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Title:

Microbial interactions in electroactive biofilms for environmental engineering applications: a role for non-exoelectrogens

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Abstract:

Microbial electrochemical systems have gained much attention over the last decade due to their potential for various environmental engineering applications ranging from energy production to wastewater treatment to bioproduction. At the heart of these systems lie exoelectrogens – microorganisms capable of exporting electrons generated during metabolism to external electron acceptors such as electrodes. The bacterial biofilm communities on these electrodes are dominated by exoelectrogens, but are nonetheless extremely diverse. So far, within the field, the main focus has been on the electroactive bacteria. However, to broaden our understanding of these communities, it is crucial to clarify how the remaining inhabitants of electrode-respiring biofilms contribute to the overall function of the biofilm. Ultimately, such insights may enable improvement of microbial electrochemical systems by reshaping the community structure with naturally occurring beneficial strains.

Keywords: Microbial electrochemical systems, electroactive bacteria, biofilms, microbial interactions, conjugative plasmids.

Synopsis: Despite their presence in electroactive biofilms, very little attention has been given to non-exoelectrogens so far. Here we argue why it is important to understand the role of these populations in electroactive biofilms.

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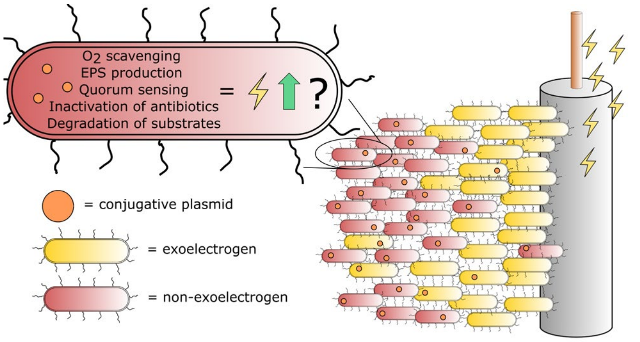

Exoelectrogens in microbial electrochemical systems

Exoelectrogens are a group of phylogenetically diverse microorganisms with the unique ability to transfer electrons to electron acceptors in the extracellular environment. This group spans all three taxonomic domains, however, most identified exoelectrogens are bacteria[1]. Especially the Proteobacteria *Geobacter sulfurreducens* and *Shewanella oneidensis* have been extensively studied due to their strong electroactive abilities. Both species reside naturally in sediments[2,3], which are often rich in minerals and low in oxygen[4]. In the absence of better (i.e. soluble) terminal electron acceptors, *Geobacter* and *Shewanella* have evolved to respire on insoluble minerals. So far, three mechanisms of extracellular electron transfer have been identified: short-range transfer where the microbe is in direct contact with the electron acceptor, long-range transfer via conductive nanowires (*Geobacter spp.*)[5], and mediated electron transfer where electron shuttles transport electrons from the microbe to a terminal acceptor (*Shewanella spp.)*[6].

Extracellular electron transfer is not just a fascinating example of bacterial resourcefulness, it is also of general interest due to its applicability in microbial electrochemical systems (MES)[1]. These systems integrate microbiology, electrochemistry, and materials science for the removal of toxic substances or synthesis of valuable compounds among others. Central to these systems are the electroactive microorganisms that degrade organics or inorganic compounds and, during this process, generate energy by passing electrons to an electrode. Often samples from wastewater treatment plants serve as inoculum since these have a high bacterial diversity[7]. The focus is usually to optimize reactor output, which is typically done by testing parameters such as pH[8], electrode material[9], and composition of organics [10,11]. However, changing these parameters not only affect the exoelectrogens, but the entire biofilm community, which is reflected in the microbial composition[8,10,11]. Since biofilms form the basis of these reactors, we believe it is critical to study the microbial communities themselves. Community analysis is for the most part limited to amplicon sequencing of 16S rRNA genes, however, reducing a community to its inhabitants does not give the full picture. It is important to understand the communal tasks of different populations, the spatial organization, as well as if and how they interact with each other. Generally, complex communities such as biofilms can facilitate the emergence of so called community-intrinsic properties: properties that only transpire in the community setting and not when the bacterial residents are not found in the community[12]. It is likely that non-exoelectrogens facilitate such community-intrinsic properties, which may ultimately stimulate the potential of the exoelectrogens in electroactive biofilms.

In MESs there is a strong selection for electroactive bacteria, and often the *Geobacter* genus is dominant[13–18]. Nevertheless, despite the strong selective pressure for exoelectrogens, the abundance of *Geobacteraceae* typically does not exceed 50% in the inner biofilm and 10% in the outer biofilm in reactors inoculated with wastewater[13,19]. In reactors continuously fed with wastewater, the resident communities in the wastewater must be expected to affect the microbial composition of the electrode biofilm over time and cause fluctuations in relative abundance, especially in the early stage of biofilm formation. Once an actual biofilm has been formed, invasion by planktonic cells is minimal[20]. In this way the biofilm itself may physically protect the electroactive bacteria, residing in the inner layers close to the electrode, from replacement and dispersion. Altogether this underlines the importance of spatial organization, microbial diversity, and the presence of non-exoelectrogens, which presumably have other important roles in the maintenance and function of the electroactive biofilms. Similar findings have been reported in numerous other studies (Table 1). However, so far, research has been focused on interactions between exoelectrogens[21]. Therefore, we argue that a better understanding of the total microbial community structure, the microbial interactions associated with non-exoelectrogens, as well as what properties are community-intrinsic is necessary for further improvement of MESs. Outside the field of electromicrobiology such a community approach has shown promise[22,23].

Table 1. Percentage of electroactive bacteria (EAB) of electrode biofilms from various inocula. Not all studies identify the bacteria to species level. When only identified to genus or in some cases family level, electroactivity was assumed if known EAB have been reported for the given genus/family. WW = wastewater, MFC = microbial fuel cell, MEC = microbial electrolysis cell.

System	**Biofilm sample**	**% EAB**	**Substrate/electron donor**	**Inoculum**	**Sampling electrode**	**Comments**	**Ref.**
MFC	Inner	72	Acetate	WW sludge	Anode		13
MFC	Outer	20	Acetate	WW sludge	Anode		13
MFC	Total	45	Acetate	Not specified	Anode		14
MFC	Total	72	Potato WW	Potato WW	Anode		11
MEC	Total	68	Potato WW	Potato WW	Anode		11
MFC	Total	44 - 86	Acetic acid, lactic acid, formic acid, succinic acid, or ethanol	WW effluent	Cathode[a]	Variation reflects different substrates.	24
MFC	Total	18	Xylose	MFC anolyte	Anode		25
MFC	Total	22 - 34	Three batch cycles with bovine/swine sewage, one batch with acetate	Bovine/swine sewage	Anode	Single-chamber aircathode MFC. Variation reflects sewage type.	26
MFC	Total	16 - 24	Three batch cycles with bovine/swine sewage, one batch with acetate	Bovine/swine sewage	Cathode[a]	Single-chamber aircathode MFC. Variation reflects sewage type.	26
MFC	Total	57 - 69	Winery/domestic WW	Winery/domestic WW	Anode	Variation reflects WW type.	27
MEC	Total	72	Acetate	MFC anolyte	Anode		28

MFC	Total	56 - 70	Acetate	Compost leachate MFC anolyte	Anode	Even though not confirmed in pure cultures, we assume electroactivity of P. acetatigenes, due to heavy domination. Variation reflects different separators.	29
MEC	Total	54 - 70	Acetate or propionate	Anaerobic digester sludge	Anode	Variation reflects substrate type and concentration	16
MEC	Total	77	Acetate	Unspecified WW	Anode		17
MEC	Total	5 - 85	Aqueous phase of bio-oil from pyrolysis of switchgrass or red oak, corn stover fermentation product, acetate/phenol mixture, or acetate	MEC anolyte	Anode	Variation reflects substrate type and different replicates	18

[a] Even though the anode is the main focus here, the microbial composition of biocathodes were also included, as cathode biofilms may also be important for future technologies.

Microbial interactions in biofilms

Generally, environmental bacteria exist in two different stages: as individual planktonic cells or as residents in multispecies biofilm communities. In most natural environments the biofilm lifestyle is dominant[30]. When residing in biofilms, bacteria interact with neighboring cells in a number of different ways, and electroactive biofilms are of course no exception.

The growth rate can, not surprisingly, be a significant determinant in shaping the bacterial composition of biofilms. Faster growing species can have a relative advantage compared to their slower growing counterparts when it comes to establishing and maintaining a position in the biofilm[31], and electroactive biofilms growing on electrodes are no different. However, even though it is an important factor, establishment in a biofilm does not only depend on growth rate. Put simply, the microbial abundance and composition is determined by how well the given species thrives in the given environment. Since MESs are designed to take advantage of the unique properties of exoelectrogens, the environment in these reactors are favorable to exoelectrogens, why they are often also the dominant populations[15]. Still, if exoelectrogens have this advantage when growing in MESs, how is there even room for non-electroactive bacteria in the biofilm? As we will discuss below, there are numerous roles to fill in order to obtain a robust biofilm, all of which are occupied by the populations suited for the task. Therefore, it is important to understand how non-exoelectrogens contribute to the establishment, maintenance, and stability of electrode respiring biofilms in order to get a more nuanced understanding of these bacterial communities. Potentially, such insights can enable natural manipulation of the reactor biofilms and, thus, enhance reactor performance.

In microbial reactors where wastewater is the substrate, the composition and concentration of nutrients and organics vary with both location and time[32,33]. This results in heterogeneity as a given substrate is utilized better by some bacteria than others, which are not necessarily the exoelectrogens. Some of the substrates in wastewater are also rather complex and not readily utilized. In biofilms, bacteria of different species are known to cooperate when degrading complex substrates, which each species by itself otherwise cannot metabolize[34,35]. However, in some cases only one species is involved in the actual degradation, shedding light on the diverse nature of microbial interactions. In a dual-species biofilm consisting of methanogens and a sulfate-reducing bacterium, it was found that even though the sulfate reducer did not directly participate in the degradation, it supplied reducing power, which enabled the methanogens to break down the compound[36]. In another case, current was generated in a microbial fuel cell from the breakdown of cellulose in a co-culture of *G. sulfurreducens* and *Clostridium cellulolyticum*. Neither of the two species could generate current in mono-cultures, but in the co-cultures cellulose was broken down by *C. cellulolyticum* to acetate, which *G. sulfurreducens* used to produce current[37]. It seems likely that other examples of such behavior exist in wastewater-driven MESs that have not yet been identified.

In addition to making substrates available, non-exoelectrogens may establish themselves in the community by consuming oxygen, e.g. coming from membrane crossover in MESs with an aerobic catholyte or air cathode. Anaerobes, such as *Geobacter spp.*, often inhabit the inner layers of the biofilm[13], whilst aerobic bacteria reside in the outer layers, where they consume the oxygen before it diffuses into the inner biofilm[38]. In this manner the anaerobes are shielded from the oxygen stress they might otherwise encounter[39] and *E. coli* has in fact been shown to do exactly this in co-cultures with *G. sulfurreducens* [40,41]. This is an illustrative example of how the success of one population in the biofilm is dependent on other inhabitants, and such interactions ultimately determine the overall productivity and survival of the community. Finally, in the context of protection, the biofilm itself and the non-exoelectrogenic residents can also neutralize toxic compounds commonly found in wastewater such as antibiotics[42] and heavy metals[43].

Not all bacteria colonize abiotic surfaces, such as electrodes in MESs, equally well. For instance, *Pseudomonas aeruginosa* is able to coexist in a biofilm with much faster growing competing bacteria, due to *P. aeruginosa'*s ability to adhere to surfaces that its competitors cannot adhere to as efficiently[44]. Extracellular polymeric substances (EPS), which makes up the matrix of the biofilm, are important for microbe cohesion but also surface adhesion[45]. Especially species of the *Pseudomonas* and *Bacillus* genera produce high amounts of EPS[46], why they can play important roles in the early development of biofilms, facilitating surface attachment and a matrix that cells can attach to. Recently efforts have also been made to promote microbe-electrode adhesion by modifying the electrode surface[47]. With this approach biofilm maturation time has successfully been shortened[48]. In another study, binding of *Shewanella oneidensis* was enhanced due to interactions between the modified electrode and a specific cell surface protein[49], however, it is unclear if the electrode is able to favor the binding of *S. oneidensis* with a mixed inoculum. Whether the matrix is produced abiotically or by bacteria, it remains an essential component of the biofilm. Therefore, good EPS producers, regardless of electroactive or not, might establish themselves in electrode-respiring biofilms by providing a matrix for expansion of the bacterial community.

Interspecies communication via quorum sensing (QS) is, in fact, also important for biofilm development and EPS synthesis[50,51]. In a microbial fuel cell inoculated with *Halanaerobium praevalens* the addition of exogenous EPS-inducing QS signaling molecules increased biofilm formation, which was accompanied by an increased power density[52]. Several studies have reported similar findings – when QS signals are added, a thicker biofilm is observed which leads to a better reactor performance[53,54]. Interestingly, the riboflavins secreted by *S. oneidensis*, which are important for mediated extracellular electron transfer, actually also stimulate biofilm formation[55]. QS signaling is, however, not only important for matrix production. In mixed-species biofilms QS signaling leads to increased abundance of *Geobacter spp.*[53], whilst QS stimulates production of redox mediators in *Pseudomonas aeruginosa*[56]. In fact, when *Pseudomonas aeruginosa* is co-cultured with *Enterobacter aerogenes*, the current generation increases substantially in MESs. Individually both species are relatively weak exoelectrogens, however, metabolites generated by *E. aerogenes* stimulate expression and secretion of redox mediators by *P. aeruginosa*, which enhance the electroactive properties of both species[57]. Altogether, this suggests multiple roles for QS in electroactive biofilms.

Even though the focus here is the role of non-exoelectrogens, we want to mention that exoelectrogens can also interact with each other. For instance, some *Geobacter* species are able to transfer electrons to other microorganisms in a process called direct interspecies electron transfer, which has been implicated in methane production in anaerobic digesters[58]. For a full review on communication between electroactive bacteria, see Paquete et al., 2022.

From the above it is clear that biofilms are dynamic communities with multiple niches to be filled, which all contribute to the overall function and stability of the biofilm. Therefore, it seems likely that bacteria that do not directly contribute to the electric properties of the biofilm can still facilitate this phenotype through other mechanisms indirectly. Whether their role is to produce EPS, make otherwise undegradable nutrients available, consume oxygen before it reaches the inner biofilm, stimulate electroactivity via quorum sensing, protect against harmful compounds, enable horizontal gene transfer, or others, remains to be answered. In the context of biofilm formation, stimulation of electroactivity, protection, and horizontal gene transfer, conjugative plasmids are important to consider since they may potentially facilitate these functions and they are, therefore, discussed in more detail below. Finally, it is important to note that some microbes may be present without affecting the electric properties of the biofilm or, of course, affecting the potential negatively. For instance, some bacteria use toxins to inhibit competitors and force their way into the community[59], and methanogens may even directly divert electrons away from the electrode for methanogenesis[60]. Such competing electrode-independent metabolisms are important to keep in mind, as not all community members are participating in creating conditions that support the exoelectrogens. Either way, understanding how the bacterial composition affects the biofilm properties is needed to advance the field.

Effect of conjugative plasmids

Bacteria divide by fission, typically yielding two isogenic progeny cells (variations occur due to mutations e.g. from DNA replication). Here, the genetic material is inherited vertically. However, bacteria may also obtain genetic material from neighboring cells via horizontal gene transfer which can occur through several different mechanisms. Here we focus on conjugation by plasmids, as these can influence both biofilm dynamics[61] and extracellular electron transfer (unpublished). During conjugation, conjugative plasmids are transferred from a donor to a recipient via a conjugative pilus. The plasmids are self-transmissible since all the genes needed for this process are encoded in the plasmid itself[62].

As cell-cell contact is required for conjugation, the rate of plasmid transfer is often higher in biofilms than in planktonic bacteria. Additionally, conjugative plasmids influence both the biofilm formation and stability by facilitating cell-surface adhesion and cell-cell contact, promoting EPS production, and protecting against antibiotics[63], which is potentially why they are often present in natural biofilms[64]. Interestingly, we recently discovered that conjugative plasmids can actually have an inhibitory effect on extracellular electron transfer in *Geobacter sulfurreducens* as the transcription of several genes including *pilA* is downregulated in plasmid-carrying cells (unpublished). *pilA* in particular caught our attention since it encodes a protein essential for electron export[5]. This suggests that there is both selection and counterselection for the spread of conjugative plasmids in electroactive biofilms. Therefore, it is important to get a better understanding of the role of conjugative plasmids in electrode/mineral respiring biofilms, as it might be a limiting factor for current production in MESs. All of this is discussed in more detail below.

Despite being extrachromosomal replicons that can transfer horizontally, the success of conjugative plasmids is typically linked to the fitness of their host. In other words, it is advantageous for the plasmids to carry traits that promote host fitness, also in biofilms. Cell-cell contact is required for conjugation but, in fact, conjugative plasmids also facilitate adhesion to non-bacterial surfaces[65]. Moreover, in natural isolates of *E. coli*, conjugative plasmids promote biofilm formation[61]. Even though the conjugative pilus seems to play a role in early biofilm formation, it is not necessarily the main facilitator of surface adhesion associated with plasmids[63]. Non-conjugative pili and fimbriae[66] as well as plasmid-stimulated EPS production[67] has also been implicated in biofilm formation and by now a connection between biofilm priming and the presence of different conjugative plasmids has been established[68–70].

Accessory plasmid genes, i.e. genes that provide the host with a novel trait that can enhance host fitness under a given selective pressure, also enhance plasmid persistence. Therefore, genes encoding e.g. resistance towards antibiotics and heavy metals are commonly encoded in conjugative plasmids[71–73]. Since microbial electrochemical systems often utilize wastewater where both antibiotics and heavy metals are present[74–76], such plasmids may be selected for in these systems. In a recent study, we found that several conjugative plasmids can inhibit nanowire-mediated extracellular electron transfer in *Geobacter sulfurreducens* (unpublished). Therefore, it seems that the benefits of conjugative plasmids are situational, and that they may be of importance for the efficiency of MESs. In the presence of stressors, such as antibiotics, plasmids providing resistance towards these are of course advantageous but in MESs they might come at the cost of reduced ability to grow on the electrode. Since growth on extracellular electron acceptors is slowed down, it is crucial to understand how plasmids spread inside electroactive biofilms under different selective pressures, in order to advance the field of wastewater driven microbial electrochemical systems. In electrode-respiring biofilms, the exoelectrogens are most abundant in the inner biofilm, where they are in close proximity to the electrode[13]. It is possible that the spread of conjugative plasmids in electroactive mixed-species biofilms is mainly limited to the non-exoelectrogens residing in the outer layers of the community. In this way, the exoelectrogens get the best of both worlds: they maintain their ability to grow on the electrode, while the outer plasmid-containing populations prevent the antibiotics (or other stressors) from reaching the inner biofilm. This is just one of the many questions we believe are important to address in order to expand our fundamental understanding of how bacterial communities develop and function inside microbial reactors.

Future perspectives

From the discussion above, it should be apparent that electroactive biofilms in MESs cannot simply be reduced to the electroactive bacteria in the community. Even though the exoelectrogens are responsible for the main phenotype needed in these systems, i.e. the ability to generate current, it is important to focus on and elucidate the contribution from the remaining species moving forward. Samples from wastewater treatment plants are extremely rich in terms of bacterial diversity[7] and, thus, it seems fair to assume that the majority of species in the biofilm earn their space by serving a communal role. Hence, there is a need to characterize community-intrinsic properties associated with elevated MES output. Therefore, we argue that mapping the role of non-exoelectrogens in electroactive biofilms is important. In other words, in order to improve a system, we need to understand it first.

The study of microbial interactions in electroactive biofilms is not straightforward. Microbes interact in a vast number of ways, which is why microbial interactions quickly become very complex to investigate and, at the same time, non-bacterial entities such as conjugative plasmids adds to the complexity even further. The initial step could be to identify non-electroactive species commonly associated with electrode-respiring bacteria. Subsequently, to reduce some but not all complexity, we suggest establishing a model system with a few non-exoelectrogens and a single exoelectrogen to mimic the biofilms found in MESs, for the study of the proposed functions of non-exoelectrogens. If in agreement with 16S sequencings from wastewater-inoculated reactors, bacteria related to species where interactive behavior has already been established should be selected. Following this, it would be necessary to validate new findings by comparing with biofilms that are more microbially diverse, which to a larger degree resembles the actual conditions of wastewater-driven MESs. In the long run this approach will provide the field with insights that will allow manipulation of electroactive biofilms for better performance. It is important to note that optimization via addition of natural strains to the biofilms is also viable for real applications installed at e.g. wastewater treatment plants, where the system is in direct contact with the environment. This is exactly why this area of research is important to explore. Genetic manipulation, whilst informative, is not suited for use in reactors that are not separate from the environment. Therefore, a natural manipulation as proposed here is a strong alternative.

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