier (Xu *et al.*, 2005) but their conductive behaviour and direct role in long-range (up to micrometre distances) extracellular electron transfer had not been reported. Similar studies done on extracellular pili-like structures (PLSs) of *S. oneidensis* and *Pseudomonas aeruginosa* indicated PLSs to be non-conductive (Reguera

Abbreviations: AFM, atomic force microscopy; BRONJ, bisphosphonate-related osteonecrosis of the jaw; MNW, microbial nanowire; PLS, pili-like structure; STM, scanning tunneling microscopy; TEM, transmission electron microscopy; TFP, type IV pili.

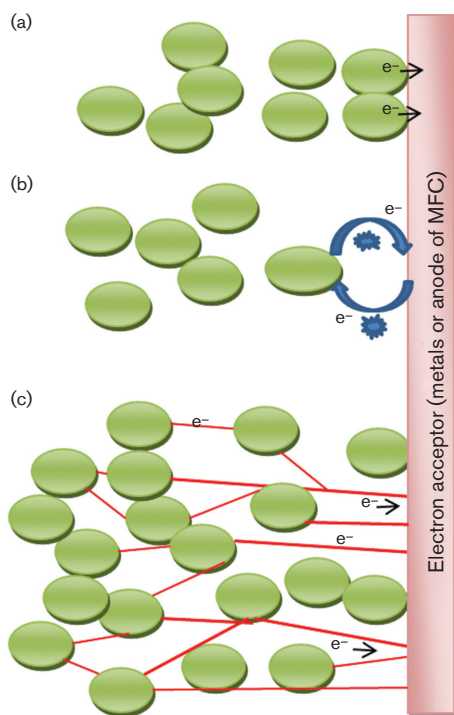


Fig. 1. Strategies by which bacteria can transfer electrons extracellularly to electron acceptors [metals or anode of microbial fuel cell (MFC)]. Bacteria can transfer electrons extracellularly by direct attachment to metal or anode surface of microbial fuel cell (a) or employ metal chelators or small molecules as a mediator for electron transfer (b) or can use MNWs (red lines) for same (c).

et al., 2005). However, previously reported non-conductive PLSs of *S. oneidensis* were conclusively proved to be electrically conductive in the following year by a different research group (Gorby *et al.*, 2006). There may be three reasons for the failure to detect MNWs in *S. oneidensis* in the first attempt, the first being cultivation conditions. Luria-Bertani, a complex medium, was used in the first study (Reguera *et al.*, 2005), compared to stressful culture conditions in the form of electron acceptor limiting conditions in the second (Gorby *et al.*, 2006), with the latter likely inducing formation of MNWs in *S. oneidensis*. The second reason could be the delicate nature of bacterial pili (5–8 nm in diameter) (Gorby *et al.*, 2006; Pelicic, 2008; Simpson *et al.*, 1976) while, lastly, there may be a tendency of some micro-organisms to produce multiple PLSs, e.g. type II pseudopili, and thus the PLSs probed by Reguera *et al.* may be different (Durand *et al.*, 2003; Gorby *et al.*, 2006).

MNWs have also been observed in the iron (Fe)-reducing bacterium *Rhodospseudomonas palustris* strain RP2 (Venkidasamy *et al.*, 2015) and in the sulfate (SO_4^{2-})-reducing bacterium *Desulfovibrio desulfuricans* (Eaktasang *et al.*, 2016). MNWs not only were observed in Fe- and SO_4^{2-} -reducing bacteria as discussed above but also were identified in the Fe-oxidizing bacterium *Acidithiobacillus ferrooxidans* (Li & Li,

2014; Valdes *et al.*, 2008). With this discovery, it was hypothesized that MNWs may connect cells to extracellular electron donors and acceptors.

Considering the role of MNWs in electron transfer, it was hypothesized that such conductive structures might be present in pathogenic microbial biofilms residing in anaerobic zones of oral cavities (Rabaey, 2010). MNWs were observed for the first time in microbial biofilms which causes bisphosphonate-related osteonecrosis of the jaw (BRONJ) (Wanger *et al.*, 2013). These MNWs were found to interconnect different cells and appeared as PLSs. This biofilm was found to be colonized by around 15 discernible bacterial morphotypes, mostly anaerobic and facultatively anaerobic, constituting genera of *Staphylococcus*, *Bacillus*, *Fusobacterium*, *Actinomyces*, *Streptococcus*, *Selenomonas* and *Treponema* but the specific MNW-producing micro-organisms among these could not be identified.

Apart from metal-reducing and pathogenic micro-organisms, MNWs have been observed in photosynthetic bacteria. Initial reports showed that *Synechocystis*, a unicellular cyanobacterium, can produce MNWs in electron acceptor (CO_2) limiting and high light conditions (Gorby *et al.*, 2006). Taking clues from this study, our group explored the possibility of MNW formations in other cyanobacteria. Some cyanobacteria become electrogenic (transfer electrons extracellularly) under high light intensity. *Synechocystis* as well as *Nostoc* sp. have been shown to exhibit such type of electrogenic behaviour (Pisciotta *et al.*, 2010). Further, *Microcystis aeruginosa* also encounter CO_2 limitation and get exposed to high light intensity when they form blooms. Thus, *Mi. aeruginosa* and *Nostoc punctiforme* might be producing MNWs, which has been confirmed by conductive atomic force microscopy (AFM) analysis (Sure *et al.*, 2015, 2016b). The discovery of MNWs in such diverse micro-organisms ranging from anaerobic, metal-reducing bacteria to photosynthetic, aerobic cyanobacteria strengthens the viewpoint that they may be pervasive in the environment. The MNW-producing micro-organisms discovered to date are shown in Fig. 2 and relevant description is given in Table 1.

Different modes of AFM including conductive AFM, scanning tunneling microscopy (STM), electrostatic force microscopy and specially designed nanofabricated electrodes are established techniques for identification and electrical characterization of MNWs produced by different micro-organisms and use of these multiple techniques has been advocated to unambiguously confirm the presence of MNWs in micro-organisms (Castro *et al.*, 2014; Gorby *et al.*, 2006; Li & Li, 2014; Malvankar & Lovley, 2014; Reguera *et al.*, 2005; Sure *et al.*, 2015; Venkidasamy *et al.*, 2015; Wanger *et al.*, 2013).

Types of MNWs

Diverse micro-organisms have been observed to produce MNWs (Fig. 2) and their composition and structure have

been found to be different from each other. According to available data, MNWs can be categorized into three types (Fig. 3).

Pili

MNWs in *G. sulfurreducens*, *Aci. ferrooxidans* and *Synechocystis* sp. have been found to be type IV pili (TFP) which are the most widespread type of pili present among bacteria (Li & Li, 2014; Pelicic, 2008; Reguera *et al.*, 2005; Sure *et al.*, 2015). Apart from common functions like adhesion and biofilm formation exhibited by most

other bacterial pili, TFP possess unique functional characteristics which include twitching motility, uptake of DNA in transformation and phage attachment (Pelicic, 2008; Proft & Baker, 2009). In addition to these functions, their electron carrying capacity further increases their significance as multifunctional extracellular structures. MNWs in *G. sulfurreducens* are polymers of PilA subunit whereas in *Synechocystis* they are composed of PilA1 (Fig. 3a, b) (Reguera *et al.*, 2005; Sure *et al.*, 2015). Though MNWs from both micro-organisms are TFP, the molecular mass of their subunits (~10 kDa for *G. sulfurreducens*, ~22 kDa for *Synechocystis*) and dimensions (width/length: 3–5 nm/

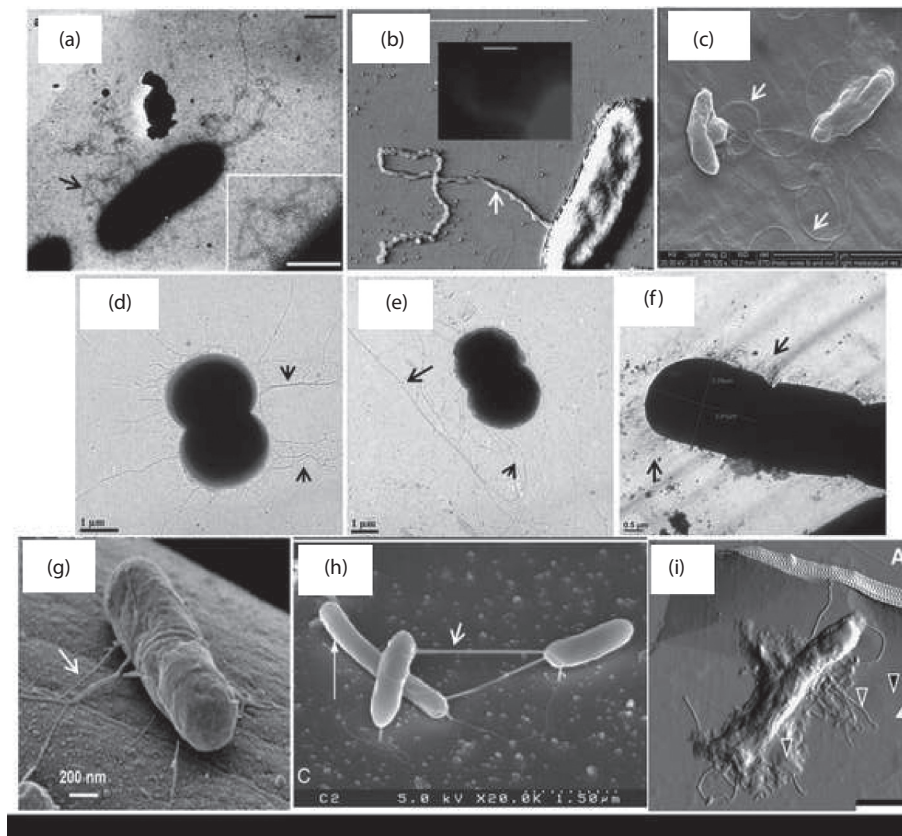


Fig. 2. Diverse micro-organisms that can produce MNWs. (a) *G. sulfurreducens*, an anaerobic, dissimilatory metal-reducing bacterium (DMRB). Inset shows nanowires at higher magnification. Scale bar, 0.2 μm [transmission electron microscopy (TEM image)] [Reprinted by permission from Macmillan Publishers Ltd: Reguera *et al.* (2005) ©2005]; (b) *S. oneidensis*, a facultative anaerobic, DMRB (AFM image). Scale bar, 2 μm . Inset shows *in vivo* fluorescence image of same cell. Scale bar, 1 μm . The image has been colour edited [Adapted from Pirbadian *et al.* (2014) ©National Academy of Sciences]; (c) *R. palustris* strain RP2, photosynthetic DMRB, metabolically versatile. Scale bar, 3 μm . [Reproduced from Venkidusamy *et al.* (2015) with permission from The Royal Society of Chemistry]; (d) *Synechocystis* PCC 6803. Scale bar, 1 μm and (e) *Mi. aeruginosa* PCC 7806 which are aerobic, unicellular photosynthetic micro-organisms (TEM images) (with kind permission from Springer Science+Business Media: Sure *et al.* (2015) ©Springer Science+Business Media). Scale bar, 1 μm ; (f) *No. punctiforme* PCC 73120, an aerobic, multicellular and filamentous photosynthetic micro-organism (Sure *et al.*, 2016b) (TEM image). Scale bar, 0.5 μm ; (g) *D. desulfuricans*, an obligate anaerobe, sulfate-reducing [Adapted from Eaktasang *et al.* (2016) ©Elsevier]; (h) *Pelotomaculum thermopropionicum* and *Methanothermobacter thermoautotrophicus* (shown with filled arrow) which are syntrophic methanogenic cocultures (scanning electron microscopy image) (Adapted from Gorby *et al.* (2006) ©National Academy of Sciences). Scale bar, 1.5 μm ; (i) unknown bacteria from BRONJ-affected bone (AFM image). The image has been colour edited [Adapted from Wanger *et al.* (2013) ©Elsevier]. MNWs are also produced by *Aci. ferrooxidans* (Li & Li, 2014) and *Aeromonas hydrophila* (Castro *et al.*, 2014) (not shown here). MNWs have been shown with open arrows in all images.

Table 1. List of MNW-producing micro-organisms

Sr. no.	Micro-organisms	Component protein of MNWs	Physiological role	Conductivity measurement (along width/length)	Description	References
1	<i>G. sulfurreducens</i>	Pilin subunit PilA	Extracellular electron transfer to insoluble electron acceptor [e.g. Fe (III)]	Along width as well as length	Metal reducing, anaerobic	Malvankar <i>et al.</i> (2011), Malvankar & Lovley (2014), Reguera <i>et al.</i> (2005)
2	<i>S. oneidensis</i> MR-1	Periplasmic and outer membrane extensions embedded with cytochromes	Not known yet	Along width as well as length	Metal reducing, facultative anaerobic	El-Naggar <i>et al.</i> (2010), Gorby <i>et al.</i> (2006), Pirkadian <i>et al.</i> (2014)
3	<i>Synechocystis</i> sp. PCC 6803	Pilin subunit PilA1	Not known yet	Along width	Photosynthetic, aerobic	Gorby <i>et al.</i> (2006), Sure <i>et al.</i> (2015)
4	<i>Pe. thermopropionicum</i>	Not known yet	Not known yet	Along width	Anaerobic, thermophilic	Gorby <i>et al.</i> (2006)
5	Multispecies biofilms observed in BRONJ	Not known yet	Not known yet	Along width as well as length	Unknown	Wanger <i>et al.</i> (2013)
6	<i>Aci. ferrooxidans</i>	Not known yet	Not known yet	Along width	Chemolithoautotrophic, acidophilic	Li & Li (2014)
7	<i>Aer. hydrophila</i>	Not known yet	Not known yet	Along width	Facultative anaerobic, heterotroph	Castro <i>et al.</i> (2014)
8	<i>Mi. aeruginosa</i>	Unnamed protein product (GenBank: CAO90693.1)	Not known yet	Along width	Photosynthetic, aerobic, forms toxic blooms	Sure <i>et al.</i> (2015)
9	<i>No. punctiforme</i>	Not known yet	Not known yet	Along width	Photosynthetic, aerobic, filamentous	Sure <i>et al.</i> (2016b)
10	<i>R. palustris</i> strain RP2	Not known yet	Not known yet	Along width as well as length	Anoxic photosynthetic, iron-respiring	Venkidasamy <i>et al.</i> (2015)
11	<i>D. desulfuricans</i>	Not known yet	Not known yet	Along width	Anaerobic, sulfate reducing	Eaktasang <i>et al.</i> (2016)

10–20 μm and 4.5–7 nm/2–10 μm for *G. sulfurreducens* and *Synechocystis*, respectively) differ from each other (Lovley *et al.*, 2009; Lovley, 2011; Sure *et al.*, 2015). In *G. sulfurreducens*, cytochromes are found to be associated with MNWs and its role in electron transfer through MNWs is disputed (Malvankar *et al.*, 2011; Strycharz-Glaven *et al.*, 2011; Strycharz-Glaven & Tender, 2012). It needs to be explored whether *Synechocystis* MNWs are embedded with cytochromes and the potential role of the latter in electron transfer. MNWs in *Aci. ferrooxidans* may be made up of PilV and PilW proteins (Li & Li, 2014). The MNWs from different micro-organisms will not always look the same and vary in width and length due to two reasons: (1) TFP have bundle forming ability as a result of which their observed width may vary; (2) length may depend on age of culture and sample preparation methods which may lead to breakage of long, delicate pili.

Extended periplasmic and outer membranes

S. oneidensis possess three different types of extracellular proteinaceous appendages: (1) Msh pili, (2) TFP and (3) flagella (Bouhenni *et al.*, 2010), but it was not clear which one of these acts as MNWs. Msh pili have been shown to be necessary for extracellular electron transfer (Fitzgerald *et al.*, 2012), while TFP and flagella have been shown to be dispensable (Bouhenni *et al.*, 2010). However, MNWs in *S. oneidensis* are made up of outer membrane vesicle chains which subsequently elongate and become MNWs (Fig. 3c) (Pirkadian *et al.*, 2014). Unlike pili and flagella, which are mostly homopolymers of a single subunit type, MNWs in *S. oneidensis* are a concoction of different cytochromes and periplasmic as well as outer membrane components. The formation of outer membrane vesicle chains and tubes has been reported in *Myxococcus xanthus* (Remis *et al.*, 2014; Wei *et al.*, 2014). Also, the ability of peptide nanotubes to convert into vesicles and vice versa is well known (Scanlon

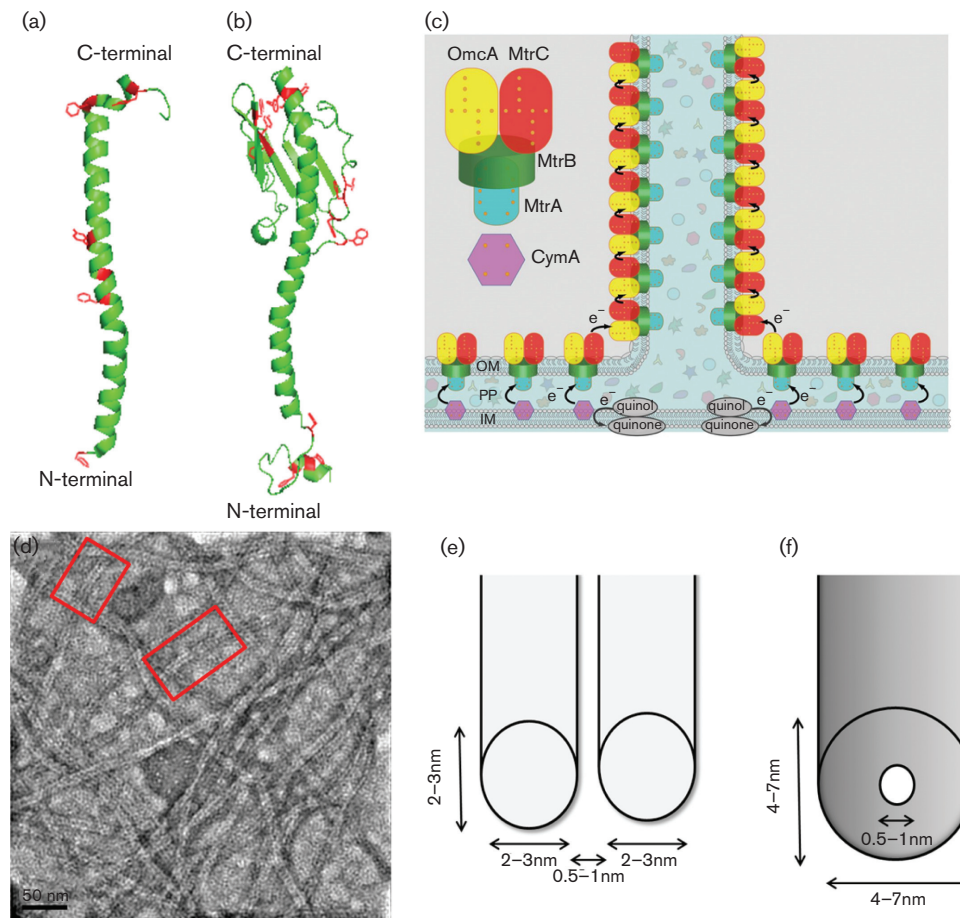


Fig. 3. Three types of MNWs observed to date. (1) MNWs made of type IV pili (TFP) as observed in *G. sulfurreducens* and *Synechocystis* are made up of subunits, PilA (a) and PilA1 (b), respectively. Both these subunits differ in their structure at C-terminus and number and positioning of aromatic amino acids (shown with red colour) [with kind permission from Springer Science+Business Media: Sure *et al.* (2015) ©Springer Science+Business Media]; (2) MNWs made of extended periplasmic and outer membranes along with cytochromes (e.g. MtrC, OmcA) as observed in *S. oneidensis* (c) [Adapted from Pirbadian *et al.* (2014) ©National Academy of Sciences]; (3) unknown MNWs as observed in *Mi. aeruginosa* and other micro-organisms. In *Mi. aeruginosa*, MNWs were found to be made up of unknown protein (GenBank: CAO90693.1), and from its TEM image (d), it seems that either it is made of two subfilaments (e) or it contains central channel (f) [with kind permission from Springer Science+Business Media: Sure *et al.* (2015) ©Springer Science+Business Media].

& Aggeli, 2008). Thus, it would not be surprising to know that MNWs in *S. oneidensis* are formed in a similar manner.

As discussed earlier, *S. oneidensis* is known to produce pili/flagella and it is puzzling why it employs a completely different strategy to produce MNWs. The role of MNWs in *S. oneidensis* physiology and metabolism is still largely unknown and deciphering it may help us understand the reason behind its completely different make-up from other MNWs. However, so far, it was not ruled out that other extracellular structures (pili and flagella) in *S. oneidensis* cannot conduct electrons. Also in their study, electrical conductivity measurements of extended membrane extensions were not done (Pirbadian *et al.*, 2014). All extracellular structures produced by *S. oneidensis* should be isolated and

studied independently for their conductive behaviour. Only then it would be appropriate to claim that MNWs produced by *S. oneidensis* are outer and periplasmic membrane extensions only and not pili or flagella.

Unknown type — MNWs whose identity needs to be confirmed

Pili-like conductive structures have been identified in *Aer. hydrophila*, *R. palustris*, *D. desulfuricans* and *No. punctiforme* but their identity has not been confirmed so far (Castro *et al.*, 2014; Eaktasang *et al.*, 2016; Sure *et al.*, 2016b; Venkidusamy *et al.*, 2015). Two distinct types of MNWs (first, short/thin MNWs of size 6–7.5 nm in diameter and 0.5–2 μm in length and second, long/thick MNWs

of size ~20–40 nm in diameter and $\geq 10 \mu\text{m}$ long) were observed in *No. punctiforme* (Sure *et al.*, 2016b). The identity of MNWs from multispecies biofilms observed in BRONJ could also not be confirmed (Wanger *et al.*, 2013). MNWs in *Mi. aeruginosa* have been found to be composed of a protein similar to an unnamed protein (GenBank: CAO90693.1) whose amino acid sequence does not match with any known protein (Sure *et al.*, 2015). Unlike others, MNWs in *Mi. aeruginosa* are wider and may be made of two subfilaments or contain central channel inside it (Fig. 3d–f) (Sure *et al.*, 2015). More elaborate studies are needed to further confirm the identities of above mentioned MNWs.

Pe. thermopropionicum produces electrically conductive flagellum-like appendages (10–20 nm in diameter) in monoculture as well as in coculture with *Me. thermoautotrophicus* (Gorby *et al.*, 2006). These flagellum-like appendages may be indeed flagella as subsequent study by other group has shown that *Pe. thermopropionicum* in cocultures with *Me. thermoautotrophicus* produce flagella which are involved in symbiosis (Shimoyama *et al.*, 2009). *G. sulfurreducens* is also known to produce flagella which were found to be non-conductive (Malvankar & Lovley, 2014).

Physiological role of MNWs

As discussed in the previous sections, each type of MNW has unique structure and composition and they may have evolved as per the physiological requirements of the micro-organisms. Some of the observed and hypothesized functions of MNWs are discussed below.

MNWs can act as a conduit between cell and extracellular electron acceptor/donors thereby mediating to and fro electron transfer. For instance, in metal-reducing micro-organisms like *G. sulfurreducens*, it was observed that MNWs can help bacteria to transfer electrons to electron acceptors (metals) available at a distance without the need of direct cell attachment or dissolved electron shuttles (Reguera *et al.*, 2005). Also in metal-oxidizing micro-organisms like *Aci. ferrooxidans*, MNWs may have the ability to transfer electrons to the cell surface, thus greatly helping cells to access electron donors at a distance (Li & Li, 2014). In anaerobic environments, photosynthetic micro-organisms can use arsenic (As) as electron donor (Kulp *et al.*, 2008) and here MNWs can play an important role to bridge the gap between the cells and any available electron donor like As. Our preliminary studies have shown that *Synechocystis* MNWs can bind and immobilize As and thus may act as a conduit of electrons between cells and As (Sure *et al.*, 2016a). Due to their ability to interact with metals, MNWs can act as a protective cellular mechanism against toxic metals (Fig. 4) (Cologgi *et al.*, 2011).

Apart from extracellular electron acceptor/donor, MNWs can also act as conduit of electrons between two different cells. For instance, occurrence of interspecies electron transfer in *Geobacter metallireducens* and *G. sulfurreducens* was observed in coculture (Summers *et al.*, 2010). Such interspecies electron transfer was also investigated in methanogenic wastewater aggregates where it was hypothesized that micro-organisms can directly transfer electrons to each other, rather than use hydrogen and formate as intermediate electron carriers (Morita *et al.*, 2011). It is hypothesized

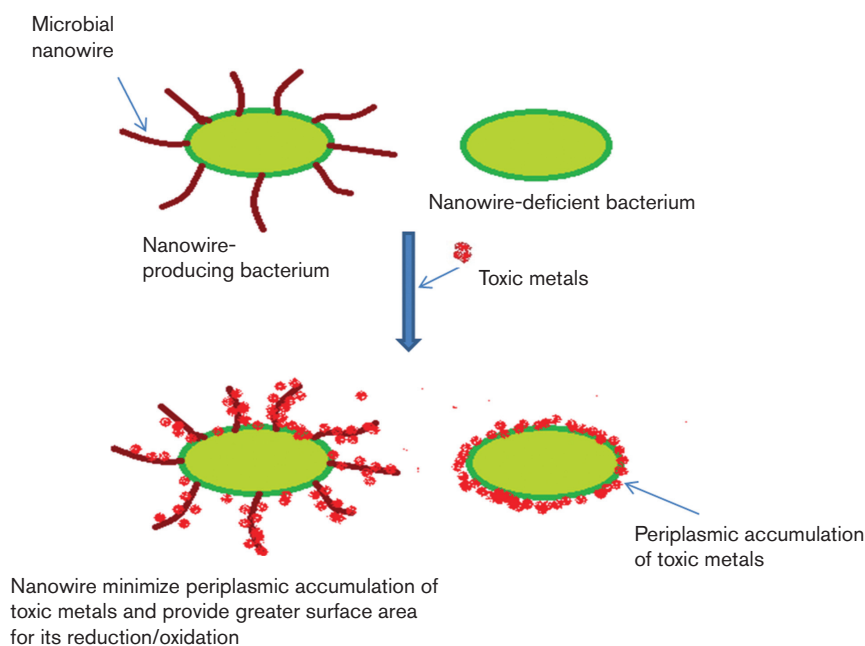


Fig. 4. Potential role of TFP in cell–metal interaction. Schematic representation of how TFP/MNWs can reduce interaction between cell membrane and toxic metals and can act as a protective barrier against latter.

that MNWs may be involved in such type of interspecies electron transfer. Apart from interspecies electron transfer, MNWs have also been implicated in electron transfer between bacteria and archaea (Wegener *et al.*, 2015). Further, cyanobacteria are an important constituent of the microbial mat and it is hypothesized that cyanobacterial MNWs can transfer electrons to other micro-organisms present in the microbial mat (Gorby *et al.*, 2006; Lea-Smith *et al.*, 2015). Such MNW-mediated electron transfers between two micro-organisms can also be part of cell communication/signalling (Reguera, 2011). For instance, it has been observed that MNWs of *G. sulfurreducens* lead to the formation of electronic networks which interconnect individual cells (Reguera, 2011). MNWs in *G. sulfurreducens* have been found to be conductive even at low voltage which is in accordance with the electronic communication occurring between cells (Reguera *et al.*, 2005; Reguera, 2011). Furthermore, a recent study has shown that MNW production in *G. sulfurreducens* is necessary for the formation of optimum electroactive and thick (more than 10 μm) biofilms (Steidl *et al.*, 2016).

Apart from these general roles, MNWs might be involved in specific functions. For example, in carbon limiting conditions, the component of photosynthetic apparatus plastoquinone gets over reduced due to unavailability of carbon to sink electrons. It is hypothesized that MNWs may help cyanobacteria to release these extra electrons present on plastoquinone so as to restrict cell damage (Gorby *et al.*, 2006; Lea-Smith *et al.*, 2015; Pisciotta *et al.*, 2010). MNWs produced in *Mi. aeruginosa* might be important for bloom formation while those of *No. punctiforme* may be involved in plant symbiosis (Duggan *et al.*, 2007; Sure *et al.*, 2015).

Mechanism of electron transfer through MNWs

With the discovery of MNWs, efforts to understand the mechanism of electron flow were commenced. The mechanism of electron transfer in MNWs has been extensively studied in *G. sulfurreducens* and *S. oneidensis* and two major mechanisms of conductivity have been proposed for MNWs: (1) metallic-like conductivity model and (2) electron hopping model. For *G. sulfurreducens* MNWs, both models have been advocated by different groups of scientists while for *S. oneidensis* MNWs, electron transfer is believed to occur by the hopping mechanism. Both major proposed mechanisms of electron transfer through MNWs are discussed here.

Metallic-like conductivity model

Elaborate experiments done in *G. sulfurreducens* showed that its MNWs have intrinsic metallic-like electrical conductivity which is comparable to carbon nanotubes and some organic conductors (Malvankar *et al.*, 2011, 2015; Malvankar & Lovley, 2012, 2014). This observation was distinct from an electron hopping mechanism observed in biological systems like

photosynthetic reaction centres (Feliciano, 2012). Some of the important properties which support the metallic-like conductivity model for *G. sulfurreducens* MNWs and biofilms are their temperature- and pH-dependent electrical behaviours (Malvankar *et al.*, 2011). This observation was also supported by structural studies where lower pH was shown to induce conformational changes in aromatic amino acids which thereby causes higher conductivity in the *Geobacter* pili (Malvankar *et al.*, 2015).

In synthetic organic metals, electron transfer is attributed to overlapping π - π orbitals of aromatic rings present in it. Proteins also contain several aromatic amino acids whose aromatic constituents can play a role similar to that of organic metals in electron transfer. To confirm the role of these aromatic amino acids in electron transfer, a *Geobacter* strain, Aro-5, was constructed (Vargas *et al.*, 2013). In *Geobacter* Aro-5, five aromatic amino acids of PilA, the pili subunit, were replaced with alanine and it was observed that the resultant modified pili showed considerable diminished electrical conductivity and ability to reduce Fe (III) compared to the pili of the control strain (Vargas *et al.*, 2013). Though this study confirmed that aromatic amino acids play an important role in conductivity of *G. sulfurreducens* pili and its biofilm, it is still unclear how modified pili are able to show residual electrical conductivity. Further, the removal of aromatic amino acids may be altering the 3D structure of pili and thereby the positioning of cytochromes on pili, which can ultimately decrease pili conductivity (Boesen & Nielsen, 2013). This is important considering the fact that tilting of molecules and interplanar distances have the potential to affect charge transport. The pilus filament model by Yan *et al.* (2015) based on *Neisseria gonorrhoeae* concludes that aromatics are too far apart to be involved in electron transport. However, experimental data generated using techniques like synchrotron X-ray micro-diffraction and rocking-curve X-ray diffraction have refuted this model and strongly supported the role of aromatic amino acids in long-distance electron transfer and reinforced the metallic-like conductivity mechanism in *Geobacter* pili (Malvankar *et al.*, 2015). Along with experimental data, modelling studies also supported the metallic conductivity model where lowest energy models of *Geobacter* pili were observed to have no central channel and a closely packed core chain of aromatic residues facilitated electron transport along the length of the pilus and conferred the potentially electrically conductive geometry to it (Xiao *et al.*, 2016).

The importance of intrinsic pilus structures of *G. sulfurreducens* in electron transfer was further studied where the *pilA* gene of *G. sulfurreducens* was replaced with the *pilA* gene from *Ps. aeruginosa* (Liu *et al.*, 2014). The resultant strain was able to produce and assemble *Ps. aeruginosa* PilA subunits into pili and interestingly these hybrid pili had the same pattern of cytochromes as that of control cells. However, the conductivity of these hybrid pili was found to be 14 times less than normal pili with significantly diminished ability to reduce iron and current generation. From these

observations, authors suggested that the intrinsic structures of *Geobacter* pili and not associated cytochromes are important for electron transfer through it. However, as the pili of *Ps. aeruginosa* have been found non-conductive in the earlier study (Reguera *et al.*, 2005), the hybrid pili here should also show non-conductive behaviour if the conductivity is 100 % related to intrinsic structure of pili. Since the pili show diminished conductivity, the basis needs to be worked out unambiguously to reach any conclusion.

The cytochromes located on *Geobacter* pili were hypothesized to be terminal reductases which transfer electrons from pili to electron acceptors like Fe and not the one playing a role in electron transport (Malvankar & Lovley, 2014). Malvankar *et al.* (2012) stressed that electron hopping does not meet the necessary biochemical requirement for electron transfer through pili. They reported that OmcS cytochromes assumed to be involved in electron transfer along the length of pili are too far from each other to carry out electron transfer as per the electron hopping model (Malvankar & Lovley, 2012). They also reported that denaturing cytochromes in *G. sulfurreducens* pilus networks and biofilms do not affect the electrical conductivity, thus ruling out any role of cytochromes in electron transfer through pili and biofilms (Malvankar *et al.*, 2011; Malvankar & Lovley, 2012). Further, STM analysis of *G. sulfurreducens* MNWs supported these findings where electron transfer is attributed to the intrinsic pilus structures and not to the cytochromes (Veazey *et al.*, 2011).

From all of the above observed results (Leang *et al.*, 2010; Malvankar *et al.*, 2011; Malvankar & Lovley, 2012), scientists refuted the electron hopping model for electron transfer in *G. sulfurreducens* MNWs and proposed the metallic-like conductivity model for same (Malvankar *et al.*, 2011; Malvankar & Lovley, 2012).

Electron hopping model

One view is emerging that electron transfer occurs by multi-step hopping in *Geobacter* and *Shewanella* MNWs and not by metallic conduction as proposed earlier. In *Geobacter* MNWs, aromatic amino acids are supposed to be involved in such electron transfer, whereas for *Shewanella* MNWs, cytochromes are believed to play this role.

As discussed in the previous section, it has been proved unambiguously that aromatic amino acids are indispensable for electron transfer through *Geobacter* MNWs. However, it is debatable whether these aromatic amino acids transfer electrons by metallic conduction or by multistep hopping. Multiple modelling studies strongly support the hypothesis that electron transfer through *Geobacter* MNWs occurs by multi-step hopping among aromatic amino acids (Feliciano *et al.*, 2015; Lebedev *et al.*, 2015; Yan *et al.*, 2015). This hypothesis was further strengthened by a recent report where experimental evidence has been provided to support multistep hopping in *Geobacter* MNWs where cryogenic STM of *Geobacter* pili showed thermal activation of the differential transversal conductance at low voltages which is in accordance with the

electron hopping mechanism (Lampa-Pastirk *et al.*, 2016). It has also been shown that metal- or redox organic cofactor-free *Geobacter* pili show carrier mobility of $3.2 \times 10^{-2} \text{ cm}^2 \text{ Vs}^{-1}$ which is too low for metallic conductivity regime where carrier mobilities of more than $1 \text{ cm}^2 \text{ Vs}^{-1}$ are required (Lampa-Pastirk *et al.*, 2016).

Quantitative measurement of electron transport across *S. oneidensis* MNWs showed that a complex electronic structure formed by its molecular constituents mediates electron transport in it (El-Naggar *et al.*, 2008). It has been proved that *S. oneidensis* MR-1 requires cytochromes, MtrC and OmcA for production of MNWs (El-Naggar *et al.*, 2010; Gorby *et al.*, 2006). Both of these cytochromes are located on the outer membrane of the cell. Scientists hypothesized that long-range electron transfer through *S. oneidensis* MNWs takes place by electron hopping where an intricate cytochrome network may be involved (Strycharz-Glaven *et al.*, 2011; Tender, 2011) and multiple experimental and modelling studies have confirmed this hypothesis (El-Naggar *et al.*, 2010; Gorby *et al.*, 2006; Leung *et al.*, 2013; Pirbadian & El-Naggar, 2012; Polizzi *et al.*, 2012). A recent study has reported that MNWs in *S. oneidensis* are composed of extended periplasmic and outer membranes embedded with cytochromes (Fig. 3c) which further supports the electron hopping model (Pirbadian *et al.*, 2014). However, in *S. oneidensis* MNWs, it is yet to be proved conclusively that cytochromes are closely spaced enough (1–2 nm) to carry out charge transport over micrometre distances. Interested readers are referred to specific reviews on this topic (Skourtis, 2013; Waleed Shinwari *et al.*, 2010).

Potential applications of MNWs

One reason behind the widespread attention gained by MNWs is their potential applications in several fields. Below we discuss some fields where MNWs can play an important role.

Bioenergy

For production of highly efficient microbial fuel cells, electron transfer should occur through biofilms so that even micro-organisms which are away from the anode can transfer electrons to it, thereby increasing total current output (Nwogu, 2007). Even for planktonic cells, long-range electron transport is necessary to improve the efficiency of microbial fuel cells. Soluble electron shuttles (natural as well as artificial) and MNWs can be useful for such long-range electron transfers (Fig. 1). Electron shuttles to be used for long-range electron transfer have their own disadvantages – natural electron shuttles have a slow diffusion rate which limits total electron flux rates while lack of long-term stability and toxicity to humans are the issues for artificial electron shuttles (Malvankar & Lovley, 2012).

MNWs thus can play an important role in improving the overall efficiency of microbial fuel cells. In *G. sulfurreducens*, these MNWs help cells to make efficient contact with electrodes by acting as a bridge between cells and electrodes,

thus enabling long-range electron transfer through biofilm (Steidl *et al.*, 2016). This long-range electron transfer thus has been shown to increase electricity production by 10 times (Reguera *et al.*, 2006). Along the same lines, MNW-producing photosynthetic micro-organisms (Gorby *et al.*, 2006; Sure *et al.*, 2015; 2016b) may be helpful in improving the efficiency of photosynthetic microbial fuel cells and microbial solar cells (Rosenbaum *et al.*, 2010; Strik *et al.*, 2011).

Methane is considered as an important renewable energy source which can be generated by anaerobic digestion of wastewater and biomass (Angenent *et al.*, 2004; De Mes *et al.*, 2003; Prochnow *et al.*, 2009). MNWs have been believed to play a role in methane production in syntrophic microbial communities (Morita *et al.*, 2011; Rotaru *et al.*, 2014; Summers *et al.*, 2010; Wegener *et al.*, 2015), which can be exploited further for improved methane production in anaerobic digesters. Interested readers are referred to specific reviews on this topic (Lovley, 2011; Malvankar & Lovley, 2014).

Bioremediation

Shewanella and *Geobacter* have been extensively studied for bioremediation of heavy metals and discovery of MNWs in these micro-organisms has further increased their potential in this field. It has been shown that MNWs can play an important role in bioremediation of a heavy metal like uranium (Cologgi *et al.*, 2011). Presence of MNWs in *G. sulfurreducens* has been shown to significantly mineralize more uranium per cell than an MNW-deficient mutant (Cologgi *et al.*, 2011). The MNWs also increase cellular tolerance to uranium by preventing its periplasmic accumulation as suggested in Fig. 4 (Cologgi *et al.*, 2011). Further, such MNWs considerably increase the total surface area available for heavy metal adsorption and subsequent detoxification. MNWs in *Synechocystis* also have been observed to precipitate arsenic (Sure *et al.*, 2016a) and chromium (Sure *et al.*, unpublished data) and thus may be helpful in their bioremediation. Readers may refer to a specific review on this topic (Lovley, 2011).

Bioelectronics

Scientists believe that MNWs will allow us to develop instruments usable in water and moist environments (Malvankar & Lovley, 2012). Furthermore, Leung *et al.* (2011) characterized *S. oneidensis* MNWs and showed that they have enough mechanical strength (Young's modulus ~1 GPa) to be used as a building block for construction of electronic devices. The MNWs can be modified using genetic and protein engineering, so different ligands (metals) can be attached to it which may help to modulate its electric behaviour (Lovley *et al.*, 2009) or can increase its electrical conductivity significantly (Tan *et al.*, 2016). In this direction, MNWs in *G. sulfurreducens* have been modified to have better conductive and adhesive properties (Reguera *et al.*, 2014). A recent study by Tan *et al.* (2016) has shown that, in *G. sulfurreducens* MNWs,

replacing C-terminal phenylalanine and tyrosine of PilA with tryptophan decreases its diameter by half and increases its conductivity by ~2000-fold. MNWs may also be used in bionanosensors (Lovley *et al.*, 2009); however, no such studies have been reported yet. Interested readers are referred to specific reviews on this topic (Amdursky *et al.*, 2014; Patolsky & Lieber, 2005; Patolsky *et al.*, 2006; Waleed Shinwari *et al.*, 2010; Wang *et al.*, 2014; Ziadan, 2012) which may inform them about how different nanowires, including MNWs, can be used for practical applications.

Potential target for pathogenic micro-organisms

MNWs have been found in pathogenic biofilms causing BRONJ and supposed to play an important role in maintenance and survival of it (Wanger *et al.*, 2013). This discovery is very important considering the fact that various human pathogenic micro-organisms like *Ne. gonorrhoeae* and *Vibrio cholerae* produce pili which are actively involved in pathogenesis (Heckels, 1989; Tacket *et al.*, 1998; Zhang *et al.*, 2000). Exoelectrogenic microbes with putative MNWs play specific role in host immune response (Ericsson *et al.*, 2015). It needs to be studied whether pili are conductive in different pathogenic bacteria and, if so, what role they play in pathogenesis. In the phenomenon called 'bioelectric effect', electrically stimulated pathogenic biofilms showed increased susceptibility to antibiotics and this may happen because of disruption of conductive filaments within them as a result of electrical stimulation (Costerton *et al.*, 1994; Wanger *et al.*, 2013). The bioelectric effect also supports the hypothesis that MNWs might be playing an important role in maintenance of pathogenic biofilms. Thus, MNWs can be a potential target for prevention and treatment of relevant diseases and future research in this direction may yield some exciting results.

Gaps in current research and future directions

The above examples suggest that microbes may have developed multiple strategies to produce MNWs as per their niche and physiological requirement. Hence, more extensive screening of micro-organisms from diverse habitats needs to be done to establish their ability to produce MNWs which may help to completely understand their abundance and role in the environment. The physiological function of most known MNWs is not identified so far except that of *G. sulfurreducens*. This is another area which can be the focus of future studies.

Comparative characterization of all known MNWs for their conductive, biochemical and mechanical properties should be done. This will significantly help us to identify the best candidate for practical applications and may also help us to produce hybrid MNWs with better functionality than any individual one. It is also of utmost importance that mechanisms of electron transfer through MNWs should be studied in MNWs produced by diverse micro-organisms (other

than *G. sulfurreducens* and *S. oneidensis*). Apart from aromatic amino acids, sulfur-containing amino acids (methionine and cysteine) are also known to act as a relay in electron transfer (Sun *et al.*, 2015; Wang *et al.*, 2009). The probable role of these sulfur-containing amino acids (if present) in electron transfer through MNWs has not been studied so far and any involvement of these amino acids in conductivity of MNWs needs to be explored.

There is also a need to develop simple methods that will allow maximum production of MNW which will be important from an application point of view. For example, the method for MNWs production in *Synechocystis* was sophisticated earlier (Gorby *et al.*, 2006), but in recent times, simple methods for maximum production of *Synechocystis* MNWs have been identified (Sure *et al.*, 2015). Most of the findings in this field to date have been generated from a few laboratories. Reproducibility, authenticity and credibility of particular data are strengthened when identical or similar results are obtained from different laboratories. This is especially true for the advanced characterization of MNWs involving determination of their electron transfer mechanisms where ambiguity still exists among researchers. So it is essential that further research be carried out to harness the true potential of this field and used to tackle contemporary problems.

Conclusions

The ability of micro-organisms to produce MNWs increases their potential to influence their surrounding environment and thus further enhances their status as 'tiny but powerful organisms'. Occurrence of MNWs in micro-organisms may be widespread and they may be employing it for diverse functions like extracellular electron transfer to metals, tolerance to toxic metals, preventing photo damage and cell communication depending upon their niche and physiological needs. The discovery of new MNW-producing micro-organisms and the identification of specific environmental conditions leading to production of MNWs is extremely important along with rigorous biochemical and electrical characterization of the same. This will help in identification of most suitable MNWs for specific practical applications in the field of bioremediation, bioenergy, bioelectronics and possibly biotherapeutics. More efforts are needed to explore the mechanism of electron flow through different MNWs which would greatly help in modulation of electro conductive and other properties of MNWs.

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