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Effect of conjugative plasmids, role of interspecies communication, and discovery of new exoelectrogens

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PhD Thesis

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Preface

The work presented in this PhD thesis was carried out between July 2019 and August 2022 at the Technical University of Denmark, in the department for Environmental and Resource Engineering. The research was supervised by Associate Professor Yifeng Zhang, and funded by the Carlsberg foundation (project Genechat, CF18-0084).

The first part of this thesis reviews the research field and introduces the primary findings of the conducted experiments. The papers listed below are found at the end of the thesis, and are referred to throughout the thesis by the number indicated below.

I **Mathias Fessler**, Jonas S Madsen, Yifeng Zhang. Microbial interactions in electroactive biofilms for environmental engineering applications: a role for non-exoelectrogens. *Environmental Science & Technology* **2022** *56* (22), 15273-15279.

II **Mathias Fessler**, Jonas S Madsen, Yifeng Zhang. Conjugative plasmids inhibit extracellular electron transfer in *Geobacter sulfurreducens*. Manuscript submitted.

III **Mathias Fessler**, Qingxian Su, Marlene M. Jensen, Yifeng Zhang. Electroactivity of the magnetotactic bacteria *Magnetospirillum magneticum* and *Magnetospirillum gryphiswaldense*. Manuscript in preparation.

In addition, the following publications and presentations, not included in this thesis, were also done during this PhD study:

1. Song Wang, Xueting Wang, **Mathias Fessler**, Biao Jin, Yanyan Su, Yifeng Zhang. Insights into the impact of polyethylene microplastics on methane recovery from wastewater via bioelectrochemical anaerobic digestion. Water Research, 221 (2022), 118844.
2. **Mathias Fessler**, Jonas S. Madsen, Yifeng Zhang. Conjugative plasmids inhibit extracellular electron transfer in *Geobacter sulfurreducens*. 8th conference of International Society for Microbial Electrochemistry and Technology, 2022. Chania, Greece. *Poster presentation*.

Acknowledgements

First of all, I would like to thank my supervisor Yifeng Zhang, who has given me the opportunity to learn and grow academically through the stimulating (and challenging!) journey a PhD is. In times of distress, which are inevitable when doing research, getting Yifeng's perspective has always been calming. I am very appreciative of Yifeng's focus on and attention to well-being, something I believe is very important in academia, which has a lot of uncertainty, pressure and stress.

I am thankful for all my fellow PhDs and other colleagues, and for the helpful discussions about confusing results, conversations about weekend plans, and everything in between. I am also grateful for the technical assistance from Lene Jensen.

I would like to acknowledge the Carlsberg foundation for the financial support that made this project possible.

Summary

At the moment the world is facing major challenges that are critical to act upon immediately, in order to prevent irreversible climate changes. Researchers across all fields are addressing this with a variety of different solutions, all contributing to a reduction in greenhouse gas emissions. These include making public transport more accessible, increased recycling, development of plant-based food alternatives, power-to-X, and resource recovery from wastewater. Even just within the field of resource recovery, there are a multitude of promising technologies under development, which can generate electricity, remove toxic compounds, and synthesize valuable chemicals. Collectively these technologies are referred to as microbial electrochemical systems (MESs), and common for them are that they use wastewater as energy input and rely on the metabolism of electroactive bacteria (EAB).

In the absence of soluble electron acceptors, EAB have evolved to respire on insoluble extracellular electron acceptors. In nature, iron oxides are often used as terminal electron acceptors. However, electrodes may replace the iron oxides, which is exactly what is taken advantage of in MESs. The electron flow from the EAB to the electrode is intrinsic for the function of the MES and, therefore, the EAB are essential for the performance of the system. Needless to say, stronger EAB result in better reactor output. Despite their importance in MESs, the knowledge on EAB is still rather limited. The overall purpose of the PhD project presented here was to improve the fundamental understanding of EAB, which will eventually lead to the construction of better performing MESs. More specifically this was addressed by (a) reviewing the field and suggesting where it should move towards in the future for better performing MESs, (b) showing that natural conjugative plasmids can inhibit extracellular electron transfer (EET), and (c) identifying new electroactive species to broaden our understanding of the phenomenon.

Firstly, this PhD project features a thorough review and perspective on how the field should move forward from here, in order to improve MESs. The study, construction and application of MESs for sustainable resource recovery and wastewater treatment is still in its infancy, why the thesis presented here suggests to look at similar fields, such as microbial ecology, for inspiration. In many ways, microbial biofilm communities growing on electrodes in reactors resemble biofilms studied in other settings. Therefore, with a basis in the already existing knowledge on microbial interactions, it is proposed to focus on interactions in electroactive biofilms with special attention to the contributions

from non-electroactive species and conjugative plasmids. It is important to establish the role of non-electroactive bacteria in these biofilms in the future, as they are often highly represented in electrode biofilms. Elucidating their contribution may present new and innovative means for optimization of MESs.
Secondly, the impact of conjugative plasmids on EET was investigated. Conjugative plasmids are commonly found in natural biofilms, where they facilitate physical stabilization, amongst other things. In this project, conjugative plasmids were originally designed to be efficient and easy-to-spread vectors of EET genes, to achieve better performing MESs, however, the conjugative plasmids actually had an inhibitory effect on electron transfer. Due to their high abundance in wastewater, addressing this negative effect was important in order to understand if and how these plasmids can limit MESs performance. By testing different electroactive species, numerous terminal electron acceptors, and using various gene knockouts it was shown that several conjugative plasmids specifically interfere with electron transfer mediated by electrically conductive cell surface nanowires. This was due to downregulated transcription of several essential nanowire genes. This is of significance, as some of the strongest electroactive bacteria use this electron export mechanism, and these species are often abundant in microbial reactors.
Finally, two species of magnetotactic bacteria were shown to be electroactive, which is the first report of electroactivity in this group of bacteria. Electroactive microbes with unique traits, such as magnetic organelles, have the potential to enable design of novel reactors, which is one of the reasons why it is important to continue to identify new EAB. Both of the magnetotactic species were able to generate current in a microbial fuel cell, and to reduce different iron oxides to a varying degree. This implicates magnetotactic bacteria in the biogeochemical iron cycle, and also suggests that they have a potential use in MESs.
In conclusion, the project presented here has added two new species to the list of known EAB, shown that conjugative plasmids substantially reduce electron export ability in nanowire-dependent EAB, and, with grounds in a thorough review of the field, proposed to look into the role of non-electroactive species in electroactive biofilms in the future. The findings reported here cannot be used in this instant to improve MESs directly. Instead, they shed light on a previously unknown inhibitor of EET and provide a deeper understanding of EET in general, which forms the basis for MES improvement in the future.

Dansk sammenfatning

I øjeblikket står verden overfor flere store udfordringer, som er vigtige at handle på øjeblikkeligt for at forhindre irreversible klimaforandringer. Forskere på tværs af felter griber dette an med en række løsninger, som alle bidrager til at reducere udledningen af drivhusgasser. Disse inkluderer lettere tilgængeligt offentlig transport, øget genbrug, plantebaserede madalternativer, power-to-X, og udvinding af ressourcer fra spildevand. Blot inden for feltet for ressourceudvinding er der en række lovende teknologier under udvikling, som kan generere elektricitet, fjerne gifte stoffer, og syntetisere brugbare kemikalier. Samlet set refererer man til disse teknologier som mikrobielle elektrokemiske systemer (MESer), og de har alle det til fælles at de bruger spildevand som energikilde og er afhængige af elektroaktive bakterier (EABer).

I manglen på opløselige elektronacceptorer har EABer udviklet sig til at bruge uopløselige ekstracellulære elektronacceptorer i stedet. I naturen bruger de ofte jernoxider som terminale elektronacceptorer. EABerne kan imidlertid også bruge elektroder i stedet for jernoxider, hvilket man udnytter MESer. Elektronflowet mellem EABerne og elektroden danner hele grundlaget for disse systemer, og derfor er de essentielle for systemernes ydeevne. Derfor siger det næsten sig selv at stærkere EABer resulterer i et bedre output fra de mikrobielle reaktorer. Til trods for deres vigtige rolle i MESer er vores viden om EABer stadig forholdsvis begrænset. Det overordnede mål med PhD projektet, der præsenteres her, var at forbedre den fundamentale forståelse of EABer, hvilket på sigt vil føre bedre MESer. Mere konkret blev dette adresseret ved at (a) gennemgå feltet og foreslå en fremtidsretning for feltet for i sidste ende at opnå MESer med forbedret ydeevne, (b) vise at naturligt forkomne konjugative plasmider kan hæmme ekstracellulær elektron transfer (EET), og (c) identificere nye elektroaktive bakteriearter for at udvide vores viden om dette fænomen.

Til at starte med indeholder dette PhD projekt en udførlig litteraturgennemgang samt et fremtidsperspektiv, for hvilken retning feltet skal bevæge sig i, for at forbedre MES outputtet fremadrettet. Forskning i, samt konstruktion og brug af MESer til bæredygtig ressourceudvinding og spildevandsrensning er stadig relativt nyt, hvorfor det i denne PhD-tese foreslås at lede efter inspiration i lignende felter, så som mikrobiel økologi. Mikrobielle biofilm, der vokser på elektroder i reaktorer, minder på mange måder om biofilm i andre miljøer. Derfor forslås det, med udgangspunkt i eksisterende viden om mikrobielle interaktioner, at fokusere på interaktioner i elektroaktive biofilm med særlig

fokus på de ikke-elektroaktive bakteriers bidrag. Fremadrettet er det vigtigt at forstå hvilken rolle de ikke-elektroaktive bakterier spiller i disse biofilm, da de ofte er velrepræsenterede i biofilm, der vokser på elektroder. En kortlægning af deres bidrag vil muligvis give anledning til nye og innovative måder at optimere MESer.

Dernæst blev konjugative plasmiders indflydelse på EET undersøgt. Konjugative plasmider findes i naturlige biofilm, hvor de bl.a. faciliterer fysisk stabilisering. I dette projekt var den oprindelige ide at bruge konjugative plasmider som effektive og let spredelige vektorer af EET gener, for at opnå bedre output fra MESer, men det viste sig at plasmiderne faktisk hæmmede EET. Grundet den høje forekomst af konjugative plasmider i spildevand, var det vigtigt at undersøge og forstå denne negative effekt, idet disse plasmider potentielt kan begrænse MESernes ydeevne. Ved at teste forskellige elektroaktive bakterier, en række terminale elektronacceptorer, og adskillige gen knockouts, blev det vist at flere konjugative plasmider specifikt forstyrrer en type elektron transfer, der afhænger af elektrisk ledende overflade pili. Dette skyldtes nedreguleret transskription af flere essentielle pilus gener. Dette er relevant, da nogle af de stærkeste EABer anvender denne elektron eksport mekanisme, og netop denne type EABer er ofte talrige i mikrobielle reaktorer. Derudover blev det vist at to arter af magnetotaktiske bakterier var elektroaktive, hvilket er første gang at det rapporteres at denne type bakterier er elektroaktive. EABer med unikke træk, så som magnetiske organeller, kan potentielt føre til design af nye typer of reaktorer, hvilket er en af grundene til at det er vigtigt at fortsætte med at identificere nye EABer. Begge de magnetotaktiske bakteriearterne kunne generere strøm i mikrobielle brændselsceller og var i stand til at reducere forskellige jernoxider i forskelligt omfang. Dette implicerer magnetotaktiske bakterier i den biogeokemiske jern cyklus, og antyder at de potentielt kan bruges i MESer.

For at opsummere så har projektet, der præsenteres her, tilføjet to nye arter til listen over kendte EABer, vist at konjugative plasmider reducerer evnen til at eksportere elektroner via pili, og med grundlag i en grundig litteraturgennemgang af feltet foreslået at kigge på ikke-elektroaktive arters rolle i elektroaktive biofilm i fremtiden. Resultaterne der beskrives heri kan på nuværende tidspunkt ikke anvendes til en direkte forbedring af MESer. De har i stedet belyst en indtil ukendt inhibitor af EET og givet en dybere generel forståelse for EET, hvilket danner et grundlag for at kunne forbedre MESer i fremtiden.

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1 Research objectives

Microbial electrochemical systems are a certain type of reactors that combine microbiology, environmental engineering, materials science and electrochemistry. These systems have a lot of potential but to reach the full potential a better understanding of the microbes inhabiting the reactors is necessary. The PhD project presented in this thesis aims at expanding fundamental knowledge of electroactive microorganisms and abiotic as well as microbial factors that can influence electroactivity of these organisms, which may ultimately lead to improved performance of microbial electrochemical systems in the future. The specific objectives are as follows:

- Review the existing knowledge on microbial interactions in biofilms and put this into the context of electroactive electrode-respiring biofilms, in order to propose new research directions within the field of microbial electrochemistry for manipulation and improvement of microbial reactors with non-electroactive bacteria (**Paper I**).
- Examine the effect of natural conjugative plasmids on extracellular electron transfer in electroactive bacteria that use either electrically conductive nanowires (*Geobacter sulfurreducens* and *Geobacter chapellei*) or membrane-bound cytochromes (*Shewanella oneidensis*) for electron export (**Paper II**).
- Establish electroactivity in magnetotactic bacteria with the purpose of novel reactor design in the future (**Paper III**).

2 Introduction

Bacteria are experts in adapting to their surroundings over time, why they are often found inhabiting seemingly uninhabitable niches in nature. This is possible due to relatively short generation times that allow rapid genome evolution, and electroactive bacteria (EAB), a unique group of microorganisms characterized by their ability to export electrons over the cell membrane, are a great example of this adaptability.

For bacteria to thrive, certain conditions are essential. First of all, a source of carbon, nitrogen and phosphorous is vital as these elements serve as cellular building blocks. Secondly, energy to fuel proliferation by assembly of these building blocks is needed. To do so bacteria transport electrons, derived from the breakdown of organics, through a series of membrane-bound cytochromes and in the process energy is generated[1]. It is crucial that the electron flow through this pathway is continuous in order to maintain growth. Therefore, a strategy to dispose electrons is just as important as the organics providing them. Oxygen can permeate the cell membrane and freely diffuse into the cell to the cytochromes of the electron transport chain, why it functions well as a terminal electron acceptor. The majority of bacterial species depend on soluble terminal electron acceptors, such as oxygen, that can cross the cell membrane. EAB, on the other hand, have evolved to use insoluble extracellular electron acceptors in a process called extracellular electron transfer (EET), to circumvent growth limitations in the absence of soluble and more easily accessible alternatives[2].

Electroactive bacteria and microbial electrochemical systems

The first observation of current producing microorganisms was reported over a century ago[3], even though it did not receive a lot of attention at that time. The discovery of *Geobacter spp.* and *Shewanella spp.* several decades ago[4,5] sparked an interest in electroactive microorganisms, which has since evolved into the broad field of microbial electrochemistry that we know today.

By adding EAB to well-designed reactors, it is possible to take advantage of the unique ability of EAB to export electrons to insoluble extracellular electron acceptors. Collectively, these reactors are referred to as microbial electrochemical systems (MESs) and may be used to generate electricity, treat wastewater, sense toxic chemicals, recover resources, desalinate water, or produce valuable compounds such as hydrogen, methane or hydrogen peroxide[2,6–10]. Regardless of the purpose, EAB are absolutely essential for the function of MESs. Most

MESs consist of two chambers: an anode chamber and a cathode chamber. In the anode chamber exoelectrogens donate electrons to the electrode, whilst electrotrophs accept electrons from the electrode in the cathode chamber[11]. For this, the electroactive microorganisms can use various EET pathways, which will be discussed further in the subsequent section.

A key feature of MESs is that they transform a waste product, i.e. wastewater, into a resource. Wastewater generally contains concentrations of organic and inorganic compounds that are adequate to sustain the growth of microorganisms[12]. In MESs, EAB contribute to wastewater treatment by metabolizing these compounds. By eliminating the majority of competing electron acceptors, for instance by creating an anaerobic environment in the anode, it is possible to direct the bulk of this microbial electron flow towards the electrode[13]. Depending on the specific purpose and configuration of the reactor, the generated current is either the final output itself, or it is used to produce the final output, even though an external power supply may also be needed[14]. In the context of MESs, wastewater serves several purposes. In addition to providing nutrients it may also function as the source of EAB. Wastewater is extremely diverse in terms of microorganisms[15], and due to the environment created inside the reactors, there is a selection for electroactive microorganisms[16], as species that can utilize the electrode as the terminal electron acceptor have a relative growth advantage over non-EAB. Therefore, EAB will usually dominate the electrode-respiring biofilm over time. However, despite this advantage, non-EAB are still present in electroactive biofilms where they might contribute to the production of current indirectly[16]. An elaboration of the roles non-EAB may have in electroactive biofilms is found in a following section below. The dominant EAB in MESs usually belong to the *Geobacter* genus[17–22], whilst the presence of *Shewanella* is rarer. These two genera use very different strategies for electron export[23–25], however, whilst this may affect the microbial abundance under certain conditions (**Paper II**), other factors are also important to consider. For instance, the carbon source strongly affects the microbial composition, as the ability to metabolize a certain substrate varies from microbe to microbe. *Shewanella* species grow well with lactate[26], which is rarely used as the substrate in microbial reactors. On the other hand, acetate, a preferred carbon source of *Geobacter spp.*, is routinely used in MESs[16] (Table 1).

Table 1. Percentage of EAB in electrode biofilms. MFC = microbial fuel cell, MEC = microbial electrolysis cell. Reprinted with permission from "Mathias Fessler, Jonas Stenløkke Madsen, and Yifeng Zhang. *Environmental Science & Technology* **2022** *56* (22), 15273-15279. DOI: 10.1021/acs.est.2c04368". Copyright 2022 American Chemical Society.

System	Biofilm sample	% EAB	Substrate/electron donor	Inoculum	Sampling electrode	Comments	Ref.
MFC	Inner	72	Acetate	WW sludge	Anode		17
MFC	Outer	20	Acetate	WW sludge	Anode		17
MFC	Total	45	Acetate	Not specified	Anode		18
MFC	Total	72	Potato WW	Potato WW	Anode		27
MEC	Total	68	Potato WW	Potato WW	Anode		27
MFC	Total	44 - 86	Acetic acid, lactic acid, formic acid, succinic acid, or ethanol	WW effluent	Cathode[a]	Variation reflects different substrates.	28
MFC	Total	18	Xylose	MFC anolyte	Anode		29
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.	30
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.	30
MFC	Total	57 - 69	Winery/domestic WW	Winery/domestic WW	Anode	Variation reflects WW type.	31
MEC	Total	72	Acetate	MFC anolyte	Anode		32
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.	33
MEC	Total	54 - 70	Acetate or propionate	Anaerobic digester sludge	Anode	Variation reflects substrate type and concentration	20
MEC	Total	77	Acetate	Unspecified WW	Anode		21
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	22

Electron export pathway in *Geobacter sulfurreducens*

Geobacter sulfurreducens is among the most proficient electroactive bacteria as determined by power density in pure cultures[2]. In addition *G. sulfurreducens* is relatively easy to cultivate compared to some EAB, and finally genetic systems for gene manipulation and knockout have made it possible to identify important genes and elucidate extracellular electron transfer pathways[34,35]. Even though researchers have worked almost two decades on mapping the EET pathway(s) in *G. sulfurreducens*, there is still some controversy regarding the specifics of electron export. Roughly speaking there are two competing models. One claims that electrically conductive pili (e-pili), composed of PilA protein monomers, permit EET[24], whilst EET in the other model relies on conductive cytochrome chains protruding from the cell surface[23]. There is compelling evidence for both models, and there is even some overlap of genes, which makes the discussion even more complicated. It is of course also a possibility that more than one EET pathway exists. All of this is discussed in more detail below.

Work done by Reguera *et at.* in the mid 2000's identified *pilA*, the main component of a surface pilus, as an essential gene for EET in *G. sulfurreducens*[36]. A *pilA* deficient strain was not able to reduce insoluble extracellular iron oxides, which led to the hypothesis that these pili were in fact conductive and responsible for this defining feature of *G. sulfurreducens*. Since then multiple studies have reported diminished EET abilities in *pilA* deletion strains[37–40] and, in addition, it has been shown that the sidechains of 5 aromatic amino acids in the PilA protein are vital for the conductivity and for reduction of both iron oxides and electrodes[41]. After the substitution of these 5 amino acids to alanines, surface pili similar to those found in the wild-type are still visible in transmission electron micrographs[41], which together with the reduced pili conductivity strongly supports the e-pili model. The PilA polymers are responsible for electron transport along the pilus, but whether or not e-pili need the aid from c-type cytochromes to transfer electrons from the pili to the actual terminal electron acceptor has also been debated. The e-pili are decorated with OmcS[41,42], a c-type cytochrome, however, deletion of *omcS* has little impact on current density[43] and abundant *omcS* expression cannot compensate for poorly conductive pili[44], making the contribution of OmcS to e-pili function questionable.

A more recent model suggests a secretory rather than conductive role for PilA, whilst the actual extracellular electron transfer is mediated by c-type cyto-

chromes. PilA remains in the periplasm, where it is responsible for translocation of OmcS and OmcZ[45]. So far conductive nanowires composed of OmcS[46,47], OmcZ[48,49] and OmcE[50] have been observed in *G. sulfurreducens*. Still, this model is consistent with the fact that *pilA* mutants show a decreased ability to export electrons[37–40], however, here it is due to reduced secretion of cytochrome nanowires. Therefore, *pilA* is central in both models, but this is also the only common ground. Both models are of course based on extensive data[23,24], why it is also possible that *Geobacter sulfurreducens* have several different electron export pathways. In support of this, several findings do not fit into both models including the observations that; (1) conductivity in *G. sulfurreducens* decreases with minor changes in the amino acid sequence of PilA[41], and (2) *Escherichia coli* can produce conductive nanowires only by expressing *pilA*[51]. Principally, these could be a result of (1) lowered cytochrome expression and/or secretion, and (2) secretion of cytochromes in *E. coli*, but this is somewhat of an assumption and at the moment there is no data to support this. Extracellular electron transfer in *G. sulfurreducens* is still intensely researched, why the exact mechanism(s) will probably be elucidated in the near future. Whilst interesting, the precise EET pathway is of less importance in the context of this thesis. Here emphasis is on the vital genes (*pilA*, *omcS*, *omcZ*, and *omcE*), rather than their role.

Extracellular electron transfer in other species

Even though species of the *Geobacter* genus have received most attention, electroactive microorganisms from other genera and even other domains of life have also been identified[2]. Whereas *Geobacter spp*. primarily export electrons to extracellular electron acceptors via long-range nanowires, other mechanisms for EET have also been observed. Short-range EET requires direct contact between the cell surface and the electron acceptor (opposed to nanowires, which can span over 50 cell lengths[52]) and, finally, mediated electron transfer is facilitated by electron shuttles that transport electrons from the cell to the extracellular acceptor, which removes the requirement for direct contact between the cell/nanowire and the electrode/mineral[53]. To keep it brief and relevant for this thesis, the focus will be on *Shewanella oneidensis*, since it is also a model organism used to study EET and, in addition, the most important EAB apart from *G. sulfurreducens* in the work presented here.

Opposed to *Geobacter* species, *S. oneidensis* is capable of both aerobic and anaerobic respiration. *S. oneidensis* encodes three terminal oxidases for aerobic respiration, one of which is a c-type cytochrome[54]. As touched upon above, c-type cytochromes are important for EET in *G. sulfurreducens*, and the same

is true for *S. oneidensis* that use an outer membrane-anchored cytochrome, MtrC, to link intracellular electron flow to extracellular electron acceptors in short-range EET. Electrons are passed from a periplasmic cytochrome, MtrA, via MtrB, which spans the outer membrane, to MtrC. Two other outer membrane c-type cytochromes (MtrF and OmcA) have also been identified[55]. All three cytochromes are involved in mineral/electrode reduction[25]. In addition to direct electron transfer, *S. oneidensis* has the ability to secrete electron shuttles that mediate EET[56], indicating that *S. oneidensis*, like *G. sulfurreducens*, might have several pathways to export electrons. Finally, in terms of relative electroactive strength, the highest reported power densities of *Shewanella spp.* and *G. sulfurreducens* are very similar (4000 mW/m^2)[2], despite relying on very different genes and pathways, suggesting that the different pathways are somewhat equal in electron transfer efficiency (different experimental setups introduce variations, which make direct comparisons problematic). However, under certain conditions, the specific EET pathway becomes very important for the EET efficiency, for instance in the presence of conjugative plasmids (**Paper II**) which will be discussed further in the results section of the thesis.

Conjugative plasmids in the natural environment

Bacterial genetic material is predominantly inherited vertically, that is, by binary cell fission where DNA replication followed by cell division yields two identical progeny cells (genetic variations occur due to DNA mutations created during replication). However, it is also possible for bacteria to acquire genetic material horizontally in a process called horizontal gene transfer (HGT)[57]. Three different modes of HGT have been identified so far: transformation, transduction and conjugation. During transformation microbes take up free DNA from the extracellular environment, whilst transduction is a process where DNA is transferred from one bacterium to another via infecting bacteriophages. Finally, neighboring microbes can directly share DNA in the form of conjugative plasmids[57]. While all three mechanisms of HGT have environmental relevance, the focus here is on conjugation.

Conjugative plasmids are large plasmids that encode all the genes needed for plasmid replication and transfer, why they are self-transmissible[58]. All conjugative plasmids have several common features that are vital for their function. An origin of transfer, relaxases that initiate and finalize conjugation at the origin of transfer, a type 4 coupling protein, and a type 4 secretion system[58]. The plasmid DNA is transferred once a donor cell attaches and brings a recipient cell into closer proximity, which happens via a conjugative pilus expressed on the surface of the donor[59].

In addition to the core genes needed for plasmid transfer, conjugative plasmids often encode accessory genes that are not essential for plasmid function, but provide the bacterial host with novel traits including resistance towards antibiotics and heavy metals[60,61]. From the perspective of the plasmids, it is advantageous to carry such genes, since they may enhance the spread of the plasmid, by allowing the bacterial host to cope with these stressors. Antibiotics and heavy metals are for instance readily detected in wastewaters[62,63], why plasmid carriage may be especially beneficial here. In fact, conjugative plasmids are also abundant in wastewaters[64,65], which are also considered hot spots for HGT[66]. Finally, conjugative plasmids not only protect against external stressors, they have also been shown to promote cell-cell contact, cell-surface contact, and production of extracellular polymeric substance (EPS)[67]; all phenotypes that facilitate formation and maintenance of biofilm communities. In MESs thick and robust biofilms are essential to maximize the performance of the system, and considering that these often utilize wastewater, it is certainly possible that conjugative plasmids play a role in the stability of electrode-respiring biofilms.

Microbial communication in biofilms

Biofilm stabilization may also be facilitated by other factors in addition to conjugative plasmids such as quorum sensing (QS). Even though bacteria are single celled they are still capable of coordinating and executing collective responses that serve the common good of the bacterial community, and QS allows exactly this[68]. QS is a way for bacteria to communicate with neighboring cells and relies on QS signals, also known as autoinducers as they often enhance their own transcription. It is the production, secretion and accumulation of QS signals in the extracellular environment that facilitate this community-wide communication[68]. Binding of the QS signals to cell receptors elicits a coordinated cellular response via expression of appropriate genes. These responses include production of virulence factors[69] or secondary metabolites[70], uptake of extracellular DNA[71], and biofilm development[72,73]. Production of EPS is necessary for biofilm formation and stability, and certain bacterial species, such as *Pseudomonas* and *Bacillus*, are known to be good EPS producers even in the absence of autoinducers[74]. Therefore, QS and e.g., *Pseudomonas spp.* and *Bacillus spp.* may play important roles in the early establishment of biofilms, regardless of if these biofilms are electroactive or not. In fact, there are numerous of functions non-electroactive bacteria may have in electroactive biofilms that can affect the overall electroactivity indirectly (**Paper I**), why it

is important to further explore and identify non-EAB associated with electroactive biofilms[16].

Discovery of new electroactive bacteria

In addition to discovering non-EAB that can facilitate the electroactive phenotype indirectly, it is also important to keep searching for new EAB. Elucidating new EET pathways will broaden the understanding of the phenomenon, and such insights may present opportunities to improve MESs in the long run.

Most of the identified electroactive microorganisms so far are anaerobes or facultative anaerobes, from the Proteobacteria phylum, that reside naturally in oxygen limited and mineral rich environments such as sediments[4,75,76]. Therefore, sediments are a good place to start when looking for electroactive microbes. However, EAB have also been observed inhabiting other niche environments, for instance the human gut[77,78] and mouth[79].

Recently, as presented as part of this thesis, *Magnetospirillum magneticum* AMB-1 and *Magnetospirillum gryphiswaldense* MSR-1 were shown to be electroactive for the first time (**Paper III**). It has already been shown that *M. magneticum* can generate current via electromagnetic induction[80], but production of current in microbial fuel cells has not been reported until now. Both *M. magneticum* and *M. gryphiswaldense* are Proteobacteria and belong to a group of bacteria, collectively referred to as magnetotactic bacteria (MTB).

Magnetotactic bacteria

MTB are characterized by internal magnetic particles, magnetosomes, that allow them to position themselves in their preferred environment by using the Earth's magnetic field for navigation[81]. This unique feature have attracted a lot of research interest after MTB swimming towards magnets under a microscope was observed several decades ago[82]. Since then intracellular vesicles containing magnetite (Fe_3O_4) or greigite (Fe_3S_4) have been found to facilitate this phenotype, also known as magnetotaxis[81]. MTB have been isolated from aquatic sediments[83,84] where they use magnetotaxis to position themselves in the interface between the oxic and anoxic zone, where they thrive best[85]. Most of the MTB identified thus far belong the Proteobacteria phylum, but species in the Nitrospirota phylum along with several other lesser phylogenetically defined species also show magnetotactic behavior[86].

M. magneticum AMB-1 and *M. gryphiswaldense* MSR-1 have become model strains in the study of MTB. Genetic manipulation tools have been developed for them[87,88], why it has been possible to identify essential genes and map a

pathway for magnetosome biogenesis. There are several major steps in magnetosome biogenesis. To begin with vesicles are formed that are either free in the cytoplasm or are attached to the cytoplasmic side of the membrane. Subsequently proteins involved in magnetosome synthesis are transported to the vesicles along with iron. Here the iron is mineralized to form the functional compound of the vesicle, which may be either magnetite of gregite. Finally, the vesicles with mineralized iron aligns to form a chain, and in the case of cell division the vesicles are positioned and distributed evenly in both progeny cells[86]. Roughly 30 genes are needed for this process and they are located together in the genome in a genomic magnetosome island[86,89]. Most bacterial movement is three-dimensional, e.g., movement via chemotaxis towards higher concentrations of substrates[90]. However, due to their magnetosomes, MTB are able to reduce some of these dimensions, since they are already positioned along the magnetic field of the Earth. In combination with oxygen sensing, this allows them to move more efficiently to zones with their preferred oxygen concentration via so-called magneto-aerotaxis[85].

MTB and magnetosomes are a great example of evolutionary adaption in microbes but their use actually extends beyond the microbial world. In the discipline of medicine MTB have been used in magnetic resonance imaging as they target tumors, and magnetosomes show promise in magnetic hyperthermia, a type of cancer treatment. In addition, magnetosomes may be used to deliver drugs, by conjugating the drugs to the magnetosomes surface[91]. Finally, MTB and magnetosomes show potential in bioremediation, cell separation, food safety, and in DNA and antigen detection[92].

3 Methodology

Strain selection

There are many known electroactive bacteria[2], why it is important to consider which strain is appropriate for the specific scientific objective in question. Here, *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR-1 were the main EAB used for several reasons (**Paper II**). First of all, both of these species have been intensely studied already, meaning that there are already protocols available for most procedures including cultivation, gene deletion[35,93], and plasmid conjugation[35,94]. Secondly, the mechanism for EET is rather well known for both species at this point[95], even though there is still some disagreements when it comes to *Geobacter sulfurreducens*[23,24]. Finally, *G. sulfurreducens* and *S. oneidensis* represent two different solutions to utilize insoluble extracellular electron acceptors, which makes it possible to asses if a given entity, conjugative plasmids in this case (**Paper II**), affects EET in general or if it is specific for at certain pathway. For routine cloning various *E. coli* strains were used (**Paper II**).

When it comes to the discovery of new electroactive microorganisms, an obvious approach is to enrich an electrode with EAB from a source that has high microbial diversity, such as sediment or sludge from at wastewater treatment plant. Over a relatively short period of time EAB will dominate the biofilm community on the electrode, and then they may be identified by sequencing[17]. However, this does not always yield identification to the species level, and the microbes can often not be cultivated in pure cultures. Therefore, this approach is not suitable when the objective is to characterize the electroactive properties in more detail. To do so it is necessary to have established a protocol for laboratory cultivation. Therefore, the combination of available protocols, data that hints at electroactivity[80], and the ability to biomineralize soluble iron, led to selection of *Magnetospirillum magneticum* and *Magnetospirillum gryphiswaldense* (**Paper III**).

Iron oxide reduction

Iron minerals are found in many different forms, each with their own reduction potential[96]. In the context of microbial electrochemistry, the reduction potential is important to consider, as it determines how easy or difficult it is for microbes to reduce the given mineral. It is common practice to synthesize readily reducible iron oxides by neutralizing a solution of $FeCl_3$ for use in experiments with electroactive bacteria[97]. It is more rare to use e.g., Fe_2O_3 with a

lower reduction potential[96], even though some studies also use this form[98]. Here, Fe_2O_3 was primarily used. This was to better mimic conditions in natural environments, where this is among the most abundant iron minerals[99–101], despite having to compromise in regards to reducibility.

Reactor configuration

The purposes of microbial electrochemical systems are very diverse and so are the reactor setups. Factors such as electrode material and size, reactor size, anolyte and catholyte, and continuous supply of medium versus batch-mode are necessary to consider in order to achieve the best performance of the given reactor.

Carbon and graphite electrodes are frequently used due to their chemical stability and low cost. In addition, carbon-based electrodes usually have a large specific surface area. However, compared to metal electrodes the conductivity of carbon electrodes is poorer and the internal resistance is higher[102]. Therefore, it is common to coat carbon electrodes with e.g. platinum, which can increase the power density significantly[103]. The power density not only depends on the physical properties of the electrode, biocompatibility is also very important. Ultimately, electroactive microorganisms are responsible for the final power output, why attachment, growth and biofilm development of these species are vital. In other words, high conductivity and low internal resistance cannot compensate for poor biocompatibility of an electrode. The reactor size itself is also important to consider, as it can severely affect the power density. Rectors with smaller volume generally perform better[104], and this is a significant issue faced when transitioning from laboratory-scale reactors to pilot-scale reactors in addition to challenges associated with high cost of fabrication and operation[105]. Here a carbon brush electrode with a large surface area (1204 cm^2)[106] was used in the anode in order to give the MTB the best possible conditions for current production (**Paper III**).

The direction of electron flow determines if an electroactive species is exoelectrogenic or electrotrophic (some are both). Exoelectrogens donate electrons to an electrode in the anode chamber, whilst electrotrophs accept electrons from the electrode in the cathode. As the MTB investigated here were hypothesized to exhibit exoelectrogenic properties, they were grown in the anode with an anolyte based on MTB medium (**Paper III**). The catholyte, on the other hand, was not predetermined. Ferricyanide ($Fe(CN)_6^{3-}$) was used in the cathode chamber in the experiments presented in this thesis, since no catalyst is needed to drive the cathode reaction and oxygen diffusion over the membrane into the anode is minimized compared air cathodes[107], which are also

frequently used in MESs. MTB are cultivated under microaerobic conditions[108], why diffusion of oxygen is less critical than when using strict anaerobes. However, it is still preferable to limit oxygen in the anode to avoid that the MTB use oxygen rather than the electrode as the electron acceptor in order to ensure the highest possible current production. Even though air cathodes have lower internal resistance, MFCs utilizing ferricyanide achieve higher maximum power densities[107].
Finally, batch-mode was chosen over a continuous supply of medium, and the experiments were terminated after the first batch. This very basic setup was used, as this was sufficient for the simple purpose of demonstrating extracellular electron transfer in MTB.

4 Results, discussion and perspectives

4.1 Microbial interactions in electroactive biofilms

Idea, purpose and hypothesis

With the observation that non-EAB are often present in electroactive biofilms, we wanted to map possible roles for non-bacterial entities and non-EAB in electroactive biofilms. Conjugative plasmids can interfere with EET (**paper II**) and, therefore, other similar interactions might exist that either suppress or stimulate electroactivity in electrode-respiring microbial communities.

We hypothesized that non-electroactive microbes can stimulate electroactivity indirectly in MESs, through multiple mechanisms. With this hypothesis in mind, the purpose was to thoroughly review research on microbial interactions, conjugative plasmids, electroactive biofilms, and interactions among EAB, in order to map potential roles for non-EAB and plasmids. Subsequently, several research directions were suggested, with the intention of intriguing and inspiring fellow researchers to pursue this area, as we firmly believe insight into the non-electroactive residents in electrode biofilms is needed to advance the field.

Results and discussion

In electroactive biofilms, EAB are usually dominant but non-EAB are also present in varying degree[16]. Table 1 shows the relative abundance of electroactive species in several microbial reactors with different configurations, substrates and inoculum. Table 1 clearly shows that electroactive biofilms are not only composed of EAB. Therefore, the question arises; what is the role of the remaining species that do not contribute directly to the performance of the system by donating/accepting electrons to/from the electrode? The answer to this seemingly simple question is not straightforward. Microbial biofilm communities are extremely complex, also when growing on electrodes in MESs, which make them difficult to study. For this reason, most community analyses do not go beyond 16S sequencing. However, this sequencing information in itself is not particularly useful, because often it simply confirms what is already known: EAB are dominant. Therefore, it is argued here that shifting the focus from the EAB to the non-EAB is one of the steps needed to advance the field. The role of the EAB is well understood, and since *Geobacter spp.* is commonly among the EAB, we have a good idea of how part of the EAB perform their role, mechanistically speaking.

At this point, the interactions between EAB and non-EAB is not completely unexplored, but it is still in its infancy. Studies so far have shown that *Clostridium cellulolyticum* facilities current production in MFCs by providing a substrate for *G. sulfurreducens*[109], *Escherichia coli* shields *G. sulfurreducens* from oxygen stress[110], and quorum sensing signaling leads to enhanced current output by stimulating secretion of redox mediators[111] and increasing abundance of *Geobacter* species[112]. Most of these findings have been reported in defined duel-species cultures, but remain to be investigated in higher complexity communities. Also, identifying more species that can carry out the same or similar tasks as described above in electroactive biofilms will indicate if these are unique observations, or if they are frequently associated with such communities.

Bacteria that are efficient EPS producers may also serve important roles in electroactive biofilms, by allowing biofilm development and providing physical stability via the EPS matrix. In addition, non-EAB may also protect the other biofilm residents from toxic compounds, such as antibiotics. In fact, conjugative plasmids can also facilitate both of these functions, as they often carry antibiotic resistance genes and stimulate EPS production and cell-cell adhesion[16]. Therefore, the presence of conjugative plasmids in electroactive biofilms is controversial, in the sense that they contribute positively with physical stability and protection but at the same time, they inhibit extracellular electron transfer in *Geobacter* species (**Paper II**).

Future perspectives

In order to achieve insight into the roles of non-EAB the initial task is identify candidates, that can facilitate the functions discussed above. This is not easily accomplished, but here two strategies are proposed. The first approach involves co-cultivation of electroactive bacteria, e.g., *Shewanella* or *Geobacter* species, and non-EAB that are known to display phenotypes that might enhance the overall power output to the electrode in MESs. These could for instance involve *Pseudomonas spp.* or *Bacillus spp.*, which are good EPS producers[74], species that display antibiotic resistance (many *Pseudomonas* species have intrinsic resistance towards one or more antibiotics)[113,114], bacteria known to secrete QS signals in biofilms[68], or strains carrying conjugative plasmids. While labor-intensive, such a screening approach provides some indication of what to expect, which makes experimental design easier.

The second approach relies on isolation of bacteria from MESs inoculated with a mixture of microbes, e.g., from a wastewater sample. Combining this with 16S sequencing permits selection of the more abundant populations that are

well represented across replicates. Selecting for bacteria that are often associated with the electrode biofilms should increase the likelihood of finding species that are advantageous for the community. Subsequently, the specific role may be mapped. The advantage of this approach is that it is not restricted to strains that are already known. On the other hand, it involves more unknowns, which makes it harder to design experiments and, additionally, the preferred species may not be possible to cultivate in pure laboratory cultures. After candidate identification and functional verification in a simple system, community complexity should be increased to better mimic conditions encountered in actual MESs. Ultimately, knowledge gained from this will allow a natural (i.e., GMO-free) manipulation of electrode-respiring communities for better reactor performance, through addition of advantageous and useful strains.

4.2 Inhibitory effect of conjugative plasmids on extracellular electron transfer

Initial purpose, preliminary results and hypothesis adjustment

As already touched upon in the introduction, the exact mechanism for extracellular electron transfer in *Geobacter sulfurreducens* is still heavily debated[23,24]. Initially, the two main purposes of this part of the PhD project was related to this debate. The purposes were to (a) find further evidence for if the PilA protein forms conductive nanowires or not, and (b) investigate if this trait could be spread to other bacterial species by heterologous expression of the *pilA* gene alone.

The list of studies that show that *pilA* is essential for EET in *G. sulfurreducens* is extensive[23,24,36,45], but the question remains whether PilA is needed for secretion of cytochrome nanowires, or if PilA is the nanowire component itself. Changing aromatic amino acids to non-aromatic amino acids in *pilA* decreases conductivity in *G. sulfurreducens*[41], truncating a surface type IV pili in *Pseudomonas aeruginosa* PAO-1 to mimic the *G. sulfurreducens* PilA protein increases the current density of *P. aeruginosa* MFCs[115], and conductive nanowires can be recovered from *E. coli* that express *G. sulfurreducens' pilA* gene[51]. Collectively this suggests that PilA can form electrically conductive pili (e-pili). On the other hand, there are also claims that PilA only plays a secretory role[45]. Therefore, to add more nuance to this debate, the aim was to examine if non-EAB could be transformed into EAB, simply by heterologous expression of *pilA*. As the intention was to spread the *pilA* gene to a large number of hosts, it was important to establish an efficient protocol for this. For this purpose traditional cloning and plasmid transformation was not viable, as most cloning plasmids for heterologous protein expression have a limited host range, bacterial competence protocols vary preventing streamlining, and availability of protocols restricts the number of possible hosts. To circumvent these issues, pKJK5, a natural conjugative plasmid[116], was used as the gene vector. This approach has not been used before, or it is at least not reported in the literature. However, it is suitable for this purpose, since it allows fast spread to a large number of hosts that are not necessarily known beforehand. Plasmid recipients may simply be isolated and identified subsequently. Conjugative plasmids are self-transmissible and only require donor and recipient to be within proximity of each other for transfer[58]. In addition, many conjugative plasmids have a

broad host range, which is also the case for pKJK5[117]. In theory, inserting the *G. sulfurreducens pilA* gene on pKJK5 would allow seamless spread of the gene and potentially the EET phenotype. Even if the host itself could not use the expressed PilA nanowires, they still might allow and enhance electron transfer through the extracellular environment of electrode-respiring biofilms. To test this hypothesis *pilA* was inserted into pKJK5 in a non-disruptive manner in a non-coding region, and the resulting plasmid was named pKJK5-PilA. Correct insertion was verified with sanger sequencing.

Before conjugating pKJK5-PilA into weak and non-electroactive bacteria, the construct was tested in the wild type *G. sulfurreducens* strain and in a *G. sulfurreducens* Δ*pilA* strain. In the wild type strain the plasmid would be expected to increase EET ability, as it is already known that overexpressing *pilA* in *G. sulfurreducens* produces higher current in MFCs, compared to the wild type[118]. In the Δ*pilA* strain, pKJK5-PilA should be able to recover the lost EET phenotype[36].

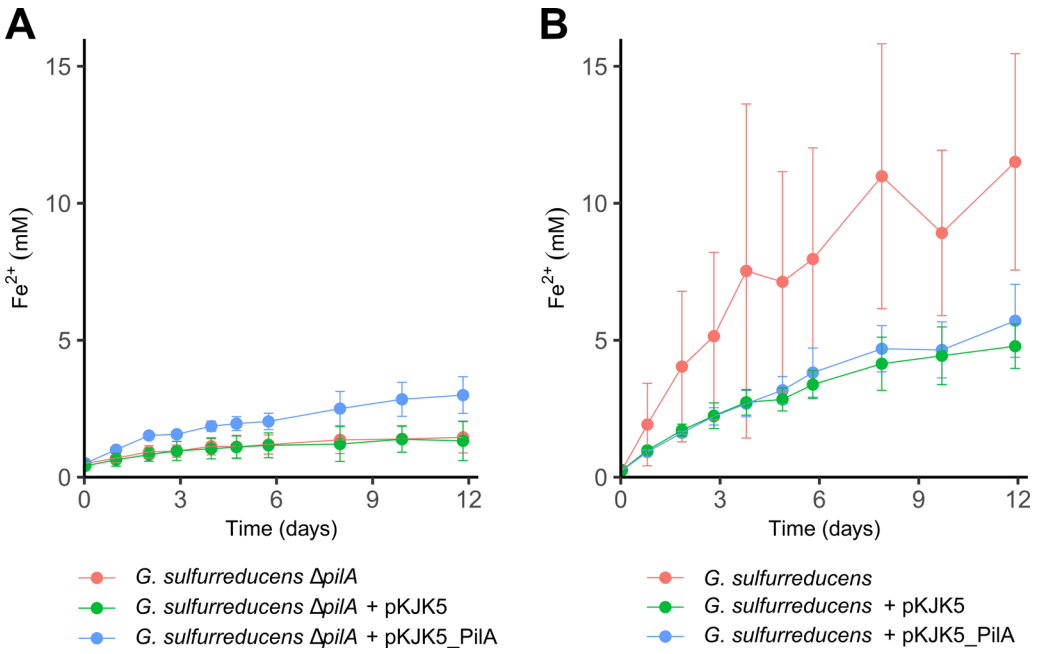

Figure 1. Reduction of Fe_2O_3 measured with ferrozine by *G. sulfurreducens* Δ*pilA* (**A**) and *G. sulfurreducens* PCA with pKJK5 with/without insertion of *G. sulfurreducens*' *pilA* gene. Error bars show standard deviation (n = 3).

As seen in Figure 1A, pKJK5-PilA did indeed increase the ability to use insoluble iron oxides as electron acceptors in the Δ*pilA* strain, confirming that PilA is expressed from the plasmid, even though *G. sulfurreducens* Δ*pilA* + pKJK5-PilA was still significantly poorer at reducing Fe_2O_3 than the plasmid-free wild type strain (Figure 1B). This could indicate that pkJK5-PilA was not able to

fully complement the genomic deletion of *pilA*. However, considering that pKJK5-PilA contains the same upstream regulatory sequence as the genomic *pilA* gene and that the *G. sulfurreducens* wild-type with either pKJK5-pilA or pKJK5 showed reduced ability to grow on Fe_2O_3 (Figure 1B), it indicates that pKJK5 inhibits EET. This was surprising but, nonetheless, a both interesting and significant observation. Conjugative plasmids are abundant in the environment[65,116,119], where they may influence EET as suggested by the preliminary results. Therefore, the initial hypothesis was revisited, and the aim instead became to examine this natural inhibitor of EET and elucidate the mechanism behind the phenomenon. *G. sulfurreducens* is among the strongest known EAB[2] and *Geobacter spp.* are often abundant in MESs[17–22], why it was important to investigate and report this finding. Moving forward the version of pKJK5 without the *pilA* insertion was used. The preliminary findings were confirmed in a follow-up experiment with 6 replicates and a control without addition of *G. sulfurreducens*. Sampling was also extended from 12 to 17 days (Figure 2).

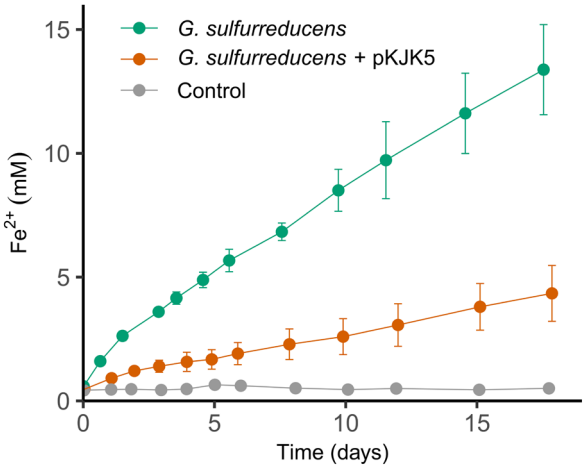

Figure 2 (from paper II). Reduction of Fe_2O_3 measured with ferrozine by *G. sulfurreducens* PCA with/without pKJK5. The control is uninoculated medium. Error bars show standard deviation (n = 6).

Results and discussion

First of all, it was necessary to asses if the pKJK5 phenotype was restricted to growth on extracellular electron acceptors, or if growth of *G. sulfurreducens* with pKJK5 was affected regardless of type of electron acceptor. When grown with either fumarate, Fe(III)-citrate or the electron mediator AQDS, pKJK5 did not have an effect (Figure 3). For reduction of these compounds *G. sulfurreducens* relies on a fumarate reductase[120], OmcB/OmcC[121], and

OmcB/OmcS/OmcT/OmcE/OmcZ[122], respectively. In opposition, PilA[24] and/or OmcS and OmcZ[45] mediate reduction of Fe_2O_3. Evidently, *pilA* is the main genetic differentiator between growth on Fe_2O_3 and the other three electron acceptors, why pKJK5 was hypothesized to interfere with transcription of *pilA* or assembly of the e-pili, possibly as a result of the conjugative pilus and the e-pilus belonging to the same family of pili[123].

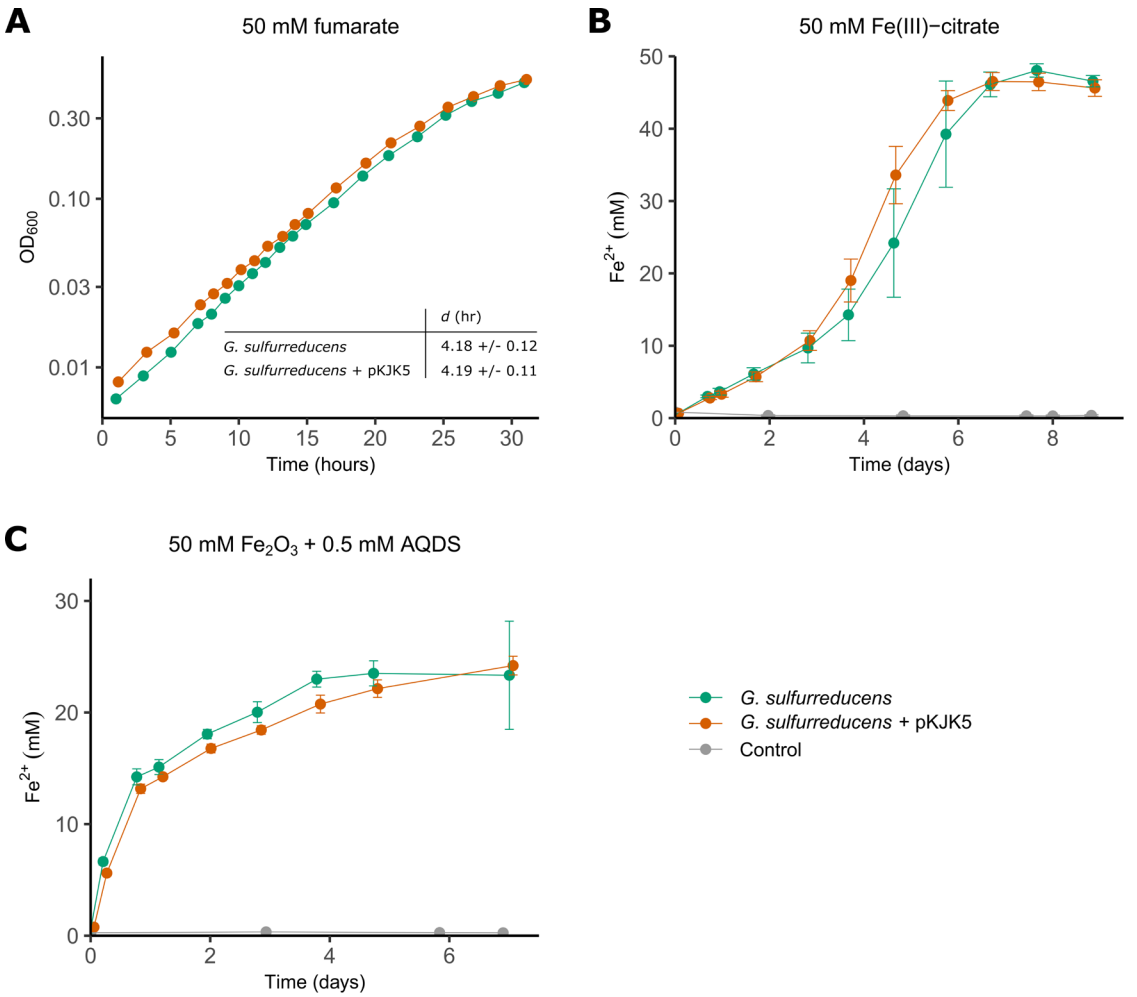

Figure 3 (from paper II). Growth and iron reduction of *G. sulfurreducens*. Growth with fumarate (**A**, n = 5) quantified by measuring optical density. One representative growth curve from five replicates is shown along with the doubling times. Reduction of Fe(III)-citrate (**B**, n = 6) and Fe_2O_3 with added AQDS (**C**, n = 6). Error bars show standard deviation.

Purification and subsequent sequencing of mRNA from *G. sulfurreducens* with and without pKJK5 revealed that transcription of several genes involved in EET, including *pilA*, was downregulated in the cells with pKJK5 (Figure 4). Interestingly, *pilA-C* was also downregulated. Together with *pilA-N* (also simply referred to as "*pilA*" throughout this thesis) *pilA-C* forms the secretory channel needed for translocation of cytochrome-based nanowires[45]. Therefore,

these results do not provide further evidence for either of the two possible EET mechanisms in *G. sulfurreducens*, as the essential genes for both mechanisms are affected by pKJK5. Nonetheless, the RNA sequencing data explains the observed phenotype in either case, as PilA is a central component of both types of nanowire.

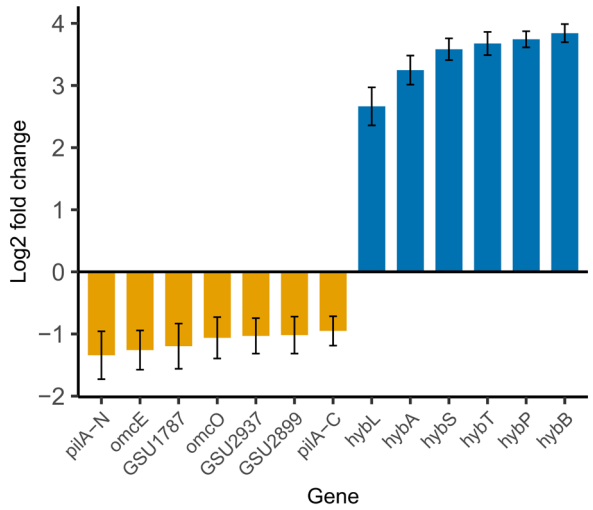

Figure 4 (from paper II). Effect of pKJK5 on the transcriptome profile in *G. sulfurreducens*. Fold change of the most highly up- and downregulated genes in the presence of pKJK5. GSU1787, GSU2937 and GSU2899 are c-type cytochromes. Error bars show standard deviation (n = 3).

Having established that pKJK5 reduces transcription of *pilA* in *G. sulfurreducens*, which in turn limits electron transfer to Fe_2O_3, the scope was broadened to include other conjugative plasmids and EAB to assess if the phenotype extended beyond a single species and a single plasmid. *Geobacter chapellei* was used as another representative of the *Geobacter* genus (and the nanowire EET pathway), whilst the use of *Shewanella oneidensis* MR-1 would indicate if the plasmid-mediated inhibition is general for EAB, regardless of species and EET mechanism. The additional plasmids tested were RP4, pB10 and RSF1010[119,124,125]. RP4 and pB10 are conjugative plasmids and RSF1010 is mobilizable. Conjugative and mobilizable plasmids are similar, but mobilizable plasmids lack some of the genes needed for transmission, why they are not self-transmissible. However, they are able to transfer along with a conjugative plasmid, if they reside in the same host cell[58].

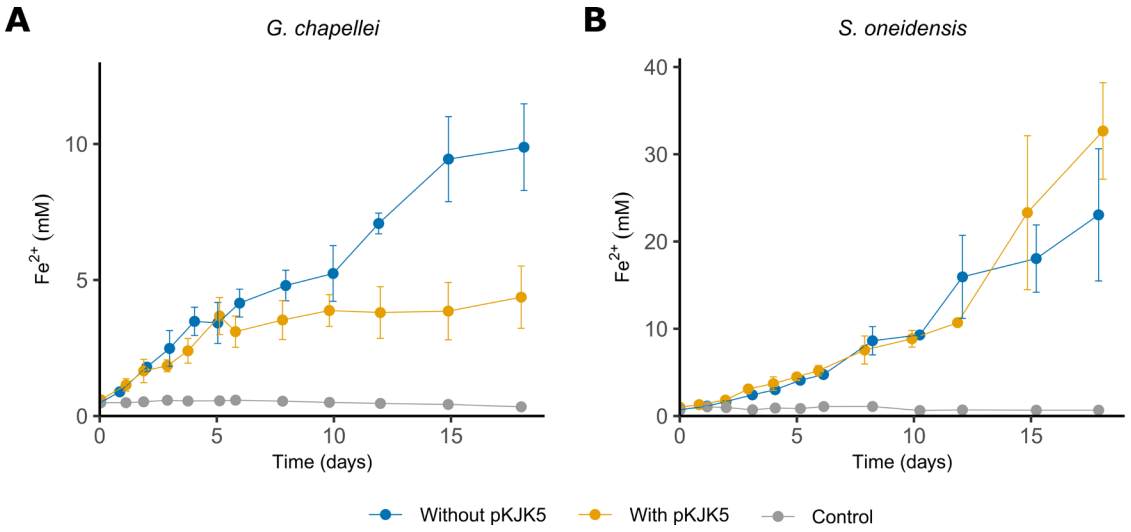

Figure 5 (from paper II). Reduction of Fe_2O_3 by *Geobacter chapellei* (**A**) and *Shewanella oneidensis* (**B**) with/without pKJK5. Controls are uninoculated cultures. Error bars show standard deviation (n = 3).

As seen in Figure 5, pKJK5 only inhibits EET in *Geobacter* species, since iron reduction is unaffected in *S. oneidensis*. Additionally, RP4 and pB10 also inhibited EET in *G. sulfurreducens*, suggesting that it is a common feature of conjugative plasmids. The presence of RSF1010, on the other hand, had no effect (Figure 6).

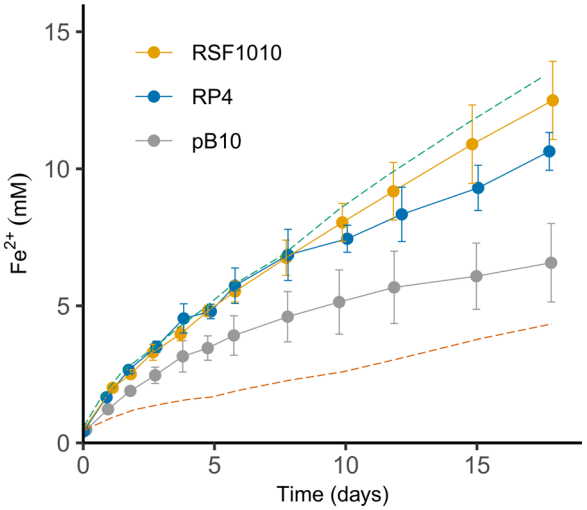

Figure 6 (from paper II). Fe_2O_3 reduction by *G. sulfurreducens* containing RSF1010, RP4 or pB10. The stippled green line shows Fe_2O_3 reduction by *G. sulfurreducens* without any plasmids (Figure 2), and the stippled orange line shows Fe_2O_3 reduction by *G. sulfurreducens* with pKJK5 (Figure 2), to ease comparison. Error bars show standard deviation (n = 6).

From the results, it is clear that conjugative plasmids inhibit extracellular electron transfer in *Geobacter sulfurreducens*. It also seems that this inhibition is limited to EAB that use nanowires for electron export, since *G. chapellei* but not *S. oneidensis* was affected by pKJK5. However, even though both the iron reduction assays and the RNA sequencing strongly indicates this, it is necessary to extend the analysis to include more EAB in the future to strengthen this conclusion.

How pKJK5 (and RP4 and pB10) downregulates transcription of *pilA*, *pilA-C* and the c-type cytochromes (Figure 4) is still unclear. We speculate that elements regulating the expression of the type 4 secretion system (T4SS) on the conjugative plasmids, which includes the conjugative pilus, might bind and downregulate *pilA* and *pilA-C*. This is supported by the fact that RSF1010, which lacks the T4SS, had no impact on Fe_2O_3 reduction (Figure 6). Still, the question remains; what is the mechanism behind the transcriptional downregulation and why are the pili genes in particular affected? The current hypothesis is based on the fact that both PilA in *G. sulfurreducens* and the conjugative pilus on pKJK5 belong to the type 4 group of pili[123]. Currently, there is disagreement on whether *pilA* is in fact a type 2 or 4 pilus. High resolution cryo-EM protein structures suggests it is a type 2 pilus[45], however, PilA is assembled into nanowires in *E. coli* with the type 4 pilus machinery[51]. Regardless, we suspect cross-regulation between the pKJK5 T4SS and *pilA* is responsible for the observed phenotype.

Usually conjugative plasmids are perceived as favorable to the host. The moderately increased metabolic burden imposed by expression of plasmid-borne genes and plasmid replication is compensated for by the favorable attributes of the plasmid. These include genes encoding antibiotic or heavy metal resistance[116,126,127], but also stimulation of EPS production for biofilm formation[67]. The relative advantage provided by the plasmid is of course situational, but even in the absence of selective pressure conjugative plasmids persist in their bacterial hosts, indicating that the disadvantages of plasmid-carriage are minimal[128,129]. The results presented here, however, show that this is not always the case, as *G. sulfurreducens*' growth is severely limited in the absence of soluble electron acceptors; a situation commonly encountered by *G. sulfurreducens* in nature. In fact, these environments specifically have driven the acquisition of conductive nanowires through evolution in *Geobacter* species[130]. The data reported in this thesis suggest that horizontal transfer of conjugative plasmids in mineral rich anaerobic environments is limited by a species boundary to some degree, since they prevent optimal proliferation of nanowire-dependent EAB. This is the first report of such a negative impact on

plasmid-carrying cells and it adds a new nuance to horizontal gene transfer. In addition, these findings may have implications for MESs. Conjugative plasmids are often observed in wastewater[65] and considering that wastewater is commonly used in microbial reactors[16], these plasmids may actually influence the microbial composition in MESs, especially if the wastewater contains residual amounts of antibiotics. Under these circumstances the presence of conjugative plasmids might lead to higher relative abundance of nanowire-independent EAB, by preventing proliferation of those EAB that are dependent on nanowires.

Future perspectives

The observation that conjugative plasmids inhibit EET in *Geobacter* species is completely new and, therefore, further investigation is needed to pinpoint the specific interaction between *Geobacter spp.* and the plasmid that leads to lowered *pilA* transcription. An examination of the impact of conjugative plasmids in electroactive biofilms is also required in order to determine if they can affect microbial composition and/or performance of MESs.

To prove/disprove the hypothesis that pKJK5 interferes with *pilA* expression through direct interaction with regulatory DNA elements, a modified *G. sulfurreducens* strain can be used. By exchanging the native *pilA* promotor region with another promotor this is possible. In regards to the specific gene(s) on pKJK5 that is/are responsible for the phenotype, transposon mutagenesis is a useful tool[131]. Constructing a pKJK5 library with different transposon insertions in pKJK5 allows identification of pKJK5 derivatives that have limited or no impact on mineral reduction. Data obtained from the modified *G. sulfurreducens* strain(s) and pKJK5 version(s) will potentially enable predictions of other EAB that are affected and the plasmids that facilitate the phenotype, based on sequence homology. This is a far more efficient approach to assess the extent of plasmid-mediated EET inhibition, than performing iron reduction assays on individual candidates. In addition, the prediction-based approach is not restricted to species that can be cultivated in the laboratory.

In the context of applied environmental engineering, it is important to clarify the impact of conjugative plasmids in MESs. Therefore, spread of pKJK5 in mixed-species biofilms growing on electrodes should be monitored, e.g., with fluorescence and flow cytometry[131]. In addition, an examination of how different concentrations of stressors, such as antibiotics, facilitate plasmid spread/loss and the potential influence on relative microbial abundance and reactor performance is needed in order to determine if conjugative plasmids are a factor to consider in these systems. In these complex biofilms, it is not

necessary for all populations to obtain plasmids for stress protection. EAB in the inner biofilm layers close to the electrode might take advantage of collective protection from plasmid-harboring populations in the outer layers[16]. Nonetheless, further experiments are needed for clarification.

4.3 Electroactivity of magnetotactic bacteria

Purpose and hypothesis

It is necessary to keep searching for new EAB in order for the field to progress. It is unlikely to identify bacteria that have stronger electroactive abilities than *Geobacter spp.* and *Shewanella spp.*, as these show up repeatedly in MESs inoculated with environmental samples[17,18,20,132], indicating that they outcompete other EAB under these selective conditions. However, there are still many reasons to continue looking for new EAB. Firstly, discovery of new electroactive species might allow design of new MESs that take advantage of species-specific properties. In addition, elucidating novel EET pathways and identifying new genes used for EET, will broaden our understanding of the phenomenon. Ultimately, this knowledge can be used in applied research when designing reactors.

Magnetotactic bacteria are a group of bacteria that biomineralize iron to form intracellular magnetic particles (magnetosomes), which are encapsulated in vesicles. These give MTB their ability to navigate in relation to the Earth's magnetic field in a process called magnetotaxis[86]. In addition, MTB and magnetosomes have been shown to generate electricity under electromagnetic induction[80]. Therefore, we hypothesized that MTB are electroactive, in addition to being magnetic. Such bacteria that possess both magnetic and electroactive properties may allow construction of novel reactors in the future.

Results and discussion

Two strains were used to represent MTB; *Magnetospirillum magneticum* AMB-1 and *Magnetospirillum gryphiswaldense* MSR-1. These two strains were chosen from a subset of MTB due to ease of cultivation and the vast amount of existing literature on these two bacteria. To test for electroactivity, *M. magneticum* and *M. gryphiswaldense* were inoculated into two-chambered MFCs. Here they were added into the anode chamber in medium without oxygen and $NaNO_3$, which is usually in *Magnetospirrilum* medium. The reason being that oxygen and $NaNO_3$ function as electron acceptors and omission of these two ensured use of the electrode as electron acceptor instead. As seen in Figure 7, both strains generated current in MFCs. However, for *M. gryphiswaldense* the acclimatization to the new environment was substantially longer and the current generated lower, compared to *M. magneticum*. Nevertheless, both species displayed electroactive properties.

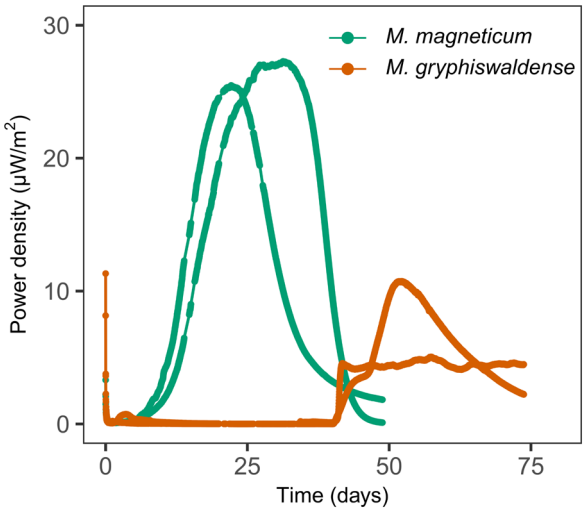

Figure 7 (from paper III). Power density generated in microbial fuel cells by *M. magneticum* and *M. gryphiswaldense*. Duplicates are shown for each strain.

Cyclic voltammetry is useful in the study of EAB, since it allows examination of oxidation and reduction processes[133]. To further characterize *M. magneticum* and *M. gryphiswaldense* in terms electroactivity, cyclic voltammetry was applied. However, after numerous attempts and several different experimental configurations no meaningful results were obtained. It remains unclear why this was the case, and after extensive troubleshooting without improvement, this direction was not pursued further.

Biomineralization is an integral part of the life style of MTB, since this process is required for magnetosome synthesis[86]. Considering that MTB already have existing iron transport and oxidation pathways used for production of magnetite (or greigite)[86], which is essentially the reaction of iron oxide reduction in reverse, and that they can transfer electrons to electrodes in MFCs, the analysis of EET was extended to include an assessment of reduction of insoluble iron oxides. Two iron minerals were tested, Fe_2O_3 and FeOOH. They differ from each other in terms of reducibility, where FeOOH is the more readily reducible of the two iron species[134]. Neither of the two MTB strains were able to reduce Fe_2O_3. On the other hand, FeOOH was reduced by *M. magneticum*, and to a very limited degree by *M. gryphiswaldense* (Figure 8).

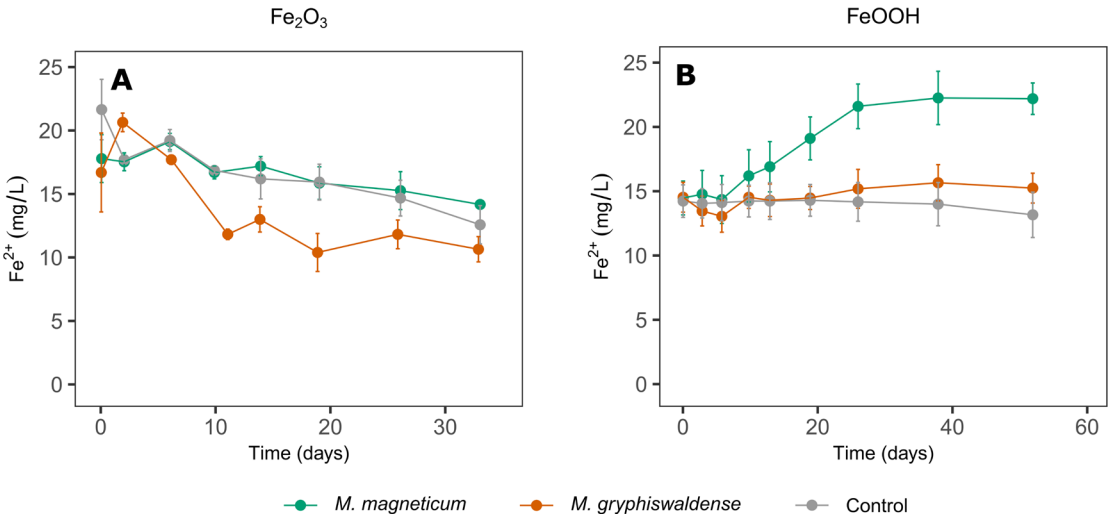

Figure 8 (from paper III). Reduction of Fe_2O_3 (**A**) and FeOOH (**B**) by *M. magneticum* and *M. gryphiswaldense* measured with ferrozine. Controls are uninoculated cultures. Error bars show standard deviation (n = 3).

Having established that both of the tested MTB were electroactive, the focus shifted to the genetic background for the phenotype. For this purpose RNA was purified from the stronger electroactive of the two, i.e. *M. magneticum*, from bacteria grown in MFCs (divided into an electrode-attached and a planktonic fraction) and in serum bottles (used for baseline gene expression). Unfortunately, it was not possible to extract RNA of sufficient quality or quantity from the MFCs, why a transcriptome profile could not be made. We suspect that the volume was too large (300 ml) and the concentration of bacteria too low. In the future, this may be solved by increasing the biomass in the reactors by increasing the number of batches or running the MFCs in continuous mode.

The results presented in this part of the thesis clearly show that *M. magneticum* and *M. gryphiswaldense* are electroactive. They were both able to generate current in MFCs, and *M. magneticum* could also reduce FeOOH. However, how these two species export electrons is still unclear. The intention from the beginning of the project was to (a) establish electroactivity in MTB and (b) map the electron pathway. The first part was achieved, but it was not possible to succeed with the second part within the time frame, due to multiple practical issues with the experiments including absence of a cyclic voltammetry signal and high quality RNA, as previously mentioned. Having said this, the work presented here still serves as a solid foundation to further explore electroactivity of MTB.

Future perspective

Moving forward, there are multiple of interesting research directions to take, to build on the fundamental finding of EET in MTB. To begin with, further effort should be put into extracting RNA, since this would be extremely informative. Most likely obtaining higher cell densities would solve the issue with suboptimal RNA quality and quantity. This would require to redesign the reactors, e.g. with a continuous supply of fresh medium. Additionally, using a different electrode in the anode chamber might also facilitate higher RNA yield, as it is difficult to efficiently recover the biofilm from a large carbon brush, from a practical perspective, simply because of the three-dimensional structure of the brush. As an alternative to transcriptomics, an analysis of potential gene candidates in *M. magneticum* and *M. gryphiswaldense* based on sequence similarity to known EET genes, such as outer membrane c-type cytochromes[47,48,50], may provide some indication of the implicated genes.

Here two MTB were used to represent this group of bacteria, however, both representatives were from the *Magnetospirillum* genus. For the initial assessment of EET this was sufficient, but to clarify if EET is a general feature of MTB or simply a general feature of *Magnetospirillum* species it is necessary to test MTB outside of this genus. Such studies would also provide information as to whether magnetosomes are involved in EET, which an existing study points towards[80].

Finally, it is also important to explore the potential of electroactive MTB for actual applications and not just for the sake of basic research. The use of magnetic electrodes would serve as an interesting starting point. Magnetism is a unique property of MTB and, therefore, it is possible to take advantage of this and design reactors around this defining ability.

5 Conclusions

The results presented here open up for a lot of future research directions. All three papers serve as foundations for new sub-fields within electromicrobiology. The two research articles function as starting points for further exploration of plasmids and MTB in MESs, since these are the first reports of plasmid-mediated inhibition of EET and electroactivity in magnetotactic bacteria, respectively. In addition, as the purpose of the literature study was to incentivize to approach the study and construction of MESs differently, all three papers require follow-up work before they may contribute to enhancing performance of MESs, even though the results are promising. The concrete conclusions are summarized below.

First of all, key challenges in progressing microbial electrochemistry from a microbiological point of view have been identified after a thorough review of the existing literature. On this basis, it has been proposed to select non-EAB that may indirectly enhance the total current output in MESs, e.g. by producing EPS, scavenging oxygen, providing substrates, or protecting from toxic compounds.

Secondly, it was demonstrated that natural conjugative plasmids inhibit extracellular electron transfer in *Geobacter sulfurreducens* and *Geobacter chapellei*, due to lowered transcription of *pilA*. Electron export in *Shewanella oneidensis*, which uses a different electron export pathway than *Geobacter* species, was unaffected. This observation might have implications for the performance and microbial structure of microbial electrochemical systems and for the transfer of conjugative plasmids in anaerobic environments where microbial mineral reduction is widespread.

Lastly, *M. magneticum* and *M. gryphiswaldense* have been added to the list of electroactive microbes. Both species generated current in MFCs, and *M. magneticum* in particular was capable of reducing FeOOH. These observations have built the foundation for the research of magnetotactic bacteria in a completely new setting.

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7 Papers

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II **Mathias Fessler**, Jonas S Madsen, Yifeng Zhang. Conjugative plasmids inhibit extracellular electron transfer in *Geobacter sulfurreducens*. Manuscript submitted.

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Perspective

Microbial Interactions in Electroactive Biofilms for Environmental Engineering Applications: A Role for Nonexoelectrogens

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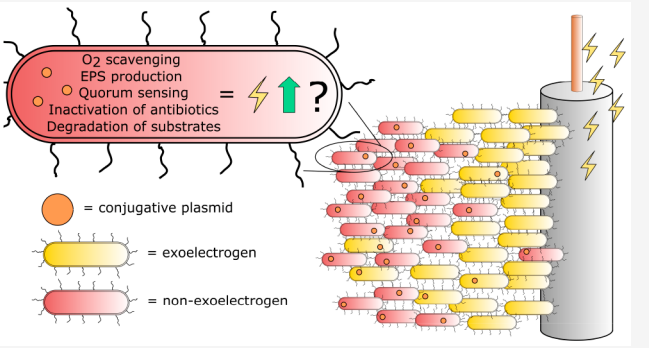
ABSTRACT: Microbial electrochemical systems have gained much attention over the past decade due to their potential for various environmental engineering applications ranging from energy production to wastewater treatment to bioproduction. At the heart of these systems lie exoelectrogens—microorganisms 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*

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 (MESs).[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 affects not only the exoelectrogens but also 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 and 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

Table 1. Percentage of Electroactive Bacteria (EAB) of Electrode Biofilms from Various Inocula[b]

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 air cathode 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 air cathode 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 was also included, as cathode biofilms may also be important for future technologies. [b]Not all studies identify the bacteria to the species level. When only identified to the genus level or in some cases the 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.

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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 nonexoelectrogens 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 nonexoelectrogens, 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 and the microbial interactions associated with nonexoelectrogens 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]

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 are 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 is favorable to exoelectrogens, which is 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 nonelectroactive 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 nonexoelectrogens 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 coculture of *G. sulfurreducens* and *Clostridium cellulolyticum*. Neither of the two species could generate current in monocultures, but in the cocultures, 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, nonexoelectrogens 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] while 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 cocultures 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 nonexoelectrogenic 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 (EPSs), which make up the matrix of the biofilm, are important not only for microbe cohesion but also for surface adhesion.[45] Especially species of the *Pseudomonas* and *Bacillus* genera produce high amounts of EPSs,[46] which is 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

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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 being 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] while QS stimulates production of redox mediators in *Pseudomonas aeruginosa*.[56] In fact, when *Pseudomonas aeruginosa* is cocultured 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 nonexoelectrogens, 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 EPSs, 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 counter-selection 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 nonbacterial 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] Nonconjugative pili and fimbriae[66] as well as plasmid-stimulated EPS production[67] have 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 toward 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

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situational and that they may be of importance for the efficiency of MESs. In the presence of stressors, such as antibiotics, plasmids providing resistance toward 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 nonexoelectrogens 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 earns 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 nonexoelectrogens 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, nonbacterial entities such as conjugative plasmids add 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 nonexoelectrogens and a single exoelectrogen to mimic the biofilms found in MESs, for the study of the proposed functions of nonexoelectrogens. 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, while 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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Notes

The authors declare no competing financial interest.

Biography

Dr. Yifeng Zhang is an associate professor working at the Technical University of Denmark. He is recognized as an international young leader in both microbial electrochemistry and bioenergy recovery from wastes. His research has included fundamental contributions and technological advancements that have generated novel insights and defined new frontiers of research and technology development. Finally, Dr. Zhang has supplied the field with innovative approaches to translate mechanistic understandings of molecule-scale phenomena to systems-scale impacts.

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Mathias Fessler, Jonas S Madsen, Yifeng Zhang. Conjugative plasmids inhibit extracellular electron transfer in *Geobacter sulfurreducens*.

Conjugative plasmids inhibit extracellular electron transfer in *Geobacter sulfurreducens*

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Conflict of Interest

The authors declare no conflict of interest.

Data availability statement

RNA sequencing data is available from NCBI (accession number: PRJNA890616).

The data is not public yet, but has been deposited and will be published if the manuscript is accepted. Until then it can be accessed by editors and reviewers via the following link:

https://dataview.ncbi.nlm.nih.gov/object/PRJNA890616?reviewer=gvrknmp7i2pjiq5rkh9haku6to

Abstract

Geobacter sulfurreducens is part of a specialized group of microbes with the unique ability to exchange electrons with insoluble materials, such as iron oxides and electrodes. Therefore, *G. sulfurreducens* plays an essential role in the biogeochemical iron cycle and microbial electrochemical systems. In *G. sulfurreducens* this ability is dependent on electrically conductive nanowires that link internal electron flow from metabolism to solid electron acceptors in the extracellular environment. Here we show that when carrying conjugative plasmids, which are self-transmissible plasmids that are ubiquitous in environmental bacteria, *G. sulfurreducens* reduces insoluble iron oxides at much slower rates. This was the case for all three conjugative plasmids tested (pKJK5, RP4 and pB10). Growth with electron acceptors that do not require expression of nanowires was, on the other hand, unaffected. Furthermore, iron oxide reduction was also inhibited in *Geobacter chapellei*, but not in *Shewanella oneidensis* where electron export is nanowire independent. As determined by transcriptomics, presence of pKJK5 reduces transcription of several genes that have been shown to be implicated in extracellular electron transfer in *G. sulfurreducens*, including *pilA* and *omcE*. These results suggest that conjugative plasmids can in fact be very disadvantageous for the bacterial host by imposing specific phenotypic changes, and that these plasmids may contribute to shaping the microbial composition in electrode-respiring biofilms in microbial electrochemical reactors.

Keywords: *Geobacter sulfurreducens*, extracellular electron transfer, nanowires, *pilA*, *omcE*, microbial electrochemical systems, conjugative plasmids, pKJK5.

Introduction

Conjugative plasmids exist in virtually all natural environments and are characterized by their ability to spread genes horizontally, which is why they play an important role in prokaryotic evolution[1,2]. They often carry advantageous traits, such as resistance to metals and antibiotics[3,4], that promote their ecological success in microbial communities. The benefits of plasmid acquisition are dictated by the environmental conditions, and depend on how the plasmid affects the host's ability to compete with surrounding microbes. Conjugative plasmids are large (often above 60 kb)[5] as they encode numerous genes specific for plasmid replication, maintenance, and transfer, which means they usually come at a metabolic cost for the host[6,7]. This cost may lead to deselection for plasmid carriage once the environment changes, however, the fitness cost plasmids impose seems to vary a great deal, as plasmids also persist in the absence of selective pressure[8,9]. So far, reduction in fitness has been related to the increased metabolic burden of maintaining the large plasmid as well as expression of plasmid-borne genes[6,7], with little focus on the impact of the immediate surroundings. Here we show that in *Geobacter sulfurreducens* conjugative plasmids can interfere with a specific phenotype, nanowire-dependent extracellular electron transfer, while imposing a minimal overall fitness burden when other electron acceptors, that do not require nanowires, are available.

Geobacter sulfurreducens is a dissimilatory metal-reducing bacterium involved in the natural metal cycle [10] and a model organism used to study extracellular electron transfer (EET). In contrast to most bacteria, electroactive bacteria such as *G. sulfurreducens* do not rely on soluble electron acceptors to get rid of electrons generated during metabolism. EET permits export of electrons to external electron acceptors such as iron(III) minerals or electrodes, in the absence of soluble alternatives. Despite the on-going discussion of the exact role of PilA, a type IV pilus protein, its importance in electron export in *G. sulfurreducens* is clear[11–14]. In the first proposed mechanism for electron export in *G. sulfurreducens*,

monomers of the PilA protein serve as the building block for the extracellular part of the pilus itself and form the basis of the electrically conductive pilus/nanowire[13]. The conductivity itself comes from stacking of the side chains of aromatic amino acids[15,16]. Deletion of the *pilA* gene severely reduces EET ability[11], whilst overexpression has the opposite effect[17], underlining the importance of these pili. Recently, however, it has been suggested that PilA is in fact involved in the secretion of nanowires and not the actual electron transfer[12]. In this model the nanowires are composed of the cytochromes OmcS[18,19], OmcZ[20,21], or OmcE[22], which give the wires their conductivity, and the decreased conductivity observed in *pilA* deletion strains is, therefore, attributed to reduced secretion of these cytochromes[12,23]. Regardless of the model, PilA has a central role in EET and in the context of the results presented here, the specific mechanism of EET is of less importance.

Due to its efficient EET ability *G. sulfurreducens* has been extensively studied and is commonly enriched in microbial electrochemical systems (MESs) inoculated with environmental samples[24,25]. MESs cover a wide variety of promising technologies, where the unique property of electroactive bacteria is used to clean wastewater and recover energy simultaneously[26,27]. Bacteria found in wastewater are rich in conjugative plasmids[28], thus, understanding the consequence of plasmid carriage on electrode-respiring bacteria is important for successful application of these systems.

In nature *Geobacter* species inhabit anaerobic iron(III)-rich environments, including freshwater sediments[29], paddy soils[30], and subsurface environments[31], where they participate in microbial dissimilatory iron(III) reduction. Additionally, *Geobacter* species are frequently found in wastewater samples[24,25]. As previously mentioned, conjugative plasmids are also widely distributed and have been isolated from similar environments[32–34], and there is evidence of natural encounters between *Geobacter* species and conjugative plasmids, in the form of horizontally acquired DNA[35–37]. Whilst these DNA uptake events could stem from transformation or transduction, they are likely to be a result of conjugation, considering that *Geobacter spp.* and bacteria carrying conjugative plasmids occupy the same

environments and conjugation is an efficient mode of horizontal gene transfer[38]. In support of this, *Geobacter lovleyi* contains a genomic island with a *tra* gene cluster[37], a set of genes encoded on conjugative plasmids needed for plasmid transfer[5].

Despite the prevalent presence of conjugative plasmids across a diverse range of natural environments, knowledge of the effects of external factors on plasmid hosts is limited. Studies have shown that extracellular quorum signals[39] and bacteriophages[40,41] can stimulate plasmid transfer. Additionally, sub-inhibitory concentrations of antibiotics may also promote conjugal transfer of transposable elements[42]. Common for these studies is that the influence of extracellular factors on plasmid transfer is the focus. Here, however, we show that the surroundings not only affect the transfer frequency, as we find that several conjugative plasmids inhibit growth of *G. sulfurreducens*, specifically when only solid extracellular electron acceptors are available. To our knowledge, this is the first report of such a drastic and negative effect only on a specific host phenotype, underlining that immediate surroundings, such as availability and nature of electron acceptors, are important to consider when assessing plasmid-host interactions. In addition, the results presented here suggest that conjugative plasmids can affect the performance of microbial electrochemical systems.

Materials and methods

Bacterial strains and cultivation conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in LB medium at 37°C if not otherwise stated. When needed 50 µg/ml of kanamycin or 100 µg/ml streptomycin was added.

G. sulfurreducens, G. sulfurreducens Δ*pilA* and *G. chapellei* were cultivated in a minimal medium with 20 mM acetate as electron donor and 50 mM fumarate as electron acceptor at 37°C and 25°C, respectively. The *G. sulfurreducens* Δ*pilA* strain was supplied by Professor Derek Lovley[11]. The medium contained the following per liter: 1.5 g NH_4Cl, 0.6 g Na_2HPO_4, 0.1 g KCl, 2.5 g $NaHCO_3$, and 10 ml/l trace element solution. The medium was bubbled with a N_2:CO_2 (80:20) gas mixture, adjusted to pH 6.8 and autoclaved. When necessary the medium was supplemented with 200 µg/ml of kanamycin or 400 µg/ml streptomycin. For solid medium 15 g/l agar was added. For the iron(III) reduction assays the fumarate was replaced with 50 mM Fe_2O_3 (Sigma-Aldrich, nanopowder, <50 nm particle size) or 50 mM iron(III)-citrate. Anthraquinone-2,6-disulfonate (AQDS) was used at a final concentration of 0.5 mM.

When cultivated aerobically, LB medium was used for *Shewanella oneidensis* MR-1. For anaerobic growth *S. oneidensis* grew with 15 mM lactate and 40 mM fumarate in minimal medium containing the following per liter[43]: 0.46g NH_4Cl, 0.225 g K_2HPO_4, 0.225 g of KH_2PO_4 , 0.117 g $MgSO_4 \cdot 7H_2O$, 0.225 g $(NH_4)_2SO_4$, 100 mM HEPES, and 5 ml/l trace element solution. The medium was bubbled with N_2 gas, adjusted to pH 7.2 and autoclaved. When needed, the medium was supplemented with 50 µg/ml kanamycin. *S. oneidensis* was grown at 25°C.

The trace element solution used for all the above contained per liter: 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.18 g $CoSO_4 \cdot 7H_2O$, 0.1 g $CoSO_4 \cdot 7H_2O$, 0.18

129 g $CoSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5 \cdot H_2O$, 0.02 g $KAl(SO_4)_2 \cdot 12H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.03 g
130 $NiCl_2 \cdot 6H_2O$, 0.3 mg $Na_2SeO_3 \cdot 5H_2O$, and 0.4 mg $Na_2WO_4 \cdot 2H_2O$.
131
132 **Plasmid construction and electroporation**
133 RP4, pB10, pKJK5 *traF*::Tn and RSF1010 were available from our strain collection. For plasmid features see
134 Table 1. pKJK5-*att*Tn7-*mcherry* (simply referred to as pKJK5 throughout the article) was constructed by
135 non-disruptive insertion of *mcherry* and a kanamycin resistance gene from pGRG36-P_{A1O4O3}-*mcherry* into
136 pKJK5-*att*Tn7 as previously described[44]. Once the insertion had been verified with Sanger sequencing, the
137 plasmid was purified with the Plasmid Midi AX kit (A&A Biotechnology) and electroporated into the *E. coli*
138 GeneHogs donor strain. We used this version of pKJK5 instead of the original isolate to ensure we could
139 assess conjugation with flow cytometry if needed.
140 pKJK5 Δ*trbC* was constructed via λred recombineering by replacing *trbC* in pKJK5-*att*Tn7-*mcherry* with a
141 chloramphenicol resistance cassette. The chloramphenicol resistance gene was PCR-amplified from pKD3
142 with primers containing sequences homologous to *trbC* (see Table 2 for primers), and the PCR products
143 were then electroporated into *E. coli* GeneHogs + pKD46 (helper plasmid with ampicillin resistance) and
144 pKJK5. Briefly, the *E. coli* GeneHogs strain with the two plasmids was grown overnight in LB at 30°C, since
145 pKD46 is heat sensitive and does not replicate at 37°C. The next day the culture was diluted 100 fold in
146 LB. After 30 minutes 100 µl 650 mM arabinose was added to induce expression of genes on pKD46 that
147 facilitate homologous recombination. The culture was grown to OD_{600} = 0.6 followed by incubation on ice
148 for 30 minutes. Cells were prepared for electroporation by washing and resuspending in 10% glycerol
149 solution. 100 ng PCR product was electroporated into the competent cells with a Bio-Rad Gene Pulser.
150 After incubation for 1 hour at 37°C in 1 ml LB, the cells were spread on LB agar plates with 50 µg/ml
151 chloramphenicol and 50 µg/ml kanamycin to select for gene disruption. At 37°C pKD46 cannot replicate,

and loss of the vector was verified by plating on LB plates with 100 µg/ml ampicillin. Correct insertion was verified with Sanger sequencing (see Table 2 for primers).

Filter mating

E. coli GeneHogs was used as the plasmid donor for the conjugative plasmids, whilst *E. coli* S17-1 was used for pKJK5 *traF*::Tn, pKJK5 Δ*trbC*, and RSF1010. Conjugations were carried out according to a previously described protocol [45]. Briefly, 1 ml outgrown O/N culture of the donor strain was washed twice in LB, then 1 ml growing (OD_{600} around 0.3 – 0.4) recipient strain was added inside an anaerobic chamber. The cell mixture was centrifuged and the pellet was resuspended in 100 µl residual supernatant and spread on a 0.22 µm filter resting on an agar plate with 0.1% tryptone, inside an anaerobic box. After at least 4 hours, the cells were transferred to an agar plate without tryptone to inhibit growth of the donor strain and the appropriate concentration of kanamycin (or streptomycin). Once colonies were visible, single colonies were transferred to liquid medium. For pKJK5, successful conjugation was also verified with PCR targeting the *tetA* gene (see Table 2 for primers).

For *S. oneidensis* filter matings were carried out aerobically on LB agar plates followed by selection on M9 agar plates with 15 mM lactate and kanamycin at 25°C.

Fe(III) oxide and Fe(III)-citrate reduction

Iron(III) oxide assays were performed in 50 ml serum bottles with 25 ml medium. The Fe_2O_3 medium was inoculated with 0.5 OD_{600} units of an overnight culture in early stationary phase. Each pair of strains, i.e. the given strain with and without the conjugative plasmid, was inoculated at the same OD_{600} and thus with the same volume, meaning that any potential carryover of small amounts of unused electron acceptor was the same for each pair. For *G. sulfurreducens* 1.35 ml of OD_{600} = 0.37 culture was added, for

G. chapellei 1.67 ml of OD_{600} = 0.30 culture was added, and for *S. oneidensis* 5 ml of OD_{600} = 0.10 culture was added. After inoculation, the cultures were incubated horizontally on a shaker.

Samples were taken by transferring 400 µl culture to 800 µl 5 M HCl. The iron was dissolved by rotating the samples for 48 hours. Samples were then stored at 4°C until all samples had been taken. At this point the Fe^{2+} concentration was measured with ferrozine in 96-well plates by mixing 10 µl sample with 75 µl ferrozine solution (2 g/l ferrozine in 25 mM HCl) and 75 µl acetate buffer (285 g/l sodium acetate in 2 M acetic acid), followed by measuring absorbance at 562 nm. A standard curve was used to convert absorbance to Fe^{2+} concentration.

For the Fe(III)-citrate experiments 400 µl culture was also mixed with 800 µl 5 M HCl, but here the Fe^{2+} concentration was measured immediately.

RNA sequencing

Cells from growing fumarate cultures were harvested in the exponential phase (at OD_{600} = 0.15) by centrifugation at 12.000 x G for 2 minutes and 4°C. The pellet was resuspended in Qiagens bacterial RNAprotect reagent, left for 5 minutes at room temperature before the cells were pelleted and flash frozen and stored at -80°C. Both conditions (i.e., *G. sulfurreducens* with/without pKJK5) were run in triplicates. Cell pellets were sent for RNA extraction and sequencing at Genewiz (Leipzig, Germany). All sequenced samples had a RIN score = 10. The reads were trimmed (Trimmomatic v.0.36), mapped (Star aligner v.2.5.2b) and counted (featureCounts from Subread package v.1.5.2) by Genewiz. Differential gene expression analysis was done with DESeq2. Genes with adjusted *p*-value < 0.05 and log2 fold change below -0.9 or above 0.9 were defined as differentially expressed.

For mapping reads to pKJK5 CLC Genomics Workbench (version 22.0.2) was used.

Statistical testing

To test if the observed differences in Fe_2O_3 reduction were statistically significant unpaired, two-tailed *t*-tests assuming heteroscedasticity were used. The threshold for significance was defined as a *p*-value < 0.05. *t*-tests were performed to test for a difference at the end of the given experiment, i.e. by comparing the last samples of the experiment, except for the growth experiments with fumarate where a difference between doubling times was tested for.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant features	Reference or source
Strains		
Geobacter sulfurreducens PCA	ATCC no. 51573	[46]
Geobacter sulfurreducens Δ*pilA*	*pilA*::Chl^R	[11]
Geobacter chapellei 172	DSM no. 13688	[47]
Shewanella oneidensis MR-1	ATCC no. 700550	[48]
Escherichia coli S17-1	*recA pro hsdR* RP4-2-Tc^R::Mu-Km^R::Tn7	[49]
Escherichia coli GeneHogs	Leucine auxotroph	Invitrogen
Escherichia coli MG1655-*lacI^q-mcherry*	Chromosomal *att*Tn7 site blocked	[50]
Plasmids		
pKJK5-*att*Tn7	Non-disruptive insertion of *att*Tn7 site	[44]
pKJK5-*att*Tn7-*mcherry* *	pKJK5-*att*Tn7::*mcherry*-Km^R	This study
pKJK5-*att*Tn7-*mcherry* Δ*trbC**	*trbC*::Chl^R	This study
pKJK5 *traF*::Tn	*traF*::Km^R	[51]
pB10::*gfp*	Str^R, *gfp*	[52]
RP4::*gfp*	Km^R, *gfp*	[53]
RSF1010::*gfp*	Km^R, P_{A1O4O3}-*gfpmut3*	[54]
pKD46	Temperature sensitive, expresses λ Red recombinase	[55]
pKD3	Source of Chl^R for *trbC* deletion	[55]
pGRG36-P_{A1O4O3}-*mcherry*	Km^R and P_{A1O4O3}-*mcherry* flanked by Tn7L and Tn7R sequences	Strain collection

*Simply referred to as pKJK5 and pKJK5 Δ*trbC* throughout the article

Table 2. Primers used in this study.

Primer name	Sequence (5'-3')	Description
trbC_KO_F	ATGCAAGCACTCTTCCCGTCATTCAGGCTCG ACCAGCGCACATGCAGATTGCAGCATTAC	Knockout of *trbC*. Red seq is complementary to seq in pKJK5, black seq anneals to pKD3 for PCR
trbC_KO_R	TTACCCCGCCACGTAGCCGCGTTCGGCCCAG CGCGTCACCGGAATTAGCCATGGTCCATA	Knockout of *trbC*. Red seq is complementary to seq in pKJK5, black seq anneals to pKD3 for PCR
trbC_seq_F	TAGTCGTTCACATCGCCAG	Seq flanking *trbC*, for sanger sequencing of deletion
trbC_seq_r	CAAGCCCGAGAACATAACC	Seq flanking *trbC*, for sanger sequencing of deletion
pKJK5_tetA_F	TCGTAATTCTGAGCACTGTCG	For verification of pKJK5 conjugation
pKJK5_tetA_R	GCAGGCAGAGCAAGTAGAG	For verification of pKJK5 conjugation

Results

pKJK5 specifically inhibits growth on iron oxides in *G. sulfurreducens*

During a preliminary study of *G. sulfurreducens'* ability to act as plasmid recipient and donor we observed that the conjugative plasmid pKJK5 slowed down growth of *G. sulfurreducens* when growing exclusively with iron oxides as terminal electron acceptors. This immediately caught our attention, as EET is one of the key characteristics of *G. sulfurreducens* responsible for the massive interest in this organism. Until now, EET in *G. sulfurreducens* has been inhibited by *pilA* and cytochrome deletions[13,14], with the purpose of mapping essential genes for electron export, but natural inhibitors of this defining feature have not been observed previously.

Initially, we assessed and quantified the impact of pKJK5 on the reduction of the iron mineral hematite (Fe_2O_3). Hematite is, together with goethite, the most abundant iron oxide in nature[56–58], why we used this as our electron acceptor, even though it is also common practice to prepare more readily reducible iron oxides in the laboratory[10,11,45]. To assess this, *G. sulfurreducens* was grown in medium with Fe_2O_3 as the sole terminal electron acceptor, and under these conditions the conjugative plasmid pKJK5 severely inhibited *G. sulfurreducens*' ability to reduce iron (Figure 1A). At the end of the experiment, after 17 days, the presence of pKJK5 led to a significant 3-fold decrease in Fe_2O_3 reduction ($P < 0.05$). The observed difference could principally be due to the increased metabolic burden of maintaining pKJK5. To clarify whether this was the case, growth of *G. sulfurreducens* on two soluble electron acceptors, fumarate and Fe(III)-citrate, was assessed (Figure 1B and 1C). Fumarate reduction takes place in the cytoplasm[59], whilst Fe(III)-citrate is reduced extracellularly by cytochromes located in the outer membrane[60]. Growth on these electron acceptors was not affected by pKJK5 (fumarate doubling time: $P > 0.05$, Fe(III)-citrate day 9: $P > 0.05$), suggesting that the plasmid interferes with the specific electron transfer mechanism for reduction of Fe_2O_3 rather than imposing a general fitness reduction. In accordance with this, the negative effect of

pKJK5 on Fe_2O_3 reduction was alleviated by adding the electron shuttle anthraquinone-2,6-disulfonate (AQDS) (Figure 1D) ($P > 0.05$, day 7). For reduction of AQDS *G. sulfurreducens* relies on several outer surface c-type cytochromes[61], rather than conductive nanowires, which allowed *G. sulfurreducens* to circumvent the nanowire-dependent electron transfer pathway otherwise needed for growth on iron oxides[11].

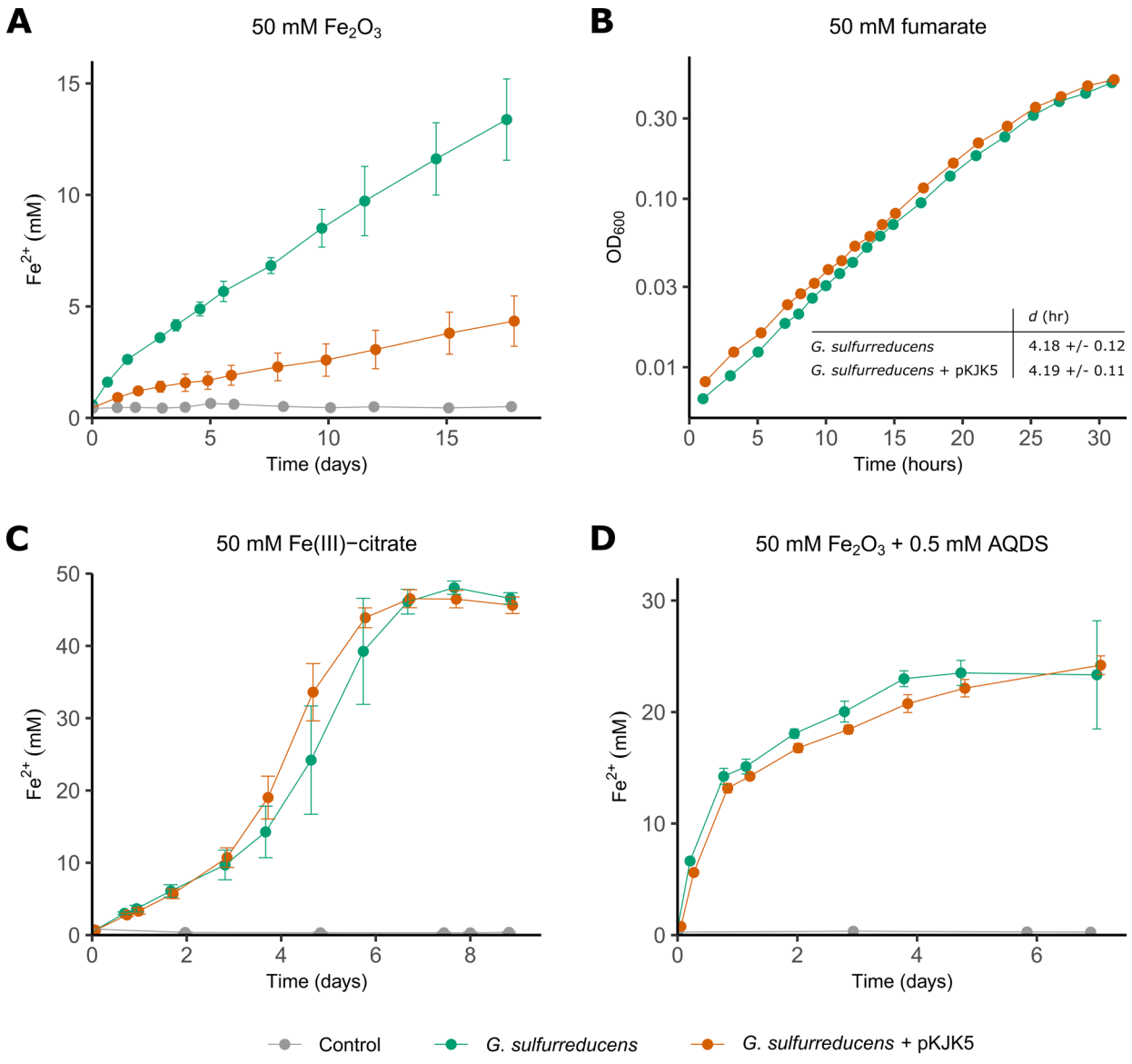

Figure 1. pKJK5 inhibits *G. sulfurreducens*' ability to reduce Fe_2O_3. *G. sulfurreducens* with and without pKJK5 was grown in medium with Fe_2O_3 (**A**, n = 6), fumarate (**B**, n = 5), Fe(III)-citrate (**C**, n = 6) or Fe_2O_3 + AQDS (**D**, n = 6) as the only electron acceptor. Growth

was either determined by measuring Fe^{2+} concentration (**A**, **C** and **D**) or OD_{600} (**B**). For growth on fumarate one representative of five replicates is shown along with doubling times with the standard deviation (SD). All the controls are uninoculated medium. Error bars show SD.

pkJK5 only interferes with extracellular electron transfer mediated by nanowires

By now, the importance of the *pilA* gene for extracellular electron transfer to minerals and electrodes in *G. sulfurreducens* is well established[11,17], despite some uncertainty on the specific mechanistic role of PilA[13,14]. When expressing pili with low conductivity, electron export to iron oxides and electrodes decreases radically[15,62]. In addition, *pilA* deletion mutants fail to accumulate OmcZ in the extracellular matrix in biofilms, which also reduces *G. sulfurreducens*' ability to generate current[63]. Whether PilA is involved in electron transport, secretion of cytochromes, or both, the PilA protein is central to both the proposed EET models and clearly essential for EET in *G. sulfurreducens*. This means that growth on insoluble electron acceptors is primarily restricted to PilA-dependent EET pathway(s). In other words, *pilA* is the main differentiator between respiration on Fe_2O_3 and respiration on fumarate, Fe(III)-citrate, and AQDS. For this reason, our attention turned to this gene. Since our initial experiments indicated that pKJK5 interfered with the microbial nanowires, we conjugated pKJK5 into a *G. sulfurreducens* strain where *pilA* had been deleted. Fe_2O_3 reduction was similar in the Δ*pilA* strain with and without pKJK5 (Figure 2A) ($P > 0.05$, day 17), which is consistent with the initial observation and indicates that pKJK5 affects PilA-dependent electron export. In agreement with previous reports, *G. sulfurreducens*' ability to transfer electrons to iron minerals was reduced but not completely lost in the *pilA* deletion strain[64,65].

G. sulfurreducens is the most well studied species in the *Geobacter* genus, but other *Geobacter* species also show electroactive properties[66] and expression of nanowires[67,68]. To determine if pKJK5's effect was common for the *Geobacter* genus or specific for *G. sulfurreducens*, iron oxide reduction by *Geobacter chapellei* was assessed. After preliminary experiments including *Geobacter chapellei*, *Geobacter metallireducens*, *Geobacter bremensis*, and *Geobacter bemidjensis*, it was decided to focus on *G. chapellei*

as it was both easy to cultivate and displayed proficient growth on Fe_2O_3. Also, *G. chapellei* is likely to use nanowires for EET based on sequence homology (NCBI protein ID = WP_214296113.1). The putative *pilA* gene in *G. chapellei* shows 79% similarity on DNA level and 88% similarity at amino acid level to the *pilA* gene of *G. sulfurreducens* (Supplementary Figure 1). In addition, all five aromatic amino acids that are essential for the conductivity of the pili are conserved in *G. chapellei*[16]. pKJK5 was conjugated into *G. chapellei* and had a similar effect on iron oxide reduction as in *G. sulfurreducens* (Figure 2B). The lowered iron reduction was also statistically significant in *G. chapellei* ($P < 0.05$, day 18). Knowing that pKJK5 did not affect growth on fumarate, ferric citrate or AQDS (Figure 1B, C and D) this strongly suggests that pKJK5 specifically interferes with the nanowires. Further evidence for this was found in the fact that pKJK5 did not inhibit mineral reduction in *Shewanella oneidensis* ($P > 0.05$, day 18), that does not use PilA-dependent nanowires to reduce external electron acceptors (Figure 2C). In *S. oneidensis*, MtrC, a c-type cytochrome anchored in the outer membrane, is the final protein in the electron export pathway[69].

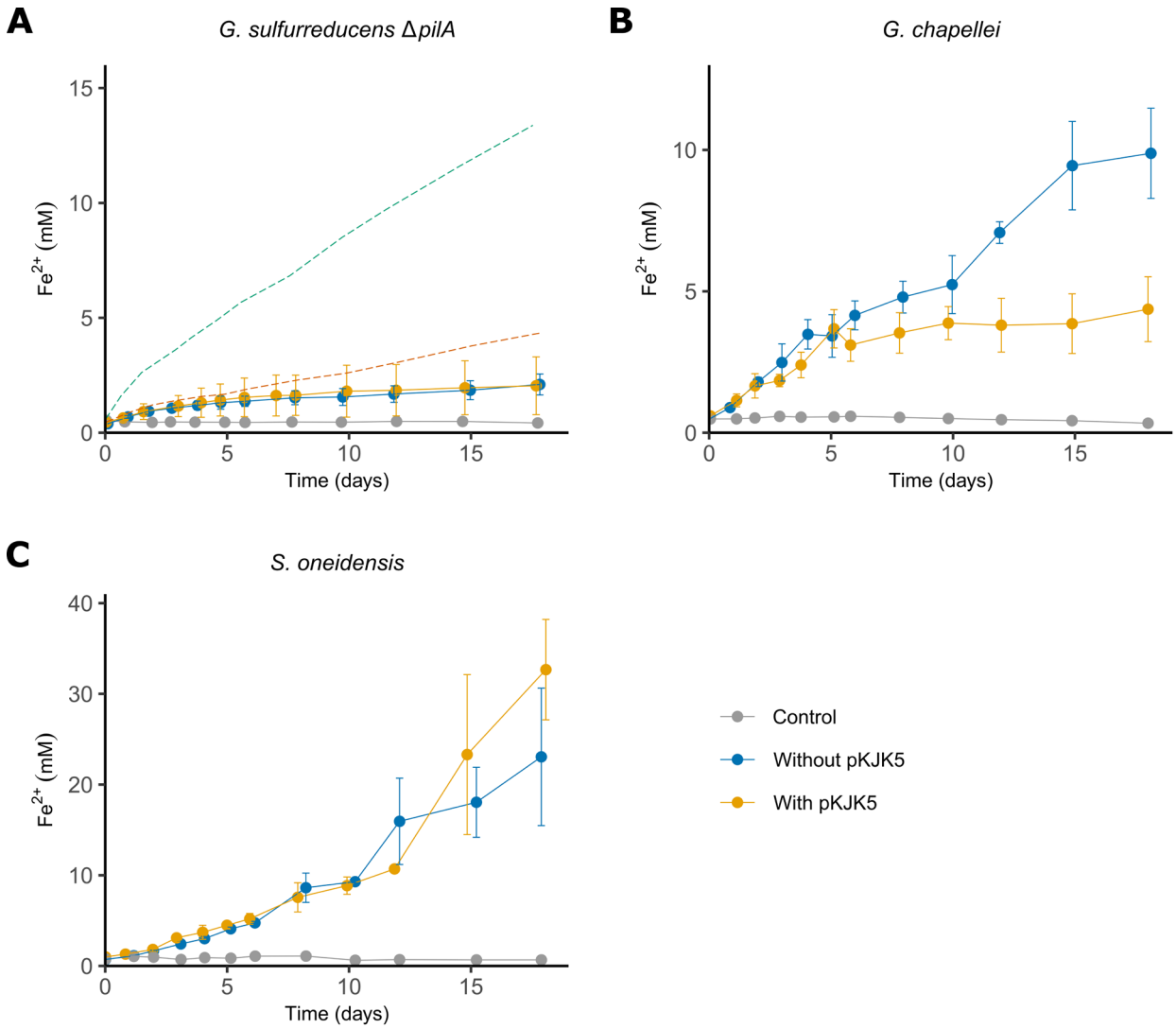

Figure 2. pKJK5 specifically inhibits *pilA*-dependent iron oxide reduction. Reduction of Fe_2O_3 by *G. sulfurreducens* Δ*pilA* (**A**, n = 6), *G. chapellei* (**B**, n = 3) and *S. oneidensis* (**C**, n = 3). Stippled lines (**A**) show Fe_2O_3 reduction of *G. sulfurreducens* WT (green) and *G. sulfurreducens* WT + pKJK5 (orange) from figure 1A to ease comparison. All the controls are uninoculated medium. Error bars show SD.

Inhibition of extracellular electron transfer is a general feature of conjugative plasmids

pKJK5 is just one of many conjugative plasmids found in nature and, therefore, it is important to establish if the observed phenotype in the two *Geobacter* species is restricted to pKJK5 or if this is a more general feature of conjugative plasmids. To do so we used three additional wild type plasmids: RP4, pB10 and

RSF1010[70–72]. The former two are conjugative plasmids belonging to the incP group like pKJK5, whilst RSF1010 is mobilizable rather than conjugative and belongs to the incQ group. Mobilizable plasmids can transfer upon cell-cell contact just as conjugative plasmids, however, as opposed to conjugative plasmids they do not encoded all the genes needed for this process themselves[5]. As seen in Figure 3A the inhibitory effect of conjugative plasmids was not only limited to pKJK5. Even though pKJK5 had the most substantial impact of the plasmids tested, similar patterns were observed for RP4 and pB10 and both plasmids led to a statistically significant decrease in Fe_2O_3 reduction ($P < 0.05$, for both plasmids on day 17). On the other hand, the growth of *G. sulfurreducens* on Fe_2O_3 was not significantly affected by the mobilizable plasmid RSF1010 (Figure 3A) ($P > 0.05$, day 17). For conjugation four elements encoded on the conjugative plasmid itself are key: an origin of transfer (oriT), relaxases that initiate the DNA transfer at the oriT, type 4 coupling proteins (T4CP), and a type 4 secretion system (T4SS), through which the DNA is transferred[5]. As opposed to conjugative plasmids, mobilizable plasmids do not encode a pilus but only the oriT and relaxase (and in some cases the T4CP), why they are not self-transmissible. Therefore, as only the conjugative plasmids had an impact on the Fe_2O_3 reduction, these findings suggest that the T4SS (which includes the conjugative pilus) could be responsible for the observed phenotype.

As the data presented so far indicated that the plasmid-mediated inhibition was specific for the nanowire electron transport pathway, the mechanism behind this became our focus. Even though the core pilin proteins are different, both the conjugative pilus and the PilA pilus in *G. sulfurreducens* belong to the family of type IV pili[73]. In addition, the conjugative pilus is one of the main differentiators between conjugative and mobilizable plasmids and, therefore, we investigated if the conjugative pilus physically interfered with PilA-mediated EET. To test this, two versions of pKJK5 were used – one with a knock out in *traF*, a gene encoding a protein involved in pilus maturation, and another with a deletion of *trbC*, the gene encoding the conjugative pilus building block[74]. Both of these pKJK5 versions were non-conjugative (data not shown), but neither of the two alleviated the effect of pKJK5 (Figure 3B) ($P > 0.05$, for both

plasmids at the end of the experiment), suggesting that the inhibition is not mediated by the actual conjugative pili. However, there are several other genes involved in biogenesis of the conjugative pilus[75], why the finding that neither the TraF nor the TrbC protein alone is responsible for the phenotype is not sufficient to dismiss the conjugative T4SS.

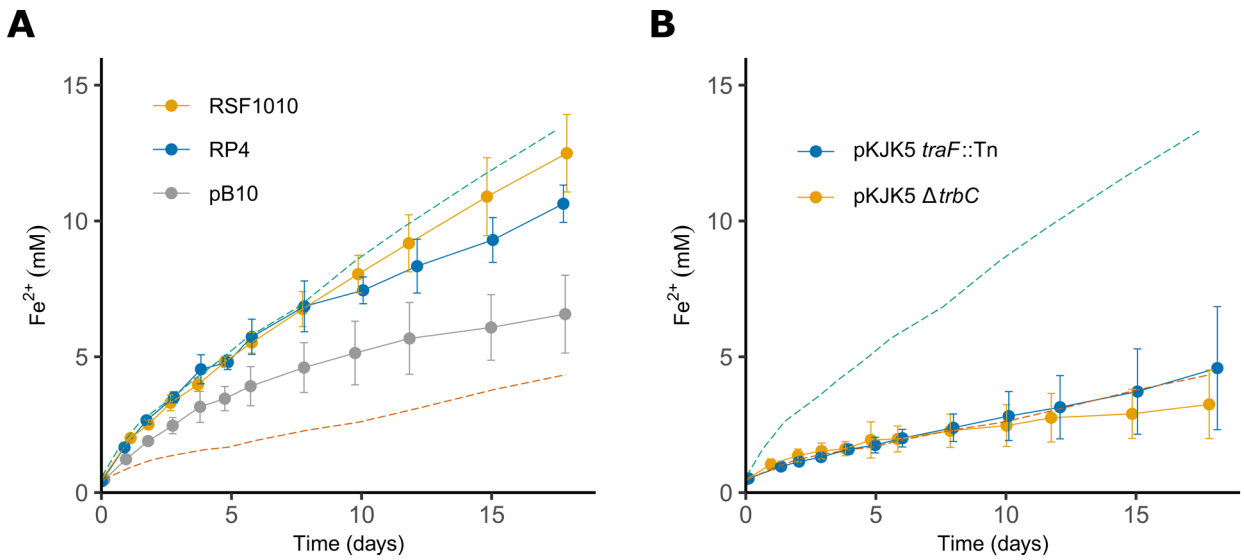

Figure 3. Inhibition of iron reduction in *G. sulfurreducens* is a general feature of conjugative plasmids, but does not depend on the conjugative pilus. Fe_2O_3 reduction by *G. sulfurreducens* with three different plasmids (**A**, n = 6); pB10 (conjugative), RP4 (conjugative) and RSF1010 (mobilzable), and with two non-conjugative versions of pKJK5 (**B**, n = 6). Stippled lines show Fe_2O_3 reduction of *G. sulfurreducens* WT (green) and *G. sulfurreducens* WT + pKJK5 (orange) from figure 1A to ease comparison. Error bars show SD.

Next, we looked into effects of pKJK5 on the host transcriptome, to determine if plasmid-borne genes interfered with expression of genes needed for EET. *G. sulfurreducens* is resistant to kanamycin, when harboring pKJK5, and is able to function as plasmid donor (data not shown), which confirms that plasmid encoded genes were expressed in *Geobacter*. In addition, the transcriptomic data presented below confirmed that pKJK5 genes were transcribed (Supplementary Table 1). pKJK5 led to differential transcription of 81 genes, after removing genes annotated as either hypothetical proteins with unknown

function or pseudogenes (Supplementary Table 2). 64 genes were transcribed at reduced levels and 17 genes were induced. The majority of differentially transcribed genes are part of basic cell metabolism, such as replication, transcription, translation and biosynthesis (see Supplementary Table 2 for full list); all processes that are also involved in maintenance of the plasmid. This is in agreement with previous findings[76]. In the context of extracellular electron transfer, the analysis showed reduced transcription of both *pilA-N* and *pilA-C* as well as five c-type cytochrome genes (Figure 4). PilA-N (also referred to simply as PilA throughout the article) is the protein that constitutes the nanowire and/or is responsible for cytochrome secretion. PilA-C is, on the other hand, non-conductive[77] and also part of the cytochrome secretion complex[12]. Evidence suggest they were once a single gene[77]. When *G. sulfurreducens* contained pKJK5, the transcription of *pilA* was reduced with 60% compared to transcription in the plasmid-free cells (adjusted *P*-value < 0.05), and the cytochromes were reduced with 58% to 51% (adjusted *P*-value < 0.05, for all cytochromes). This strongly implies why *G. sulfurreducens*' ability to reduce iron minerals diminishes in the presence of pKJK5. Two of the five downregulated cytochromes (OmcE and OmcO) are located in the outer membrane, cytochrome GSU2937 is predicted to localize in the periplasm[78], whilst the cellular location of the remaining two unnamed cytochromes is unknown. When *omcE* is deleted the ability of *G. sulfurreducens* to reduce iron oxides is limited[10], and recently OmcE was in fact found to assemble into conductive filaments[22], similar to OmcS and OmcZ filaments[19,21]. Of the 17 genes that were induced, six genes in particular were highly upregulated. Based on sequence homology all of these, except for *hybT*, encode proteins of a periplasmic membrane-bound [NiFe]-hydrogenase, an enzyme that catalyzes reversible conversion of H_2 to protons and electrons.

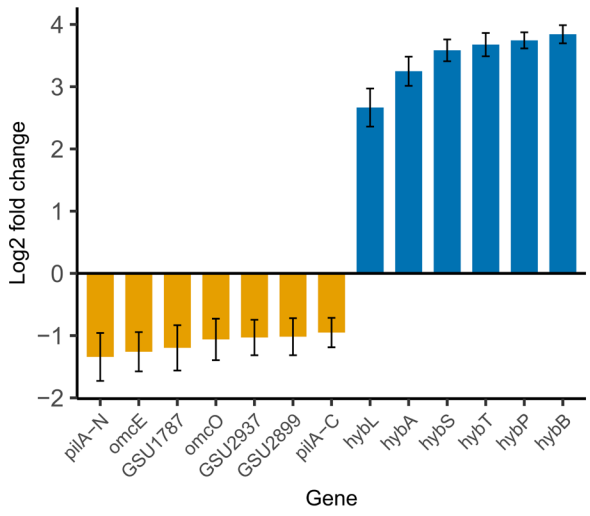

Figure 4. pKJK5 downregulates transcription of *pilA* and several cytochromes in *G. sulfurreducens*. Gene transcription of *G. sulfurreducens* with pKJK5 compared to gene transcription of *G. sulfurreducens* without pKJK5. GSU1787, GSU2937 and GSU2899 encode c-type cytochromes. Error bars show SD (n = 3). For full table of differentially transcribed genes see Supplementary Table 2.

Discussion

The results presented here demonstrate that pKJK5 inhibits *G. sulfurreducens*' growth on Fe_2O_3 and that this is due to reduced transcription of *pilA* and several c-type cytochromes. However, what causes this reduced transcription is not clear from the RNA sequencing. Considering that PilA and the conjugative pilus are both type IV pili, we speculate that regulation of *pilA* transcription is recognized by elements regulating transcription of the conjugative pilus, which would explain the lower transcription of both *pilA-N* and *pilA-C* (Figure 4). Meanwhile, the reduced transcription of c-type cytochromes is more surprising. Previous reports show upregulation of OmcE, OmcO and GSU2937 in response to iron oxide-dependent growth[78], but it is not clear how or why pKJK5 affects the transcription of these genes. At this time, it is best explained as an indirect effect of pKJK5, in the sense that these cytochromes are somehow indirectly coupled to *pilA* expression. Considering that PilA is needed for secretion of OmcS and OmcZ[12], this might also be the case for some of the cytochromes that are downregulated in our differential transcription

368 analysis (Figure 4), such as OmcE, which is known to form nanowires[22]. If PilA is responsible for secretion
369 of these cytochromes it seems plausible that their expression is coupled to the expression of *pilA*, in order
370 to prevent wasting resources on synthesis of cytochromes in situations where they cannot translocate to
371 the outside of the cell. However, with such cross-regulation *omcS* and *omcZ* would also be expected to
372 show up in the gene expression analysis as these depend on *pilA* for secretion[12]. Downregulation of these
373 two cytochrome genes was observed, but was not statistically significant (Supplementary Table 2).
374 Interestingly, whilst deletion of *omcE* in *G. sulfurreducens* has no effect on conductivity when respiring on
375 electrodes[79], iron oxide reduction is slower without OmcE[10]. Therefore, the reduced transcription of *omcE*
376 we observe here may also contribute to the poor reduction of Fe_2O_3. OmcO, on the other hand, is not
377 essential for iron oxide reduction[78], and the remaining three cytochromes have not yet been examined.
378 Since hematite reduction was inhibited by all three conjugative plasmids tested, but not the mobilizable
379 plasmid RSF1010 (Figure 3A), this suggests that the inhibition is caused directly or indirectly by one or
380 more factors encoded as part of the IncP-1 backbone which is similar between pKJK5, pB10 and RP4.
381 Further investigations are needed for identification of the exact mechanism.
382 As for the increased transcription of the *hyb* genes, we also consider this an indirect effect. The *hyb* genes
383 encode a periplasmic [NiFe]-hydrogenase and we suspect that these genes are also linked to *pilA* and/or
384 cytochrome expression, simply because this seems more plausible than pKJK5 directly regulating *hyb*
385 expression. In *G. sulfurreducens* the *hyb* operon couples hydrogen oxidation to reduction of both soluble
386 and insoluble electron acceptors[80], and upregulation of [NiFe]-hydrogenases is linked to growth on iron
387 minerals[78]. Here, the observed *hyb* upregulation might be a response to the pKJK5-mediated nanowire
388 downregulation, as these hydrogenases present an alternative route for electron disposal, i.e., by
389 conversion of electrons and protons to H_2. We want to note that to obtain sufficient biomass for RNA
390 sequencing, the RNA was purified from cultures grown with fumarate and not hematite. We believe this
391 to be an acceptable compromise as the results of the transcription analysis fit well with the phenotypes

observed when *G. sulfurreducens* grew with Fe_2O_3. This also suggests that the transcription of pKJK5 genes was not affected by the type of electron acceptor.

Often acquisition of conjugative plasmids is associated with a benefit for the bacterial host, such as resistance to antibiotics or heavy metals. However, here we report the opposite, conjugative plasmids severely limit the growth of *G. sulfurreducens* and *G. chapellei*, specifically when respiring on insoluble electron acceptors. Granted, this negative effect is highly dependent on the surrounding environment, however, the inhibition is specific to the very environment *Geobacter* species have specialized to inhabit. Our results suggest that when a plasmid protects against an environmental stressor, there is both a selection and counter-selection for plasmid uptake by *Geobacter spp.*, given that the availability of soluble electron acceptors is scarce. In sediments, this means that the availability of electron acceptors may, in fact, be an indirect determinant of conjugal transfer efficiency by preventing proliferation of nanowire-dependent plasmid recipients.

In addition to their potential influence in natural environments, conjugative plasmids may also have an impact on the community structure in artificial systems, namely in microbial electrochemical systems. The configuration and purpose of MESs is very diverse, but common for all these systems are that electroactive bacteria are essential[27]. Whether they respire on the anode, cathode, or both, electron flow between the chambers is an integral part of the reactors. For this reason, the selective pressure for electroactive species is strong and, therefore, it is usually sufficient to inoculate with a diverse mixture of bacteria. Ultimately, electroactive species will dominate the electrode biofilm, why wastewater samples are often used as the inoculum due to their high bacterial diversity[26,81]. Additionally, to achieve sustainable operation, most reactors are designed to run using wastewater as a source of organics. Consequently, there is a continuous entry point for conjugative plasmids, as these are abundant in wastewater[28,82,83].

As we have shown here, conjugative plasmids repress the transcription of *pilA* and numerous cytochromes, why it is certainly plausible that such plasmids influence the microbial composition in MESs.

For *Geobacter* species, commonly enriched in MESs[24,84,85], our results suggest there is a trade-off between the ability to grow on electrodes and the potential positive attributes plasmids can provide, such as the ability to withstand the residual amounts of antibiotics that are found in wastewater[86,87]. In support of this, wastewaters with higher concentrations of antibiotics show increased abundance of antibiotic resistance genes[88]. Additionally, conjugative plasmids are implicated in biofilm formation and stabilization[89], underlining their usefulness for the bacterial communities, which complicates the situation even further. In the context of MES community composition, our results also indicate that spread of conjugative plasmids in MESs favor growth of electroactive bacteria that do not rely on nanowires, such as *Shewanella* species. Having said this, biofilms are very complex. Different species fill different roles in biofilms and, therefore, all members of the biofilm do not necessarily need the plasmid even if the surroundings contain residual amounts of antibiotics. In electrode-respiring biofilms, *Geobacter* is more abundant in the inner layers than in the outer layers[24], which is not surprising. This means that toxic or anti-bacterial compounds might never reach the inner biofilm, as they may be removed by plasmid-containing cells in the outer layer. Effectively this gives *Geobacter* species protection without compromising EET ability.

At this point, it is important to note that we are not claiming that conjugative plasmids are a major determinant of microbial community structure in MESs. We argue that they may play a part and that environmental factors are important to consider in regard to MESs community dynamics; thriving in these systems is not simply a question of whether an organism is electroactive or not.

Having established that an important group (IncP) of conjugative plasmids inhibits extracellular electron transfer in pure cultures of *G. sulfurreducens* and *G. chapellei* it is important to better mimic conditions encountered both in nature and MESs, moving forward. This will make it possible to assess the significance of conjugative plasmids in multispecies electroactive biofilms and, thus, better understand how and if they influence MES performance.

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Author contributions

M.F., J. S. M., and Y.Z. conceptualized the project and wrote the manuscript. M.F. performed experiments. Y.Z. secured funding.

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A

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G.chapellei       AGGAGCACAACCAAGGAAAGGAGAAACACATGTTACAGAA 40
G.sulfurreducens  AGCAGCAAAAAGAA-GAAAGGAGACACTTATGCTTCAGAA 39

G.chapellei       AATGAGAAACAGAAAAGGTTTTACCCTGATCGAGCTGCTG 80
G.sulfurreducens  ACTCAGAAACAGGAAAGGTTTCACCCTTATCGAGCTGCTG 79

G.chapellei       ATCGTTGTTGCGATCATCGGTATCCTGGCTGCCGTTGCCA 120
G.sulfurreducens  ATCGTCGTTGCGATCATCGGTATTCTCGCTGCAATTGCGA 119

G.chapellei       TCCCGCAGTTTTCATCCTATCGCGTTAAGGCTTACAACAG 160
G.sulfurreducens  TTCCGCAGTTCTCGGCGTATCGTGTCAAGGCGTACAACAG 159

G.chapellei       TGCTGCATCCAGTGATTTGAGGAACTTGAAAACAGGTTTA 200
G.sulfurreducens  CGCGGCGTCAAGCGACTTGAGAAACCTGAAGACTGCTCTT 199

G.chapellei       GAGGCCGCTTTTTCTGAT 218
G.sulfurreducens  GAGTCCGCATTTGCTGAT 217
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B

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G.chapellei       MLQKMRNRKGFTLIELLIVVAIIGILAAVAIPQFSSYRVK 40
G.sulfurreducens  MLQKLRNRKGFTLIELLIVVAIIGILAAIAIPQFSAYRVK 40

G.chapellei       AYNSAASSDLRNLKTGLEAAFSDNQYYP 68
G.sulfurreducens  AYNSAASSDLRNLKTALESAFADDQTYP 68
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Supplementary Figure 1. DNA (**A**) and amino acid (**B**) alignment of presumed e-pili gene in *G. chapellei* (WP_214296113.1) and *G. sulfurreducens pilA* gene (GSU1496). The amino acids marked in yellow are the five aromatic amino acids that are essential for conductivity. The alignments were made with NCBI blast, and the coloring was done with the Color Align Conservation online tool (https://www.bioinformatics.org/sms2/color_align_cons.html).

Supplementary Table 1

Gene name	Total gene reads (with pKJK5)	Total gene reads (without pKJK5)
trfA	16753	0
ssb	9068	0
trbA	572	0
trbB	43105	0
trbC	12077	0
trbD	7669	0
trbE	68163	0
trbF	17839	0
trbG	26445	0
trbH	13420	0
trbI	60619	0
trbJ	43625	0
trbK	2937	0
pKJK5_14	4669	0
trbL	59950	0
trbM	36217	0
trbN	23956	0
trbO	3672	0
trbP	12682	0
upf30.5	10987	0
pKJK5_21	11131	0
pKJK5_22	9842	0
parA	4418	0
pKJK5_24	3637	0
pKJK5_25	3981	0
pKJK5_26	3328	0
pKJK5_27	5940	2
intI1	3965	0
dfrA	2527	0
aadA11b	4358	1
qacEdelta1	1507	0
sul1	4538	0
pKJK5_33	1921	0
istB	1479	0
istA	1485	0
tetR	9385	0
tetA	658	0
pKJK5_38	201	0
pKJK5_39	175	0
traC	90725	0
traD	4448	0
traE	36732	0
traF	3716	0
traG	25551	0
traI	10235	0
traH	1772	0

traJ	2161	0
traK	6199	0
traL	12024	0
traM	4048	0
upf54.4	6591	0
upf54.8	1458	0
kfrA	8255	0
korB	49083	0
incC2	0	0
incC1	57412	0
korA	0	0
kleF	1667	0
kleE	13779	0
kleB	5743	0
kleA	5665	0
korC	3230	0
klcB	6385	0
klcA	8631	0

Supplementary Table 2

Gene_ID	Base_mean	log2(FC)	StdErr	Wald-stats	p-value	p-adj	GSU annotation	Gene name (if available)	GenBank description
gene-GS_RS00205	52385,91234	-0,9412114	0,309428	-3,041779868	0,002351838	0,040317226	GSU0038		lipoprotein
gene-GS_RS00265	332,2730512	-1,1395415	0,362661	-3,142163544	0,001677043	0,032842304	GSU0049		Hypothetical protein
gene-GS_RS00300	1038,577703	-0,9124089	0,278449	-3,276755308	0,001050073	0,024041152	GSU0056		antitoxin
gene-GS_RS00845	1815,652135	-1,3725503	0,312831	-4,387507718	1,14657E-05	0,001281349	GSU0165		Hypothetical protein
gene-GS_RS00850	317,4293903	-1,2194112	0,219764	-5,548725429	2,8776E-08	7,70311E-06	GSU3475		Hypothetical protein
gene-GS_RS01995	598,9300074	-1,1531511	0,255631	-4,511001271	6,45E-06	0,000871785	GSU0401	mcp40H-12	methyl-accepting chemotaxis sensory transducer, class 40H
gene-GS_RS02000	153,8108496	-1,3691432	0,24459	-5,597697047	2,17218E-08	6,29932E-06	GSU0402		hemerythrin family protein
gene-GS_RS02005	551,4664745	-0,9113275	0,247235	-3,686071697	0,000227742	0,009329382	GSU0403	cheY64H-1	response receiver CheY associated with MCPs of classes 40H and 40+24H
gene-GS_RS02180	56,69591247	1,2558837	0,384138	3,269354862	0,00107793	0,024358423	GSUR007		tRNA-Pro
gene-GS_RS02450	86961,31602	-1,009613	0,303109	-3,330859103	0,000865784	0,020793848	GSU0490	ato-1	succinyl:acetate coenzyme A transferase
gene-GS_RS02455	48627,951	1,04643079	0,338938	3,087384875	0,00201926	0,037472176	GSU0491	rhlE-1	ATP-dependent RNA helicase RhlE
gene-GS_RS02745	472,1290609	-0,9663293	0,241227	-4,00588916	6,17846E-05	0,003707077	GSU3487		Hypothetical protein
gene-GS_RS02755	3037,768357	-1,2738086	0,362969	-3,509414252	0,000449095	0,014606075	GSU0552		reverse transcriptase
gene-GS_RS02795	3179,185212	-1,1350571	0,339434	-3,34396503	0,000825901	0,020529551	GSU0561		pseudogene
gene-GS_RS02960	1340,044231	1,25231517	0,302743	4,136560307	3,52551E-05	0,002610376	GSU3489		Hypothetical protein
gene-GS_RS02965	1251,599334	1,00087584	0,318915	3,138375543	0,001698871	0,032864858	GSU0596		response receiver
gene-GS_RS02970	489,9710252	1,62113596	0,234286	6,919469397	4,53338E-12	1,57762E-09	GSU0597		Hypothetical protein
gene-GS_RS03075	9841,491215	-1,2592417	0,315772	-3,987822717	6,66825E-05	0,00380418	GSU0618	omcE	cytochrome c
gene-GS_RS03270	1331,708471	-1,2043685	0,331405	-3,634126276	0,000278924	0,010535607	GSU0655	rpoH	RNA polymerase sigma-32 factor RpoH
gene-GS_RS03375	290,634186	0,91786088	0,273716	3,353335623	0,000798438	0,020372489	GSU0677		ABC transporter, membrane protein
gene-GS_RS03620	111,5770158	0,93651026	0,202994	4,613492487	3,95959E-06	0,000626334	GSU0725		Hypothetical protein
gene-GS_RS03630	218,1566487	1,26217751	0,265928	4,746304282	2,07167E-06	0,000388618	GSU0727		lipoprotein
gene-GS_RS03790	140,1469557	-1,4279166	0,329773	-4,330004122	1,49107E-05	0,001526149	GSU3497		Hypothetical protein
gene-GS_RS03800	445,6023208	-1,1488306	0,333643	-3,443294597	0,000574673	0,017240188	GSU3500		Hypothetical protein
gene-GS_RS03820	314,4083695	-1,2602965	0,339369	-3,713645788	0,000204295	0,008829137	GSU3502		Hypothetical protein
gene-GS_RS03825	600,9317739	-1,0963048	0,320701	-3,418467239	0,000629749	0,018262721	GSU3503		Hypothetical protein
gene-GS_RS03830	562,739875	-1,0715728	0,302539	-3,541935218	0,000397203	0,013685807	GSU3504		Hypothetical protein
gene-GS_RS03915	17141,88047	0,99579908	0,223738	4,450744846	8,5573E-06	0,00106355	GSU0780	fdhD/mobA-2	formate dehydrogenase accessory protein FdhD and molybdopterin nucleotidyltransferase
gene-GS_RS03930	16567,42785	3,58379971	0,175159	20,4602273	4,87107E-93	5,65044E-90	GSU0782	hybS	periplasmically oriented, membrane-bound [NiFe]-hydrogenase small subunit
gene-GS_RS03935	11052,99159	3,24797715	0,234207	13,86795551	9,90603E-44	6,89459E-41	GSU0783	hybA	periplasmically oriented, membrane-bound [NiFe]-hydrogenase iron-sulfur cluster-binding subunit
gene-GS_RS03940	11949,95342	3,8425111	0,146148	26,29197425	2,369E-152	4,1221E-149	GSU0784	hybB	periplasmically oriented, membrane-bound [NiFe]-hydrogenase integral membrane subunit
gene-GS_RS03945	24107,95367	2,66446581	0,306202	8,701662407	3,27057E-18	1,4227E-15	GSU0785	hybL	periplasmically oriented, membrane-bound [NiFe]-hydrogenase large subunit
gene-GS_RS03950	6164,784927	3,74415171	0,129607	28,88851832	1,6643E-183	5,7917E-180	GSU0786	hybP	periplasmically oriented, membrane-bound [NiFe]-hydrogenase maturation protease
gene-GS_RS03955	1582,852463	3,67594251	0,187397	19,61577485	1,13396E-85	9,86543E-83	GSU0787	hybT	twin-arginine translocation pathway protein, TatA/TatE family
gene-GS_RS03960	1425,443108	2,81427341	0,232361	12,11165046	9,16316E-34	5,31463E-31	GSU0788		Hypothetical protein
gene-GS_RS04175	119,2891611	0,90668192	0,225275	4,024773696	5,70301E-05	0,003575252	GSU0829		efflux pump, RND family, membrane fusion protein
gene-GS_RS04820	550,5810155	-1,6218702	0,394781	-4,108277351	3,98621E-05	0,002831024	GSU0956		Hypothetical protein
gene-GS_RS04830	387,0519698	-1,0488743	0,312784	-3,3533517	0,000798392	0,020372489	GSU0959		Hypothetical protein
gene-GS_RS06255	223,0953886	-1,1137821	0,331272	-3,362137055	0,000773417	0,020372489	GSU1256		SCO family protein
gene-GS_RS06315	182,8315596	-1,0623111	0,222726	-4,769584399	1,84606E-06	0,0003779	GSU1268		helix-turn-helix transcriptional regulator, LysR family
gene-GS_RS06750	834,977733	0,94406041	0,222515	4,242689795	2,20857E-05	0,001956409	GSU0761		transposase of ISGsu7
gene-GS_RS06755	1161,39666	-1,0040095	0,321432	-3,123552665	0,001786819	0,034165547	GSU1357		Hypothetical protein
gene-GS_RS06770	4013,293655	-1,1178154	0,351882	-3,176674365	0,001489742	0,029967066	GSU1360		Sir2 superfamily protein
gene-GS_RS06780	2112,611194	-1,487822	0,313789	-4,741466552	2,12177E-06	0,000388618	GSU1362		Hypothetical protein
gene-GS_RS06785	2826,776481	-1,4405256	0,390818	-3,685925693	0,000227873	0,009329382	GSU1363		RNA-directed DNA polymerase
gene-GS_RS06880	83,641039	-0,9130551	0,238601	-3,826709375	0,000129868	0,006549849	GSU1383		3'-to-5' exonuclease
gene-GS_RS07445	37389,07038	-1,3421472	0,384998	-3,486114012	0,000490092	0,015365049	GSU1496	pilA-N	geopilin domain 1 protein
gene-GS_RS07450	33898,84079	-0,9509288	0,23639	-4,022708064	5,75328E-05	0,003575252	GSU1497	pilA-C	geopilin domain 2 protein
gene-GS_RS07505	1186,801524	-1,2426198	0,356713	-3,483525389	0,000494856	0,015375889	GSU1508		Hypothetical protein
gene-GS_RS07510	972,8350103	-1,2766511	0,351529	-3,631704869	0,000281555	0,010535607	GSU1509		glycosyltransferase
gene-GS_RS07520	1452,907225	-1,1817394	0,35953	-3,286904359	0,001012952	0,023608696	GSU1510.1		glycosyltransferase
gene-GS_RS07685	2704,240639	-1,1652346	0,353106	-3,299960427	0,000966985	0,022737206	GSU1540		lipoprotein
gene-GS_RS08220	3065,097852	-1,3452394	0,368813	-3,647480118	0,000264825	0,010472617	GSU1647		Hypothetical protein
gene-GS_RS08350	1293,901963	-1,679505	0,359782	-4,668117944	3,03971E-06	0,000503724	GSU1673		Hypothetical protein

gene-GS_RS08565	535,184006	-1,5416195	0,368739	-4,180783	2,90507E-05	0,002407058	GSU1715		Hypothetical protein
gene-GS_RS08925	1737,968067	-1,1966512	0,36421	-3,285610494	0,001017616	0,023608696	GSU1787		cytochrome c
gene-GS_RS08935	1213,36915	-1,002996	0,222529	-4,507249937	6,56733E-06	0,000871785	GSU1789		GDP-mannose--undecaprenyl-phosphate mannosyltransferase
gene-GS_RS08940	1595,210416	-1,1659466	0,379282	-3,074088148	0,00211147	0,038516202	GSUR032		tRNA-Asp
gene-GS_RS08945	4864,982967	-1,1612094	0,392572	-2,957955916	0,003096864	0,048721142	GSUR033		tRNA-Val
gene-GS_RS08950	12557,78725	-1,2898312	0,384135	-3,357758069	0,000785774	0,020372489	GSUR034		tRNA-Asp
gene-GS_RS08980	715,7795584	-1,415371	0,33169	-4,267147717	1,97988E-05	0,001862158	GSUR036		tRNA-His
gene-GS_RS08985	4361,000848	-1,4779741	0,338499	-4,366252476	1,26396E-05	0,001332907	GSUR037		tRNA-Arg
gene-GS_RS08990	369,5689623	-1,3205541	0,301388	-4,381573609	1,17825E-05	0,001281349	GSUR038		tRNA-Pro
gene-GS_RS09085	50,64941814	-1,2836546	0,393152	-3,265031327	0,00109452	0,024573733	GSUR040		tRNA-Met
gene-GS_RS09100	1530,59712	-1,1059563	0,368381	-3,002207056	0,002680298	0,044416367	GSU1814	divIC	septum formation initiator family protein
gene-GS_RS09280	1007,828996	-1,3293844	0,323152	-4,113801537	3,89196E-05	0,002821672	GSU1850		Hypothetical protein
gene-GS_RS09285	1716,435912	-1,3600809	0,361178	-3,765676589	0,000166099	0,007811127	GSU3542		teichoic acid biosynthesis glycosyltransferase
gene-GS_RS09290	2575,61899	-1,3304645	0,3557	-3,740414445	0,000183717	0,008303057	GSU1851		glycosyltransferase, WbuB-like family
gene-GS_RS09295	2384,220601	-1,5997998	0,377432	-4,238643331	2,24875E-05	0,001956409	GSU1852		membrane protein
gene-GS_RS09305	1618,26579	-1,6860378	0,358799	-4,699116753	2,61289E-06	0,000454643	GSU1853		membrane protein
gene-GS_RS09315	2103,830773	-1,0576133	0,354797	-2,980897876	0,002874046	0,046595469	GSU1855		polysaccharide chain length determinant protein
gene-GS_RS09325	1776,085577	-1,2435812	0,373554	-3,329055624	0,00087141	0,020793848	GSU3545		Hypothetical protein
gene-GS_RS09345	782,0188594	-1,8722544	0,381121	-4,912488779	8,99275E-07	0,000213746	GSUR042		tRNA-Glu
gene-GS_RS09350	3010,408682	-1,1865013	0,328363	-3,613383097	0,000302228	0,010940161	GSUR043		tRNA-Gln
gene-GS_RS10635	6618,238811	-1,1655752	0,312556	-3,729174772	0,000192108	0,008570967	GSU2119		integrative genetic element Gsu56, integrase
gene-GS_RS10640	297,4236013	-1,3559139	0,374979	-3,615968102	0,000299227	0,010940161	GSU2120	ihfA-2	integration host factor, alpha subunit
gene-GS_RS10735	938,8319416	-1,5015102	0,390745	-3,842686592	0,000121695	0,006227912	GSU3560		Hypothetical protein
gene-GS_RS10740	35,16637367	-1,502851	0,412641	-3,642031537	0,000270495	0,010535607	GSU2138		pseudogene
gene-GS_RS10800	141,3019932	-1,081694	0,344832	-3,136873492	0,001707598	0,032864858	GSU3564		Hypothetical protein
gene-GS_RS10805	138,6186845	-1,6636237	0,377584	-4,405972103	1,05311E-05	0,001262118	GSU3565		Hypothetical protein
gene-GS_RS10960	2309,350978	-0,9368066	0,219083	-4,276035993	1,9025E-05	0,001839088	GSU2183		Fic family protein
gene-GS_RS11265	2882,512522	-1,0384562	0,342173	-3,034888318	0,002406248	0,040659703	GSU2244		glycosyltransferase
gene-GS_RS11990	639,4522339	-1,0989088	0,349785	-3,141671318	0,001679865	0,032842304	GSU3574		pseudogene
gene-GS_RS12005	286,7076355	-1,6105539	0,350545	-4,594430317	4,33933E-06	0,00065656	GSU3575		Hypothetical protein
gene-GS_RS12010	551,803026	-1,3111613	0,327763	-4,000337169	6,32523E-05	0,003730813	GSU3576		lipoprotein
gene-GS_RS12015	583,4733513	-1,2295122	0,360153	-3,413865038	0,000640483	0,0183356	GSU3577		pseudogene
gene-GS_RS12025	585,973104	-1,199076	0,339229	-3,53470711	0,000408227	0,013792537	GSU3578		Hypothetical protein
gene-GS_RS12030	595,6339391	-1,4269419	0,376753	-3,787472033	0,000152188	0,00725498	GSU3579		Hypothetical protein
gene-GS_RS12035	759,5896392	-1,5223726	0,39383	-3,865561416	0,000110834	0,005843972	GSU2395		Hypothetical protein
gene-GS_RS12065	1096,425959	-0,9760364	0,305441	-3,195502371	0,001395876	0,02926295	GSU3580		lipoprotein
gene-GS_RS12185	398,6578796	1,00546785	0,223388	4,500987565	6,76385E-06	0,000871785	GSU2424		Hypothetical protein
gene-GS_RS12275	3105,682415	-1,6286514	0,39035	-4,172283241	3,01562E-05	0,002440552	GSU2469		Hypothetical protein
gene-GS_RS12410	704,9725969	-0,9202944	0,288324	-3,191871778	0,001413541	0,029381355	GSU3584		Hypothetical protein
gene-GS_RS12415	1071,336872	-1,3695722	0,376882	-3,633953875	0,000279111	0,010535607	GSU2442		RelA/SpoT domain protein
gene-GS_RS12425	130,4674378	1,52838895	0,311424	4,907740193	9,21318E-07	0,000213746	GSU2471		RNA-directed DNA polymerase and maturase, group II intron origin
gene-GS_RS12435	675,9645946	-1,2860475	0,351478	-3,658968801	0,000253232	0,010129287	GSU2473	(VapB)	antitoxin, AbrB family
gene-GS_RS12690	7178,884095	-1,0032241	0,327057	-3,067430498	0,002159077	0,038751039	GSU2526		membrane protein
gene-GS_RS12755	9452,537348	-0,9367996	0,314122	-2,982278704	0,002861114	0,046595469	GSU2539		carboxynorspermidine/carboxyspermidine dehydrogenase
gene-GS_RS13000	2079,623626	-0,9967865	0,297257	-3,353284566	0,000798586	0,020372489	GSU2588	lpdA-2	dihydrolipoamide dehydrogenase
gene-GS_RS13010	990,0603467	-1,2147332	0,381514	-3,18398059	0,001452648	0,029709792	GSU3593		Hypothetical protein
gene-GS_RS13015	561,7636644	-1,1687526	0,291553	-4,008708056	6,10518E-05	0,003707077	GSU2590		Hypothetical protein
gene-GS_RS13025	502,9603649	-1,1714276	0,378432	-3,095477489	0,001964963	0,036763822	GSU2592		Hypothetical protein
gene-GS_RS13045	894,5371388	-1,1509245	0,356083	-3,232178819	0,001228502	0,026719908	GSU3595		Hypothetical protein
gene-GS_RS13050	351,2088004	-1,0451094	0,29503	-3,542386594	0,000396524	0,013685807	GSU3596		Hypothetical protein
gene-GS_RS13055	1114,55396	-1,6226579	0,390299	-4,157478423	3,2178E-05	0,002544985	GSU2596		lipoprotein
gene-GS_RS13195	1774,689988	-1,0825186	0,340649	-3,177811034	0,001483914	0,029967066	GSU2622		ensor cyclic diguanylate phosphodiesterase, HAMP and GAF domain-containing, putative heme-binding site
gene-GS_RS13700	1226,882137	-1,2359722	0,317951	-3,887300902	0,000101365	0,005511726	GSU2727		Hypothetical protein
gene-GS_RS13755	362,4092422	-1,367777	0,317908	-4,302433839	1,68932E-05	0,001679668	GSU2741		transcriptional regulator, TetR family
gene-GS_RS13805	6607,28064	1,08103762	0,307129	3,519818677	0,000431842	0,014291658	GSU2750		Hypothetical protein
gene-GS_RS13900	2132,623706	-1,0694174	0,316878	-3,374853686	0,000738549	0,020237418	GSU2770		lipoprotein
gene-GS_RS13915	734,4623545	-1,4243308	0,352635	-4,039106133	5,36553E-05	0,003457785	GSU2773		Hypothetical protein

gene-GS_RS13920	454,2872641	-1,3021423	0,326147	-3,992505561	6,53788E-05	0,00379197	GSU2774		Hypothetical protein	
gene-GS_RS14045	12,26504858	1,1627816	0,393893	2,952021602	0,003157008	0,049046381	GSU2799	(NifB)	nitrogenase molybdenum-iron cofactor biosynthesis radical SAM domain iron-sulfur cluster-binding oxidoreductase	
gene-GS_RS14155	64,0761428	0,97402776	0,28636	3,401410477	0,000670391	0,018838725	GSU2822	gnfR	nitrogen fixation transcript antitermination response regulator, ANTAR domain-containing	
gene-GS_RS14230	8819,579496	-1,0519918	0,341426	-3,081169965	0,002061889	0,037964943	GSU3611	rpmJ	ribosomal protein L36	
gene-GS_RS14325	29991,92756	-1,044431	0,341889	-3,054879027	0,002251513	0,039269707	GSU2853	rpsS	ribosomal protein S19	
gene-GS_RS14445	7458,654452	-1,140353	0,307123	-3,71301593	0,000204804	0,008829137	GSUR052		tRNA-Thr	
gene-GS_RS14585	2525,837884	-1,0174634	0,297375	-3,42147838	0,000622817	0,018213465	GSU2899		lipoprotein cytochrome c	
gene-GS_RS14650	2839,255014	-1,0612636	0,333027	-3,186720352	0,001438958	0,029630621	GSU2912	omcO	cytochrome c	
gene-GS_RS14770	1200,261073	-1,3447886	0,358572	-3,750404941	0,000176549	0,008150046	GSU2936		Hypothetical protein	
gene-GS_RS14775	2153,397904	-1,0301092	0,285265	-3,611065797	0,000304941	0,010940161	GSU2937		cytochrome c	
gene-GS_RS15170	852,1715379	-0,9335068	0,313707	-2,975723797	0,00292298	0,047092454	GSU3019		dehydrogenase, E1 protein, alpha and beta subunits	
gene-GS_RS15175	493,9977248	-0,9662399	0,318386	-3,034810642	0,002406867	0,040659703	GSU3020		acyltransferase, left-handed parallel beta-helix (hexapeptide repeat) family, lipoyl attachment domain-containing	
gene-GS_RS15365	678,0070518	-1,0752058	0,290631	-3,699553983	0,000215979	0,009146519	GSU3060		transcriptional regulator, TetR family	
gene-GS_RS15455	10733,34998	-1,1232214	0,343555	-3,269410765	0,001077717	0,024358423	GSU3078	mraZ	cell division protein MraZ	
gene-GS_RS15970	275,6988269	-1,154603	0,382401	-3,019350079	0,002533176	0,042586732	GSU3183		Hypothetical protein	
gene-GS_RS16515	12007,98155	-1,177812	0,318585	-3,697014675	0,00021815	0,009146519	GSU3298		transcriptional regulator with cupin-like beta-barrel domain	
gene-GS_RS16730	14018,20018	-1,1363354	0,339621	-3,345896755	0,000820169	0,020529551	GSU3339	groES	chaperonin GroES	
gene-GS_RS17080	2211,34951	2,34603064	0,214338	10,94549533	6,9835E-28	3,4718E-25	GSU3409		Hypothetical protein	
gene-GS_RS17085	1005,997789	2,18933519	0,266652	8,210455175	2,20352E-16	8,52026E-14	GSU3410		Hypothetical protein	
gene-GS_RS17525	159,2335068	-1,1635042	0,310403	-3,748367243	0,00017799	0,008150046	GSU0281*		sensor histidine kinase	*GS_RS17525 only covers part of GSU0281
gene-GS_RS17570	8185,563333	-1,1667381	0,349956	-3,333954821	0,000856206	0,020793848	No annotation		Hypothetical protein	
gene-GS_RS17575	435,2365944	-1,3144595	0,357457	-3,677249783	0,000235762	0,009540143	GSU1381		Hypothetical protein	

Paper III: Manuscript in preparation.

Mathias Fessler, Qingxian Su, Marlene M. Jensen, Yifeng Zhang. Electroactivity of the magnetotactic bacteria *Magnetospirillum magneticum* and *Magnetospirillum gryphiswaldense*.

Title:

Electroactivity of the magnetotactic bacteria *Magnetospirillum magneticum* and *Magnetospirillum gryphiswaldense*

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Abstract:

Magnetotactic bacteria reside in sediments and are named after their ability to navigate via internal magnetic particles. Here, we show that two magnetotactic species, *Magnetospirillum magneticum* and *Magnetospirillum gryphiswaldense*, are electroactive. They were both able to generate current in microbial fuel cells with maximum power densities of 27 $\mu W/m^2$ and 11 $\mu W/m^2$, respectively. In the presence of the electron shuttle resazurin both species could also reduce Fe_2O_3, an iron oxide that is abundant in the environment. In addition, *M. magneticum* also displayed reduction of the iron oxide FeOOH. The work presented here adds *M. magneticum* and *M. gryphiswaldense* to the growing list of known electroactive bacteria, and implies that electroactivity might be common for magnetotactic bacteria.

Keywords: Magnetotactic bacteria, *Magnetospirillum magneticum*, *Magnetospirillum gryphiswaldense*, extracellular electron transfer, microbial fuel cells.

1. Introduction:

The growing interest to replace fossil fuels with renewable energies has increased the development of sustainable biotechnological processes. Microbial fuel cells (MFCs) are transducing devices where microorganisms use an anode as an electron acceptor for their anaerobic respiration of organic material, converting chemical energy of organic compounds into electrical energy [1]. Although the feasibility of MFCs for electric energy production requires significant improvement, MFCs have been successfully applied to treat wastewater and remove toxic compounds [2,3]. Numerous of microorganisms are specialized in extracellular electron transfer (EET), i.e., electron transfer reactions beyond the cell surface, which is central to the function of MFCs [1]. The generation of current in MFCs depends on electroactive microorganisms (EAM) that use their unique ability to export electrons to solid-state electron acceptors, such as iron oxides [4] or electrodes [1].

Bacteria from the *Geobacteraceae* and *Shewanellaceae* families are to date the most used in MFCs because of their well-documented EET mechanisms and ability to generate high power densities [1,5–7]. The deduction of different EET mechanisms mainly derive from studies with different wild type strains of *Geobacter* or *Shewanella* species, e.g. *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR-1 [4,7]. During direct long-range EET, often found in *Geobacter* species, cells establish physical contact with the solid electron acceptor trough electrically conductive nanowires extending from the cell surface [5,8,9]. On the other hand, *Shewanella* species usually rely on direct cell-electrode contact [10]. Common for both types of EET are conductive proteins, such as outer-membrane multiheme *c*-type cytochromes and conductive pili. *Shewanella spp.* are also capable of indirect EET where electron transfer is mediated by flavins, which are self-produced diffusible redox-active molecules [11]. In fact, it is possible to mimic this EET strategy artificially and enhance current output of MFCs by adding synthetic redox mediators such as resazurin, AQDS, neutral red and humic acid to the reactor medium [12–14].

Bacteria with electroactive properties are not only limited to metal-reducers in the *Geobacter* and *Shewanella* genera and are not confined to specific environments either [1,15,16]. Due to practical reasons, the search for new EAM is often done in pure cultures with strains previously isolated on liquid media. However, the maintenance of EAM in laboratory cultures with soluble electron acceptors may lead to a decrease or even loss of EET capability over time, why it has been suggested to isolate new electroactive strains with *in situ* electrodes [17]. Regardless of isolation strategy, identifying and mapping EET mechanisms in bacteria outside the *Geobacter* and *Shewanella* genera will potentially broaden our understanding of this phenomenon. Discovery of novel electroactive genera can clarify how

representative the different EET mechanisms are, lead to identification of new electron transfer pathways, and potentially increase the success of microbial electrochemical technologies.

A group of microorganisms, collectively referred to as magnetotactic bacteria (MTB), contain intracellular magnetic nano-crystal particles, which function as a biological compass that allows MTB to migrate along redox gradients according to the Earth's geomagnetic field [18]. The so-called magnetosomes are membrane-enclosed vesicles containing iron oxide and/or iron sulfide in the form of magnetite (Fe_3O_4) or greigite (Fe_3S_4), respectively [19]. In order to synthesize magnetosomes, MTB sequester soluble iron [20] from the surroundings and, therefore, they play an essential role in global iron cycling [21,22]. Magnetite biomineralization relies on precipitation of soluble Fe^{2+} and Fe^{3+}, however, the exact mechanism for uptake of extracellular iron to magnetite formation remains unclear, even though a number of genes, such as *mamB*, *mamM* and *nirS*, are known to be important [20].

Magnetotactic bacteria are a phylogenetically, morphologically and metabolically diverse group of prokaryotes, and many remain uncultured [23]. In this study, *Magnetospirillum magneticum* strain AMB-1 and *Magnetospirillum gryphiswaldense* strain MSR-1 were selected as model MTB to explore, for the first time, their potential for electroactive behavior. Both bacteria were isolated from freshwater sediments and are Gram-negative affiliated with the *Alphaproteobacteria* [24,25]. *M. magneticum* has previously been found to generate electricity by electromagnetic induction [26]. *M. magneticum* can convert mechanical energy into electrical energy by pumping the MTB or purified magnetosomes through a solenoid, by applying Faraday's law of electromagnetic induction. The heterotrophic and facultative anaerobic lifestyle of *M. magneticum* and *M. gryphiswaldense* and their strong dependence on iron for the production of their magnetosome led us to examine their potential for EET to electrodes and solid iron oxides. Hence, the purpose of the study presented here was to screen two magnetotactic bacteria to determine if MTB are electroactive. In addition, we investigated their ability to reduce two types of iron oxides and to use electron mediators during the reduction of hematite.

2. Materials and methods:

2.1. Strains and medium

The strain *Magnetospirillum magneticum* AMB-1 was kindly supplied by of Dennis Bazylinski, UNLV, Las Vegas. *Magnetospirillum gryphiswaldense* MSR-1 was purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Both species were cultivated in *Magnetospirillum* medium unless otherwise stated in the different experimental setups described below. The growth medium contained 0.68 g/liter KH_2PO_4, 0.85 g/liter sodium succinate · 6 H_2O, 0.575 g/liter sodium tartrate · 2 H_2O, 0.05 g/liter sodium acetate, 0.17 g/liter $NaNO_3$, 0.11 g/liter NH_4Cl, 0.1 g/liter yeast extract, 2 ml/liter ferric quinate solution ($FeCl_3$ · 6 H_2O 4.5 g/liter, quinic acid 1.9 g/liter), and 5 ml/liter Wolfe's mineral solution (DSMZ recipe no. 141). The media was flushed with N_2 and pH adjusted to 6.75 prior to autoclavation at 121°C for 20 minutes.

For the growth experiments the medium described above was used, and cultures were inoculated from fresh overnight cultures. Growth was quantified by measuring optical density at 600 nm. Cultures were grown in sealed serum bottles containing 25 ml of growth medium with a 35 ml headspace. Oxygen was added to a final concentration of 1% through a 0.22 µm filter.

2.2. MFC design and operation

The electroactive potential of the two *Magnetospirillum* strains was tested in sterile H-shaped MFCs. Each MFC reactor was assembled by using two 250 ml glass bottles as two half cells. The half cells were separated by a cation exchange membrane. Carbon brushes (The Mill-Rose Company, Ohio, US) were pretreated at 450°C for 30 minutes and subsequently used as anodes [27]. The surface area of the anodes were 1204 cm^2 [27]. The cathode was a 4 cm by 4 cm titanium mesh connected to a titanium wire. Both the anode and cathode were placed in the center of each half cell. The assembled MFCs without anolyte and catholyte were sterilized by autoclavation at 121°C for 20 minutes. The anode chambers of the MFCs were filled with sterile growth medium as described above in section with $NaNO_3$ omitted. The catholyte was a solution of 50 mM phosphate buffer (pH 7) containing 50 mM $K_3[Fe(CN)_6]$. The anode chamber was closed with a gas-tight butyl rubber stopper, whilst the cathode was left open for aeration. The anolyte was flushed with N_2 through a 0.22 µm filter to ensure anoxic conditions. The MFCs were operated at 30°C in batch mode and inoculated from fresh overnight serum bottle cultures. The cells were concentrated by centrifugation and the cell suspension was diluted to OD_{600} = 3.2. 1 ml cell suspension was used as inoculum. The cathode and anode were connected with a 1000 Ω resistor and voltage was recorded automatically every 30 minutes. Duplicate reactors were run in parallel.

2.3. Iron reduction and electron mediators

To test the growth of the two *Magnetospirillum* strains with different types of iron oxides as terminal electron acceptors in serum bottle cultures, $NaNO_3$ and O_2 in the media was replaced by either 25 mM FeOOH or 25 mM hematite (Fe_2O_3, Sigma-Aldrich, nanopowder, <50 nm particle size). FeOOH was made as described previously by neutralizing a $FeCl_3$ solution with NaOH until the pH reached 7 [28]. The solution was washed before being added to the medium. In experiments with electron mediators (AQDS, humic acid, neutral red, resazurin) they were added to a final concentration of 5 µM. The cultures were incubated for ~30 days in the dark at 25°C. For analysis of reduced iron, 200 µl samples were taken from the serum bottles with added FeOOH at regular intervals. The samples were added directly into 200 µl of 1M HCl. The samples were incubated in the dark at 25°C for 24 hours and Fe^{2+} concentrations were measured. For Fe_2O_3, 400 µl culture was mixed with 800 µl 5 M HCl. Tubes were rotated for 2 days to dissolve Fe_2O_3 prior to quantification of Fe^{2+}. The Fe^{2+} concentrations were determined by using ferrozine. Briefly, 10 µl sample was mixed with 75 µl ferrozine solution (2 g/liter ferrozine, 5 ml/liter 5 M HCl) and 75 µl acetate buffer (285 g/liter sodium acetate, 116 ml/liter acetic acid) in a 96-well plate, followed by measuring absorbance at 562 nm with a plate reader (BioTek Synergy Mx).

2.4. Statistical testing

Statistical significance was determined by comparing to the given control cultures with a one-tailed t-test assuming unequal variance. The threshold for significance was a *p*-value below 0.05.

3. Results and discussion

3.1. Current generation in MFCs

As mentioned above *M. magneticum* AMB-1 as well as magnetosomes purified from this strain can convert mechanical energy to electrical energy by applying Faraday's law of electromagnetic induction [26], which is why we turned our attention towards magnetotactic bacteria in the search for new EAM. To begin with, we assessed *M. magneticum* AMB-1's ability to convert chemical energy to electrical energy in a MFC. In our MFC setup, *M. magneticum* was grown in the anode chamber, where the only available electron acceptor was the electrode. Therefore, *M. magneticum* could only proliferate in these reactors by exporting electrons generated during cell metabolism over the cell membrane to the electrode. In the reactors, *M. magneticum* generated current immediately upon inoculation (Figure 1A). The current continued to increase until it peaked after approximately 30 days at 27 $\mu W/m^2$. The other magnetotactic strain tested here, *Magnetospirillum gryphiswaldense* MSR-1, was also able to generate current in the MFCs, even though the current was slightly lower and the acclimatization period was substantially longer (Figure 1A). *M. gryphiswaldense* might not be as strong of an electroactive as *M. magneticum*, as seen by the lower and slower current output, however, part of the explanation for the observed difference in current may also be due to the fact that *M. gryphiswaldense* in general has a higher doubling time than *M. magneticum*, as seen by the growth in MTB medium with $NaNO_3$ as the electron acceptor (Figure 1B). Under these conditions the doubling times for *M. magneticum* and *M. gryphiswaldense* were 3.97 hours (±0.14, n = 4) and 5.67 hours (±0.18, n = 4), respectively. In addition, the medium does not permit equal cell density of the two species. *M. gryphiswaldense* does not reach the same cell density as *M. magneticum* (Figure 1B), which most likely also contributes to the lower power density of *M. gryphiswaldense*. Nevertheless, both *Magnetospirrilum* species showed electroactive properties, suggesting that electroactivity might be common for MTB, however, investigation of MTB of other genera than *Magnetospirillum* is required to support this.

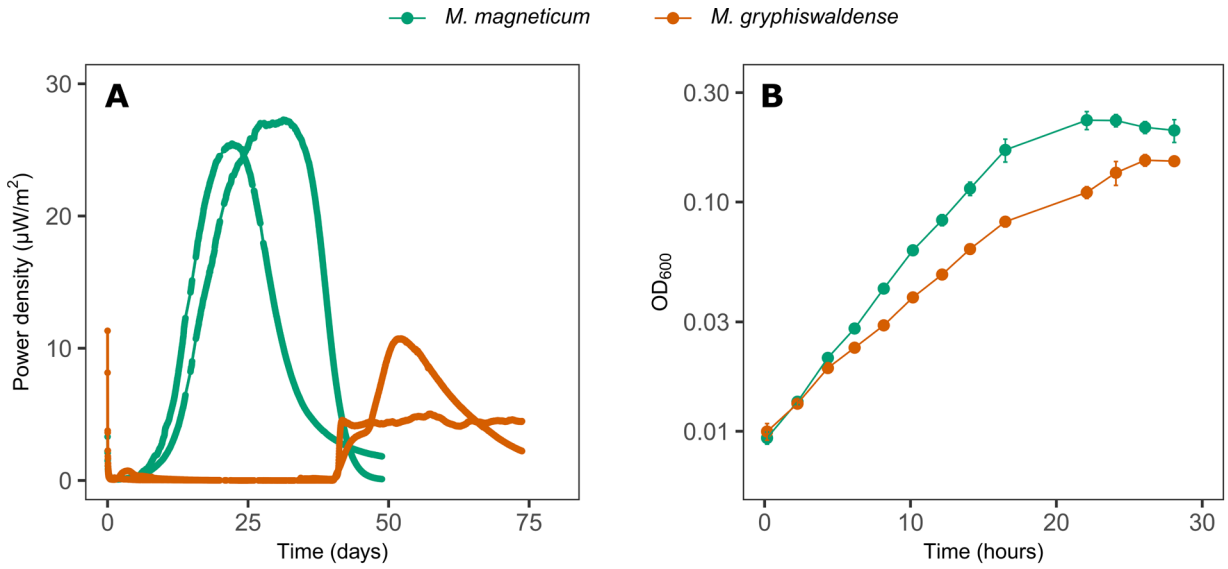

Figure 1. Power density of *M. magneticum* and *M. gryphiswaldense* in microbial fuel cells (**A**, n = 2), and growth in serum bottles with $NaNO_3$ and 1% O_2 (**B**, n = 4). Error bars show standard deviation.

The maximum power densities reached here by *M. magneticum* and *M. gryphiswaldense* were 27 μW/m^2 and 11 μW/m^2, respectively, which compared to other electroactive species is quite low [1]. However, it is important to note that the purpose of the study presented here is to identify electroactive bacteria within the group of MTB, why the MFCs have not yet been optimized to yield the highest possible power density. This is important to consider when comparing to the power densities of electroactive species that have already been extensively studied and used in microbial reactors [1]. In the future, to reach the full electroactive potential of MTB, factors such as the medium composition, cell density, and electrode material must be examined. For instance, it might also be possible to reach higher current output by taking advantage of the unique property of MTB by using magnetic electrodes.

3.2. Reduction of naturally occurring iron oxides

Having established that both MTB strains were electroactive, it was important to determine if this property was restricted to electrodes in MFCs or if it could also be applied for reduction of other external electron acceptors. For this purpose two different electron acceptors were used: Fe_2O_3 (hematite) and β-FeOOH (akaganeite). Fe_2O_3 is one of the most abundant iron oxides in natural environments [29], and it is therefore likely that *M. magneticum* and *M. gryphiswaldense* encounter this mineral in nature. However, Fe_2O_3 has a relatively low reduction potential [30], meaning that it requires adequate electron export mechanisms to sustain microbial growth. FeOOH, on the other hand, is less abundant in nature but more suitable for microbial reduction as it has a higher reduction potential [31]. Neither of the two MTB were

able to reduce Fe_2O_3 (Figure 2A). On the contrary, *M. magneticum* was able to reduce FeOOH (Figure 2A). The Fe^{2+} concentration in the *M. magneticum* cultures continued to increase until 26 days after inoculation, and after this the Fe^{2+} levels remained stable. At day 26 the Fe^{2+} concentration was 22 mg/L compared to 14 mg/L in the uninoculated control, a difference that was statistically significant ($P < 0.05$, day 52). Minimal FeOOH reduction was observed in the *M. gryphiswaldense* cultures (Figure 2B) ($P < 0.05$, day 52). This is consistent with the initial observations from the MFCs indicating only weak electroactive properties, since the iron(III) reduction of *M. magneticum* was limited to FeOOH, the most readily reduced iron oxide of the two tested, and here iron(III) reduction was still relatively poor compared to *Geobacter sulfurreducens* [31]. In line with the first assessment of relative EET strength among the two MTB, FeOOH reduction was very limited and Fe_2O_3 reduction was completely absent in *M. gryphiswaldense*. Collectively, this suggests that MTB participate in the natural iron cycle both by internalizing soluble ferrous and ferric iron for magnetosome synthesis [21] and by reducing insoluble iron oxides extracellularly.

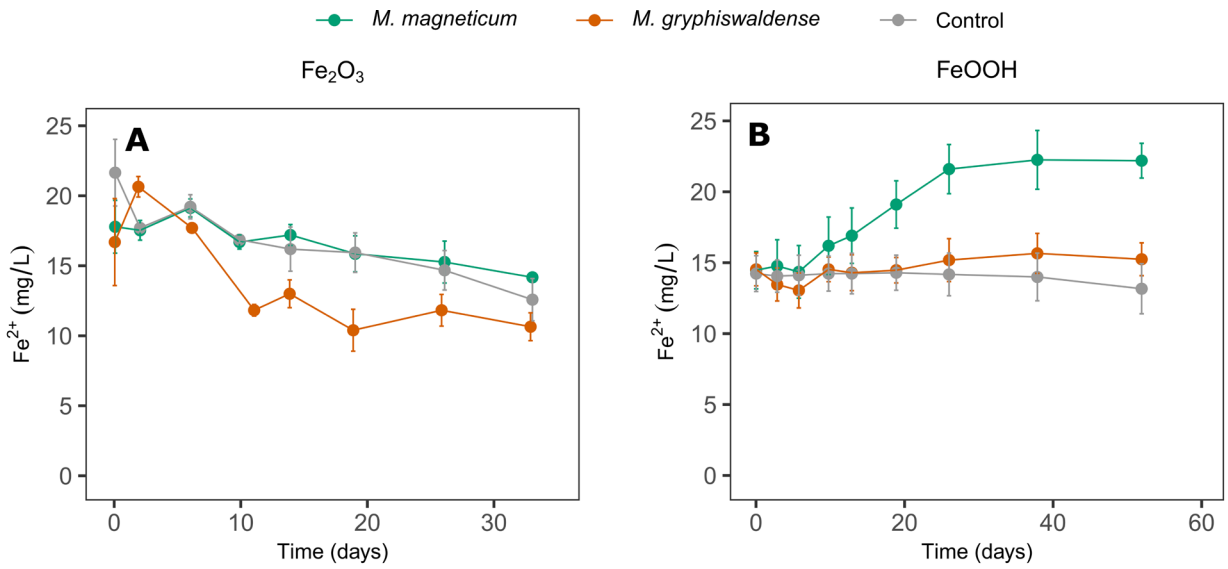

Figure 2. Reduction of Fe_2O_3 (**A**, n = 3) and FeOOH (**B**, n = 3) by *M. magneticum* and *M. gryphiswaldense*. Error bars show standard deviation and the controls are uninoculated medium.

3.3. Fe_2O_3 reduction mediated by electron shuttles

In natural environments it is common for microbes to produce electron shuttles to mediate extracellular electron transfer [32]. Electron shuttles can transfer electrons between the bacterial cells and the extracellular electron acceptor, thus allowing an alternative electron pathway that does not require direct

reduction or contact with the extracellular acceptor. Once in the oxidized state, after reduction of e.g., iron oxides, they can be reused. Therefore, as soon as these shuttles are present in the extracellular environment in their oxidized state they are available for use by the surrounding bacteria. This means that species that do not necessarily synthesize shuttles themselves may still be able to use this electron pathway since the shuttles are not restricted to the bacteria that synthesize and secrete them [32]. To test if electron shuttles could enhance iron(III) reduction by *M. magneticum* and *M. gryphiswaldense*, they were grown with Fe_2O_3 and four different electron shuttles: resazurin, humic acid, neutral red, and anthraquinone-2,6-disulfonate (AQDS) [12,33,34]. Fe_2O_3 was chosen over FeOOH due to its higher abundance in nature. Increased iron(III) reduction was observed in the cultures with added resazurin for both MTBs, whilst the effect of the remaining three shuttles was negligible (Figure 3). This is consistent with the previous findings that not all shuttles enhance EET in *Shewanella* [35]. Iron reduction in *M. gryphiswaldense* increased until day 6 reaching a Fe^{2+} concentration of 25 mg/l, and after this the Fe^{2+} levels stayed rather constant. On the other hand, the Fe^{2+} concentration in the *M. magneticum* culture increased continuously throughout the experiment, ending at 40 mg/l after 33 days. At the end of the experiment both MTB cultures with resazurin were significantly different from the control culture ($P < 0.05$, for both species on day 33). Even though iron oxide reduction is still rather limited compared to stronger EAM such as *Geobacter spp.* and *Shewanella spp.*, these results suggest that MTB are also able to reduce Fe_2O_3 in sediments, given that appropriate electron shuttles are present.

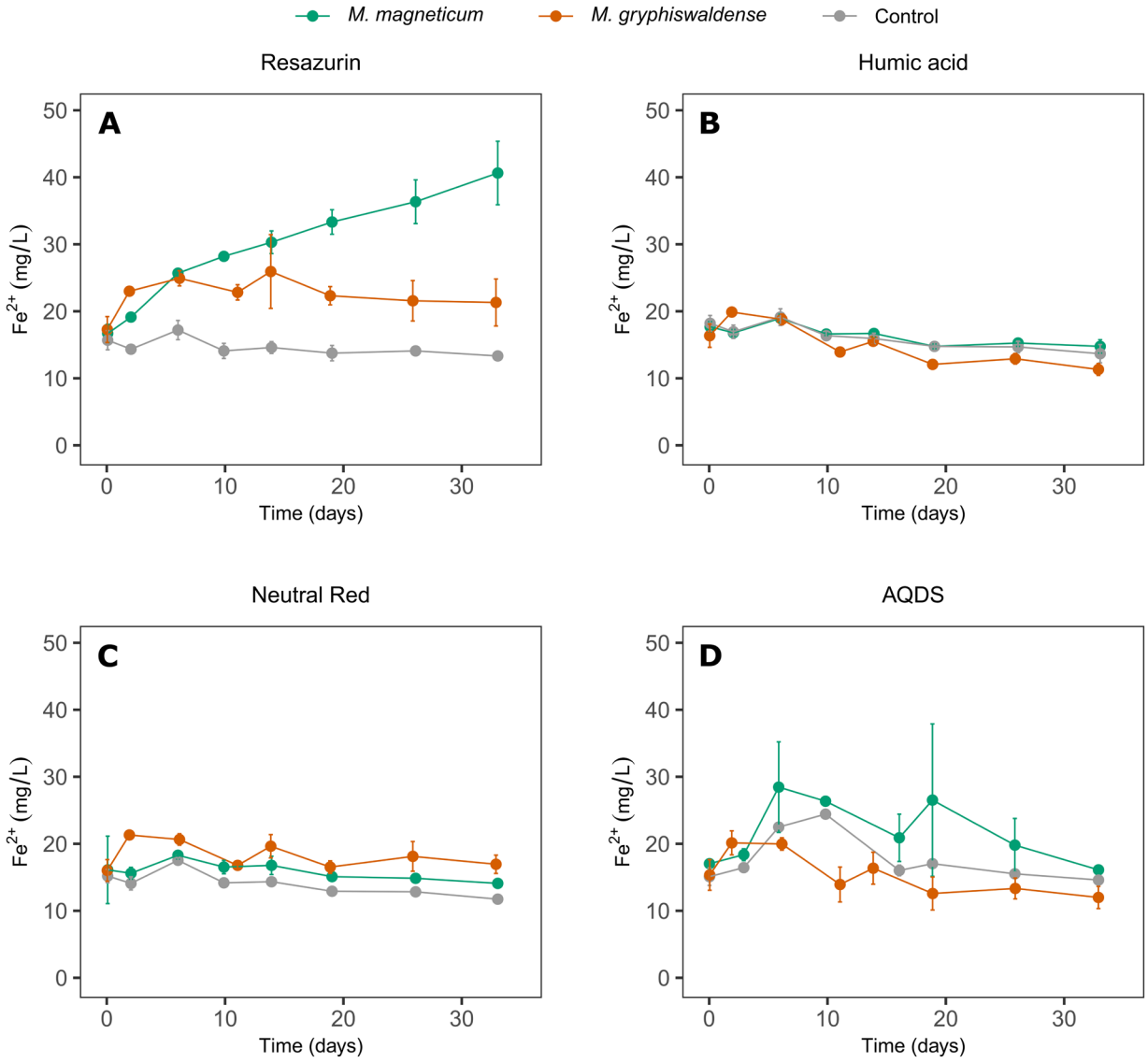

Figure 3. Fe_2O_3 reduction by *M. magneticum* and *M. gryphiswaldense* in cultures with resazurin (**A**, n = 3), humic acid (**B**, n = 3), neutral red (**C**, n = 3), and AQDS (**D**, n = 3). Error bars show standard deviation and the controls are uninoculated medium.

3.4. Relevance and future perspectives

The results presented here show that MTB possess electroactive properties. Of the two species tested here, *M. magneticum* displayed stronger ability to reduce extracellular electron acceptors, even though they were both relatively weak compared to bacteria from other genera [1]. In order to properly assess the electroactivity of *M. magneticum* in relation to other EAM, however, it is necessary to optimize MFC cultivation conditions for MTB. In the setup presented here, the power density is quite low (Figure 1A), but at the same time, so is the cell density (Figure 1B). Therefore, the full potential of the large surface area of the anode is most likely not utilized in the setup presented here, which may make the two MTB

seem weaker than they are in reality, in regards to electroactivity. Higher cell densities are key to achieve this, which e.g., a continuous input of medium will allow. In addition, it seems that *M. magneticum*'s ability to reduce electrodes in MFCs cannot be applied to all external electron acceptors. Despite their natural ability to internalize and utilize soluble iron for magnetosomes synthesis, *M. magneticum* was poor at reducing insoluble Fe_2O_3 to sustain growth, when we supplied this as the only terminal electron acceptor. On the other hand, *M. magneticum* was able to reduce FeOOH, and in the presence of electron shuttles it could also reduce Fe_2O_3.

Until now, MTB have not been identified in mixed species reactor biofilms. However, they might still be present since bacteria in these mixed biofilms are not always identified to species level and in some cases not even to genus level. Even though the power densities of the two MTB used here are insufficient for these species to dominate in mixed species reactors, other factors such as oxygen concentration may give MTB a relative advantage. Oxygen availability is not uniform across microbial biofilms. The inner biofilm is often anoxic, whilst the outer biofilms is oxic, with a gradient existing between the two [36]. As opposed to *Geobacter spp.*, *M. magneticum* and *M. gryphiswaldense* can still grow in the presence of oxygen, why they can inhabit parts of the biofilm that anaerobic EAM such as *Geobacter spp.* cannot. In other words, thriving in an electrode-respiring biofilm does not simply depend on relative strength of electroactivity as external factors such as oxygen concentration also contribute to shaping the bacterial community, which can allow the proliferation of weaker EAM.

Maximum current output is one aspect that is important to investigate moving forward, identifying the genes involved is another. Genes of electron export pathways in numerous EAM including *Geobacter sulfurreducens* and *Shewanella oneidensis* are already known, and in order to place MTB on this spectrum it is essential to identify the genes used. This will add to the existing knowledge within the field, and indicate how well the different electron export pathways are represented in nature. Despite the ability of purified magnetosomes and *M. magneticum* to convert mechanical energy to electricity [26], it is not obvious that magnetosomes are involved in EET at this point, since *M. magneticum* and *M. gryphiswaldense* show different performance in the fuel cells and iron(III) reduction assays. If magnetosomes are implicated in electron export, more similar current and iron(III) reduction profiles would be expected. However, minor changes in amino acid sequence may have substantial impact on EET, which, for instance, is the case in *G. sulfurreducens* [37]. Therefore it is not possible to exclude magnetosome involvement either. For clarification of this, further examination of the EET mechanism of MTB is required.

4. Conclusion

Two magnetotactic bacteria were shown to be electroactive, as they could both produce current in MFCs and reduce insoluble iron oxides. Electroactivity, which has not previously been demonstrated in bacteria from this group, adds further to the uniqueness of these species. Moving forward optimization of reactor design and elucidation of the electron export pathway will broaden the understanding of both magnetotactic and electroactive bacteria.

Acknowledgements

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