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Microbial Nanowires: An Electrifying Tale

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Corresponding Author:	Mandira Kochar, PhD The Energy and Resources Institute New Delhi, INDIA
First Author:	Sandeep K Sure
Order of Authors:	Sandeep K Sure
	Leigh M Ackland, PhD
	Angel A Torriero, PhD
	Alok Adholeya, PhD
	Mandira Kochar, PhD
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Review Paper

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Authors

Sandeep Sure,¹ M. Leigh Ackland,² Angel A.J. Torriero,² Alok Adholeya¹ and Mandira Kochar¹

¹TERI-Deakin Nanobiotechnology Centre, TERI Gram, The Energy and Resources Institute, Gual Pahari, Gurgaon Faridabad Road, Gurgaon, Haryana 122 001, India

²Centre for Cellular and Molecular Biology, Deakin University, 221 Burwood Highway, Burwood, Melbourne, Victoria 3125, Australia.

Correspondence

Mandira Kochar

Email: mandira.malhotra@gmail.com or mandira.kochar@teri.res.in

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Abstract

Electromicrobiology has gained momentum in the last ten years with advances in microbial fuel cells and the discovery of microbial nanowires (MNWs). The list of MNWs producing microorganisms is growing and providing intriguing insights into the presence of such microorganisms in diverse environments and the potential roles MNWs can perform. This review discusses the MNWs produced by different microorganisms, including their structure, composition and role in 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 are not only poised to change the way we see microorganisms but may also impact the fields of bioenergy, biogeochemistry and bioremediation, hence their potential applications in these fields are highlighted here.

Introduction

Microorganisms are known to produce sophisticated nanomachines like bacterial flagellar nanomotor 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 the 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.* discovered extracellular electrically conductive protein nanofilaments in *Geobacter sulfurreducens* and termed them microbial nanowires (MNWs) ((Reguera *et al.*, 2005). This discovery opened many new avenues of research in nanotechnology and microbiology. This review deals with MNWs produced by diverse microorganisms and discusses several important aspects of MNWs including their types, role, mechanism of electron transfer and potential applications.

Discovery of microbial nanowires 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 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 (upto μm distances) extracellular electron transfer had not been reported. Similar studies done on extracellular pili-like structures (PLS) of *S. oneidensis* and *Pseudomonas aeruginosa* indicated PLS to be non-conductive (Reguera *et al.*, 2005). However, previously reported non-conductive PLS 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 (5-8nm in diameter) (Gorby *et al.*, 2006; Pelicic, 2008; Simpson *et al.*, 1976), while lastly, there may be a tendency of some microorganisms to produce multiple pili like structures e.g. type II pseudopilus and thus the PLS 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, *Rhodopseudomonas palustris* strain RP2 (Venkidusamy *et al.*, 2015), and in sulphate (SO_4^{2-}) reducing bacterium *Desulfovibrio desulfuricans* (Eaktasang *et al.*, 2016). MNWs were not only observed in Fe and SO_4^{2-} reducing bacteria as discussed above, but they were also 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 PLS. This biofilm was found to be colonized by around fifteen discernible bacterial morphotypes, mostly anaerobic and facultatively anaerobic, constituting genera of *Staphylococcus*, *Bacillus*, *Fusobacterium*, *Actinomyces*, *Streptococcus*, *Selenomonas* and *Treponema* but the specific MNWs producing microorganisms amongst these could not be identified.

Apart from metal reducing and pathogenic microorganisms, MNWs have been observed in photosynthetic bacteria. Initial reports showed that *Synechocystis*, a unicellular cyanobacterium, can produce MNWs in electron acceptor (CO₂) limiting and high light conditions (Gorby *et al.*, 2006). Taking clues from this study, our group explored the possibility of MNWs formation 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 behavior (Pisciotta *et al.*, 2010). Further, *Microcystis aeruginosa* also encounter CO₂ limitation and get exposed to high light intensity when they form blooms. Thus, *M. aeruginosa* and *N. punctiforme* might be producing MNWs which has been confirmed by conductive atomic force microscopy (AFM) analysis (Sure *et al.*, 2015, Sure *et al.*, 2016b). The discovery of MNWs in such diverse microorganisms ranging from anaerobic, metal reducing bacteria to photosynthetic, aerobic cyanobacteria strengthen the viewpoint that they may be pervasive in the

environment. The MNWs producing microorganisms 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 microorganisms and use of these multiple techniques have been advocated to unambiguously confirm the presence of MNWs in microorganisms (Castro *et al.*, 2014; Gorby *et al.*, 2006; Li & Li, 2014; Malvankar *et al.*, 2014; Reguera *et al.*, 2005; Sure *et al.*, 2015; Venkidusamy *et al.*, 2015; Wanger *et al.*, 2013).

Types of microbial nanowires

Diverse microorganisms 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*, *A. ferrooxidans* and *Synechocystis* sp. have been found to be 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 while same is PilA1 for *Synechocystis* (Fig. 3a-b) (Reguera *et al.*, 2005; Sure *et al.*, 2015). Though MNWs from both microorganisms are TFP, the molecular weight (MW) of their subunits (~10kDa for *G.*

sulfurreducens while ~22kDa for *Synechocystis*) and dimensions (width/length - 3-5nm/10-20µm and 4.5-7nm/2-10µm 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.*, 2011a; Malvankar *et al.*, 2011b; 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 *A. ferroxidans* may be made up of PilV and PilW proteins (Li & Li, 2014). The MNWs from different microorganisms 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) Concerning the length, it 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 has been shown 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 elongates and become MNWs (Fig. 3c) (Pirbadian *et al.*, 2014). Unlike pili and flagella, which are mostly homopolymers of single subunit, 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 & Aggeli,

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

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 behavior. 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 – Microbial nanowires whose identity need to be confirmed

Pili-like conductive structures have been identified in *A. hydrophila*, *R. palustris*, *D. desulfuricans* and *N. 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 6-7.5nm in diameter and 0.5-2µm in length and second, long/thick MNWs of size ~20-40nm in diameter and ≥10µm long were observed in *N. 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 *M. 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 *M. 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. *Pelotomaculum thermopropionicum* produces electrically conductive flagellum-like appendages (10-20nm in diameter) in monoculture as well in coculture with *Methanothermobacter thermoautotrophicus* (Gorby *et al.*, 2006). These flagellum-like appendages may be indeed flagellas as subsequent study by other group have shown that *P. thermopropionicum* in cocultures with *M. 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 *et al.*, 2014).

Physiological role of microbial nanowires

As discussed in the previous sections, each type of MNWs has unique structure and composition and they may have evolved as per the physiological requirements of the microorganisms. 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 microorganisms 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 microorganisms like *A. 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 microorganisms 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 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 microorganisms can directly transfer electrons to each other, rather than use hydrogen and formate as intermediate electron carrier (Morita *et al.*, 2011). It is hypothesized 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 microorganisms present in the microbial mat (Gorby *et al.*, 2006; Lea-Smith *et al.*, 2015). Such MNWs mediated electron transfer between two microorganisms can also be part of cell communication/signaling (Reguera, 2011). For instance, it has been observed that MNWs of *G. sulfurreducens* leads 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, recent study has shown that MNWs production in *G. sulfurreducens* is necessary for the formation of optimum electroactive and thick (more than 10µm) 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 (PQ) gets over reduced due unavailability of carbon to sink electrons. It is hypothesized that MNWs may help cyanobacteria to release these extra electrons present on PQ so as to restrict cell damage (Gorby *et al.*, 2006; Lea-Smith *et al.*, 2015; Pisciotta *et al.*, 2010). MNWs produced in *M. aeruginosa* might be important for bloom formation while those of *N. punctiforme* may be involved in plant symbiosis (Duggan *et al.*, 2007; Sure *et al.*, 2015).

Mechanism of electron transfer through microbial nanowires

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 mechanism 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 group of scientists while for *S. oneidensis* MNWs, electrons transfer is believed to occur by hopping mechanism. Both major proposed mechanisms of electron transfer through MNWs have been discussed here.

Metallic like conductivity model

Elaborate experiments done by Malvankar *et al.* 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.*, 2011b; Malvankar *et al.*, 2012; Malvankar *et al.*, 2014; Malvankar *et al.*, 2015). 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 metallic like conductivity model for

272 *G. sulfurreducens* MNWs and biofilms are their temperature and pH dependent electrical
273 behavior (Malvankar *et al.*, 2011b). This observation was also supported by structural studies
274 where lower pH was shown to induce conformational changes in aromatic amino acids which
275 thereby causes higher conductivity in the *Geobacter* pili (Malvankar *et al.*, 2015).

276 In synthetic organic metals, electron transfer is attributed to overlapping π - π orbitals of
277 aromatic rings present in it. Proteins also contain several aromatic amino acids whose
278 aromatic constituents can play a role similar to that of organic metals in electron transfer. To
279 confirm the role of these aromatic amino acids in electron transfer, a *Geobacter* strain, Aro-5
280 was constructed (Vargas *et al.*, 2013). In *Geobacter* Aro-5, five aromatic amino acids of
281 PilA, the pili subunit were replaced with alanine and it was observed that the resultant
282 modified pili showed considerable diminished electrical conductivity and ability to reduce Fe
283 (III) compared to the pili of the control strain (Vargas *et al.*, 2013). Though this study
284 confirmed that aromatic amino acids play an important role in conductivity of *G.*
285 *sulfurreducens* pili and its biofilm, it is still unclear how modified pili are able to show
286 residual electrical conductivity. Further, the removal of aromatic amino acids may be altering
287 the 3D structure of pili and thereby the positioning of cytochromes on pili, which can
288 ultimately decrease pili conductivity (Boesen & Nielsen, 2013). This is important considering
289 the fact that tilting of molecules and interplanar distances have the potential to affect charge
290 transport. The pilus filament model by Yan *et al.* based on *Neisseria gonorrhoeae* concludes
291 that aromatics are too far to be involved in electron transport (Yan *et al.*, 2015). However,
292 experimental data generated using techniques like synchrotron X-ray microdiffraction and
293 rocking-curve X-ray diffraction have refuted this model and strongly supported the role of
294 aromatic amino acids in long distance electron transfer and reinforced metallic like
295 conductivity mechanism in *Geobacter* pili (Malvankar *et al.*, 2015). Along with experimental
296 data, modeling studies also supported the metallic conductivity model where lowest energy

models of *Geobacter* pili were observed to have no central channel and 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 pili structure of *G. sulfurreducens* in electron transfer was further studied where the pilA gene of *G. sulfurreducens* was replaced with the pilA gene from *P. aeruginosa* (Liu *et al.*, 2014). The resultant strain was able to produce and assemble *P. aeruginosa* PilA subunits into pili and interestingly these hybrid pili had the same pattern of cytochromes as that of control cells. But the conductivity of these hybrid pili was found to be 14 times lesser than normal pili with significantly diminished ability to reduce iron and current generation. From these observations, authors suggested the intrinsic structures of *Geobacter* pilus and not associated cytochromes, are important for electron transfer through it. However, as the pili of *P. 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 *et al.*, 2014). Malvankar *et al.* 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 *et al.*, 2012). They also reported that denaturing cytochromes in *G. sulfurreducens* pili networks and biofilms does not affect the electrical conductivity, thus ruling out any role of cytochromes in electron transfer through pili and biofilms (Malvankar *et al.*, 2011b; Malvankar *et al.*, 2012). Further, STM analysis of *G. sulfurreducens* MNWs

supported these findings where electron transfer is attributed to the intrinsic pili structure, and not to the cytochromes (Veazey *et al.*, 2011).

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

Electron hopping model

One view is emerging that electron transfer occurs by multistep 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 earlier 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 modeling studies strongly support the hypothesis that electron transfer through *Geobacter* MNWs occurs by multistep 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 electron hopping mechanism (Lampa-Pastirk *et al.*, 2016). It has also been shown that metals or redox organic cofactor free *Geobacter* pili show carrier mobility of $3.2 \times 10^{-2} \text{ cm}^2/\text{Vs}$ which is too low for metallic conductivity regime where carrier mobilities of more than $1 \text{ cm}^2/\text{Vs}$ 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 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 (1-2nm) to carry out charge transport over μm distances. Interested readers are referred to specific reviews on this topic (Skourtis, 2013; Waleed Shinwari *et al.*, 2010).

Potential applications of microbial nanowires

One reason behind the widespread attention gained by MNWs is the potential applications of this 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 microorganisms which are away from 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 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 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 have been shown to increase electricity production by 10 times (Reguera *et al.*, 2006). On the same line, MNWs producing photosynthetic microorganisms (Gorby *et al.*, 2006; Sure *et al.*, 2015; Sure *et al.*, 2016b) may be helpful in improving the efficiency of photosynthetic MFC and microbial solar cells (Rosenbaum *et al.*, 2010; Strik *et al.*, 2011).

Methane is considered as 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, 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 microorganisms have further increased their potential in this field. It has been shown that MNWs can play 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 a 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 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.* characterized *S. oneidensis* MNWs and showed that they have enough mechanical strength (Young's modulus ~1 GPa) to use it as a building block for construction of electronic devices (Leung *et al.*, 2011). 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 behavior (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.* 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 (Tan *et al.*, 2016). MNWs may also be used in bionanosensors (Lovley *et al.*, 2009), however no such prior art 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; Ziad, 2012) which may sensitize them about how different nanowires, including MNWs can be used for practical applications.

Potential target for pathogenic microorganisms

MNWs have been found in pathogenic biofilms causing BRONJ and supposed to play important role in maintenance and survival of it (Wanger *et al.*, 2013). This discovery is very important considering the fact that various human pathogenic microorganisms like *Neisseria gonorrhoeae* and *Vibrio cholera* 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 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

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 microorganisms 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 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 to identify the best candidate for practical applications and may also help 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 microorganisms (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 MNWs which will be important from application point of view. For example, 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 till date have been generated from few labs. Reproducibility, authenticity and credibility of particular data gets strengthened when identical or similar results are obtained from different labs. This is especially applied to the advanced characterization of MNWs involving determination of electron transfer mechanisms through them 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 use it to tackle contemporary problems.

471 **Conclusions**

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

485 **Conflict of Interest**

486 No conflict of interest to declare

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838 **Table 1:** List of MNWs producing microorganisms

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Sr. No	Microorganisms	Component Protein of MNWs	Physiological Role	Conductivity Measurement (Along width/length)	Description	References
1	<i>Geobacter 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> , 2011b; Malvankar <i>et al.</i> , 2014; Reguera <i>et al.</i> , 2005)
2	<i>Shewanella 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; Pirbadian <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>Pelatomaculum thermopropionicum</i>	Not known yet	Not known yet	Along width	Anaerobic, thermophilic	(Gorby <i>et al.</i> , 2006)
5	Multispecies biofilms observed in bisphosphonate-related osteonecrosis of the jaw (BRONJ)	Not known yet	Not known yet	Along width as well as length	Unkonwn	(Wanger <i>et al.</i> , 2013)
6	<i>Acidithiobacillus ferrooxidans</i>	Not known yet	Not known yet	Along width	Chemolithoautotrophic, acidophilic	(Li and Li, 2013)
7	<i>Aeromonas hydrophila</i>	Not known yet	Not known yet	Along width	Facultative anaerobic, heterotroph	(Castro <i>et al.</i> , 2014)

8	<i>Microcystis 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>Nostoc punctiforme</i>	Not known yet	Not known yet	Along width	Photosynthetic, aerobic, filamentous	(Sure <i>et al.</i> , 2016b)
10	<i>Rhodopseudomonas palustris</i> strain RP2	Not known yet	Not known yet	Along width as well as length	Anoxic photosynthetic, iron respiring	(Venkidusamy <i>et al.</i> , 2015)
11	<i>Desulfovibrio desulfuricans</i>	Not known yet	Not known yet	Along width	Anaerobic, reducing sulfate	(Eaktasang <i>et al.</i> , 2016)

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Figure Legends

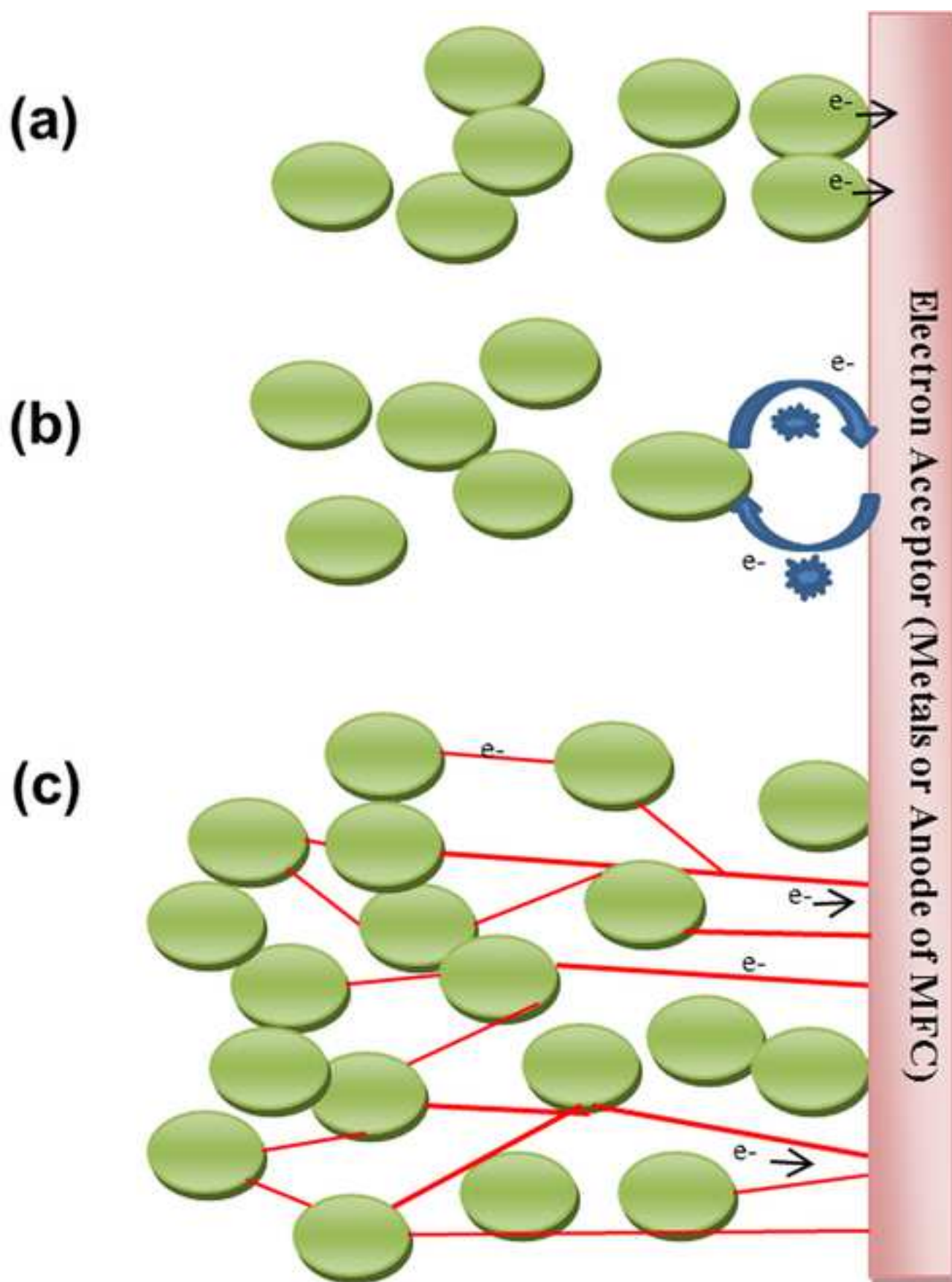
Fig. 1. Strategies by which bacteria can transfer electrons extracellularly to electron acceptors (metals or anode of microbial fuel cell). Bacteria can transfer electrons extracellularly by direct attachment to metal or anode surface of microbial fuel cell (a) or employ metal chelators or small molecules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

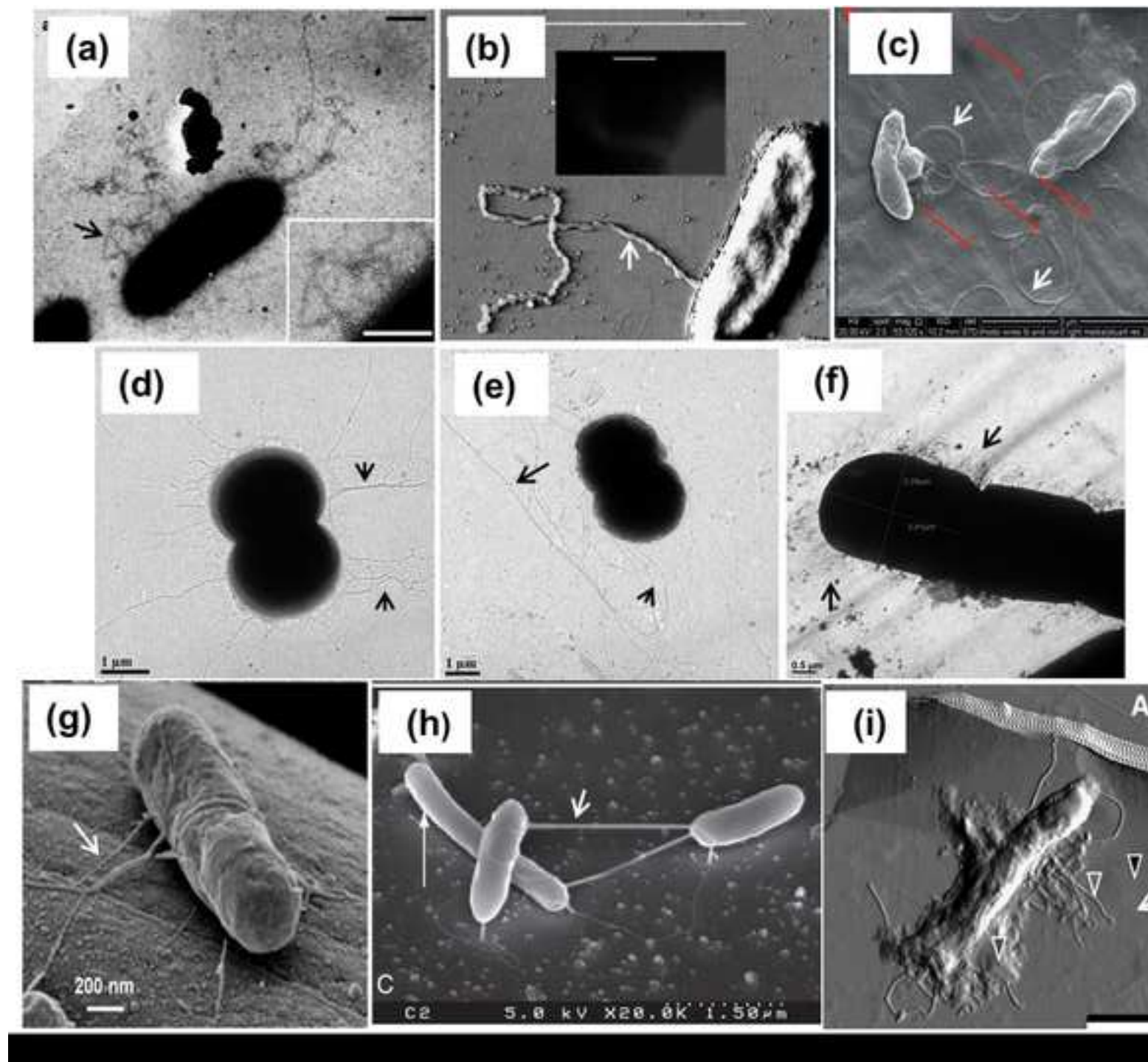
Fig. 2. Diverse microorganisms that can produce microbial nanowires (MNWs). (a) *Geobacter sulfurreducens*, an anaerobic, dissimilatory metal reducing bacteria (DMRB). Inset shows nanowires at higher magnification. Scale bar, 0.2µm (TEM image) (Reprinted by permission from Macmillan Publishers Ltd: Nature, Reguera *et al.*, 2005©2005); (b) *Shewanella oneidensis*, a facultative anaerobic, DMRB (AFM image). Inset shows in vivo fluorescence image of same cell. The image has been color edited (Adapted from Pirbadian *et al.*, 2014©National Academy of Sciences); (c) *Rhodospseudomonas palustris* strain RP2, photosynthetic DMRB, metabolically versatile (Reproduced from Venkidusamy *et al.*, 2015 with permission from The Royal Society of Chemistry); (d) *Synechocystis* PCC 6803 and (e) *Microcystis aeruginosa* PCC 7806 which are aerobic, unicellular photosynthetic microorganisms (TEM images) (With kind permission from Springer Science+Business Media: Suret *et al.*, 2015©Springer Science + Business Media); (f) *Nostoc punctiforme* PCC 73120 an aerobic, multicellular and filamentous photosynthetic microorganism (Suret *et al.*, 2016) (TEM image); (g) *Desulfovibrio desulfuricans*, an obligate anaerobe, sulphate reducing (Adapted from Eaktasang *et al.*, 2016©Elsevier); (h) *Pelotomaculum thermopropionicum* and *Methanothermobacter thermoautotrophicus* (shown with filled arrow) which are syntrophic methanogenic cocultures (SEM image) (Adapted from Gorby *et al.*, 2006©National Academy of Sciences); (i) Unknown bacteria from BRONJ affected bone

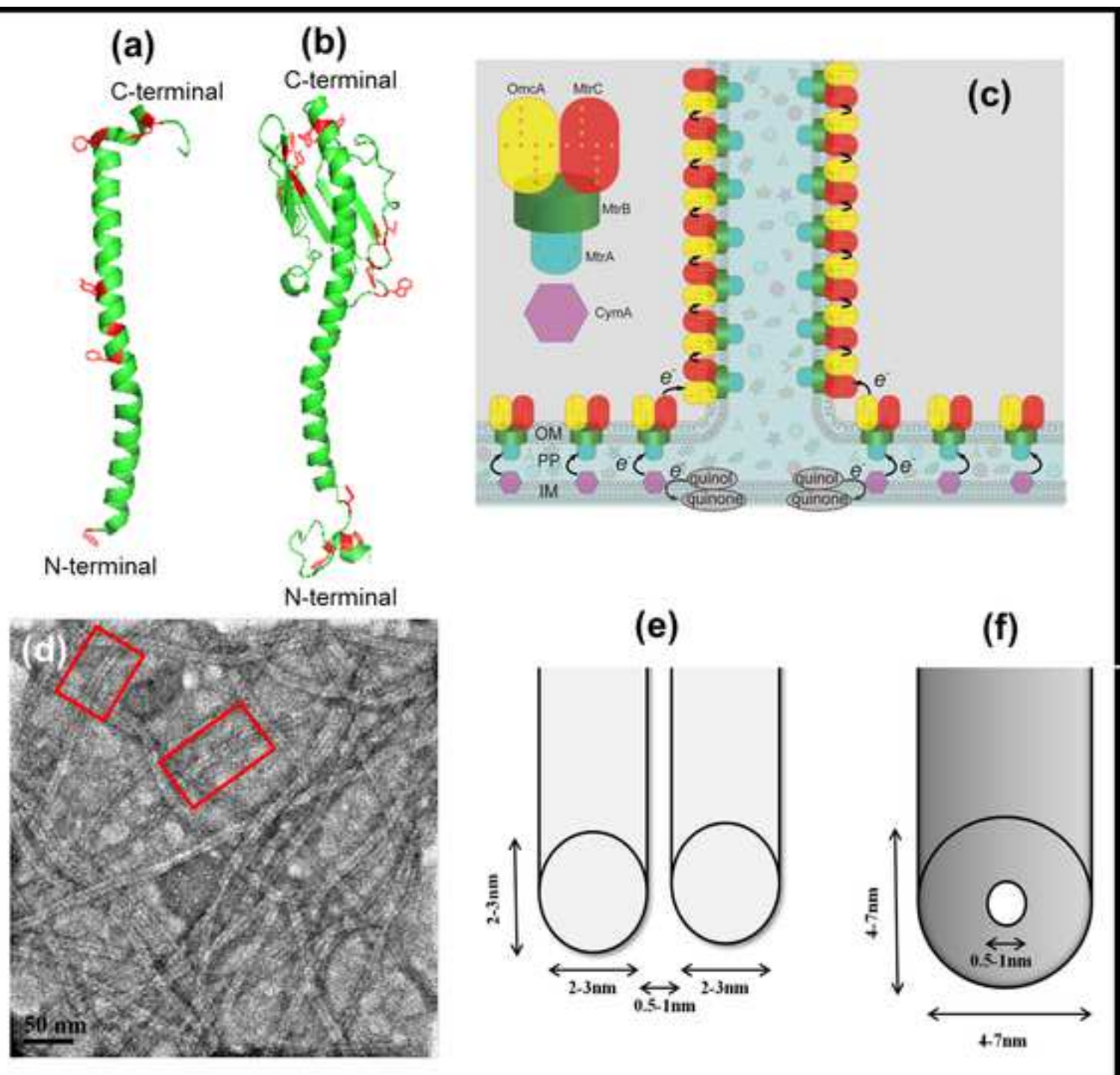
(AFM image). The image has been colour edited (Adapted from Wanger *et al.*, 2013©Elsevier). MNWs are also produced by *Acidithiobacillus ferrooxidans* (Li & Li, 2014) and *Aeromonas hydrophila* (Castro *et al.*, 2014) (not shown here). MNWs have been shown with open arrows in all images.

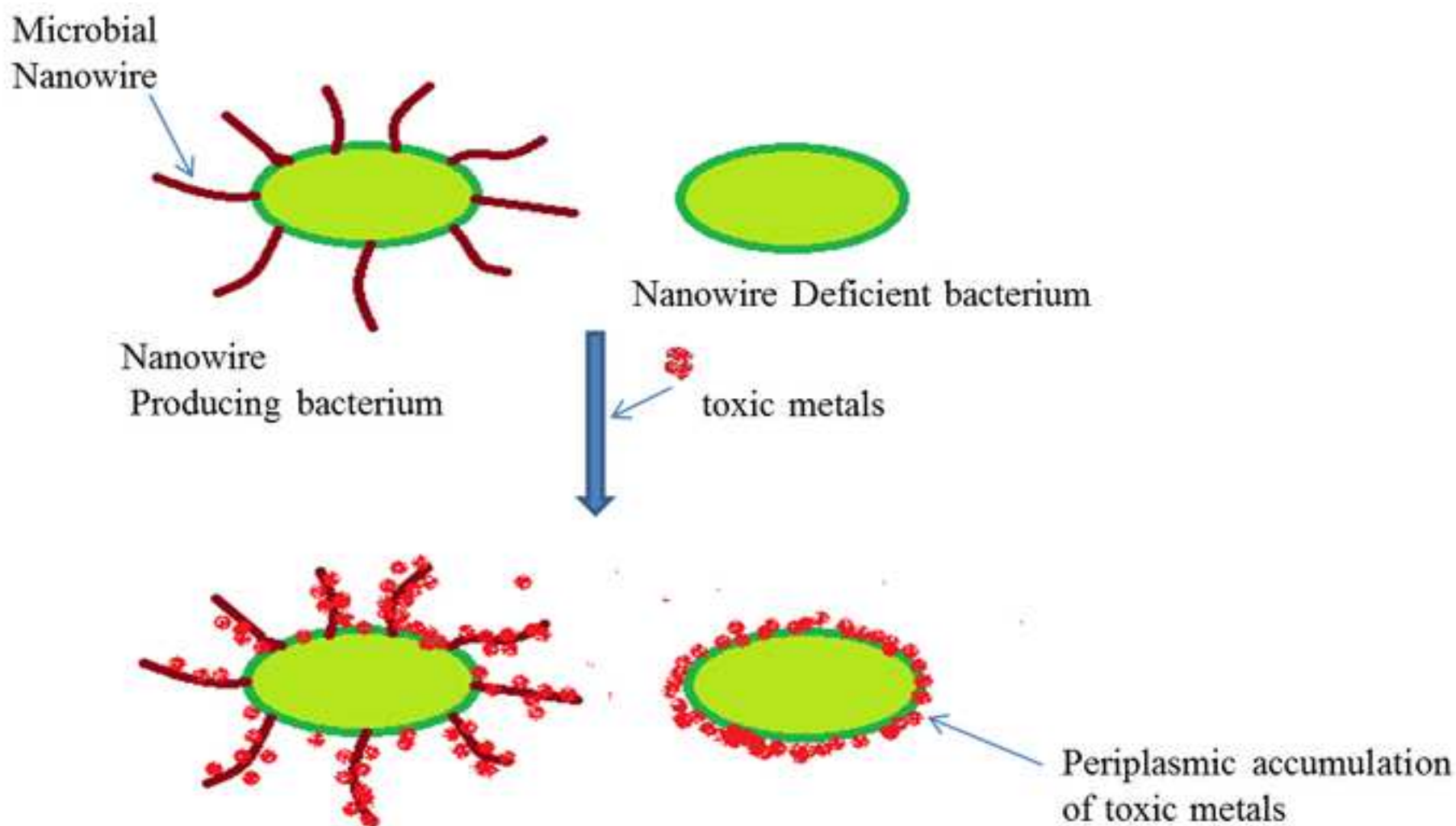
Fig. 3. Three types of microbial nanowires (MNWs) observed to date. 1) MNWs made of type IV pili (TFP) as observed in *Geobacter sulfurreducens* and *Synechocystis* are made up of subunits, PilA (a) and PilA1 (b), respectively. Both these subunits differ in their structure at C-terminal and number and 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 *Shewanella oneidensis* (c) (Adapted from Pirbadian *et al.*, 2014©National Academy of Sciences); 3) Unknown MNWs as observed in *Microcystis aeruginosa* and other microorganisms. In *M. aeruginosa*, MNWs was found to be made up of unknown protein (GenBank : CAO90693.1) and from its TEM image (d), it seems that it is either made of two subfilaments (e) or contains central channel (f) (With kind permission from Springer Science+Business Media: Sure *et al.*, 2015©Springer Science + Business Media).

Fig. 4. Potential role of Type IV pili (TFP) in cell-metal interaction. Schematic representation of how TFP/microbial nanowires can reduce interaction between cell membrane and toxic metals and can act as a protective barrier against latter.









Nanowire avoid periplasmic accumulation of toxic metals and provide greater surface area for its reduction/oxidation