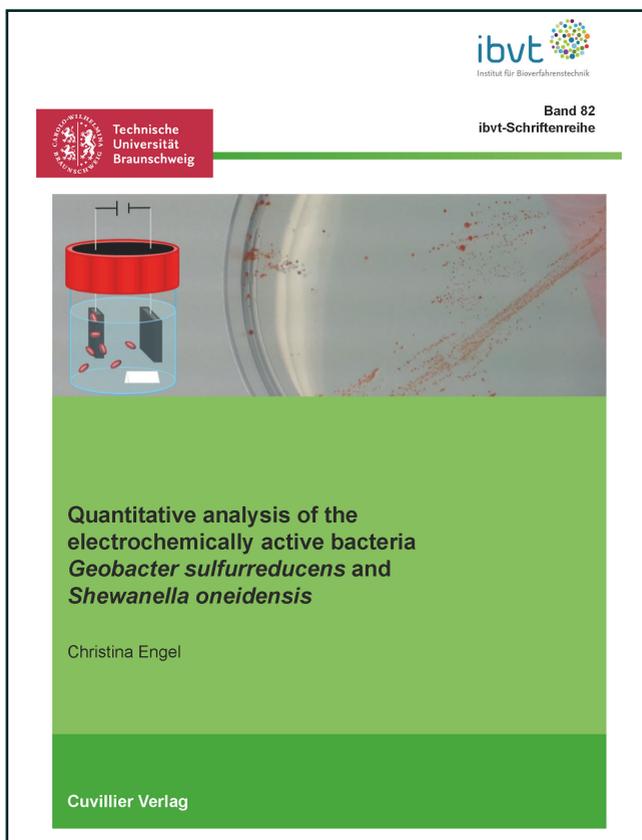




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# Quantitative analysis of the electrochemically active bacteria *Geobacter sulfurreducens* and *Shewanella oneidensis*



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# 1 Introduction

About 10 billion cubic meters of municipal wastewater are being produced every year in Germany (WS-1, 2018). Its treatment requires over 4,000 GWh of electricity, which constitutes roughly 20 % of the municipal energy demand (WS-2, 2018). Aeration of activated sludge tanks has the largest share of energy consumption in wastewater treatment (WS-2, 2018). As worldwide CO<sub>2</sub> emissions are constantly increasing and have reached 33,444,000,000 tons in 2017 (WS-3, 2018), there is both a need to reduce the energy demand as well as to enhance the proportion of CO<sub>2</sub>-neutral, renewable energy sources.

One way of combining the need to purify municipal wastewater as well as making a contribution to renewable energy sources and energy savings is to couple wastewater treatment to either the electricity production in microbial fuel cells (MFCs) or the hydrogen production in microbial electrolysis cells (MECs) (Du et al. 2007; Logan et al. 2008). In these bioelectrochemical systems (BESs), a special type of bacteria called electrochemically active bacteria is serving as biocatalyst for the conversion of organic matter to electricity (Logan 2009). As this conversion is an anaerobic process, a large proportion of the energy demand of wastewater treatment plants can be conserved (Gu et al. 2017). At the same time, the energy resulting from the conversion of organic matter is used to produce electricity or the clean fuel hydrogen gas (H<sub>2</sub>) (Du et al. 2007; Logan et al. 2008). The way in which electrochemically active bacteria transfer electrons to an anode, which is the ultimate cause of the electricity generation, has been studied for roughly the past two decades and continues to be subject of investigation by researchers around the world.

Understanding how electrochemically active bacteria function on a molecular level and how they interact in complex communities makes it possible to improve MFCs and MECs. Therefore, two very well studied electrochemically active model organisms, *Geobacter sulfurreducens* and *Shewanella oneidensis* (Caccavo et al. 1994; Venkateswaran et al. 1999), were used in this work to investigate a few specific research questions:

- What is the long-term behaviour of interactions between electrochemically active bacteria in a mixed culture?
- Which anode potential provides the optimal conditions for bacterial performance in MECs?
- How does *G. sulfurreducens* handle oxygen intrusion into its living environment?



By trying to answer these questions, this thesis intends to provide some further knowledge for the improvement of BESs.

### 1.1 Redox reactions – the energetics of electron transfer

Redox reactions occur between two chemical species where electrons are transferred from one species in a reduced state (*red*) to another in an oxidised state (*ox*) (Binnewies et al. 2004). They consist of two half-reactions, the reduction and the oxidation (Binnewies et al. 2004). The removal of a number of  $n$  electrons ( $e^-$ ) from one species ( $A$ ) is called the oxidation (equation 1.1) while the uptake of the electrons from the second species ( $B$ ) is called the reduction (equation 1.2) (Binnewies et al. 2004).

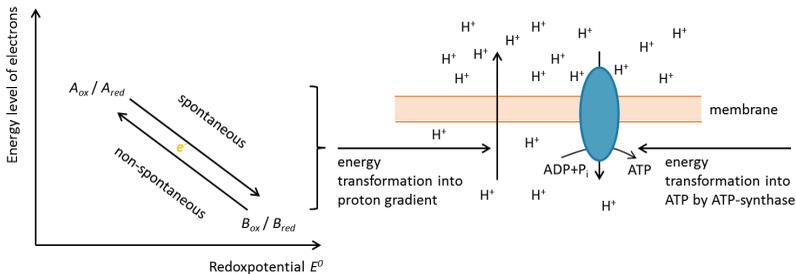


Electrons present in a reduced species have a certain energy level (Binnewies et al. 2004). This energy level is quantified by the reduction or redox potential  $E^\circ$  (Binnewies et al. 2004). A high energy level is represented by a low redox potential and vice versa (Binnewies et al. 2004). The redox potential is not an absolute value, but rather has to be reported in comparison to another half-reaction (Binnewies et al. 2004). The half-reaction  $2H^+ + 2e^- \leftrightarrow H_2$  has been defined to have a redox potential of  $E^\circ = 0 \text{ V}_{\text{SHE}}$  (SHE = standard hydrogen electrode) and by default all other potentials are reported in relation to it (Binnewies et al. 2004). Redox potentials are dependent on temperature, pH and the concentration of as well as ratio between reduced and oxidised species (Binnewies et al. 2004). Therefore, standard reaction conditions are defined for this half-reaction which are  $H_2$  at a partial pressure of 1,000 hPa, a proton activity of 1 mol/L and a temperature of 25 °C (Binnewies et al. 2004). In biological systems, it is more common to report redox potentials at neutral pH, which is indicated by an apostrophe ( $E'^\circ$ ) (de Bolster 1997; Acworth 2003).

Transfer of electrons from species A to species B will only occur spontaneously if the energy level of electrons in  $B_{red}$  is lower than the energy level in  $A_{red}$  (Binnewies et al. 2004). In other words, electrons are only transferred spontaneously from components with a low redox potential to components with a higher redox potential (Binnewies et al. 2004). This principle is shown in **Figure 1.1**. When a redox reaction takes place, the difference in energy levels of



electrons between the two reaction partners is converted into other forms of energy, for example heat, new chemical bonds or electricity (Binnewies et al. 2004; Nelson and Cox 2017). Every living organisms is making use of this potential energy from redox reactions as shown schematically in **Figure 1.1** (Acworth 2003). In general, electrons residing in substrates used by cells have a low redox potential (Nelson and Cox 2017). Substrates are converted intracellularly by enzymes, whereby electrons are transferred in multiple reaction steps until they are finally transferred to a terminal electron acceptor with a high redox potential (Nelson and Cox 2017). For example, in aerobic organisms the terminal electron acceptor is oxygen (Nelson and Cox 2017). The half-reaction  $\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$  has a redox potential of  $E^{\circ'} = 0.815 \text{ V}_{\text{SHE}}$ , thus the energy gain from the transfer of electrons to this acceptor is high (Wood 1988). The transfer of electrons is catalysed by a number of enzymes residing in the bacterial membrane which use the potential energy from the electron transfer to translocate protons from the cell interior to the periplasm (Nelson and Cox 2017). The thus created proton gradient is converted into chemical bond energy, usually in the form of ATP (see **Figure 1.1**) (Acworth 2003; Nelson and Cox 2017).



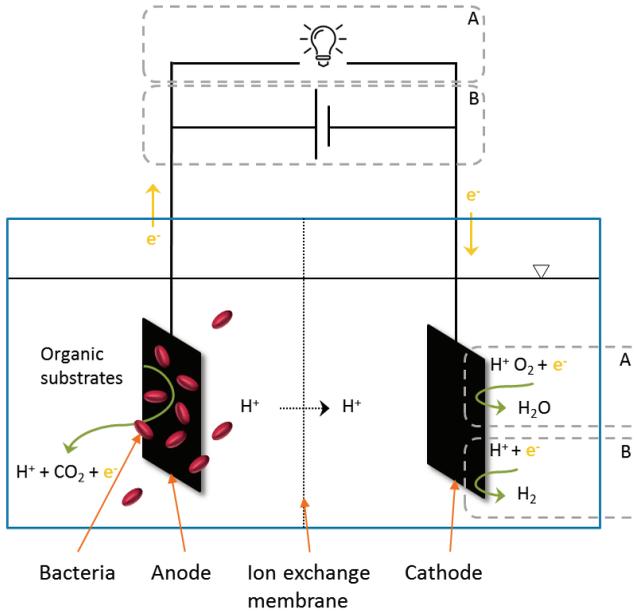
**Figure 1.1:** Scheme of energy conversion from substrates metabolised via redox reactions into a proton gradient and subsequently into ATP.

## 1.2 Bioelectrochemical systems – making use of the energy from redox reactions

In bioelectrochemical systems (BESs) the two half-reactions of a redox reaction are occurring separately at an anode (oxidation) and a cathode (reduction). In contrast to classical electrochemical systems, the redox reactions are in part carried out by microorganisms. In general, there are two different types of BES, microbial fuel cells (MFCs) which produce



energy, and microbial electrolysis cells (MECs) which require an additional energy source (see **Figure 1.2**) (Lovley 2006; Logan et al. 2008; Logan 2009).



**Figure 1.2:** Scheme of (A) microbial fuel cell (MFC) and (B) microbial electrolysis cell (MEC) (adapted from Lovley (2006) and Logan et al. (2008)).

In MFCs, bacteria are used to produce electricity from the energy stored in organic substances (Lovley 2006; Logan 2009). Hereby, bacteria reside in the anodic compartment of the MFC and transfer electrons to an anode (see **Figure 1.2 A**, Lovley, 2006; Logan, 2009). Electrons are then conducted through an external electric circuit to the cathode compartment, where the reduction of e.g. oxygen to water is occurring (Lovley 2006; Logan 2009). The potential difference between these half-reactions is positive so that electrical energy is gained (Lovley 2006; Logan 2009). The prospect of MFCs lies in their ability to purify complex wastewater streams while at the same time turning the therein contained chemical bond energy directly into electricity (Bond and Lovley 2003; Logan 2009; Hallenbeck et al. 2014). Anodic and cathodic compartments have to be separated to ensure that bacteria transfer electrons to an electrode



(Lovley 2006; Du et al. 2007). Otherwise, bacteria would transfer electrons directly to oxygen without delivering part of the energy stored in organic substrates in the form of electrical energy (Lovley 2006). A separation is usually realised with a membrane that allows protons to diffuse to the cathodic chamber (Lovley 2006). The membrane constitutes an internal resistance for the system, which can create limitations for MFC performance (Logan 2009; Hallenbeck et al. 2014).

In MECs, electrons released by bacteria are used for other reactions, for instance the production of  $H_2$  from protons (see **Figure 1.2 B**). The redox potential of the reaction  $2H^+ + 2e^- \leftrightarrow H_2$  is low ( $-0.414 V_{SHE}$  at pH 7) so that the half-reactions taking place at the anode and the cathode usually have a negative potential difference (Logan et al. 2008). This means they are not spontaneous (Logan et al. 2008). Rather, an external voltage has to be applied in these systems that provides the energy for the reaction (Logan et al. 2008; Rosenbaum et al. 2010). The need of additional energy to drive the reaction forward also means that the reaction will not take place spontaneously within the liquid medium even if anode and cathode compartments are not separated (Logan et al. 2008). Thus, the resistant, separating membrane can be eliminated (Logan et al. 2008).

Mostly,  $H_2$  is produced by electrolysis of water. This process requires a high cell voltage of 2.3 V on average to be applied to an electrolysis cell (Call et al. 2009). In MECs operated with acetate or lactate, the theoretical amount of voltage needed is only 0.14 V or -0.011 V, respectively (Rosenbaum et al. 2010). In practice, higher potentials of about 0.5 V to 0.7 V are needed to achieve worthwhile  $H_2$  production rates, but this still constitutes a huge energy conservation compared to classical electrolysis of water (Call et al. 2009; Rosenbaum et al. 2010).

It is also possible that, instead of the anode, bacteria are present at the cathode where they take up electrons and use them to form reduced, high-energy compounds, often by  $CO_2$  fixation (Hallenbeck et al. 2014; Rosenbaum and Franks 2014). This is called microbial electrosynthesis (MES) (Rosenbaum and Franks 2014). Usually the anodic half-reaction of MES is the cleavage of water to protons and oxygen (Rosenbaum and Franks 2014). The potential of MECs lies in their ability to store electrical energy in a chemical form. This is advantageous for power plants with inconsistent productivity like solar arrays or wind turbines (Rosenbaum and Franks 2014).



### 1.3 Electrochemically active bacteria

As stated in chapter 1.1, bacteria use redox reactions to gain energy for cell metabolism and growth by transferring electrons present in the substrates they consume to a terminal electron acceptor (Nelson and Cox 2017). Typically, terminal electron acceptors are soluble molecules that are reduced intracellularly (Babauta et al. 2012a). But there are also bacteria capable of transferring electrons through their cell membranes and periplasm to an insoluble electron acceptor (Babauta et al. 2012a). Bacteria capable of performing this extracellular electron transfer (EET) are termed electrochemically active bacteria (Babauta et al. 2012a).

Natural insoluble electron acceptors encountered by electrochemically active bacteria are different forms of metal oxides often found in sediments of water bodies (Santos et al. 2015). Electrochemically active bacteria use in principle the same transfer mechanisms to reduce a number of different metal ions (Santos et al. 2015). In terms of these transfer mechanisms, anodes in bioelectrochemical systems are quite similar to insoluble metal oxides (Babauta et al. 2012a). Therefore, anodes can serve as terminal electron acceptor to bacteria, which allows the production of electricity.

Among all electrochemically active bacteria, two have been studied in detail due to their ability to respire insoluble metal oxides (Hallenbeck et al. 2014). The first and possibly most important one is *Geobacter sulfurreducens*, which belongs to the  $\delta$ -proteobacteria (Caccavo et al. 1994). In MFCs inoculated with wastewater that contains an undefined mixture of hundreds of microorganisms, *Geobacter* spp. quickly become dominant within the anodic biofilm, which is the reason why *G. sulfurreducens* has been studied by many researchers (Logan 2009). *G. sulfurreducens* was described first by Caccavo et al. (1994) to be an obligately anaerobic, non-motile, Gram-negative, rod-shaped bacterium. It is capable of reducing different forms of Fe(III), Mn(IV), U(VI), elemental sulfur, fumarate and malate (Caccavo et al. 1994; Mehta et al. 2005; Shelobolina et al. 2007). As carbon and energy source, *G. sulfurreducens* utilises acetate (Caccavo et al. 1994). Other possible electron donors are H<sub>2</sub> and lactate (Brown et al. 2005; Call and Logan 2011). Unlike most bacteria, *G. sulfurreducens* is capable of oxidising acetate completely to CO<sub>2</sub> under anaerobic conditions, which makes this bacterium especially interesting for the use in wastewater-treatment-MFCs as a complete degradation of organic substrates is the aim in wastewater treatment (Bond and Lovley 2003).

Another well-studied organism is *Shewanella putrefaciens* MR-1, which was found in 1988 by Myers and Nealson (1988) and reassigned *Shewanella oneidensis* in 1999 (Venkateswaran et



al. 1999). It belongs to the  $\gamma$ -proteobacteria and can reduce iron, manganese and uranium oxides as well as elemental sulfur (Venkateswaran et al. 1999). Possible electron donors are lactate, succinate and fumarate (Venkateswaran et al. 1999). *S. oneidensis* cells are Gram-negative and rod-shaped, but unlike *G. sulfurreducens* this bacterium is a facultative anaerobe (Venkateswaran et al. 1999). Under aerobic conditions, the complete oxidation of the carbon source to CO<sub>2</sub> is possible, but under anaerobic conditions, acetate is the fermentative end product from lactate or pyruvate (Meshulam-Simon et al. 2007). Cell growth under anaerobic conditions is only supported with lactate as carbon source, but *S. oneidensis* has been shown to be metabolically active and produce H<sub>2</sub> gas when pyruvate serves as the sole energy source (Meshulam-Simon et al. 2007).

Often, electrochemically active bacteria form biofilms to maintain close proximity to their insoluble electron acceptors (Babauta et al. 2012a). Both *S. oneidensis* and *G. sulfurreducens* possess this ability, but *G. sulfurreducens* forms thicker and area-wide biofilms (Babauta et al. 2012a). Electrochemically active biofilms need to be conductive as electron transfer from cells in upper biofilm layers has to be possible (Leang et al. 2013). A high biofilm conductivity is also important for the application of electrochemically active biofilms in MFCs or MECs as it reduces charge transfer resistance at the anode-biofilm interface and results in higher current densities and power outputs (Malvankar et al. 2012; Leang et al. 2013).

#### 1.4 Extracellular electron transfer by *G. sulfurreducens* and *S. oneidensis*

The mechanisms for extracellular electron transfer (EET) have been intensively studied in both *G. sulfurreducens* and *S. oneidensis* (Marsili et al. 2008a; Bond et al. 2012; Pirdadian et al. 2014). While both species display similarities in their transfer mechanisms, there are also fundamental differences (Santos et al. 2015). Generally speaking, EET can be divided into two different mechanisms (Borole et al. 2011; Babauta et al. 2012a). The first is based on c-type cytochromes present in the membrane of bacteria and in the extracellular matrix of electrochemically active biofilms (Babauta et al. 2012a). This cytochrome-based transfer is often termed direct electron transfer due to the close proximity usually maintained between the cells and the electron acceptor (Borole et al. 2011). *G. sulfurreducens* is especially known for its direct transfer mechanisms, in which conductive pili play an important role next to c-type cytochromes (Bond et al. 2012). *S. oneidensis* also uses c-type cytochromes, but it additionally employs a second mechanism that is based on soluble mediators (Marsili et al. 2008a; Breuer

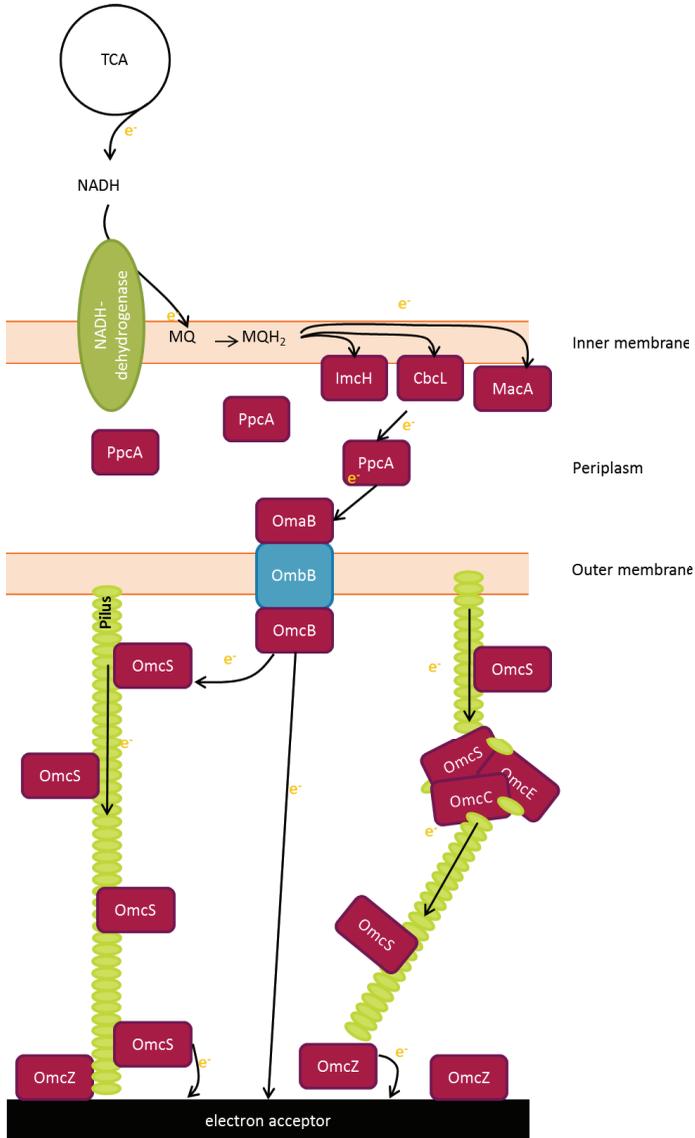


et al. 2015). This is also termed indirect electron transfer (Borole et al. 2011). In both cases, electron transfer starts within the cytoplasm of the cells during the metabolisation of organic substrates.

The EET mechanisms of *G. sulfurreducens* are schematically depicted in **Figure 1.3**. Acetate is metabolised by *G. sulfurreducens* via the tricarboxylic acid (TCA) cycle and degraded completely to CO<sub>2</sub> (Galushko and Schink 2000). The hereby released electrons are temporarily stored in the form of NADH. NADH is oxidised by the type I NADH dehydrogenase located in the inner membrane, which simultaneously transports protons into the periplasm, thus creating a proton gradient for ATP synthesis (Mahadevan et al. 2006). The NADH dehydrogenase transfers electrons to menaquinon, which is present within the inner membrane (Caccavo et al. 1994; Galushko and Schink 2000; Butler et al. 2006).

Several proteins residing in the inner membrane (ImcH, CbcL) and at the periplasmic side of the inner membrane (MacA) oxidise menaquinol back to menaquinon to transfer electrons onto c-type cytochromes located within the periplasm (Levar et al. 2014; Santos et al. 2015; Zacharoff et al. 2016). While ImcH was shown to be necessary for the reduction of high redox potential electron acceptors ( $\geq 0.0 V_{\text{SHE}}$ , (Levar et al. 2014)), CbcL is involved in transfer to acceptors with redox potentials  $\leq -0.1 V_{\text{SHE}}$  (Zacharoff et al. 2016). From here, electrons are transferred to periplasmic c-type cytochromes of the PpcA-family, of which at least five different types are present in the periplasm (Santos et al. 2015).

Electrons are transferred from PpcA-family cytochromes through the outer membrane by a porin-cytochrome protein complex that incorporates OmaB or its homologue OmaC (octoheme c-type cytochrome on the periplasmic side), OmbB/C (a porin) and OmcB/C (dodecatheme c-type cytochrome on the outer membrane side). OmcB was shown to be necessary for the reduction of insoluble Fe(III) in *G. sulfurreducens* (Leang et al. 2003; Liu et al. 2014). But the deletion of OmcB did not impair current production in BES (Holmes et al. 2006; Nevin et al. 2009). Possibly, the homologue OmcC was able to compensate OmcB in these studies.



**Figure 1.3:** Schematic representation of the extracellular electron transfer (EET) mechanisms within *G. sulfurreducens* biofilms (Santos et al. 2015; Ordóñez et al. 2016; Reguera 2018).



If a bacterium is closely attached to an anode, electrons can be transferred directly from the outer membrane (Breuer et al. 2015). But since *G. sulfurreducens* forms multi-layer biofilms of up to 200  $\mu\text{m}$  thickness, most cells are not in direct contact with the anode and electron transfer has to be realised through the biofilm (Babauta et al. 2012b). Since electrons can only be transferred from lower to higher redox potentials, there needs to be a redox potential gradient within the biofilm (Babauta et al. 2012b). It has been shown that OmcB is more abundant in cells farther than 10  $\mu\text{m}$  away from the electrode (Stephen et al. 2014). Even when a potential is applied to the electrode which is high enough to allow for complete oxidation of OmcB, a portion of OmcB in these cells will always stay in the reduced state (Stephen et al. 2014). Contrary to this, OmcB located less than 10  $\mu\text{m}$  from the electrode surface is only found oxidised (Stephen et al. 2014). As stated in chapter 1.1, the redox potential is dependent on the ratio between reduced and oxidised forms of a chemical species. Thus, with a greater proportion of reduced OmcB present in upper layers of the biofilm, a redox gradient is maintained ensuring electron flow from upper layers to the electrode (Stephen et al. 2014).

This potential driven electron flow can be realised by the existence of conductive type IV pili composed of PilA in *G. sulfurreducens*. Deletion of the corresponding gene *pilA* led to the inability of *G. sulfurreducens* to reduce insoluble electron acceptors. Thus, it could be concluded that pili are indeed a necessary part in EET (Reguera et al. 2005). The mode of conductivity induced by PilA is still under intensive debate (Lovley 2017; Reguera 2018), but it is quite likely that electron hopping between residues of aromatic amino acids in PilA are responsible for the observed metallic-like conductivity (Reguera 2018). Another c-type cytochrome, OmcS, was found to be located along the pili (Leang et al. 2010). While the spacing of individual OmcS proteins was too high to allow for an explanation of the conductivity of the pili by cytochrome-hopping, deletion of OmcS led to the inability of *G. sulfurreducens* to reduce insoluble electron acceptors (Mehta et al. 2005; Leang et al. 2010). Thus, OmcS is thought to be responsible for the transfer of electrons from the pili to the insoluble electron acceptor rather than for providing the pili conductivity (Leang et al. 2010). A *G. sulfurreducens* mutant that produced more PilA and OmcS formed more conductive and more stable biofilms on anodes compared to the wild type (Leang et al. 2013). These biofilms also produced higher current densities in potential controlled experiments and higher power outputs in MFCs (Leang et al. 2013). Within biofilms, complexes of OmcC, OmcS, OmcE and other unidentified cytochromes have been found that likely serve as relay for electrons and that are interconnected by PilA (Ordóñez et al. 2016).