

Chapter 18

Electrodes as Electron Acceptors, and the Bacteria Who Love Them

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When Faraday wanted to explain the forces of nature, he chose the example of a candle. The disappearance of paraffin and production of water was visible in any laboratory or lecture hall (Faraday 1988). However, when the topic turned to electricity, Faraday questioned his ability to provide a tangible example, saying, “I wonder whether we shall be too deep to-day or not”. Electron movement clearly is a less obvious phenomenon, something that occurs invisibly between molecules, within the candle flame, or at the moment a glass rod is rubbed with wool (Faraday 2000).

Microbial metabolism also conceals from view the electrical flow that powers living systems. We measure the products of electron trafficking; gas evolution, accumulation of reduced metals, the generation of heat. But because electrons must be carefully passed tiny distances, between proteins and redox centers within the confines of an insulating membrane, gaining direct access to this flow seems unlikely. Despite these barriers, it has recently become common to place electrodes in anaerobic environments, and collect a current of electrons at the expense of microbial activity (Reimers et al. 2001; Tender et al. 2002, 2008; Liu et al. 2004; Logan 2005), or monitor electrons flowing out of bacteria using electrodes as respiratory electron acceptors (Bretschger et al. 2007; Marsili et al. 2008a, b; Richter et al. 2009). These demonstrations show that a window into life’s electron flow exists.

The fact that some bacteria can direct electrons far beyond the cell surface elicits ideas for energy generation, bioremediation, and sensing, which all draw from the use of an electrode as one half of a bioelectrochemical reaction. The challenges related to harnessing bacteria for fuel-cell like devices have been detailed in multiple reviews (Logan and Regan 2006; Du et al. 2007; Rabaey et al. 2007; Schroder 2007; Lovley 2008a, b), and the field has even produced its first text (Logan 2008). But, at the heart of all of these technologies are the bacteria, who evolved this ability for other purposes, such as reduction of metals or redox-active compounds in

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the environment. This chapter will discuss what respiration to a seemingly artificial electrode, and this tangible electron flow, can tell us about organisms active in biogeochemical cycling.

Is an Electrode a Defined Habitat?

Electrodes, especially as they are currently used in the cultivation of microorganisms, are not all the same. The surface chemistry of graphitic, glassy, or other carbon materials can differ widely, roughness can influence available surface area (McCreery 2008), and three dimensional structures (flat versus fibrous) can affect diffusion. For example, the electrodes used for microbial fuel cell research can be ammonia-treated carbon brushes (Cheng and Logan 2007; Logan et al. 2007), gold (Richter et al. 2008), carbon cloth (Nevin et al. 2008), stainless steel (Dumas et al. 2008), or carbon-coated titanium (Biffinger et al. 2008). The electrochemical potential of these surfaces to accept electrons can be precisely controlled (via a potentiostat), or allowed to drift (as in a fuel cell). These variables are somewhat analogous to the challenges in comparing data between laboratories studying metal oxide respiration, in terms of how surface charge, surface area, passivation-influenced reactivity, and accessibility of pore spaces can alter outcomes (Roden and Urrutia 2002; Roden 2006).

Another set of variables that affect measurements of microbial activity lie in the device used to house the electrode. As negative charge moves into the electrode and travels via a wire to a counter electrode (the cathode), positive charge must as quickly migrate this same distance, but through the biofilm and electrolyte (Torres et al. 2008a, b). This is again where porosity and three-dimensional effects can alter the environment, and the resistance imposed between the two electrodes can lead to incorrect interpretations of bacterial capability.

A demonstration of this effect was a set of comparisons by Liang et al. (2007), who inoculated reactors containing identical electrodes, but arranged in three different configurations that impacted charge equilibration between electrodes (commonly quantified as “internal resistance”). The rate these electrodes could collect current from bacteria varied more than 20-fold, yet the bacterial inoculum and conditions were otherwise identical. Dewan et al. (2008), illustrated this under even more controlled conditions. Simply changing the configuration of electrodes (such as the ratio of electrode surface areas), dramatically altered how a pure culture would perform. From these examples, one can see how confusion may arise, in terms of comparing bacterial abilities; there are cited examples of “power output” by bacteria in fuel cells that vary over 100-fold (as high as 5 W/m² of electrode to as low as 0.03 W/m²) (Liang et al. 2007; Dewan et al. 2008; Zuo et al. 2008a; Yi et al. 2009). This likely reflects differences in internal resistance or electrode configuration of the devices, rather than isolation of bacteria capable of strikingly different electron transport rates.

Thus, the “electrode” is not a fixed or defined environment, but an electron acceptor that can vary widely in surface charge, porosity, and electron acceptor potential, which is incubated like any other electron acceptor in a medium controlled for salinity, microaerobic versus strictly anaerobic conditions, mixing, and other factors. The diversity of possible electrode-based experiments, largely conducted in fuel-cell like devices, has led to isolation of a wider variety of organisms known to direct electrons beyond their outer surface, compared to experiments with Fe(III) as the electron acceptor. In addition, as researchers have focused their attention on controlling the electrode environment more precisely, specific abilities related to extracellular electron transfer have become more apparent.

What Bacteria Can Use Electrodes as Electron Acceptors?

A long list of organisms have demonstrated a qualitative ability to produce electrical current, although the factors described above discourage direct comparison. Even *E. coli* (Zhang et al. 2008b), and yeast (Prasad et al. 2007), have been persuaded to produce some measure of current at electrodes, although these observations are typically linked to cell lysis (and release of redox-active compounds) or evolution under laboratory pressure. In fact, before metal-reducing bacteria were discovered, microbial-electrode research largely focused on fermentative growth of organisms such as *Proteus* (Kim et al. 2000), or *Bacillus* (Choi et al. 2001), which could divert a small percentage of their metabolism to reduction of soluble redox-active mediators, which could then be oxidized by electrodes. These observations do not necessarily mean that organisms such as *E. coli* (Zhang et al. 2008b), yeast (Prasad et al. 2007), *Clostridia* (Park et al. 2001; Prasad et al. 2006), and *Klebsiella* (Zhang et al. 2008a), are using extracellular electron acceptors such as Fe(III) in their environment, but it may indicate a competitive advantage exists from disposal of a small number of redox equivalents under anaerobic conditions.

A second way to approach this question is 16S-based rDNA surveys of electrodes used to enrich for bacteria under various conditions (Lee et al. 2003; Pham et al. 2003; Back et al. 2004; Holmes et al. 2004a, b; Kim et al. 2004; Phung et al. 2004; Logan et al. 2005; Jong et al. 2006; Kim et al. 2006, 2007; Jung and Regan 2007; Catal et al. 2008; Ishii et al. 2008a, b; Mathis et al. 2008; Park et al. 2008; Wrighton et al. 2008; Zuo et al. 2008b). It should be straightforward to ask who survives or is enriched in such devices, and use this to create a list of putative electrode-reducing bacteria. However, in many of these studies, oxygen leakage into the electrode chamber from the cathode can support growth of a subpopulation of aerobic heterotrophs, partial flux of electrons through the anaerobic food chain to methanogenesis may support a normal anaerobic community, and substrates used to enrich the bacteria may be partially fermentable (e.g. glucose, lactate, ethanol), supporting a mixed community of fermentative and respiratory organisms. Thus, it is not surprising that such surveys find a general enrichment of multiple low

G+C gram positive fermentative and syntrophic organisms, many *Proteobacteria* with known metal-reduction and respiratory abilities, and *Bacteriodes* commonly found in anaerobic habitats.

Sifting through these observations, however, a few trends can be noted. Sequences related to *Geobacteraceae* are commonly enriched when simple fatty acids (such as acetate) are used as the electron donor, especially when the medium is buffered with CO₂ (Bond et al. 2002; Holmes et al. 2004a, b; Jung and Regan 2007; Chae et al. 2008; Ha et al. 2008; Ishii et al. 2008c). Such findings are consistent with enrichments that repeatedly obtain *Geobacter* using acetate as the electron donor and Fe(III) as the electron acceptor (Lovley et al. 1993; Coates et al. 1996; Straub et al. 1998; Snoeyenbos-West et al. 2000; Bond et al. 2002; Kostka et al. 2002; Nevin et al. 2005), and with the fact that *Geobacter* requires CO₂ to build C3 metabolites from acetyl-CoA, and synthesize amino acids (Mahadevan et al. 2006; Tang et al. 2007; Sun et al. 2009). Thus, the metal-reduction machinery of *Geobacter* appears well-suited to also competing for electrodes as insoluble electron acceptors, especially when other strategies are not available.

Perhaps more interesting are cases where *Geobacteraceae* are not enriched on electrodes, even when similar surfaces and inocula have been used. Or, even when *Geobacter* is present, certain genera seem to be reported in multiple 16S rDNA libraries, indicating that they are able to take advantage of the electrode in some way. Examples include communities where *Pseudomonas* spp. (Rabaey et al. 2004), *Gammaproteobacteria* (Rabaey et al. 2004, 2005), *Rhizobiales* (Ishii et al. 2008b), *Azoarcus* and *Dechloromonas* (Kim et al. 2007), and Firmicutes (Jung and Regan 2007; Mathis et al. 2008; Wrighton et al. 2008) have either dominated or represented significant percentages of the electrode-attached population. In many of these cases, follow-up studies have isolated new pure cultures with electrode-reducing capabilities. These findings suggest that passing electrons to the outer surface may confer an advantage, even in nitrate-reducing or fermentative habitats, and that use of an electrode as bait allows us to catch organisms that would otherwise be missed when using metal reduction as the sole selective pressure.

Examples of isolates that support this idea include *Rhodopseudomonas palustris* DX-1 (Xing et al. 2008), *Dechlorospirillum* VDY (Thrash et al. 2007), *Ochrobactrum anthropi* YZ-1 (Zuo et al. 2008b) the prosthecae-like strain Mfc52 (Kodama and Watanabe 2008), and the thermophillic Gram-positive *Thermincola* sp. strain JR (Wrighton et al. 2008). Electron transfer sustained by these organisms to electrodes appears to be robust, both in terms of overall rates, and in terms of their ability to form biofilms. In addition, as most were obtained using the electrode as the enrichment tool, these isolates were truly competitive for the electrode as a primary source of energy generation. As most fundamental information regarding electron transfer to external acceptors is based on organisms such as *Geobacter* and *Shewanella*, further data showing the molecular basis and physiological role of extracellular electron transfer in these isolates may shed new light on the ecological role of this widespread ability.

What Does It Take to Use an Electrode?

When growth on an electrode is being described, it is important to consider the events taking place. Electrode reduction requires oxidation of a substrate (often in multiple steps), transfer of electrons across membranes, surface attachment, possible secretion of mediators, and, in the case of biofilms, cell-cell contact. Key questions that arise in the study of electron transfer to metals, or electrodes, involve which steps are rate-limiting, and how each step responds to changing in driving force (as either substrate concentration or voltage). Figure 18.1 shows a simplified view of these interactions.

For all cells, the rate of oxidation will be sensitive to the supply and concentration of donor, but only the first layer of electrode-interacting cells mediates electron transfer. If this basal layer of cells can pass electrons to the electrode directly, there will be a relationship between the electrode potential and the rate electrons traverse their final hop from proteins to the electrode. While the rate of enzymatic catalysis in response to substrate availability is typically visualized as a Michaelis-Menten system (reaction reaches half $V_{\max}$ at concentration $[K_m]$, and saturates at $V_{\max}$), electron transfer rates respond according to Butler-Volmer theory, where the rate of the forward reaction increases exponentially with the difference in donor and acceptor potentials.

Thus, while an enzyme has a maximal turnover rate constant (k_{cat}), reached at high substrate concentrations, electron transfer is described by a rate constant achieved at

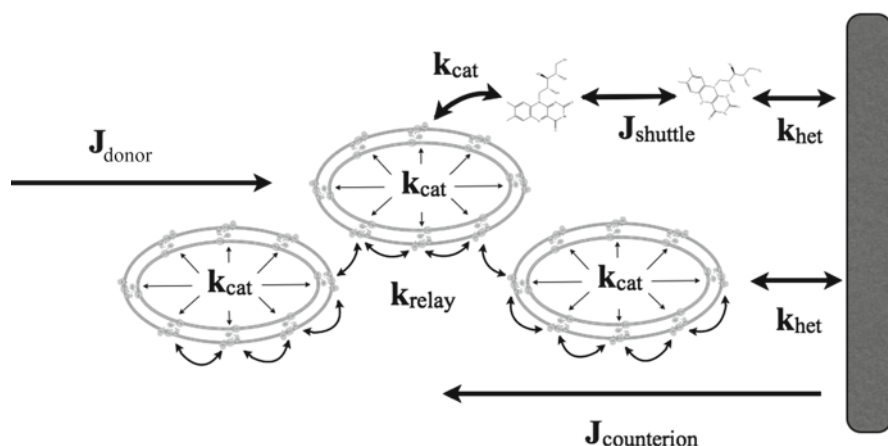


Fig. 18.1 Cartoon model of steps which may control the overall phenotype of current flux to electrodes. Electron donor flux into biofilm leads to oxidation of substrate, followed by electron transfer to electron shuttles or directly to the electrode surface. More distant cells rely on a relay between cells and electrodes, and all electron movement must be compensated by outward flux of ions. Fluxes (J) are limited by diffusion rates and concentration gradients, while enzymatic reaction rates (k_{cat}) are governed by substrate availability. Reaction rates at electrodes (k_{het}) respond to changes in electrode potential

the midpoint potential of the redox species (k_{het}), that can be increased nearly 50-fold simply by application of a small (~ 0.2 V) potential. This difference in responses usually means that an enzymatic reaction transferring electrons to an electrode can be easily brought to the point where enzyme turnover rate (k_{cat}) is rate-limiting, relative to the rate electrons can hop from the enzyme to the electrode, if sufficient potential is applied. In other words, if the electrical connection is good, one would expect application of a small potential to pull the system to a point where the maximum turnover rate of the cell's enzymatic machinery is reached. If the connection is poor, each increase in potential at the electrode would be met with an increase in current, as there remains an excess of enzymatic capacity waiting to be used.

If the organism is utilizing a soluble mediator to shuttle electrons to the electrode, the Butler-Volmer relationship will still control mediator-electrode reaction rates, and perhaps Michaelis-Menten kinetics will describe cell-mediator kinetics, but, the mediator needs to diffuse to the electrode. Unlike electron transfer, which responds to applied potential, diffusion is a function of the compound's mobility, and concentration. Thus, as with direct electron transfer, application of a small potential can easily accelerate surface oxidation rates, and leave delivery of electrons to the electrode (diffusion) as the rate-limiting step. In such a case, the primary mechanism of increasing flux to the electrode is to increase mediator concentrations.

If cells are to stack on top of each other, rates of current production can scale significantly. However, for this to occur, a mechanism of longer-range electron transfer is required. In addition, cells now attach to other cells, rather than the electrode. Thus, new factors must come into play; relay of electrons from cell to cell (perhaps via cytochrome-to-cytochrome collisions, mediators, or conductive macromolecules [Reguera et al. 2005; Gorby et al. 2006, 2008; Juarez et al. 2009]), and cell-cell aggregation rather than surface attachment. If long-range transfer is via soluble mediators, the relatively slow rates of diffusion restrict this strategy from being useful over distances greater than a few microns (Picioreanu et al. 2007, 2008). Basic diffusion calculations strongly support the conclusion that when organisms (such as *G. sulfurreducens*) form >20 μm thick biofilms, and the rate of respiration per unit biomass is increasing as cells are added to these biofilms, some non-soluble mediator based mechanism for relaying electrons to the electrode must be active. A TEM image of an actual *Geobacter* biofilm, showing the intimate cell-cell contact throughout the film, compared to the low percentage of cells that form the actual connection with electrodes, is shown in Fig. 18.2. This image shows how, when we think of bacteria "growing on electrodes", the majority of the bacteria are actually not in contact with the electrode, but rely on each other (or each other's outer surface) as conduits.

Finally, as cells grow thicker on the electrode, there is the question of flux out of the biofilm. Recent modeling (Torres et al. 2007, 2008a, c), and confocal microscopy experiments (Franks et al. 2009) have suggested that the flux of protons or positive charge out of the biofilm can ultimately become limiting for some organisms. In other words, even if the bacteria have solved the problem of attaching, bringing electrons to their outer surface, relaying these negative charges quickly

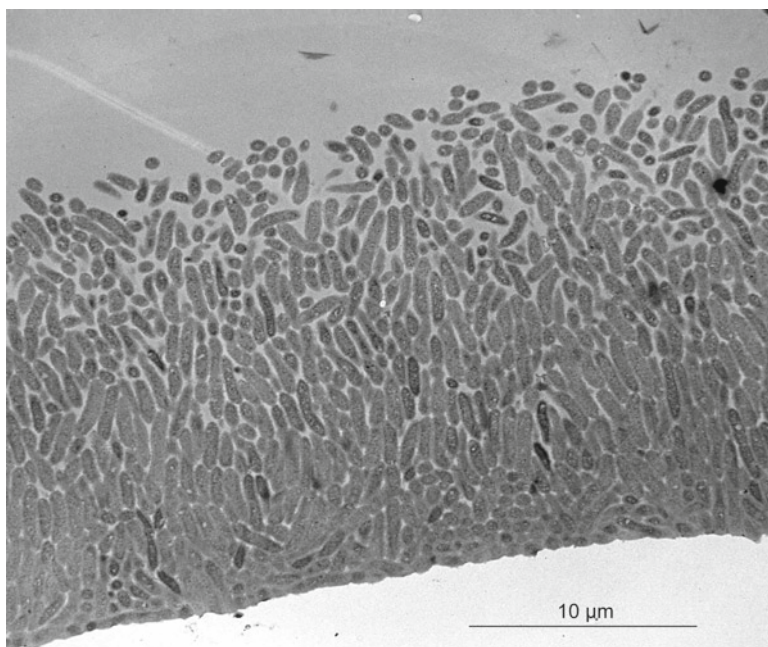


Fig. 18.2 TEM cross section of a *G. sulfurreducens* biofilm, grown using an electrode as the electron acceptor (3,400 × magnification). Cells at the *bottom* of the image are the only cells in contact with the electrode. The electron transfer rate per cell in this biofilm was consistent with each cell respiring at a similar rate, implying a mechanism for relaying electrons from more distant cells to the surface (Image credit; E. LaBelle and G. Ahlstrand)

between cells, and getting them to the electrode, they will eventually outpace the rate positive charge can escape the biofilm. Evidence for this issue can be seen in the recent evolution of a laboratory strain (KN400), which had an increased capacity for electrode reduction, while growing as thinner biofilms (Yi et al. 2009).

Some Examples of the Relationship Between Potential and Electron Transfer Rates

The mixture of enzymatic, diffusional, and electrochemical laws that govern electron transfer to electrodes can result in organisms capable of similar rates of electron transfer to electrodes, but at very different potentials. This is reminiscent of the classic descriptions of anaerobic microbial communities defined not by their *ability* to use hydrogen, but the threshold below which they cannot metabolize hydrogen (Lovley 1985; Lovley and Goodwin 1988). Some real and theoretical current-voltage curves are shown for illustration (Fig. 18.3).

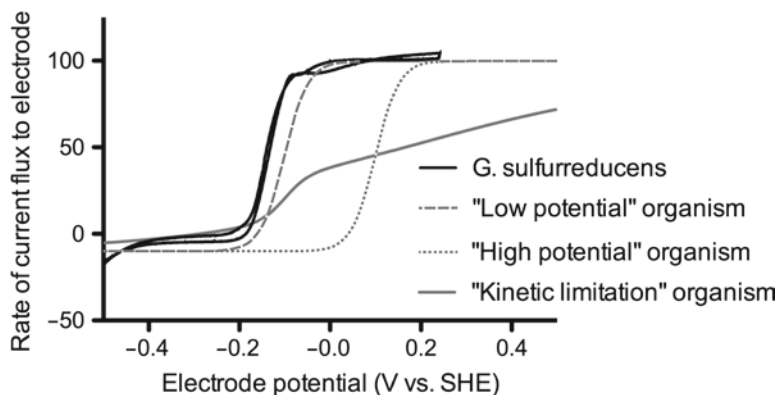


Fig. 18.3 Three theoretical slow scan rate current-potential curves, and representative data from *G. sulfurreducens* (thick black line). If the potential of the electrode is raised to a high enough potential, all organisms could be described as having a similar rate of electron transfer to electrodes. However, at lower potentials, some of these organisms are unable to transfer electrons, and show a different phenotype. For example, at -0.1 V, the low potential organism (dashed line) would respire faster than the high potential (dotted line) or kinetic limitation (grey line) organism

To obtain this data, bacteria are typically grown on electrodes under conditions where electron donor is in excess (e.g., higher concentrations do not result in faster respiration rates), and the potential of the electrode is held sufficiently positive as to be non-limiting (e.g., higher potentials do not result in faster respiration rates). The potential is then swept from high to low potential slowly (1 mV/s), and this slow change in potential allows sampling of the rate of respiration across a range of applied potentials. Because the observed rates are being controlled by the electrode potential, they should be similar regardless of the direction of the potential sweep.

In the examples shown in Fig. 18.3, there are two organisms (“high potential” and “low potential”) who have identical maximum rates of current flux to the electrode. If the electrode were held at a sufficiently high potential (>0.2 V), then these would both appear similar, and would be expected to compete equally at an electrode. However, when the electrode potential is held at -0.1 V, the “high potential” organism cannot transfer electrons to the electrode, while the “low potential” organism could respire at half its maximal rate. Thus, in a device such as a fuel cell, where the potential is allowed to drift (and typically equilibrates to a low potential), the “low potential” organism would be highly favored, and would out compete other strains which have similar *rates* of electron transfer, but require a higher electron acceptor potential. Similarly, if these organisms were competing for an oxidized iron mineral with a low potential, the “low potential” organism would be favored.

Both of these theoretical organisms respond similarly to changes in driving force. The switch from no electron transfer to a maximal rate occurs over a narrow window, which is defined by the Nernst equation. The flat plateau seen as the potential is raised higher suggests that electron transfer rate from terminal

cytochromes (which is accelerated by potential) is not rate-limiting, but more likely, a key step in substrate oxidation within the cell, or a key step in electron transfer within the membrane has become rate-limiting. This behavior, with a characteristic threshold near -0.2 V, and a half-maximal current around -0.15 V, is what is typically observed for *G. sulfurreducens* (Marsili et al. 2008b). This sigmoidal wave-like shape is what would be expected if the connection between bacteria and electrodes was facile, and some step in acetate oxidation or transfer of electrons to the outer membrane by cells represented the slowest step.

Such findings seem contrary to the notion that reduction of external metals is slow, an assumption based on slow growth rates of bacteria on insoluble metals versus chelated metals. These observations with electrodes suggest it is the process of finding metal particles, reducing them, and finding others, while fighting against the competing forces of passivation and limiting surface area – which cause slower growth rates, not slow rates of surface electron transfer per se. Thus, when an electrode is provided as a constant, unchanging electron acceptor, cells grow and respire as fast as when soluble acceptors are present.

A second possible type of response is also shown, where there is an initial rise in electron transfer rate, followed by a more general linear response. This could be caused by many factors affecting the kinetics of electron transfer. Proteins may be heterogeneously oriented at the electrode, with each requiring a different driving force for optimal electron transfer, or, electron transfer from proteins could have a slow rate constant, and require significant overpotential to drive the reaction. In any case, there appears to be some resistance to electron transfer, which can be overcome with potential, leading to a “kinetic limitation” in electron transfer. With enough driving force, even this organism could produce the same rate of electron transfer as the other examples.

This example again shows how a report of the overall rate of electron transfer can have less ecological meaning than the potential at which that rate is achieved. While the “kinetic limitation” organism looks, in the figure, to be rather sluggish, and to have a low rate of electron transfer versus the “high potential” organism, consider the case where the electrode is at -0.15 V. Under these conditions, the “kinetic limitation” and “low potential” organisms would be capable of similar electron transfer rates, and would out compete organisms requiring a higher potential. In the environment, the “high potential” organism would be expected to grow well in the presence of chelated metals, or high potential metal acceptors (perhaps Mn(IV)), but would not be thermodynamically capable of reducing lower potential metal oxides.

Similarities with the Thermodynamics of Metal Reduction

In the environment, Fe(III) minerals likely have a potential to accept electrons near 0 V or lower (Straub et al. 2001). Thus, to generate energy, an organism using donors near a potential of -0.3 V (typical of simple fatty acids) only has, at best, a

small potential drop to use in energy generation. The fact that *Geobacter* can transfer electrons to external acceptors at potentials as low as -0.22 V, and reaches its maximal rate of electron transfer by nearly -0.1 V, shows how little energy this organism ‘keeps for itself’, while moving electrons through a proton-pumping electron transport chain, to reach the outer surface.

For the reduction of a -0.22 V acceptor to still be favorable, while linked to oxidation of a donor with a reduction potential of -0.28 V (the E° of acetate), a ΔE of approximately 0.06 V per electron remains for proton pumping and ATP generation. In the case of acetate oxidation (an eight electron oxidation), this is equivalent to ($\Delta G = -nF\Delta E$), and predicts that only -46 kJ/acetate is available for ATP generation by *Geobacter*. This is consistent with models and yield data that estimate the ATP/acetate of *Geobacter* to be approximately 0.5 ATP/acetate (Mahadevan et al. 2006; Sun et al. 2009). Clearly, while higher potential acceptors exist (either as freshly precipitated ferrihydrite, or higher potential electrodes), this evidence suggests *G. sulfurreducens* is most competitive when both donors and acceptors are limiting, and near the thermodynamic threshold for growth.

Bacteria which couple very little of their net electron flow to ATP production should therefore dominate when the selective pressure is a low electrode potential. This ability of organisms to grow near thermodynamic equilibrium explains why anodes of fuel cells self-equilibrate to potentials near -0.2 V versus SHE, and why this can lead to powerful enrichment of *Geobacter*-like organisms. It also shows that there may be little that can be done in terms of discovering bacteria capable of creating stronger potentials, so long as the same donors are used as fuel. If the potential of the cathode, using oxygen as an acceptor, is fixed (e.g., at $+0.4$ to $+0.5$ V versus SHE), finding a bacterium that requires 0.05 V less overpotential for their own growth would increase the overall power output less than 10%.

Conclusions

Following a rush of initial findings showing the repeatability of electricity generation by bacteria in fuel cell-like devices, it has become clear that a wider diversity of bacteria are capable of extracellular respiration to electrodes than enrichments with Fe(III) may have suggested. Certainly, new isolates are yet to be discovered, especially from systems incubated at higher and lower temperature, pH, and salinities. As researchers have begun to recognize the factors that can influence measurements of electron transfer rates by these organisms, they have been able to dissect various adaptations which contribute to the overall phenotype of electron transfer to electrodes. Clear differences in strategy (direct versus mediators), complexity (thinner layers versus conductive biofilms), and potential-dependent behavior suggest that these abilities exist for competition in different niches, or to utilize different electron acceptors in the environment. The use of electrodes to study respiration offers a degree of control that should prove very useful in the future isolation and study of these bacteria.

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