Power output and columbic efficiencies from biofilms of *Geobacter sulfurreducens* comparable to mixed community microbial fuel cells

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Summary

It has been previously noted that mixed communities typically produce more power in microbial fuel cells than pure cultures. If true, this has important implications for the design of microbial fuel cells and for studying the process of electron transfer on anode biofilms. To further evaluate this, *Geobacter sulfurreducens* was grown with acetate as fuel in a continuous flow 'ministack' system in which the carbon cloth anode and cathode were positioned in close proximity, and the cation-selective membrane surface area was maximized in order to overcome some of the electrochemical limitations that were inherent in fuel cells previously employed for the study of pure cultures. Reducing the size of the anode in order to eliminate cathode limitation resulted in maximum current and power densities per m^2 of anode surface of 4.56 A m^{-2} and 1.88 W m^{-2} respectively. Electron recovery as current from acetate oxidation was *c.* 100% when oxygen diffusion into the system was minimized. This performance is comparable to the highest levels previously reported for mixed communities in similar microbial fuel cells and slightly higher than the power output of an anaerobic sludge inoculum in the same ministack system. Minimizing the volume of the anode chamber yielded a volumetric power density of 2.15 kW m^{-3}, which is the highest power density per volume yet reported for a microbial fuel cell. *Geobacter sulfurreducens* formed relatively uniform biofilms 3–18 μm thick on the carbon cloth anodes. When graphite sticks served as the anode, the current density (3.10 A m^{-2}) was somewhat less than with the carbon cloth anodes, but the biofilms were thicker (*c.* 50 μm) with a more complex pillar and channel structure. These results suggest that the previously observed disparity in power production in pure and mixed culture microbial fuel cell systems can be attributed more to differences in the fuel cell designs than to any inherent superior capability of mixed cultures to produce more power than pure cultures.

Introduction

It has been proposed that microbial communities that can oxidize organic wastes or renewable biomass with electron transfer to the anodes of microbial fuel cells may be able to produce sufficient energy to power a diversity of electronic devices (Shukla *et al.*, 2004; Lovley, 2006a). However, with the exception of sediment microbial fuel cells, which can power electronic monitoring devices by extracting electrons from aquatic sediments (Reimers *et al.*, 2001; Bond *et al.*, 2002; Tender *et al.*, 2002), the potential practical applications of microbial fuel cells have yet to be realized because of their low power output. Re-engineering fuel cells to increase anode surface area, improve cathodic reactions and reduce internal resistance have yielded more power (Angenent *et al.*, 2004; Rabaey *et al.*, 2005a; Rabaey and Verstraete, 2005; Logan *et al.*, 2006). However, when the energy costs of pumping materials through the fuel cells and/or stirring the systems are considered, it is doubtful that these systems produce more power than they consume. Thus, further substantial improvements are required for most postulated applications of microbial fuel cells.

The mixed communities colonizing the anodes of microbial fuel cells are generally complex, making it difficult to elucidate how current is made in such systems. To date, attempts to simplify the study of these complex communities with representative pure cultures have been disappointing because the pure cultures have generally produced substantially lower power densities than mixed cultures (Table 1). The most direct example of this is the study of Rabaey and co-workers in which isolates obtained from the anode of a mixed community microbial fuel cell

Table 1. Maximum current densities, power densities and columbic efficiencies where mediators have not been added to representative freshwater pure and mixed culture fuel cells.

System design	Anode material	Cathode material	Exchange membrane	Current density	Power density[a]	Columbic efficiency (%)	Culture/inoculum	Reference
Dual chambered stacked cell	Reticulated vitreous carbon (RVC)	RVC 50 mM FeCN	Nafion 117	44 mA m^{-2}	3 W m^{-2} 500 W m^{-3}	8.3	*Shewanella oneidensis* strain DSP10	Ringeisen *et al.* (2006)
Dual chambered H-cell	Solid graphite	Graphite	Nafion 117	50 mA m^{-2}	9.3 mW m^{-2} 0.25 W m^{-3}	56.2	*Shewanella oneidensis*	Lanthier *et al.* (2007)
Dual chambered H-cell	Solid graphite	Solid graphite with 50 mM FeCN	Nafion 117	65.4 mA m^{-2}	13.1 mW m^{-2} 0.36 W m^{-3}	95	*Geobacter sulfurreducens*	Bond and Lovley (2003)
Dual chambered stacked cell	Solid graphite	Solid graphite Xwith 100 mM FeCN	Ultrex	130 mA m^{-2}	1.67 mW m^{-2} 77 mW m^{-3}	b	*Pseudomonas aeroginosa*	Rabaey *et al.* (2005b)
Upflow chamber	RVC	RVC	Ultrex	516 mA m^{-2}	170 mW m^{-2} 8.7 W m^{-3}	0.7–8.1	Anaerobic digestor sludge	He *et al.* (2005)
Single chamber	Carbon cloth non-wet proof	Air Cathode Carbon cloth non-wet proof coated carbon and PTFE	None	1.05 A m^{-2}	766 mW m^{-2} 19.1 W m^{-3}	32	Wastewater	Cheng *et al.* (2006)
Dual chambered channelled stacked cell	Carbon paper	Carbon cloth (0.5 mg cm^{-2}, 10% Pt)	Nafion 117	1.3 A m^{-2}	309 mW m^{-2} 77 W m^{-3}	65	Wastewater	Min and Logan (2004)
Dual chambered stacked cell	Carbon fibre	Carbon fibre 50 mM FeCN	Nafion 117	3.2 A m^{-2}	1.6 W m^{2} 1.4 W m^{-3}	40–45	Anaerobic digestor sludge	This study
Dual chambered stacked cell	Carbon fibre	Carbon fibre 50 mM FeCN OR Air cathode carbon (4.0 mg of Pt cm^{-2})	Nafion 117	4.6 A m^{-2}	1.9 W m^{-2} 43.3 W m^{-3} optimized anode 2.15 kW m^{-3}	100	*Geobacter sulfurreducens*	This study
Dual chambered stacked cell	Solid graphite	Graphite 100 mM FeCN	Ultrex	6 A m^{-2}	3.6 W m^{-2} 216 W m^{-3}	65–89	Anaerobic digestor sludge enrichment	Rabaey *et al.* (2003)
Dual chambered stacked cell (6×)	Solid graphite	Solid graphite with 50 mM FeCN	Ultrex	c	c 280 W m^{-3}	29	Anaerobic and aerobic digestor sludge	Aelterman *et al.* (2006)

a. Power densities calculated using the anode surface area and the anode chamber volume.
b. Not given.
c. Granular anode, no surface area given.
Other tables containing similar data(Angenent *et al.*, 2004; Moon *et al.*, 2005; Rabaey and Verstraete, 2005; Rabaey *et al.*, 2005a; Chang *et al.*, 2006; Freguia *et al.*, 2007).

(Rabaey *et al.*, 2005b) generated power densities much lower than the mixed community (Rabaey *et al.*, 2004).

If pure cultures are unable to produce as much current as mixed communities, this raises the important ecological question of whether, multi-species microbial interactions are required for the highest current densities. It would also stymie efforts to study the mechanisms for high-current-density production because: (i) it would be difficult to replicate mixed culture communities and maintain a stable community composition and (ii) functional analysis with approaches, such as genetic manipulation and gene expression studies, which are readily tractable with pure cultures growing on anodes (Holmes *et al.*, 2006; Reguera *et al.*, 2006), would be substantially more technically difficult with mixed cultures.

It was suggested that a culture of *Shewanella oneidensis* could produce high power densities (Ringeisen *et al.*, 2006). However, in that study, the culture was grown in a large reservoir with oxygen available as an electron acceptor and the culture was continuously recycled through a small-volume anode chamber. This had several important consequences: (i) the effective volume of the anode culture was much larger than what was used in the power per volume calculation, (ii) it is likely that the planktonic cells in the recycling culture were responsible for most of the current production, via reduction of the excreted electron shuttle, riboflavin (Lanthier *et al.*, 2007; von Canstein *et al.*, 2008; Marsili *et al.*, 2008) and (iii) oxygen in the reservoir could have served as the primary electron acceptor supporting growth. Furthermore, columbic efficiency was less than 10%. None of these conditions are representative of how microbial fuel cells colonized by mixed communities function. Furthermore, the current density of 44 mA m^{-2} in the *S. oneidensis* system (Ringeisen *et al.*, 2006) is substantially less than that typically reported for mixed culture systems (Table 1). A subsequent study found similar low current densities for *S. oneidensis* (Lanthier *et al.*, 2007). These studies also demonstrated that *S. oneidensis* only incompletely oxidizes its fuel, lactate, leaving two-thirds of the electrons in unutilized waste products. Furthermore, *Shewanella* species have not been reported to be important components of the anode community harvesting current from complex organic matter. For all these reasons, this system cannot be considered as a pure culture model of mixed culture microbial fuel cells that produce much higher current densities and effectively recover a high percentage of the electrons available in fuel as electricity.

Current production by *Geobacter* species is of interest because closely related microorganisms have been found to be the predominant organisms on the anodes of fuel cells harvesting electricity from aquatic sediments and complex wastes (Bond *et al.*, 2002; Tender *et al.*, 2002; Holmes *et al.*, 2004a; Gregory *et al.*, 2005; Jung and Regan, 2007). Furthermore, *Geobacter* species have the unique ability, shared by only a few other pure cultures (Bond *et al.*, 2002; Chaudhuri and Lovley, 2003; Holmes *et al.*, 2004b), to completely oxidize organic substrates to carbon dioxide with an anode serving as the sole electron acceptor (Bond *et al.*, 2002; Bond and Lovley, 2003). Such metabolism is necessary to account for the high columbic efficiencies that are observed with microbial fuel cells oxidizing complex wastes. A genetic system (Coppi *et al.*, 2001), a genome-based *in silico* model (Mahadevan *et al.*, 2006) and substantial physiological data are available for *Geobacter sulfurreducens* (Lovley *et al.*, 2004), the most highly studied species of *Geobacter*. Previous studies have demonstrated that it is possible to monitor gene expression of *G. sulfurreducens* growing on anodes and to evaluate the growth and metabolism of various mutants (Holmes *et al.*, 2006). Therefore, studies of *G. sulfurreducens* offer the possibility of providing insights into the mechanisms for electron transfer to anodes and optimizing this process via improved fuel cell design and/or genetic engineering.

Previous studies with *G. sulfurreducens* (Bond and Lovley, 2003) suggested that, as with other pure cultures, current densities in fuel cells (65 mA m^{-2}) were low compared with mixed cultures. However, when the anodes of the fuel cells were poised with a potentiostat to overcome potential electrochemical limitations and acetate was continuously provided to prevent electron donor limitations, current densities as high as 2.26 A m^{-2} of anode surface area were obtained (Reguera *et al.*, 2006). Although poising the anode is an artificial condition, this result was important because it suggested that if limitations to electron and/or proton flow in *G. sulfurreducens* fuel cells could be eliminated, then this pure culture might be capable of relatively high power densities.

Here we report that *G. sulfurreducens* is capable of producing power densities in microbial fuel cells comparable to those reported with various mixed cultures while maintaining high columbic efficiency. The results suggest that previously noted differences in the power production of mixed communities versus pure cultures can, at least in the case of *G. sulfurreducens*, be attributed to differences in fuel cell design. The fact that mixed cultures are not necessarily required for high current densities at high columbic efficiency is an important ecological principle and provides the opportunity to study mechanisms for electron transfer at high current densities with a genetically tractable pure culture.

Results

High-density power production

When *G. sulfurreducens* was inoculated into the ministack fuel cell with an air cathode, there was an increase in
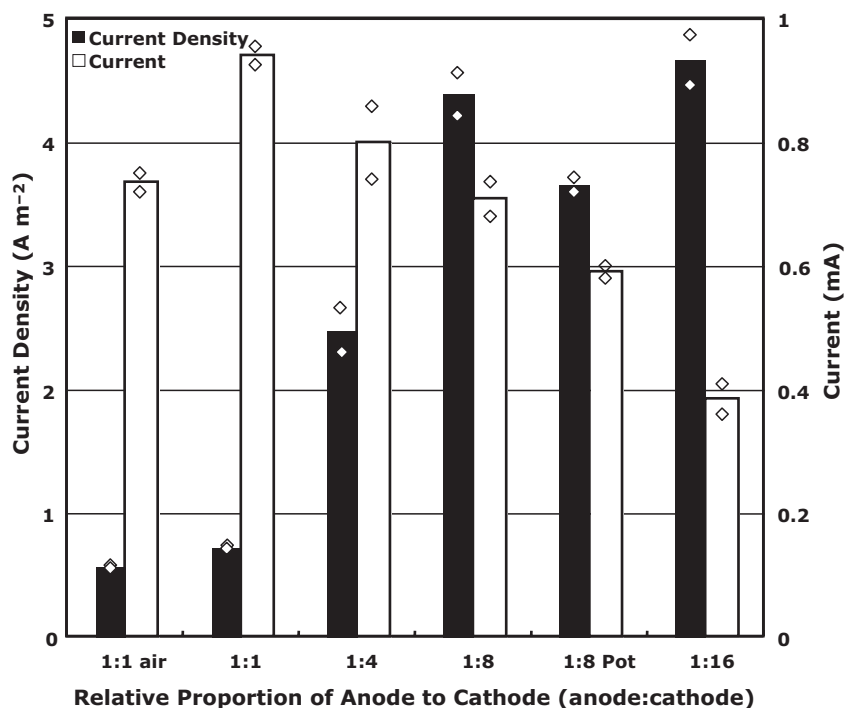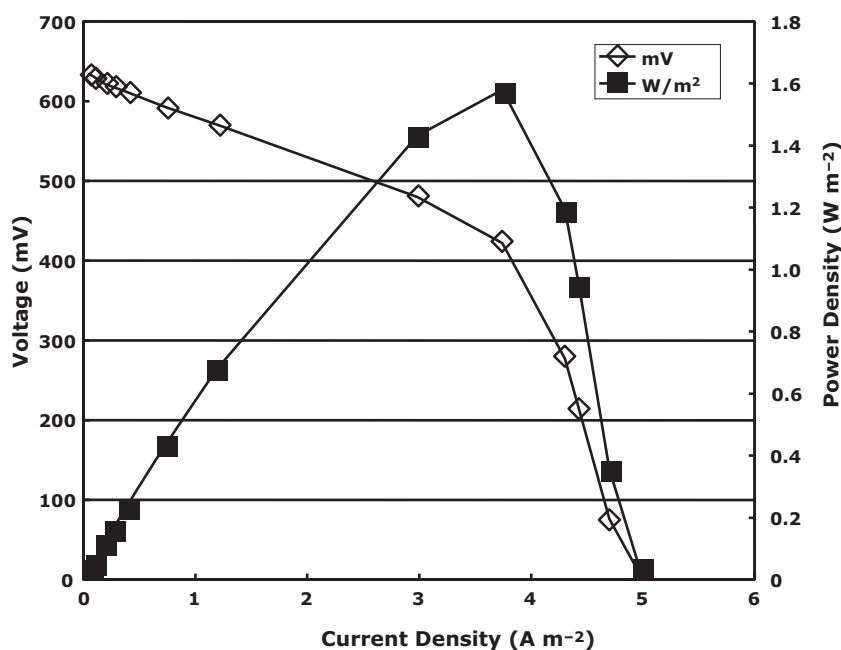
Fig. 1. Current and current density in *G. sulfurreducens* fuel cell with carbon fibre anodes of decreasing size. Cathodes were carbon fibre in 50 mM ferricyanide, unless denoted air, indicating an air cathode. Resistance was 560 ohm. Pot designates that the anode was poised at +300 mV versus an Ag/AgCl reference. Bars are average of duplicates, diamonds indicate the values of the duplicates.

current over time that stabilized at *c.* 0.7 mA (Fig. 1), which is equivalent to 0.57 A m^{-2} and 0.24 W m^{-2}. Once the maximum current was reached, it could be maintained indefinitely as long as fuel was provided to the anode chamber. When the platinum-loaded air cathode was replaced with carbon cloth and the cathode chamber was filled with ferric cyanide (50 mM), current production was slightly higher (0.9 mA; 0.7 A m^{-2}; 0.39 W m^{-2}; Fig. 1), suggesting that electrochemical limitations, rather than the ability of *G. sulfurreducens* to transfer electrons to the anode, was limiting current production in the air cathode ministack.

In order to further evaluate potential electrochemical limitations, the size of the anode was reduced in systems in which the cathode was the ferric cyanide system (Fig. 1). As expected, as the size of the anode was decreased, the total current decreased. However, the current density increased until the anode was 1/8 of the original size. At this anode size, the current density could not be improved by poising the anode potential with a potentiostat, suggesting that the microbial reactions at the anode, not the cathode, were limiting current production. Similar current densities with the 1/8-sized anode were also observed when an air cathode was substituted for the ferric cyanide-filled cathode chamber (data not shown). The current densities of *c.* 4.6 A m^{-2} in these non-cathode limited systems with 560 ohm external resistance represent a power density of *c.* 1.9 W m^{-2} and a power per anode chamber volume of *c.* 43 W m^{-3}. Substantially, lower or higher resistance did not yield increased power

(Fig. 2). When sewage sludge was used as the inoculum, the power density (1.6 W m^{-2}) was slightly less than that of the pure culture. When the volume of the anode chamber was reduced to 336 μl, the power that *G. sulfurreducens* produced per volume of anode chamber increased to 2.15 kW m^{-3} under optimal conditions of an external resistance of 200 Ω [as determined by a current-voltage (IV) curve] and a flow rate of 0.5 ml min^{-1}.

Further studies with the full-sized anode chamber demonstrated that, once the ministacks had stabilized at maximum power, acetate concentrations in the effluent remained stable over time (*c.* 8.8 mM). This made it possible to calculate a rate of acetate consumption from the difference in acetate concentrations in the influent and effluent and the flow rate of the acetate feed. In each case, triplicate determinations (mean ± standard deviation) were made with duplicate fuel cells. Comparison of acetate consumption and electrons being recovered as electricity indicated that *c.* 55% (56.8 ± 0.03; 54.6 ± 0.01) of the electrons from the acetate being consumed were converted to electricity with a FeCN cathode. In order to determine what proportion of acetate consumed, not recovered as electricity, might be attributed to incorporation of acetate into cells over time, the acetate feed was switched from growth medium to bicarbonate buffer to prevent cell growth. Under these conditions, 65% (65.3 ± 8.1; 65.4 ± 2.4) of the electrons in the acetate consumed were recovered as current. To evaluate the potential impact of oxygen leaking through the plastic and into the anode chambers, the ministacks were placed in

Fig. 2. Current density–voltage and current density–power relationships for *G. sulfurreducens* fuel cell obtained by varying the resistance between anode and cathode. The electrodes were an 1/8-sized carbon fibre anode and a full-sized carbon fibre cathode. The cathode was submerged in 50 mM ferricyanide.

an anaerobic chamber or the plastic housing was replaced with a glass system. Under these conditions, efficiencies were *c.* 100% in the presence of growth medium (100.9 ± 8.0; 98.1 ± 2.9) or buffer (102 ± 12.1; 109.8 ± 9.0).

Anode biofilms

Biofilms completely coated the fibres of the carbon cloth anodes (Fig. 3A). The biofilms on the full-sized anodes were confluent layers 2.2–8.5 μm thick (Fig. 3B). When cathode limitation was eliminated with the 1/8-sized anodes, the biofilms were thicker (3–18 μm) than on the full-sized anodes (Fig. 3C). Like the biofilms in the cathode-limited system, the biofilms in the system without cathode limitation were relatively uniform. However, in some instances, small pillars (10 μm maximum height) were observed, primarily in regions where the biofilm had overgrown in the spaces between the carbon fibres.

In contrast, previously described biofilms on solid graphite stick anodes in H-cell microbial fuel cells poised with potentiostats had substantial pillar and channel structure (Reguera *et al.*, 2006). In order to investigate this difference further, a solid graphite stick with the same nominal dimensions (0.635 cm × 1.27 cm) as a 1/8-sized carbon cloth anode was used as the anode in a ministack fuel cell. The current density/power density was 3.1 A m^{-2} /(0.9 W m^{-2} 20 W m^{-3}) which is somewhat less than with carbon cloth anodes with the same geometric dimensions. This may be due to the greater texture of the carbon cloth surface providing more surface for colonization. The biofilms on the solid graphite anode

were highly differentiated with pillars *c.* 50 μm high (Fig. 4), similar to that when the same material was previously used in poised system (Reguera *et al.*, 2006). The BacLight reagent used to visualize the cells has been suggested to yield green fluorescing cells when the cells are viable and red cells when the cells are non-growing (Renye *et al.*, 2004; Teal *et al.*, 2006). On the graphite stick anodes, the exterior of the biofilms had a substantial percentage of red cells whereas cells closest to the anode were primarily green. In contrast, green and red cells were more evenly mixed in the carbon fibre biofilms. Carbon fibre anodes were also employed as an anode in a H-cell system and poised with a potentiostat in order to mimic the incubation system in which highly differentiated biofilms were first observed on solid graphite anodes (Reguera *et al.*, 2006). The biofilms on the carbon fibre anodes were undifferentiated in a manner similar to that observed in the ministack system.

Discussion

These results demonstrate that a pure culture of *G. sulfurreducens* can produce electrical power at levels that are comparable to those observed in mixed culture microbial fuel cells. The power density per anode surface reported here for *G. sulfurreducens*, 1.9 W m^{-2}, is comparable to the 1.5 W m^{-2} reported to be the highest a mixed microbial community produced in a similar continuous flow/air-cathode microbial fuel cell (Cheng *et al.*, 2006), and to the 1.6 W m^{-2} obtained in this study with a sewage sludge inoculum in the same ministack system employed in the studies with *G. sulfurreducens*. Further-
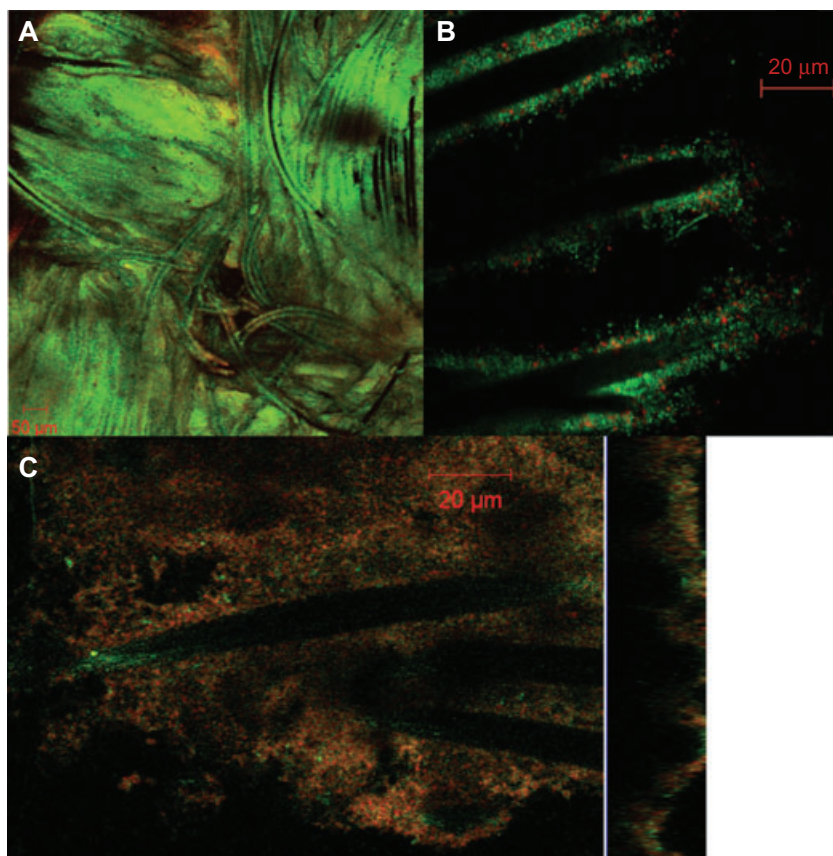
Fig. 3. Confocal laser scanning microscopy of *G. sulfurreducens* on carbon fibre anode surfaces. Three-dimensional top projection of 1/8-sized anode at 100× magnification (A), full-sized (B) and 1/8-sized (C) anodes at 400× magnification. Large panels in B and C represent a single slice through the carbon fibre anode (black) and attached biofilm (green and red). The smaller panel on the right in C represents a single slice through the anode and biofilm perpendicular to the slice shown in the large panel on the left.

more, the results demonstrate that as previously noted in all-glass systems (Bond and Lovley, 2003), when care is taken to exclude oxygen, the *G. sulfurreducens* microbial fuel cells can be operated at very high columbic efficiency.

Significance of high power densities with pure cultures

The ability to produce high current densities with a pure culture is important because the community composition of mixed cultured microbial fuel cells, commonly inoculated from sewage sludge, is generally poorly defined and can change over the time that the fuel cell is being operated (Rabaey *et al.*, 2004). In these cases, it is not clear which microorganisms in the mixed culture are the primary contributors to current production and which microbes may play more minor supporting roles in the anode community. Without the ability to study the organisms in the community in pure culture or defined mixed cultures, it is difficult to make definitive conclusions about their physiology or to predict how the microorganisms will respond to changes in operating parameters in the fuel cell. In contrast with a pure culture, such as *G. sulfurreducens*, it is possible to study the physiology of the organism while growing on an anode on a genome-wide scale

(Holmes *et al.*, 2006) and to genetically alter the organism to evaluate which proteins might be important for electron transfer and growth on anodes (Holmes *et al.*, 2006; Reguera *et al.*, 2006). There are large differences in the genome scale gene expression in low current- versus high current-producing biofilms (Holmes *et al.*, 2006; K.P. Nevin, submitted); therefore, it is critical to examine high-power-density systems.

Furthermore, the results demonstrate that it is not necessary to postulate that multi-species interactions are required for high power densities in microbial fuel cells. In addition to having implications for the ecology of microbial fuel cells harvesting electricity from complex wastes in open environments, this finding may simplify the engineering of microbial fuel cells to be used as self-contained units for applications such as powering mobile electronic devices. The use of defined microbial catalysts for such applications will make it possible to more readily predict and modify the properties of the systems. Furthermore, with pure cultures, there is the possibility of genetically engineering the microorganisms for enhanced electricity production. This is an important consideration because the lack of any previous evolutionary pressure on microorganisms to produce electricity suggests that they are unlikely to be optimized for this process (Lovley, 2006a,b).
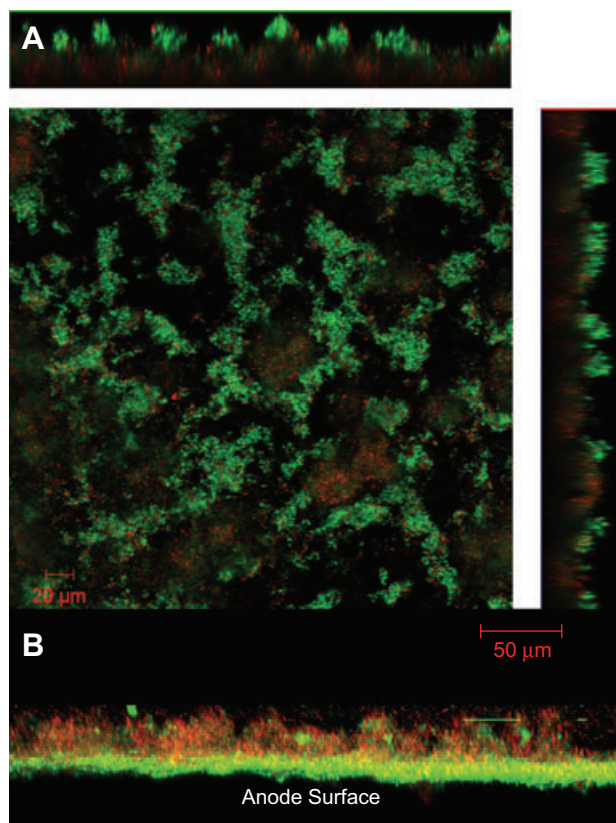
Fig. 4. Confocal laser scanning microscopy of *G. sulfurreducens* on a solid graphite anode surface with the same dimensions as the 1/8-sized carbon cloth anode shown in Fig. 3.
A. Large panel single slice through biofilms, top and right panels perpendicular slices.
B. Three-dimensional side projection.

Biofilm formation

Initial studies with *G. sulfurreducens* found that cells attached to the anode surface were responsible for power production (Bond and Lovley, 2003). The cells formed a virtual monolayer on the anode (Bond and Lovley, 2003), consistent with the concept that *Geobacter* species directly transfer electrons onto insoluble electron acceptors (Lovley *et al.*, 2004). However, in subsequent studies in which potentiostat-poised *G. sulfurreducens* fuel cells produced more power, *G. sulfurreducens* biomass accumulated on the anodes in direct proportion to the current level (Reguera *et al.*, 2006). This resulted in thick biofilms with extensive pillar structures up to 50 μm in height. Thus, most of the cells were no longer in direct contact with the anode, but they were still viable and effectively contributing to power production (Reguera *et al.*, 2006). Thick biofilm formation and high levels of current production are dependent upon the production of pili. This has been attributed (Reguera *et al.*, 2006) to the pili serving as electrical conduits through the anode biofilm as the result of their electrical conductivity (Reguera *et al.*, 2005), but

the pili may also have a structural role in biofilm formation (Reguera *et al.*, 2007).

The results shown here demonstrate that the structure of anode biofilms is dependent upon the anode material. Solid graphite anodes deployed in the ministacks under fuel cell conditions formed thick biofilms with pillar structures similar to those previously observed in potentiostat-poised systems. The biofilms on the carbon cloth anodes also increased in thickness as electrochemical limitations were eliminated and power density increased. Subsequent studies have demonstrated a direct correlation between biofilm thickness on carbon cloth anodes and power production (K. Nevin, unpubl. data). However, even under non-cathode limiting conditions, in either fuel cell mode or when the anode was poised with a potentiostat, the biofilms on carbon cloth were much thinner than those on solid graphite and lacked the extensive pillars found on solid graphite. These differences in biofilm structure on the two anode materials might be attributed to the enhanced transport of electron donor through the mesh of carbon fibres versus transport to the solid graphite surface. A better understanding of how anode geometries influence the structure and function of anode biofilms may help improve fuel cell designs.

In summary, with an appropriately designed microbial fuel cell, *G. sulfurreducens* is capable of sustaining high power densities with high columbic efficiencies. It is expected that continued examination of the mechanisms of electron transfer through the anode biofilm and other aspects of *Geobacter* physiology will lead to further optimization of power production by this organism.

Experimental procedures

Innocula

For pure culture studies, *G. sulfurreducens* strain PCA (ATCC 51573, DSMZ 12127) was obtained from our laboratory culture collection. *Geobacter sulfurreducens* was grown with 10 mM acetate as the electron donor in either 55 mM Fe(III) citrate freshwater medium (Coates *et al.*, 1998) or with fumarate (40 mM) in NBAF medium (Coppi *et al.*, 2001) with resazurin omitted and with 1.3 mM Fe(II) chloride added.

Anaerobic digester sludge from the Pittsfield, Massachusetts waste water treatment plant served as the mixed culture inoculum. The sludge from the digestor was allowed to flow for a few seconds ($> 30 \, l \, min^{-1}$) prior to collection into a sterile, anaerobic bottle. The sample was transported on ice to the laboratory and used immediately to inoculate fuel cells.

Growth in fuel cells

A 'ministack' fuel cell (Fig. 5) was constructed from two plastic methanol fuel cell stacks (http://www.fuelcellstore.com) or from glass pieces of the same size and configuration as the
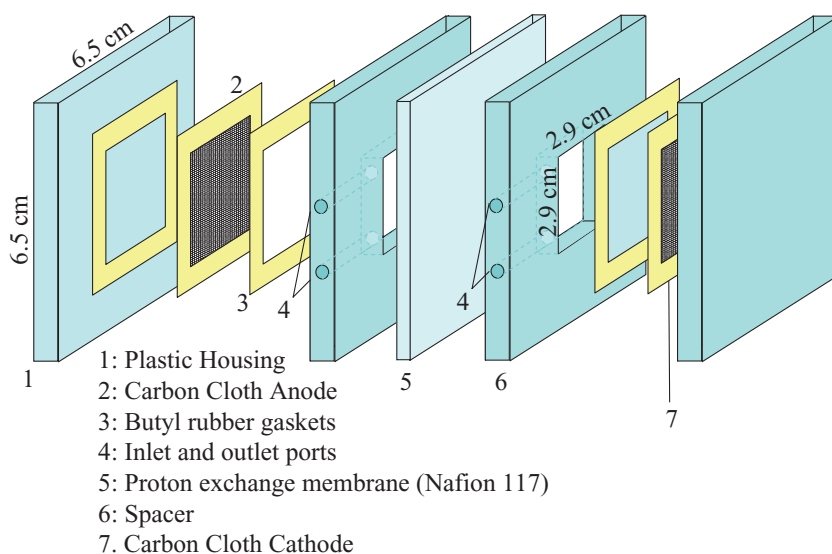
Fig. 5. Diagram of the ministack fuel cell.

1: Plastic Housing
2: Carbon Cloth Anode
3: Butyl rubber gaskets
4: Inlet and outlet ports
5: Proton exchange membrane (Nafion 117)
6: Spacer
7. Carbon Cloth Cathode

plastic parts. Anode and cathode volumes were 7 ml (2.9 cm on each side and 0.85 cm deep). Gaskets were butyl rubber. The two chambers were separated with a cation selective membrane (Nafion 117). Anodes (typically 2.54 cm × 2.54 cm, but as small as 0.635 cm × 0.635 cm) were graphite cloth (0.3 mm thick, grade GC-14, Electro-lytica, Amherst, NY). Solid graphite anodes were 1.27 cm × 0.635 cm × 2 mm thick (grade G20, Graphite Engineering and Sales, Greenville, MI). Air-breathing cathodes were gas diffusion electrodes (2.54 cm × 2.54 cm; 4.0 mg of Pt cm^{-2}, http://www.fuelcellstore.com) hot-pressed to Nafion 117 membrane. In some instances, the cathode was graphite cloth (2.54 cm × 2.54 cm) submerged in 50 mM ferricyanide, the reservoir was 200 ml, open to the air and re-circulated at a dilution rate of 00.85 h^{-1}. When noted, the anode was poised at 300 mV versus Ag/AgCl with a potentiostat (Amel Instruments, Milan, Italy). The counter electrode was carbon cloth submerged in the freshwater medium. Connections were with either platinum or stainless steel wires (0.25 mm diameter). A 560 ohm resistor was placed in the anode-cathode circuit, unless otherwise noted. The chambers were sterilized by UV irradiation; 10 min each side (Spectrolinker XL-1500 UV Crosslinker. Spectronics Corp., Westbury, NY), then flushed with 1 l of sterile water, filled with acetate-fumarate medium and connected to a 200 ml acetate-fumarate medium reservoir that re-circulated medium through the anode chamber at a dilution rate of 00.85 h^{-1}. The chamber was inoculated 10% with late log phase cells into the medium reservoir. Once the culture A$_{600}$ reached 0.2, the media reservoir was replaced with one containing acetate, but no fumarate. Once current production began, the medium was no longer re-circulated and new medium was continuously supplied to the anode chamber at a dilution rate of 00.17 h^{-1}, and the effluent was sent to a waste container. Sterility of ministacks was assured by 16S rRNA clone library analysis (Holmes *et al.*, 2004a) of the fuel cell at end of run and 100% of clones were the inoculated strain.

For the mixed culture study, 10% of the anaerobic digestor sludge served as the inoculum. As with the pure culture, the medium was initially recycled but, once current production

began, the anode chamber received a continuous supply of fresh medium containing 10 mM acetate. Maximal current production was sustained after 15 days and was maintained for as long as 60 days.

For studies designed to optimize the power density per volume, the depth of the anode chamber was reduced to 0.4 mm by removing the spacer and using thinner butyl rubber gaskets, providing 336 μl of anode volume. There was one inlet in the centre of the anode endplate and four outlets at each corner of the anode chamber. The anode (carbon fibre) and air-breathing cathode (gas-diffusion; 10.0 mg of Pt cm^{-2}) were both 2.54 cm × 2.54 cm. Inoculation and running conditions were the same as with the larger volume anode chambers.

Fuel cell voltage was measured across the resistor with a Keithley datalogger connected to a computer running Excelink. Current measurements were collected directly from potentiostat outputs every second with a Power Laboratory 4SP connected to a Macintosh computer, and data were logged with Chart 5.0 software (ADI instruments, Mountain View, CA).

Other analyses

Acetate and other organic acids were determined via HPLC with a fast-acid column (Bio-Rad, Hercules, CA) with an eluent of 8 mm H$_2$SO$_4$ and UV detection at 210 nm. Fe(II) was measured via the ferrozine method (Lovley *et al.*, 1988).

Confocal microscopy

In order to examine biofilms on the anode surfaces, the fuel cells were disassembled and the carbon cloth anode was removed without touching its surface. Anodes were then dipped in freshwater medium to remove any loose cells or debris that were not part of the attached biofilm. Anode biofilms were fluorescently stained with the LIVE/DEAD BacLight Bacterial Viability Kit (L7012) (Molecular Probes,

Eugene, OR). Manufacturer's instructions were followed with the following exceptions: dyes were mixed in freshwater medium rather than bacterial suspension and, after the incubation step, samples were soaked again in freshwater media for 5 min to remove excess dye. After staining and while still wet, anodes were placed gently, so as not to disturb the biofilm, onto a few drops of ProLong Antifade agent (P7481; Molecular Probes, Eugene, OR) that had been added to the surface of a glass coverslip. Biofilm structures were examined with confocal laser scanning microscopy with a Zeiss LSM510 Meta inverted microscope equipped with a 10×, 25×, 40× or 63× objective lens. Two- and three-dimensional images were prepared and biofilm thicknesses were calculated using the Zeiss LSM Image Browser v.4.0.0.157. For each sample, average biofilm thickness was calculated by examining between two and seven fields of view, measuring the biofilm at three or more points along each observable carbon cloth fibre, for a total of more than 20 points.

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