Weak electricigens: a closer look at the underdogs

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Abstract

Electroactivity appears to be a phylogenetically diverse trait independent of cell wall classification, with both Gram-negative and Gram-positive electricigens reported. While numerous electricigens have been observed, the majority of research focuses on a select group of highly electroactive species. Under favorable conditions, many microorganisms can be considered electroactive, either through their own mechanisms or exogenously-added mediators, producing a weak current. Such microbes should not be dismissed based on their modest electroactivity. Rather, they may be key to understanding what drives extracellular electron transfer in response to transient limitations of electron acceptors or donor, with implications for the study of certain pathogens. Due to their low electroactivity, such populations are difficult to grow in bioelectrochemical systems and characterise with electrochemistry. Here, we provide a critical review of recent research on weak electricigens, their relevance to microbial ecology and bioelectrochemical systems, and an assessment of the methods commonly used to observe them.

Keywords: Electricigens, Biofilms, Bioelectrochemistry, Extracellular Electron Transfer, Microbial Fuel Cells

1. Introduction

Microorganisms capable of electronically interacting with conductive surfaces have been described by various names; electroactive microorganisms, exoelectrogens, electric bacteria and electricigens, as they will be referred here (Koch & Harnisch, 2016; Logan, 2009; Lovley, 2006; Nealson, 2017). Electricigens are defined by their ability to carry out extracellular electron transfer (EET). As the name hints, EET involves the movement of electrons between the internal cellular environment and a conductive solid beyond the cell boundary, which can act as either an electron acceptor or donor. The conductive solid usually takes the form of either an electrode, as is common in the laboratory, or a metal, which typifies the natural environment that has driven the evolution of this survival strategy (Lovley, 2008). EET can be viewed as a survival strategy employed when soluble electron acceptors or donors are not available at sufficient concentrations, enabling biomass synthesis and/or cellular maintenance to proceed (Hernandez & Newman, 2001). EET can occur under both anodic conditions (the electrode/metal is reduced) and cathodic conditions (the electrode/metal is oxidised). Most studies refer to anodic EET, as this mode is common amongst electricigens and is easily observed in short-term experiments. Conversely, cathodic EET was only reported after the discovery of anodic EET (Clauwaert et al., 2007), is much less frequent, and is characterised by slow kinetics (Yang et al., 2015).

Both anodic and cathodic EET are facilitated by a number of specific mechanisms (Schroder et al., 2015), with electricigens typically employing one or more at a time. Focusing on the anodic flow of electrons from the interior to the exterior of the cell: direct electron transfer (DET) is the movement of electrons across outer-membrane proteins, typically c-type cytochromes, to a solid external electron acceptor (Okamoto et al., 2011). A related mechanism is the conduction of electrons across pilus-like appendages termed nanowires, which extend from the cell membrane and close the distance between the cell and the external electron acceptor (Gorby et al., 2006). Finally, the last described mechanism is mediated electron transfer (MET), whereby the cell secretes soluble redox shuttles that carry electrons to a nearby acceptor and diffuse back to the cell upon oxidation, facilitating a revolving door strategy of electron transport (Marsili et al., 2008; von Canstein et al., 2008; Wang et al., 2010). Recent research shows that the lines distinguishing these three modes of EET are somewhat fuzzy, as intermediate strategies such as adsorption of redox mediators to the cell surface (Okamoto et al., 2013) or immobilisation of mediators in the biofilm matrix (Xiao & Zhao, 2017) are also possible. While these are the main modes of EET described to date, it is important to note that these findings may not be exhaustive, and could be a consequence of the narrow range of electricigens identified, with a recent review identifying 94 species described to date (Koch & Harnisch, 2016). Several reports suggest that the electricigens are much more abundant in nature (Cournet et al., 2010) and are present in most ecosystems (Chabert et al., 2015). In spite of this, the majority of in-depth bioelectrochemical studies still focus on strong electricigens present in niche environments, with little mechanistic insight available for the majority of alternative electricigens discovered. In this review, the recent developments in electricigen enrichment and characterization are summarised, with particular regard to weakly electroactive populations and communities, who may find application in bioprocesses, biosensors and bioremediation; a path related to, but distinct from, the traditional power-generation envisioned for strong electricigens.

2. Strong vs. weak electricigens

A previous review (Doyle & Marsili, 2015) identified enrichment conditions as a possible reason for the relative lack of diversity seen in microbes capable of EET. The authors would here like to further build upon that discussion by suggesting that screening protocols may also play a large part in underestimating electricigen abundance in a culture, as strains producing a low current may quickly be ruled out. From an applied perspective, this elitist approach to quantifying electroactivity makes sense; the more efficient the microorganism at converting organic matter to current, the more interesting it may prove to the researcher or engineer keen to develop microbial fuel cells (MFCs). However, this approach limits observation of what can be termed weak electricigens; microbes engaging in EET on a smaller scale. There is not yet an agreed definition of weak electricigens. Figure 1 outlines the key physiological differences between microorganisms that lay on the extreme ends of the current-producing spectrum. Operatively, we can classify a microorganism as a weak electricigen if it produces a small current or has low coulombic efficiency (CE). As current production and CE depend on a multitude of parameters, such as electrode size, carbon source and the extent an anaerobic environment is maintained, the authors have opted not to provide specific cut-off numbers which separate the strong from the weak. Instead, it is sufficient to say a weak electricigen will produce a significantly smaller current (at least 10-fold) than its strong counterparts, *Geobacter* and/or *Shewanella*, when tested in the same bioelectrochemical system. As such, observation of weak electricigens often requires a more specific set of electrochemical techniques, discussed in Section 5.1. Weak electricigens are not specialised for EET, but are capable of carrying out EET in a very specific set of environmental conditions, such as limitation of their preferred electron acceptor (e.g., oxygen, nitrate, or other soluble acceptors).

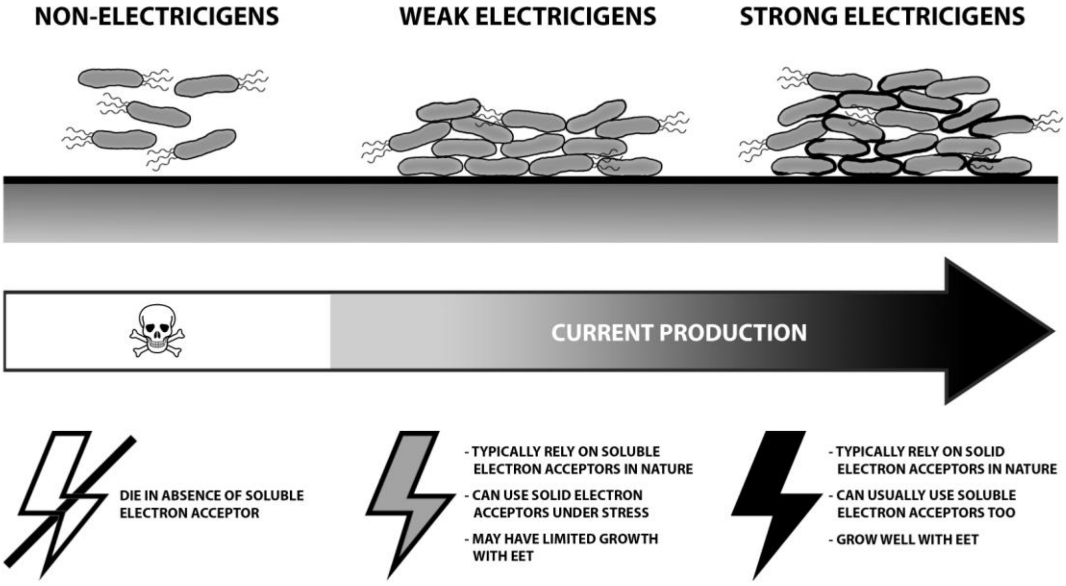

Figure 1. Graphical summary of the key physiological differences between weak and strong electricigens. Image credit: Kulanan Phanviroj.

Many of the strongest electricigens are strict anaerobes that can use oxidised metals as their sole electron acceptor indefinitely (Caccavo et al., 1994; Lovley et al., 1993). Such conditions are often accompanied by slow growth rates that could pose challenges in real-world applications, e.g., generating sufficient biomass to provide a time-sensitive signal in a biosensor. This may not be a limitation of weak electricigens, who are by definition not as specialised and therefore likely to grow more easily in a range of different environments. As an extension of this, weak electricigens may be able to compensate for a specific metabolic shortcoming of a strong electricigen, enabling a more stable bioprocess in the context of a mixed community.

It is important to note that not all strong electricigens are fastidious; electrogenic members of *Shewanella* are facultatively anaerobic with a diverse metabolism (Ong et al., 2014) and amenable to genetic manipulation on the lab bench (Coursolle & Gralnick, 2012). Furthermore, as the deep ocean serves as a common habitat to this genus, psychrophilic (Zhao et al., 2006), thermophilic (Ghosh et al., 2003) and piezophilic (Toffin et al., 2004) strains have been reported, indicating broader adaptation than *Geobacter*. Nonetheless, weak electricigens likely have an even broader metabolic diversity and, despite their small current output, warrant further investigation in order to develop novel EET-based devices. In pure culture, industrial applications using this niche of weak electricigens may still be challenging due to the inherent inefficiency of their electron transfer. However, in the absence of a strong counterpart, they may prove very useful.

It must be noted that there is always a trade-off by generalists; the weak electricigen may well be a “jack of all trades”, but might not be considered a master of any, and therefore will likely not thrive while forced to carry out EET. Indeed, one possibility worth exploring is that certain weak electricigens engaging in EET may merely be able to survive, and not divide, during these conditions, enabling cell maintenance but not biomass production.

3. The role of weak electricigens in mixed microbial communities and potential applications

In terms of the role of weak electricigens in nature, where microbes typically exist as part of a multi-species community, it can be hypothesised that weak electricigens may engage in oxygen scavenging, which maintains an anaerobic environment for strong electricigens, in addition to performing complex substrate degradation, thus providing simple sugars for strong electricigens to oxidise further. Such metabolic interactions have been previously demonstrated (Bourdakos et al., 2014; Zhang et al., 2013). Once these duties have been performed, the weak electricigen may find itself without oxygen and engage its modest EET abilities in order to survive. Such quick adaptability may enable a weak electricigen to be co-cultured with a strong electricigen in the context of operating a biosensor, e.g. *E. coli* maintaining an anaerobic environment for *Geobacter*, who would otherwise succumb to an oxygen-induced death.

Biotechnological applications are more likely to be possible using mixed communities where both strong and weak electricigens are present. For example, a mixed microbial community successfully degraded cellulose in a sediment microbial fuel cell (Sajana et al., 2014). Contrary to what was initially demonstrated, the use of a strong electroactive community does not provide any advantage in terms of long-term power production (Ewing et al., 2017). It is likely that a more balanced weak electroactive community will give higher power and more stability with time, particularly for field applications.

Potential applications unrelated to bioelectricity production have been scarcely reported. The concept of electrical-aided white biotechnology was first defined by Harnisch et al. (2015). Recently, Moscoviz et al. (2017) demonstrated that both microbial community structure and metabolic patterns were affected when a mixed fermentative community was exposed to cathodic conditions. Application of a negative redox potential changed the fermentation products of *Clostridium acetobutylicum*, resulting in higher butanol production from fructose-based feedstock (Chen et al., 2017). Similar observations have also been reported for wastewater, where application of a positive redox potential caused a shift of the microbial community to methanogenic bacteria and higher methane production (Guo et al., 2017). Understanding the microbial ecology of weak electricigens in electro-fermentation and other reactions will allow control of the final product(s) of such processes, with broader possibilities when viewed from the vantage point of synthetic biology (Song et al., 2014).

Industrial applications and bioremediation are often linked to extreme environments, such as mining wastewater low in pH, with preliminary studies using weak electricigens yielding promising results (Ni et al., 2016). As a consequence of strong electricigens' high level of adaptedness to conditions that are conducive to EET, they may not prove tolerant in such environments. For example, cytochrome conformation in *Geobacter,* an obligate anaerobe, is pH-sensitive (Morgado et al., 2017). Whereas the acidophile *Acidithiobacillus* successfully produced electricity from tetrathionate at low pH (Sulonen et al., 2016). However, only a small portion of the electron flow was collected at the electrode, demonstrating that low CE is a typical trait of weak electricigens.

Other potential applications include biorecovery of metals from electronic waste (Villares, 2017), antibiotic biosensors (Ward et al., 2014) viability sensors (Doyle et al., 2017; Selim et al., 2017) and even the need for extremophilic weak electricigens, i.e. those resilient to extremes of pressure, temperature, or pH, as reviewed by Dopson et al. (2016).

4. Discovering weak electricigens

Strong electricigens are usually found in sediments, particularly those that are nutrient-limited, rich in metals, and/or anaerobic. However, weak electricigens may not occupy a specific ecological niche, and their presence could be more broadly distributed across nature, with EET becoming a viable, though less energetically favourable, mode of respiration in response to stressful conditions where soluble electron acceptors (or, theoretically, donors) are limited. Possible candidate weak electricigens include aerobic species, pathogens, and even commensals inhabiting the human gut where iron concentrations can be locally high (Abbaspour et al., 2014).

As many enrichment studies utilising acetate or lactate as electron donor and carbon source have returned a high abundance of *Geobacter* and *Shewanella*, respectively, the hunt for novel weak electricigens may necessitate alternative medium compositions. Since high power outputs will not be a reasonable expectation from a weak electricigen pure culture, investigations could focus on answering why such microbes turn to this mode of respiration at all, in addition to detection of novel mechanisms of EET which may differ from those employed by strong electricigens.

4.1 Screening of existing pure cultures for EET activity

Rather than isolating electricigens from scratch, a novel approach can be to test existing microbial pure cultures for electrogenic abilities. Should EET be detected in these species, it is likely to fall under the umbrella of weak electroactivity, and may be indicative of a survival strategy utilised by the microbe in response to a limitation that occurs occasionally in its natural environment. Examples of such unexpected electricigens are discussed in Section 6. During such screening procedures, it is important to modify the species' preferred growth conditions so that growth is encouraged but theoretically limited in some way, e.g. soluble electron acceptor removed (or donor, if checking for cathodic EET) and a poised electrode introduced instead. In terms of preparing candidate lists, one example could be to consider which species are associated with metal-rich environments or are resistant to oxidative stress. Such criteria may describe certain pathogens, e.g., those associated with hemochromatosis patients who have high internal iron concentrations (Khan et al., 2007).

4.2 Enrichment of weak electricigens

In addition to screening existing pure cultures, novel isolates can be obtained through standard enrichment experiments. While the MFC design is a reactor configuration possible for such experiments, these devices are optimised for power output from electricigens. Since this, by definition, will not be a viable application for weak electricigens, they will not be discussed in this review. Instead, the recommendation is potentiostat-controlled set-ups capable of detecting small current outputs reliably; a design that has demonstrated reliable results (Pierra et al., 2015). Electrochemical cells consist of a three-electrode set-up where the working electrode is maintained at a controlled potential, measured against a reference electrode, and balanced by a counter electrode (Bard & Faulkner, 2001). The working electrode can be poised at either anodic or cathodic potentials, enabling observation of both directions of EET.

In terms of the working electrode dimensions, macroelectrodes e.g., 1 x 1 cm carbon felt, provide a large surface area for bacterial attachment, enabling sufficient biomass for DNA and RNA extraction post-experiment (Doyle et al., 2017). The large surface area can also be useful in collecting current, however any variations in electrode size will reduce the repeatability of results due to the inherently small current output. Furthermore, such large electrodes have drawbacks when it comes to certain electrochemical techniques, as discussed in Section 5.

Another option is mini-electrodes, such as screen-printed electrodes (SPE), whose working electrodes are a few millimeters in diameter. Advantages of SPE include reliability and small diffusional limitations, which favour the development of a thin, conductive biofilm (Sismaet et al., 2016; Ward et al., 2014) However, this technology is cumbersome for characterisation of anaerobic microorganisms, as the small volume of the sample favors oxygen diffusion, in addition to low biomass yields available for subsequent genetic analysis. Therefore, a combination of both macroelectrodes and SPE may be the optimum design for a study.

5. Characterisation of weak electricigens

Electrochemical characterisation of weak electricigens differs from that of strong electricigens, as the main measurable output (current) is small and poorly repeatable in many experimental set-ups. Furthermore, with the notable exception of *Pseudomonas aeruginosa* (Wang et al., 2013), the concentration of microbially-secreted redox-active species in weak electricigens can be extremely low, if present at all. This leads to low biofilm conductivity, making direct biofilm electrochemistry challenging.

One might be tempted to adopt large electrodes and high surface area materials, like carbon felt or nanotube-coated graphene, to increase current output in a bioelectrochemical device, hoping to improve direct electrochemistry. However, while these materials do maximise current output for weak electricigens, they lead to high capacitive currents during voltammetry, which hides the signature of redox-active species, increases electrochemical noise and lowers repeatability. Thick biofilms are also not desirable when impedance experiments are performed, as the specific conductivity of weak electricigen biofilm is too low to be measured (Kato Marcus et al., 2007). Therefore, to electrochemically characterise weak electricigens it is best to opt for high sensitivity electrochemical methods on small electrodes, which minimise diffusional limitations.

In contrast with the study of strong electricigens, characterisation of weak electricigens often requires addition of redox mediators and other wiring agents, in addition to modified electrode surfaces to detect small currents and to detect the accumulation of non-conductive biofilms. Careful consideration should be given to the biological or chemical degradation of these redox mediators, particularly in long-term experiments.

In addition to the electrochemical analyses, the most informative studies will also consist of metabolic or genetic data, in order to deepen our understanding of fundamental mechanisms of EET. Experiments with single species biofilms do not pose particular challenges. However, current output of complex microbial communities depends on the metabolic network and on interspecies electron transfer (Villano et al., 2010). Amperometry and chronocoulometry of weak electricigen consortia can be effectively performed together with metagenomics, metatranscriptomics and proteomics, whose results can clarify the role of individual species in the overall current output. In these experiments, particular attention should be used to standardise the volume reactor/electrode surface area ratio and the fluid dynamics, as changes in these parameters result in differing contribution to the EET by the planktonic biomass rather than the biomass in the biofilm. In this section, a summary of some of the techniques at the disposal of the bioelectrochemist is provided.

5.1 Electrochemical methods

5.1.1 Amperometry and chronocoulometry

Direct measurement of current output remains the principal method to determine electroactivity with time, due to its low cost and amenability to high throughput. Given the low current density of weak electricigens, measurement of charge (i.e. the integration of the current output; a feature typically available in standard electrochemical software) may be more informative (Kim et al., 2013; Wang et al., 2010).

Repeatability of the electrochemical signal is of paramount importance, as it enables statistical analysis to detect meaningful differences. Such analysis is extremely important when working with weak electricigens due to the low signal. The required repeatability can be obtained with small electrodes fabricated with standard methods, such as lithographic films, vapour-deposited films, SPE and associated modifications.

5.1.2 EIS with and without redox probe

Electrochemical impedance spectroscopy of weak electricigens can be very informative, as it allows detection of biofilm formation and other interfaces through the monitoring of interfacial capacitance. In fact, capacitance is unrelated to electroactivity, as recently demonstrated (Babauta & Beyenal, 2014). EIS is largely considered a non-destructive technique, particularly for low applied potentials, thus it is the ideal tool to monitor biofilms of weak electricigens in long-term experiments. EIS is particularly effective when the electricigen biofilm produces redox mediators or avails of exogenously-added redox mediators for the EET process. In this case, the change in biofilm conductivity can be tracked back to a change in metabolic activity (Paredes et al., 2014) or biofilm matrix composition (van Duuren et al., 2017).

EIS returns an average impedance of the whole surface. Since weak electricigen biofilm communities are likely heterogeneous at the μm scale, local EIS might be preferable to determine the microstructure of biofilms in relation to their electroactivity (Moreira et al., 2014). However, local EIS is labour-intensive, thus may not be suitable for routine characterisation of biofilms. EIS can be integrated with other microscopy and spectroscopy methods to provide a more accurate picture of the local electroactivity in biofilms. While little research has been carried out in this direction, it is likely that a higher resolution method will produce detailed knowledge of EET in weak electricigen biofilms.

For very weak electricigens, the non-conductive biofilm appears as a large capacitive element, as is typical for non-conductive polymer films. Additional information on the formation of weak electricigen biofilms can be obtained with a strong redox probe, such as KFeCN6 (Zhang et al., 2014b). The redox probe diffuses to the electrode only in regions free from biofilm, so the process of biofilm formation or removal can be monitored through the attenuation or increase of the redox probe signal with respect to the control.

5.1.3 Cottrell analysis

The analysis of thin *Geobacter* biofilms through the Cottrell equation has been recently proposed (Zhang et al., 2017). However, it is not clear if this method will work also with weak electricigens or if the Cottrell analysis offers additional insight with respect to EIS. Experimentation with such under-explored electrochemical approaches may improve existing analysis of weak electricigens.

5.1.4 Why voltammetry often does not work

Direct voltammetry of weak electricigens is often not very informative, as these microorganisms tend to lack a clear voltammetric signature, either under turnover or non-turnover conditions. There are several examples of weak electricigen voltammetry in earlier studies (Park et al., 2001), but it has mainly been used for qualitative purposes (e.g., showing some electroactivity) rather than for the quantification of electroactive species (which are often unknown) involved in the EET process.

5.2 Genetic analysis

Genetic analysis of weak electricigens can be performed on both single species populations and mixed-species communities extracted from an electrode (Kouzuma et al., 2013). However, it is important to note that in the latter case (metagenomics), the abundance of weak electricigens may be low, with electrodes dominated by strong electricigens such as *Geobacter* and weak electricigens such as *Enterobacter* and *Aeromonas* present but undetected (Doyle et al., 2017). Therefore, optimum insight may be gathered by sequencing weak electricigens after they have been isolated in pure culture.

5.2.1 Genomics

In previous years, sequencing technologies often came with prohibitive price tags and lengthy sample preparation steps. However, the development of new platforms such as the relatively low-cost and compact MinION sequencer from Oxford Nanopore, which can fit into the palm of the hand and attach directly to a portable laptop, have marked a new era in genetic sequencing (Ashton et al., 2015; Judge et al., 2015). Usable both on the lab bench and in the field, sequencing was recently carried out using the device aboard the International Space Station. However, due to larger error rates when compared to other available platforms (Laver et al., 2015; Mikheyev & Tin, 2014), researchers interested in single nucleotide polymorphisms or phylogenetic analysis based on the 16s rRNA gene alone will need to select their method of sequencing with care. Regardless of sequencing method chosen, assembly of novel electricigen genomes and deposition in online genome repositories will enhance our understanding of the abundance and mechanisms of such microbes.

5.2.2 Transcriptomics

While genomics provides all of the genes associated with an electricigen, only transcriptomics, which profiles RNA expression, can reveal which genes are upregulated or downregulated during EET. By switching from a soluble to a solid electron acceptor, and profiling RNA before and after, a list of candidate genes suspected of EET involvement can be generated, with similar studies previously conducted (Ishii et al., 2015; Ishii et al., 2013). Such an approach is likely to enhance our understanding of the specific mechanisms associated with weak electroactivity, e.g. an increase in expression of genes associated with a soluble electron shuttle in support of mediated electron transfer.

5.2.3 Mutants to identify mechanism

Once a weak electricigen has been identified, either via enrichment or through screening of existing microbes, establishing a genetic system can be mechanistically informative (Bretschger et al., 2007; Kim et al., 2008). Gene knockouts can empirically demonstrate which cellular components or products play a role in electroactivity and can be cross-referenced with the list of suspected candidates generated through transcriptomics.

5.2.4 Other considerations

Due to the low signal output and conductivity, electrochemical characterisation of weak electricigens must be integrated with other methods borrowed from biofilm microbiology, such as spectroscopy, confocal laser scanning microscopy with metabolic/redox stains, atomic force microscopy and metabolomics (Franks et al., 2010; Reguera et al., 2005; Song et al., 2016). Surface-enhanced Raman scattering was recently proposed for rapid identification of electricigens in small volumes (Wang et al., 2016). The details of the Raman spectrum allow, in principle, to identify strong electricigens from non-electricigens. However, the applicability of this method to weak electricigens has not been tested. Non-invasive characterisation of strong electricigens has been achieved through confocal Raman spectroscopy (Virdis et al., 2012). However, these and other spectroscopic methods do not measure the electoractivity directly, thus may be more useful for detailed characterisation of known (strong) electricigens.

Outside of a controlled laboratory environment, weak electricigens might occur as dense and thick microbial mat. CLSM and other microscopy methods are often limited to the analysis of thin biofilms (100 µm). Thus, special methods must be developed to determine the electrochemical characteristics and the 3D structure of these biological formations (Santini et al., 2015).

Finally, the duration of experiments with weak electricigens must be considered carefully. While it is true that long-term experiments increase cumulative charge output, thus facilitating analysis of the EET processes, such experiments result in a dynamic and complex biofilm, with poor repeatability, as is well-known for MFCs (Larrosa et al., 2009). The adoption of specially designed characterisation methods in short-term experiments increases experimental throughput while decreasing the likelihood of these errors.

6. Model weak electricigens

While electroactivity has been observed in both Gram-positive and Gram-negative microbes, the thick, non-conductive membrane of Gram-positives makes them generally much less electroactive than Gram negatives. This was demonstrated early on by Matsunaga and Nakajima (1985) where the application of the same (high) redox potential resulted in a higher current for the

Gram-negative than for the Gram-positive. As a result of a thicker membrane, it is likely that Gram-positive bacteria show DET rather than MET (Modestra & Mohan, 2014).

However, electroactivity also depends on biofilm formation and the presence of extracellular redox mediators, eventually immobilised in the biofilm. Here some of the recent work on weak electricigens, both Gram-negative and Gram-positive, is discussed with particular regard to studies that have provide mechanistic information about the EET process occurring.

6.1 Pseudomonas aeruginosa

Since the discovery of phenazines and their role in EET, *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen also common in the environment (Alhede et al., 2014), has become a model weak electricigen. This species is often used as biofilm-forming model organism for its pathogenicity and antimicrobial resistance (Blair et al., 2015). *P. aeruginosa* is capable of anodic EET in MFCs (Rabaey et al., 2005). The most investigated EET mechanism avails of phenazines, redox–active pyrazines with high antimicrobial activity. *P. aeruginosa* produces several phenazines at high concentrations (μM) (Wang et al., 2013). Phenazines such as pyocyanin are produced mostly during aerobic growth, thus providing a simple strategy for maximising the current output with alternating aerobic/anaerobic conditions (Yong et al., 2017). Furthermore, phenazine production is affected by redox potential (Seviour et al., 2015) and often controlled by quorum sensing (Cabeen, 2014)

The current output when *P. aeruginosa* is grown as biofilms in potentiostat-controlled electrochemical cells is low, approximately 10 μA/cm^2. The current output is very sensitive to mutations that affect the biofilm matrix, as the matrix determines the retention of phenazines, and hence the MET rate (Qiao et al., 2017)

Pseudomonas biofilms formation can be monitored with several electrochemical methods. Among them, EIS provides sensitive results for initial biofilms, where the increase of the double layer capacitance and diffusion element with time corresponds to the accumulation of cells in the planktonic and biofilm phases (Piasecki et al., 2013). Impedance measurement enables non-mucoids and wild type to be distinguished from each other, as the non-mucoid strains shows a more conductive phase angle (Ward et al., 2014). While it is certainly true that the impedance spectrum is different across various mutants of *P. aeruginosa*, it is necessary to design experiments that normalise for phenazine production, as the latter change broadly across mutants (Wang et al., 2013) and phenazine redox state depends on the oxidation potential of the electrode at which biofilm is grown (Seviour et al., 2015). After such normalisations, the unicity of EIS *P. aeruginosa* spectra with respect to other non-electricigens present in biomedical samples may even facilitate identification of the species in a polymicrobial sample. However, the electrochemical signature of *P. aeruginosa* is similar to that of other weak electricigens. Thus, impedance analysis could be coupled with a species-specific separation method (e.g., immunomagnetic beads) to allow precise identification of the weak electicigen (Varshney & Li, 2009).

It has been shown that redox cycling of phenazines in *P. aeruginosa* enhances its anaerobic survival (Glasser et al., 2014), thus providing a possible ecological explanation for weak electroactivity in mixed microbial communities. Considering that phenazines are often toxic to other components of the microbial community, this mechanism might result in increasing *P. aeruginosa* abundance and increased likelihood of infection.

The weak electroactivity of *P. aeruginosa* can be used for sensing purposes, similarly to what has been already reported for strong electricigens. For example, production of phenazines in *P. aeruginosa*, specifically pyocyanin, was used to determine the toxicity of water containing the contaminant 3, 5-dichlorophenol (Yu et al., 2017).

Electrochemical analysis can be used also to determine the susceptibility of *P. aeruginosa* to antibiotics. Webster et al. (2015) showed that production of pyocyanin in a thin biofilm grown on SPE was greatly reduced in the presence of antibiotics. While the applicability of similar methods to mixed microbial consortia must be demonstrated, it is likely that electrochemistry can help improve common methods for the determination of antibiotic resistance in clinical settings.

6.2 Bacillus

Members of the *Bacillus* genus are Gram-positive, spore-forming bacteria which can thrive in many environments due to their broad physiological abilities in addition to playing a role in pathogensis (Turnbull, 1996). The weak electroactivity of the has been repeatedly reported. *Bacillus thuringiensis* DRR-

1 from cow rumen produced a small potential and current when cultivated in an MFC with unspecified rich medium (Jothinathan & Wilson, 2017). *B. cereus* was also cultivated on an MFC anode and produced a high current output (Islam et al., 2017). In both studies, the potential was not controlled or measured, as is typical for MFCs. However, the cyclic voltammogram of *B. cereus* shows a strong peak, either due to the bacterial biomass or to the reduced carbon compounds in the growth medium. *B. subtilis* in an MFC with glucose as carbon and energy source and 2,4-dichlorophenol as pollutant produced a significant current output(Hassan et al., 2016). Such recent studies demonstrate that *Bacillus* spp. can perform EET in an MFC. However, accurate experiments in potentiostat-controlled electrochemical cells are needed to understand the mechanism and ecological relevance of EET in the *Bacillus* genus. The few studies available demonstrate a high potential for EET, but the relevance of biofilms in EET has not been assessed. *Bacillus* spp. may also play another role in co-culture bioelectrochemical devices. A recent study using a co-culture of *Shewanella* and *Bacillus* showed that *Bacillus* sp. RH33 produces a high amount of flavins but cannot use them effectively for EET. However, when co-cultured with *Shewanella oneidensis* MR-1, the power output increased significantly (Liu et al., 2017). Similar observations were made by Wu et al. (2014), who reported another flavin-secreting *Bacillus* capable of EET. On the other hand, a flavin-utilising *B. megaterium* has been recently reported, showing the versatility of riboflavin as biocompatible redox mediator (You et al., 2018)

6.3 Enterococcus

Enterococci are Gram-positive bacteria that frequently inhabit the gastrointestinal tract and can be pathogenic. Some are metabolically versatile and especially resistant to redox stress (Djorić & Kristich, 2015) which is a typical trait of electricigens. *E. faecalis* has been shown to produce electricity in an MFC when artificially supplied with redox mediators (Zhang et al., 2014a). A relative of *E. gallinarum* was demonstrated to use iron as an electron acceptor but the impact on growth was not determined (Kim et al., 2005). More recently, *E. faecalis* was wired to an electrode maintained at oxidative potential with an Os-redox polymer, showing a sustainable current production (Pankratova et al., 2017). Such findings indicate that the genus is an interesting candidate for the screening protocols outlined in Section 4.1.

6.4 Other Gram-positives

For the sake of simplicity, the studies investigating EET in Gram-positive bacteria can be divided into three groups: a) Gram-positives that avail of soluble redox mediators produced by other bacteria to carry out EET; b) Gram-positives that produce their own mediators or can achieve DET; c) Gram-positives capable of MET through exogenous mediators, either present in the environment or added in the laboroatory.

Although group a) is less interesting in view of its application, is relevant in terms of microbial ecology. In fact, mediators produced by a given microorganism can be used by other strains, present as an interspecies collaboration. Notable examples include *Brevibacillus* sp. PTH1, which was found capable of MET in an MFC when in the presence of microbially-produced phenazines from *P. aeruginosa* (Pham et al., 2008).

Group b) includes true weak electricigens such as *Lactococcus lactis*, which secretes a soluble quinone to achieve EET in MFCs (Freguia et al., 2009). *Micrococcus luteus* was one of the first Gram-positives found capable of cathodic EET without mediators, likely using membrane redox compounds (Cournet et al., 2010). Another strain capable of anodic EET was *Thermincola ferriacetic*a, which likely transfers electrons through the biofilm without the need for soluble redox mediators (Marshall & May, 2009). In a later study, Wrighton et al. (2011) found that *Thermincola potens* was capable of direct extracellular reduction of iron with c-type cytochromes playing a role in this process, with multiheme c-type cytochromes later implicated (Carlson et al., 2012).

Group c) includes many potential electricigens, and in certain cases the inherent biological benefit of the EET capabilities are not clear. One example of electroactivity in the presence of exogenously-added mediators was seen when a strain of *Corynebacterium* procued a current at an MFC anode when supplemented with the artificial redox mediator AQDS (Liu et al., 2010). On the other hand, certain species can use Fe(III) as an electron sink and redox *mediato if other electron acceptors are depleted. A Clostridium strain related to C. butyricum* could derive a current from glucose in the presence of amorphous ferric oxyhydroxide (Park et al., 2001). This EET mechanism is particularly interesting for its biogeochemical implications, as Fe(III) is a very common chemical species in the environment.

7. Outlook

Further diversity to explore - The recent research reviewed here demonstrates that electricigen diversity is probably higher than what has been discovered to date. In fact, it is likely that many species avail of EET to increase their energy transfer and chance of survival under challenging environmental conditions. Specific experiments can be designed to screen existing microbes for electrogenic abilities in addition to enriching novel weak electricigens, particularly from extreme environments.

Developing experimental methods - Conventional MFC-based set-ups cannot be applied to the analysis of weak electricigen biofilms because of the low current output. Methods common in surface analysis and redox film interface should be adapted to biofilms to gaininsight into the EET process and mechanisms. Integration with molecular biology and systems biology to create artificial electricigen biofilms will play a key role in this research.

Metagenomics and metatranscriptomics – An increase of sequencing technologies applied to this area will help resolve whether novel EET mechanisms exist for weak electricigens. Additionally, the role of weak electricigens in supporting strong electricigens in mixed-species bioprocesses can be clarified

Relevance of weak electricigens in nature – While the role of strong electricigens in biogeochemical processes has been thoroughly explored, particularly in deep subsurface environments, the relevance of weak electricigens has not yet been assessed yet. It is likely that these microorganisms contribute to metal cycling in aerobic and surface environments, and may also play a role in a medical context.

Applications of weak electricigens – Current applications include include bioremediation, biosensors, and laboratory models to test novel antimicrobial agents. However, applications to white biotechnology are very promising, as applied redox potential changes the outcome of fermentation processes.

8. Conclusions

Here, weak electricigens have been reviewed, with an emphasis on their ability to enhance our understanding of extracellular electron transfer. A case was made to look beyond traditional power generation and to consider electroactivity from the vantage point of the microorganism in order to clarify what drives evolution of this trait amongst microbes that may frequently have other modes of respiration at their disposal.

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