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Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*

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Summary

Whole-genome analysis of gene expression in *Geobacter sulfurreducens* revealed 474 genes with transcript levels that were significantly different during growth with an electrode as the sole electron acceptor versus growth on Fe(III) citrate. The greatest response was a more than 19-fold increase in transcript levels for *omcS*, which encodes an outer-membrane cytochrome previously shown to be required for Fe(III) oxide reduction. Quantitative reverse transcription polymerase chain reaction and Northern analyses confirmed the higher levels of *omcS* transcripts, which increased as power production increased. Deletion of *omcS* inhibited current production that was restored when *omcS* was expressed *in trans.* Transcript expression and genetic analysis suggested that OmcE, another outer-membrane cytochrome, is also involved in electron transfer to electrodes. Surprisingly, genes for other proteins known to be important in Fe(III) reduction such as the outer-membrane *c*-type cytochrome, OmcB, and the electrically conductive pilin 'nanowires' did not have higher transcript levels on electrodes, and deletion of the relevant genes did not inhibit power production. Changes in the transcriptome suggested that cells growing on electrodes were subjected to less oxidative stress than cells growing on Fe(III) citrate and that a number of genes annotated as encoding metal efflux proteins or proteins of unknown function may be important for growth on electrodes. These results demonstrate for the first time that it is possible to evaluate gene expression, and hence the metabolic state, of microorganisms growing on electrodes on a genome-wide basis and suggest that OmcS, and to a lesser extent OmcE, are important in electron transfer to electrodes. This has important implications for the design of electrode materials and the genetic engineering of microorganisms to improve the function of microbial fuel cells.

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Introduction

The discovery of electricigens, microorganisms that can directly transfer electrons derived from organic matter oxidation to electrodes (Lovley, 2006), has raised the possibility of developing microbial fuel cells that can efficiently harvest electricity from a variety of organic matter sources. In a microbial fuel cell microorganisms catalyse the oxidation of organic or inorganic compounds and electrons derived from this oxidation are donated to the anode of the fuel cell. These electrons then flow from the anode, through the device to be powered, or a resistor in experimental studies, and onto the cathode. Electrons at the cathode combine with oxygen and protons derived from the organic matter oxidation, typically to produce water. Generally the anode and cathode chambers are separated with a cation-selective membrane that allows the net passage of protons from the anode to cathode chamber while diminishing the diffusion of oxygen from the cathode to anode chambers.

Although the possibility that microbial cultures might generate an electrical current has been known since early in the 20th century (Potter, 1911), without appropriate electricigens, power production in early microbial fuel cells was limited by at least one of two factors (Lovley, 2006): (i) the need to add a mediator that could serve as an electron shuttle to promote electron transfer between the microbes and the electrodes; and (ii) the use of microorganisms that could only incompletely oxidize their organic fuel, thus extracting only a portion of the electrons available. The requirement for added mediators is a detriment because most mediators are toxic to humans, frequently lack long-term stability, and are not cost-effective in open systems because of the need to constantly replenish them. The incomplete oxidation of organic fuels results in obvious inefficiencies.

To date, the only microorganisms known to not require the addition of exogenous mediators and to be able to

completely oxidize their organic fuels to carbon dioxide with nearly quantitative direct electron transfer to electrodes are members of the *Geobacteraceae* (i.e. *Desulfuromonas*, *Geopsychrobacter* and *Geobacter* species), *Rhodoferax ferrireducens* and *Geothrix fermentans* (Bond *et al*., 2002; Bond and Lovley, 2003; Chaudhuri and Lovley, 2003; Holmes *et al*., 2004a; Bond and Lovley, 2005). Mechanisms for electron transfer in *Geobacteraceae* are of particular interest because: (i) *Geobacteraceae* are the predominant microorganisms that naturally colonize electrodes harvesting electricity from highly anoxic wastes such as organic-rich sediments (Bond *et al*., 2002; Tender *et al*., 2002; Holmes *et al*., 2004b) and animal wastes (Gregory *et al*., 2005); (ii) *Geobacteraceae* can oxidize a variety of organic compounds to carbon dioxide with an electrode serving as the sole electron acceptor (Bond *et al*., 2002; Bond and Lovley, 2003; Holmes *et al*., 2004a); and (iii) the complete genome sequence (Methé *et al*., 2003) and a genetic system (Coppi *et al*., 2001) for *Geobacter sulfurreducens* are available, which makes it possible to systematically evaluate mechanisms for electricity production and to potentially engineer improved electricity-producing strains.

Although *Geobacter* species are very efficient in converting organic compounds to electricity, the practical application of *Geobacter*-based microbial fuel cells has been limited by the fact that, like all previously described microbial fuel cells, the rate of current production is relatively slow (Lovley, 2006). Attempts to optimize this process have been severely constrained by a lack of information on the mechanisms by which *Geobacter* species transfer electrons to electrodes.

Unlike Fe(III) reduction, there probably has not been previous evolutionary pressure on microbes to produce electricity (Lovley, 2006). Thus, it has been proposed that microorganisms, such as *Geobacter* (Bond *et al*., 2002; Bond and Lovley, 2003; Holmes *et al*., 2004a; Lovley *et al*., 2004) and *Shewanella* (Kim *et al*., 2002) species, that can transfer electrons to Fe(III) and electrodes may use similar mechanisms for electron transfer to both electron acceptors. Both Fe(III) oxides and electrodes represent insoluble, extracellular electron acceptors and thus Fe(III)- and electrode-reducing microorganisms must have a strategy for transferring electrons that are released from central metabolism in the cytoplasm to the outside of the cell. It has been suggested that outer-membrane proteins, most notably *c*-type cytochromes, are involved in electron transfer to extracellular Fe(III) (Lovley *et al*., 2004). More recently, it has been suggested that, in *G. sulfurreducens*, electrically conductive pili are the electrical conduit between the cell surface and Fe(III) oxides (Reguera *et al*., 2005)

The purpose of the studies reported here was to evaluate gene expression of *G. sulfurreducens* growing with an electrode as the sole electron acceptor in order to identify proteins that might be important in electron transfer to electrodes. The results demonstrate that the transcript levels of genes for several outer-membrane *c*-type cytochromes are higher during growth on electrodes than during growth on the soluble electron acceptor, Fe(III) citrate. Genetic studies suggest that these cytochromes are important for electron transfer to electrodes.

Results

Whole-genome microarray comparisons of G. sulfurreducens *cells grown on electrodes versus soluble electron acceptors*

In order to gain insight into the physiology of *G. sulfurreducens* cells grown on current-harvesting electrodes, gene transcript levels in cells grown via electron transfer to an electrode were compared with cells grown with Fe(III) citrate as the electron acceptor with a whole-genome DNA microarray (Methé *et al*., 2005). A total of 474 genes (197 upregulated, 277 downregulated) exhibited significant changes in expression (see supplementary data; Tables S1 and S2). The majority of genes with higher transcript levels during growth with electrodes as the electron acceptor were annotated as coding for proteins involved in energy metabolism, heavy metal transport, or hypothetical proteins (Table S1). Genes with lower transcript levels on electrodes coded primarily for proteins involved in energy metabolism, transport and binding, hypothetical proteins, or proteins with unknown functions (Table S2).

The gene with by far the greatest increase in transcript abundance during growth on electrodes encodes the outer-membrane *c*-type cytochrome, OmcS (Table 1, Table S1). This was followed by *omcT*, which appears to always be co-transcribed with *omcS*, while *omcS* can also be transcribed monocistronically (Mehta *et al*., 2005). OmcS is required for growth of *G. sulfurreducens* on Fe(III) and Mn(IV) oxide, whereas OmcT is not required for growth on either of these insoluble electron acceptors (Mehta *et al*., 2005). Transcript levels for another gene that encodes an outer-membrane *c*-type cytochrome involved in electron transfer to Fe(III) and Mn(IV) oxides (Mehta *et al*., 2005), OmcE, were also slightly higher during growth on electrodes (Table 1, Table S1). However, there was not an increase in transcript levels for other genes that have previously been shown to be important in Fe(III) oxide reduction. For example, the genes for the outer-membrane *c*-type cytochrome, OmcB (Leang *et al*., 2003), the outer-membrane proteins OmpB (Mehta *et al*., 2006) and OmpJ (Afkar *et al*., 2005), and the electrically conductive pili considered to be the electrical conduit between the cell and Fe(III) oxides (Reguera *et al*., 2005) were not differentially expressed on electrodes.

Table 1. Genes that encode electron transport proteins that were significantly upregulated in *G. sulfurreducens* cells grown on current-harvesting electrodes compared with cells grown with Fe(III) citrate provided as the electron acceptor.

Locus ID	Annotation	Gene name	M (log_2)	Fold-change	*q*-Value
GSU2504	Cytochrome *c* family protein	*omcS*	4.28	19.46	2.19×10^{-6}
GSU2503	Cytochrome *c* family protein	*omcT*	2.93	7.62	4.79×10^{-5}
GSU2937	Cytochrome *c* family protein	–	1.52	2.87	0.002
GSU0784	Nickel-dependent hydrogenase, membrane protein	*hybB*	1.14	2.21	0.001
GSU0594	Cytochrome *c* family protein	–	1.02	2.03	0.005
GSU0783	Nickel-dependent hydrogenase, iron-sulphur cluster-binding protein	*hybA*	1.01	2.02	0.001
GSU0618	Cytochrome *c* family protein	*omcE*	0.98	1.98	4.42×10^{-5}
GSU0782	Nickel-dependent hydrogenase, small subunit	*hybS*	0.88	1.84	0.001
GSU2797	Electron transfer flavoprotein, beta subunit	*etfB*	0.83	1.78	6.64×10^{-6}
GSU0745	NAD-dependent dehydrogenase subunit	–	0.67	1.59	9.17×10^{-5}
GSU0743	NAD-dependent dehydrogenase subunit	–	0.67	1.59	0.0005
GSU2494	Cytochrome *c* family protein	–	0.58	1.50	0.009
GSU2934	Cytochrome *c* family protein	–	0.41	1.33	0.006
GSU0741	NAD-dependent dehydrogenase subunit	–	0.40	1.32	0.002

The values represent the fold-difference in expression between the two conditions according to LIMMA analysis.

Table 2. Genes encoding heavy metal efflux transport proteins that were upregulated in *G. sulfurreducens* cells grown on the electrode compared with cells grown with Fe(III) citrate provided as the electron acceptor.

Locus ID	Annotation	M (log_2)	Fold-change	*q*-Value
GSU1340	ABC transporter, permease protein	2.25	4.77	0.0002
GSU1330	Metal ion efflux outer-membrane protein family protein, putative	1.73	3.33	0.001
GSU1338	Heavy-metal-associated domain protein	1.65	3.14	0.0005
GSU1341	ABC transporter, ATP-binding protein	1.37	2.59	0.002
GSU1332	Heavy metal efflux pump, CzcA family	1.21	2.31	0.004
GSU2137	Metal ion efflux outer-membrane protein family	0.96	1.94	0.001
GSU2136	Heavy metal efflux pump, CzcB family	0.91	1.88	0.0002
GSU2135	Heavy metal efflux pump, CzcA family	0.79	1.73	0.0006
GSU0829	Heavy metal efflux pump, CzcB family	0.76	1.69	0.0006
GSU0830	Heavy metal efflux pump, CzcA family	0.67	1.59	0.0007
GSU0828	Metal ion efflux outer-membrane protein family	0.54	1.45	0.002

The values represent the fold-difference in expression between the two conditions according to LIMMA analysis.

Four putative *c*-type cytochrome genes that have yet to be characterized, GSU2937, GSU0594, GSU0618 and GSU2934, had slightly higher transcript levels during growth on electrodes, as did subunits for several components of [NiFe] hydrogenases (Table 1). With the exception of *omcS* and *omcT*, the gene with the greatest increase in transcript abundance during growth on electrodes was GSU2780, which codes for a hypothetical protein (Table S1). A number of genes that encode proteins involved in the heavy metal stress response (Nies, 1995; Nies, 1999) were also upregulated on the electrode (Table 2).

The genes with the greatest decrease in transcript levels during growth on electrodes were associated with oxygen reduction and/or oxidative stress (Cabiscol *et al.*, 2000; Lin *et al.*, 2004; Rodionov *et al.*, 2004) (Table 3). For example, transcripts for genes encoding subunits of cytochrome *d* ubiquinol oxidase, expected to be involved in oxygen uptake, had the largest decline (Junneman, 1997). There were also significant decreases in transcript levels for thioredoxin peroxidase and superoxide dismutase, among others (Table 3).

Levels of OmcS, OmcT and OmcE transcripts during current production

The apparent increase in transcript levels for *omcS* and *omcT* was further investigated in a more quantitative manner. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses demonstrated that levels of transcripts for *omcS* and *omcT* increased significantly as *G. sulfurreducens* colonized the surface of electrodes and current production increased (Fig. 1A). At higher current, levels of transcripts for OmcS and OmcT stabilized, but remained high. In contrast, the relative level of several 'housekeeping' genes remained relatively constant. Northern hybridization analysis of mRNA harvested from the electrode at 2.0 mA of current further confirmed that *omcS* and *omcT* mRNA transcript levels were higher in cells grown on electrodes versus Fe(III) citrate (Fig. 1B).

Table 3. Genes encoding proteins that have been shown to be involved in the oxidative stress response of *G. sulfurreducens* or other anaerobic bacteria that were significantly downregulated in *G. sulfurreducens* cells grown on electrodes compared with Fe(III) citrate grown cells.

Locus ID	Annotation	Gene name	M (log_2)	Fold-change	*q*-Value
GSU1640	Cytochrome *d* ubiquinol oxidase, subunit I	*cydA*	–3.70	–13.0	5.86×10^{-10}
GSU1641	Cytochrome *d* ubiquinol oxidase, subunit II	*cydB*	–3.27	–9.62	1.28×10^{-8}
GSU3246	Thioredoxin peroxidase	–	–2.94	–7.65	5.86×10^{-10}
GSU1158	Superoxide dismutase	*sodA*	–2.43	–5.40	3.71×10^{-8}
GSU0843	NADH oxidase, putative	–	–0.60	–1.51	0.0001
GSU3294	Rubredoxin-oxygen oxidoreductase	–	–0.47	–1.39	0.001

The values represent the fold-difference in expression between the two conditions according to LIMMA analysis.

Two transcripts of *c*. 1.4 kb and 3 kb were detected with the *omcS* probe, whereas only one transcript of *c*. 3 kb was detected with the *omcT* probe, consistent with previously described patterns of transcription (Mehta *et al*., 2005) discussed above.

Levels of transcripts for *omcE* were also further analysed because of the somewhat higher transcript levels suggested by microarray analysis and the previously described (Mehta *et al*., 2005) role of OmcE in reduction of another insoluble electron acceptor, Fe(III) oxide. Levels of *omcE* transcripts, as determined with quantitative RT-PCR, followed a pattern similar to that observed with *omcS* and *omcT* transcripts with initial increases in transcript levels, followed by sustained elevated transcript levels at higher current (Fig. 2).

Genetic analysis of role of outer-membrane cytochromes in electricity production

In order to evaluate the potential role of the outer-membrane cytochromes that appeared to have higher expression levels during growth on an electrode versus growth on Fe(III) citrate, electricity production in mutants deficient in these genes was examined. Studies were conducted in microbial fuel cells that lacked a potentiostat because in this system the current consistently stabilized at 0.8 mA with the wild-type cells (Fig. 3) for periods of over a week. This made it possible to reliably compare power production between mutant and wild-type cells.

The previously described (Mehta *et al*., 2005) mutant in which *omcS* and *omcT* were deleted produced significantly less current than the wild type (Fig. 3) and the current remained low as the culture was re-fed fresh medium several times (data not shown). When *omcS* was expressed *in trans*, the current was comparable to the wild type (Fig. 3). Expressing *omcT* in the double mutant did not restore electricity production (data not shown).

The previously described (Mehta *et al*., 2005) mutant in which *omcE* was deleted initially produced substantially less power than wild-type cells (Fig. 3). However, when the medium was replaced after the first 200 h of incubation, power production gradually increased and continued to increase with each change of medium until power production was slightly less than that of the wild type.

It was hypothesized that the adaptation of the *omcE* deletion mutant to produce power over time might be attributed to increased production of OmcS to compensate for the loss of the other outer-membrane *c*-type cytochrome. However, it has not yet been possible to quantify protein levels on electrode-grown cells. Therefore, a triple mutant was constructed in which *omcS*, *omcT* and *omcE* were deleted. This mutant had characteristics similar to the *omcS* and *omcT* double mutant and did not adapt over time (data not shown).

OmcB is an outer-membrane cytochrome in *G. sulfurreducens* that is required for the reduction of soluble, chelated Fe(III) as well as insoluble Fe(III) oxides (Leang *et al*., 2003). As noted above, there was no apparent upregulation of OmcB expression during growth on electrodes. Consistent with this observation, a mutant in which *omcB* had been deleted (Leang *et al*., 2003) produced power as well as the wild type (Fig. 3). In a similar manner, expression of genes for the electrically conductive pili of *G. sulfurreducens* was not upregulated during growth on electrodes and a *G. sulfurreducens* mutant strain in which the gene encoding PilA, the structural pilin protein, was deleted (Reguera *et al*., 2005) was able to produce electricity as well as the wild-type strain (data not shown).

Discussion

These results provide the first insight into the mechanisms by which *G. sulfurreducens* transfers electrons onto electrodes and the physiological state of cells during growth on electrodes. The microarray analysis demonstrated that the expression of genes for several outer-membrane *c*-type cytochromes is higher in cells growing on electrodes than those growing with the soluble electron acceptor, Fe(III) citrate. Analysis of mutants deficient in some of these outer-membrane *c*-type cytochrome genes indicated that they were required for optimal electricity production. As detailed below, this result might have been predicted from the role of these cytochromes in Fe(III)

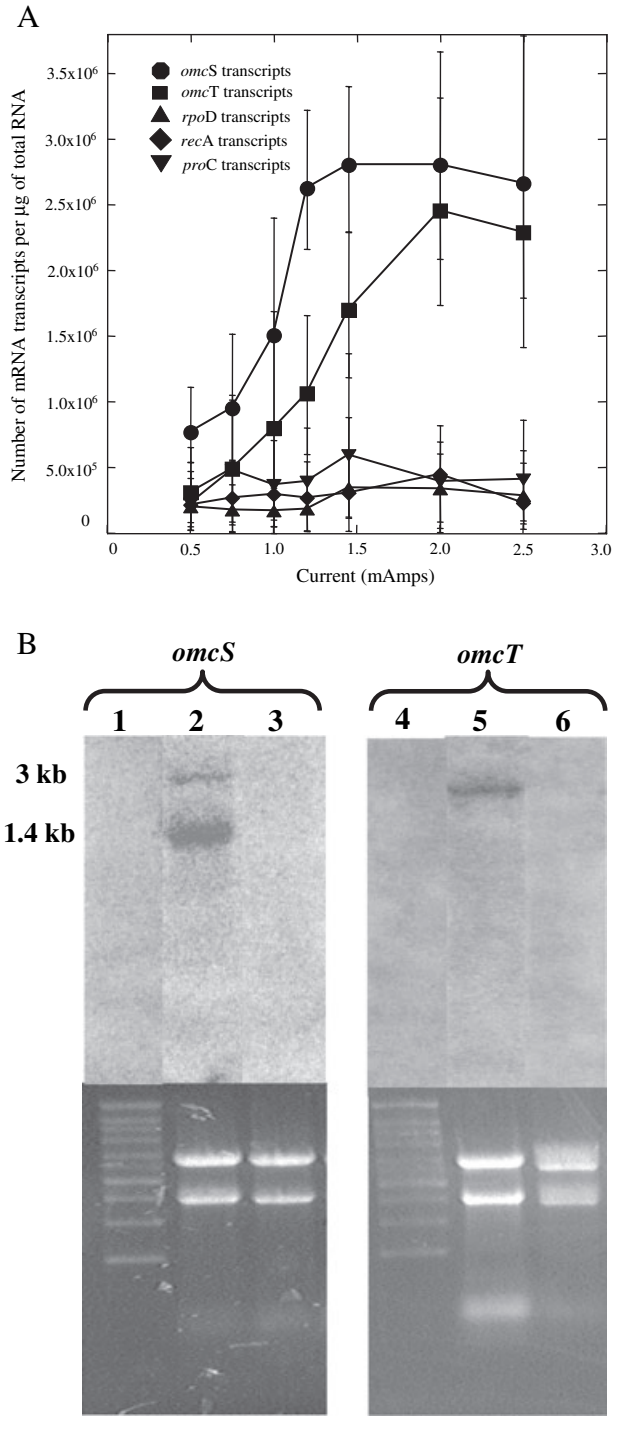

Fig. 1. A. Number of *omcS*, *omcT*, *recA*, *rpoD* and *proC* mRNA transcripts expressed by *G. sulfurreducens* cells grown on a current-harvesting electrode at different rates of current determined with quantitative RT-PCR analysis. Each point is the average of triplicate samples.
B. Northern blot analyses comparing *omcS* and *omcT* expression profiles in *G. sulfurreducens* cells grown on either an electrode or soluble Fe(III) citrate. Total RNA (5 µg) was isolated from cells grown with acetate (10 mM) provided as the electron donor and either a current-harvesting electrode poised at +200 mV (in reference to an Ag/AgCl electrode) (middle lanes) or Fe(III) citrate (55 mM) (final lanes) as the electron acceptor, and hybridized with ^{32}P-labelled probes for *omcS* or *omcT*. Lanes 1 and 4, RNA mass ladder; lanes 2 and 5, RNA extracted from cells colonizing the current-harvesting anode when the current reached 2.0 mA; lanes 3 and 6, RNA extracted from cells grown with Fe(III) citrate provided as the electron acceptor. Lower panels consist of pictures of agarose gels showing that similar quantities of RNA from each sample were transferred to the membranes.

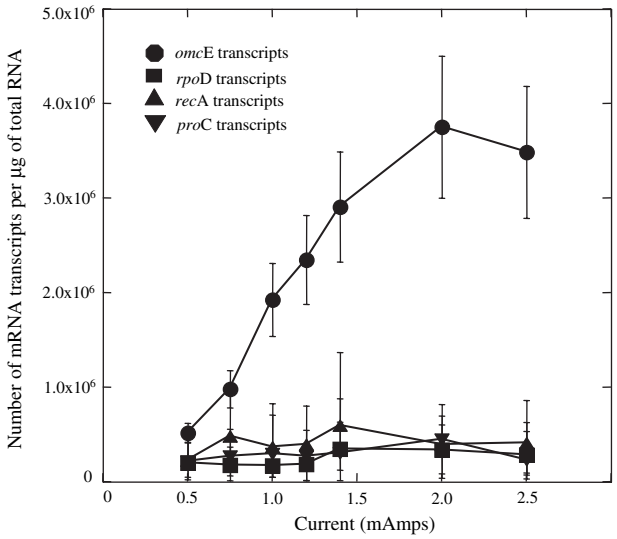

Fig. 2. Number of *omcE*, *recA*, *rpoD* and *proC* mRNA transcripts expressed by *G. sulfurreducens* cells grown on a current-harvesting electrode at different rates of current determined with quantitative RT-PCR analyses. Each point is the average of triplicate samples.

oxide reduction, but it is notable that other electron transport components known to be important in Fe(III) oxide reduction were not required for electricity production. Thus, there may be substantial differences between extracellular electron transfer to electrodes and Fe(III). Furthermore, the results suggest that the physiological state of cells growing on electrodes differs from that of cells growing on Fe(III) citrate in other important ways.

Outer-membrane c-type cytochromes involved in electron transfer to electrodes

Both the microarray and quantitative RT-PCR results indicated that levels of transcripts for *omcS*, *omcT* and *omcE* are higher in cells growing on electrodes. OmcS, OmcT and OmcE are predicted to be hexahaem *c*-type cytochromes localized in the outer membrane of *G. sulfurreducens* (Mehta *et al.*, 2005). OmcS and OmcE were first identified in *G. sulfurreducens* because they were the two most abundant proteins that could be readily sheared from the outer surface of cells grown on Mn(IV) oxide (Mehta *et al.*, 2005). Analysis of deletion mutants and their complements demonstrated that OmcS and OmcE, but not OmcT, were required for optimal Fe(III) and Mn(IV) oxide reduction but not for the reduction of Fe(III)

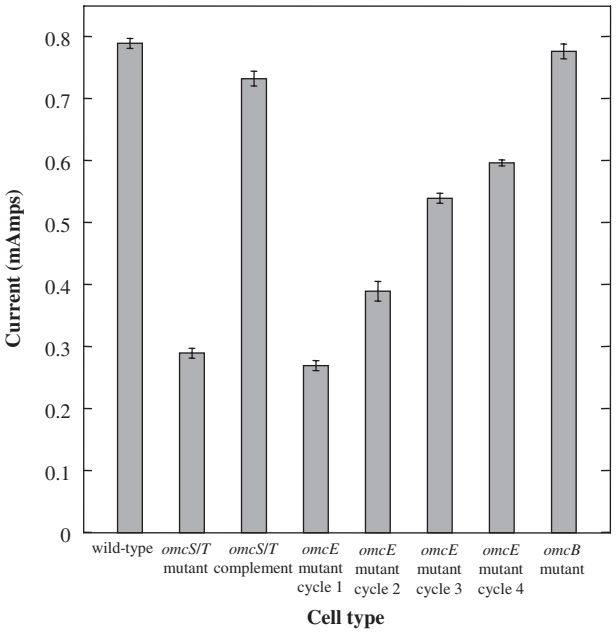

Fig. 3. Current generated by microbial fuel cells inoculated with the *G. sulfurreducens* wild-type strain, the *omcS*/*omcT* deletion mutant, the *omcS*/*omcT* deletion mutant in which *omcS* was expressed *in trans*, the *omcE* deletion mutant (each cycle represents the current produced after 200 h incubation followed by removal of the medium from the anode chamber and replenishment with fresh medium), or the *omcB* deletion mutant. The results shown are from representative fuel cells of triplicate fuel cells run for each treatment.

citrate. Over time, the *omcE* mutant adapted to reduce Fe(III) oxide, but at a much slower rate than the wild-type (Mehta *et al*., 2005). The *omcS* mutant, on the other hand, never adapted to growth on Fe(III) oxide (Mehta *et al*., 2005). The phenotype of the *omcS*- and *omcE*-deficient mutants and the complemented strains during growth on electrodes suggest that OmcS and OmcE also play an important role in electron transfer to electrodes. As with electron transfer to Fe(III) oxide, OmcS appears to be more essential for electron transfer to electrodes than OmcE. Over time, the mutant deficient in OmcE was able to adapt to produce electricity at rates only slightly lower than those of the wild type, whereas adaptation was not observed in the mutant deficient in OmcS.

The actual role of OmcS and OmcE in electron transfer to Fe(III) oxide or electrodes can not yet be definitively determined. Although OmcS and OmcE are clearly exposed on the outer surface of *G. sulfurreducens* they appear to only be intermediaries in electron transfer to Fe(III) oxides (Mehta *et al*., 2005). This conclusion is based on the observation that Fe(III) oxides appear to associate primarily with the electrically conductive pili of *G. sulfurreducens* rather than the outer surface of the cell and that pili are required for optimal Fe(III) oxide reduction (Reguera *et al*., 2005). In contrast, the pilin-deficient mutant generated electricity as well as the wild type. This difference may be due to the manner in which *G. sulfurreducens* interacts with the electrode. Previous studies (Bond and Lovley, 2003) have suggested that the cells are firmly attached in a near-monolayer structure that is rarely more than a few cells thick. This apparent tight association between cells and the electrode may alleviate the need for electron transfer at a distance via pili, which appears to be an important feature of Fe(III) oxide reduction by *G. sulfurreducens*. If this is the case, then OmcS and OmcE might be able to directly transfer electrons to the electrodes. The absolute requirement for OmcS for effective electricity production would suggest OmcS serves as the primary electrical contact between the cell and electrode surfaces. However, without any direct evidence of OmcS and electrode contact this remains speculation.

Another surprising finding was the fact that the outer-membrane *c*-type cytochrome, OmcB, which is required for optimal electron transfer not only to Fe(III) oxide, but also to Fe(III) citrate (Leang *et al*., 2003; Leang *et al*., 2004), was not required for electricity production. This observation and the production of electricity in the absence of pili, which are also required for Fe(III) oxide reduction (Reguera *et al*., 2005), demonstrates that even though Fe(III) oxides and electrodes both represent extracellular electron acceptors, there may be significant differences between the pathways involved in electron transfer to Fe(III) and electrodes.

Other differences in metabolic state during growth on electrodes versus Fe(III) citrate

In addition to increased expression of genes for several *c*-type cytochrome genes that appear to be important in electron transfer to electrodes, significant differences in expression of a number of other genes also suggest that the metabolic state of *G. sulfurreducens* cells growing with an electrode as an electron acceptor is different from cells growing on Fe(III) citrate. For example, the lower transcript levels for genes encoding proteins involved in oxygen consumption and oxidative stress responses suggest that *G. sulfurreducens* may be under less oxidative stress when growing on electrodes compared with Fe(III) citrate. Reasons for this require further evaluation, but this might reflect the ability of the cells concentrated at the electrode surface to maintain a more consistent low redox potential in this environment versus the redox potential of the bulk stirred medium. Alternatively, some components in Fe(III) citrate medium may initiate an oxidative stress response as cells growing with fumarate as the electron acceptor also had lower transcript level of some of these same genes when compared with cells grown on Fe(III) citrate (Methé *et al*., 2005).

There may also be several explanations for the increase in expression of several genes annotated as encoding heavy metal efflux systems. For example, metal contaminants in the graphite may have elicited this response. An alternative explanation, especially for the putative metal ion efflux outer-membrane protein, is that the annotation of this gene is incorrect and this outer-membrane protein plays a role in outer-membrane electron transfer or cell attachment processes. Further genetic analysis of the role of these proteins as well as the *c*-type cytochromes of unknown function that were upregulated in electrode-grown cells is warranted.

In addition to the genes referred to above that have previously been characterized in other bacterial species, but not fully investigated in *G. sulfurreducens*, there were also a number of genes that were differentially expressed in cells grown on current-harvesting electrodes that have not yet been described in any organism. For example, 50 hypothetical proteins and 20 proteins with unknown function were upregulated in cells grown on electrodes, and 50 hypothetical proteins and 45 proteins with unknown functions were downregulated on electrodes. The potential role of many of these proteins on the electrode is currently being investigated.

Implications

Optimizing the power output of microbial fuel cells requires an understanding of both the physiological state of the microorganisms growing on the electrode surface and the mechanisms by which electrons are transferred to electrodes. The results presented here suggest that the physiological state of cells producing electricity may be significantly different than that of cells growing on alternative electron acceptors. Furthermore, although some of the components that are necessary for electron transfer to Fe(III) oxides are also required for electricity production, others are not. These results emphasize the need to directly evaluate the metabolism of cells growing on electrodes to understand the microbiology of this process. The studies reported here demonstrate for the first time that microbial metabolism during growth on electrodes can be evaluated on a genome-wide scale.

Although further confirmation is clearly needed, the available results suggest that outer-membrane *c*-type cytochromes, rather than pili, are likely to be the electrical contact between *G. sulfurreducens* and the electrode surface. This is an important distinction because it is unlikely that the graphite anodes that are typically employed in microbial fuel cells are optimal for promoting electron transfer from cells. Thus, electrode design is a major area of microbial fuel cell research and materials that might interact best with pili are unlikely to also be the optimal surfaces for interacting with cytochromes. Further study of microbe–electrode interactions are also likely to provide other insights into potential strategies for engineering fuel cells or the electricigens themselves.

Experimental procedures

Bacterial strains and culturing conditions

A wild-type strain of *G. sulfurreducens*, strain DL1 (ATCC 51573), and its double mutant (*omcS1* and *omcS2*::*kan*) used for this work were obtained from our laboratory culture collections. Cells were grown under anaerobic conditions in a previously described bicarbonate-buffered, defined medium (Lovley and Phillips, 1988) provided with acetate (10 mM) as the electron donor and Fe(III)-citrate (55 mM) or fumarate (20 mM) as the electron acceptor under $N_2 : CO_2$ (80:20) at 30°C.

Electrode system

Geobacter sulfurreducens cells were first grown in medium with acetate (10 mM) provided as the electron donor and either Fe(III)-citrate (55 mM) or fumarate (40 mM) as the electron acceptor. Cells were then pelleted via centrifugation, washed, and re-suspended in anoxic medium lacking electron donor or acceptor. This cell suspension served as an inoculum for the anaerobic anodic chamber (250 ml of medium) of a two-chambered electrode system constructed as previously described (Bond *et al.*, 2002; Bond and Lovley, 2003) with electrodes consisting of 2.34 cm by 7.02 cm by 1.17 cm sticks of unpolished graphite (grade G10; Graphite Engineering and Sales, Greenville, MI). The electron acceptor provided for growth in the anode chamber consisted of either Fe(III)-citrate (55 mM) or an anode poised with a potentiostat (AMEL Instruments, Milan, Italy) at a constant potential of +200 mV (in reference to an Ag/AgCl electrode).

Mutants were tested in a fuel cell system where anaerobic conditions were maintained in the anodic chamber through continual flushing with $N_2 : CO_2$ (80:20), while the cathode chamber stayed aerobic by flushing with filtered sterile water-saturated air as previously reported (Chaudhuri and Lovley, 2003). The anode and cathode were connected via a 560 Ω resistor, and the cathode chamber contained Tris-buffer (30 mM; pH 7) amended with potassium ferricyanide (50 mM) to enhance O_2 reduction at the cathode.

In order to colonize mutants on the anode surface, cells were first grown in the anodic chamber with acetate (10 mM) as the electron donor and fumarate (20 mM) as the electron acceptor. Once early stationary phase was reached, the cultured medium was replaced anaerobically with fresh bicarbonate-buffered growth medium amended with electron donor; acetate (10 mM).

Analytical techniques

Cell current and voltage were measured with a precision multimeter (Keithley, Model 2000, Keithley Instruments, OH, USA). The anode potential was measured against a Ag/AgCl reference electrode (LF-2, 2 mm OD; Electrolytica, NY).

Acetate concentrations were determined with an HP series 1100 high-pressure liquid chromatograph (Hewlett Packard, Palo Alto, CA) on a Fast Acid Analysis column (Bio-Rad laboratories, Hercules, CA) with an eluent of 5 mM H_2SO_4 and absorbance detection at 210 nm.

Fe(III) reduction was monitored by measuring the formation of Fe(II) over time with a ferrozine assay in a split-beam dual-detector spectrophotometer (Spectronic Genosys2; Thermo Electron Corporation, Mountain View, CA) at an absorbance of 562 nm after a 1 h extraction with 0.5 N HCl as previously described (Lovley and Phillips, 1987; 1988).

MRNA extraction from the electrode

Cells were harvested from the current-harvesting electrodes for microarray analyses when current reached *c.* 0.5 mA. For quantitative RT-PCR analysis, cells were harvested at 0.5, 0.75, 1.0, 1.2, 1.4, 2.0 and 2.5 mA of current. Once the desired current was obtained, electrodes were removed from the anodic chamber, rinsed with RNA protect (Qiagen), and vigorously scraped with a sterile razor blade into 100 ml of RNA protect, producing a graphite slurry. The biomass on the surface of electrodes that were not exposed to current was not high enough to extract sufficient quantities of mRNA therefore it was necessary to supplement the graphite slurry generated from these chambers with planktonic cells. Graphite suspensions were then transferred to prechilled 50 ml conical tubes and centrifuged at 4000 r.p.m. for 15 min at 4°C. The supernatant was discarded and pellets were flash frozen in an ethanol/dry ice bath and stored at −80°C. Prior to RNA extraction, the pellet was re-suspended in 3 ml of TPE buffer (100 mM Tris-HCl, 100 mM KH_2PO_4, 10 mM EDTA; pH 8.0), and aliquoted into eight separate 2 ml screw cap tubes. RNA was extracted from these cell pellets as previously described (Holmes *et al.*, 2004c). All RNA products were treated with DNA-free (Ambion) according to the manufacturer's instructions, and the MICROBExpress kit (Ambion) was used to remove *c.* 55% of the contaminating ribosomal RNA from all mRNA samples prior to quantitative RT-PCR analyses.

Using this RNA extraction method, high-quality RNA was extracted from the surface of an electrode. All samples had A_{260}/A_{280} ratios of 1.8–2.0, indicating that they were of high purity (Ausubel *et al.*, 1997). In order to confirm the fact that PCR products generated from cDNA template did not result from the amplification of contaminating DNA, all PCR analyses included negative controls with RNA that had not been subjected to RT-PCR.

Microarray analysis

All microarray analyses were performed at the Institute for Genomic Research (TIGR; http://www.tigr.org) as previously described (Methé *et al.*, 2005). Whole-genome DNA microarrays were conducted comparing RNA extracted from *G. sulfurreducens* cells grown with either acetate (10 mM) provided as the electron donor and a poised electrode harvested at 0.5 mA of current provided as the electron acceptor or acetate (10 mM) as the electron donor with Fe(III) citrate (55 mM) provided as the electron acceptor. Total RNA (5 μg) from each condition was used to generate cDNA labelled with cyanine 3 or cyanine 5 (Cy3/Cy5) fluorescent dyes.

Results from microarray hybridizations were analysed via two independent statistical approaches: the significance analysis of microarrays (SAM) and linear models for microarray analysis (LIMMA) mixed model algorithms. Differentially expressed genes were identified by linear models with *q*-values by applying the LIMMA statistical software package (Smyth and Speed, 2003; Smyth, 2005) (http://bioinf.wehi.edu.au/limma/) and the software *q*-value (http://genomine.org/qvalue/) as described (Storey and Tibshirani, 2003; Rodriguez *et al.*, 2004; Smyth, 2004; Smyth *et al.*, 2005).

In the LIMMA mixed model, biological replicates are treated as a randomized block to allow for modelling correlations among technical replicates within biological replicates. The *P*-values obtained from the model are then adjusted for multiple testing following the *q*-value procedure (Storey and Tibshirani, 2003) to control the false discovery rate (FDR). Genes with *q*-values smaller than 0.01 were considered differentially expressed, which results in an FDR of 1%.

Signal intensities from the six hybridizations performed for each of the three biological replicates were also analysed separately using SAM (Tusher *et al.*, 2001) as previously described (Methé *et al.*, 2005). Genes that were identified by this method in at least two of three biological replicates were considered to be differentially expressed. Approximately 60% of the genes that were determined to be differentially expressed with the LIMMA software package were also identified in at least two of three biological replicates by SAM analysis. There was a considerable amount of variation among signal intensities for replicate hybridizations from each sample. The LIMMA software package specifically models variation caused by biological replicates. Therefore, results obtained from LIMMA analyses were considered more reliable. The microarray data from these experiments have been submitted to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) in MIAME format (E-TIGR-83).

Quantification of gene expression with quantitative RT-PCR

Quantitative RT-PCR analysis was performed to verify results obtained from the microarray experiments. Three genes that encode the *c*-type cytochromes, OmcS, OmcT and OmcE, were selected for further analyses. Several housekeeping genes, *recA*, *proC* and *rpoD*, that have been shown to be constitutively expressed in either *G. sulfurreducens* (Jara *et al.*, 2003; Holmes *et al.*, 2004c; Holmes *et al.*, 2005) or other bacterial species (Brady and Csonka, 1988; Vandecasteele *et al.*, 2001; Marlowe *et al.*, 2002; Vandesompele *et al.*, 2002; Savli *et al.*, 2003), and were not detected in the microarray analyses were selected as external controls for quantitative RT-PCR.

Primers were designed for quantitative RT-PCR analysis according to the manufacturer's instructions, and representative products from each of these primer sets were verified by sequencing. Primers targeting the *omcS*, *omcT*, *omcE*, *recA*, *proC* and *rpoD* genes were designed from the *G. sulfurreducens* genome sequence (Methé *et al.*, 2003). The following primers were used to amplify gene fragments from *omcS*, *omcT* and *omcE* genes: omcS1108f (5′-CGCT

TCAACCTGGCATACG-3′) and omcS1197r (5′-CTGGAGGG ACGAGGTATTGG-3′); omcT1110f (5′-CGCTTCAACCTGGC ATACG-3′) and omcT1267r (5′-TGGTAAGGAGCAAACTTG TCAGC-3′); omcE68f (5′-AGCCGCCAGCATCAAGAATA-3′) and omcE249r (5′-TGGACGAGTAGAGGGTGAAGGT-3′). The constitutively expressed housekeeping genes, *recA*, *proC* and *rpoD*, were amplified with the following primer sets: recA660f (5′-GTGAAGGTGGTCAAGAACAAGGT-3′) and recA737r (5′-GGAAATGCCCTCACCGTAGTAA-3′); proC2f (5′-CATGCTGAAGGGAAGCACTCT-3′) and proC77r (5′-GGCCAGCAGCCCTTTGAT-3′); and rpoD1132f (5′-TCATGA AGGCGGTGGACAA-3′) and rpoD1210r (5′-GCCTGTCGA ATCCACCAAGT-3′).

The DuraScript enhanced avian RT single-strand synthesis kit (Sigma) was used to generate cDNA from extracted *omcS*, *omcT*, *omcE*, *recA*, *rpoD* and *proC* transcripts as previously described (Holmes *et al*., 2004c). Once the appropriate cDNA fragments were generated by RT-PCR, quantitative RT-PCR amplification and detection was performed with the GeneAmp 5700 sequence detection system (PE Biosystems, Foster City, CA). Optimal TaqMan PCR conditions were determined using the manufacturer's guidelines. Each PCR consisted of a total volume of 50 μl and contained 3–9 μl of the appropriate primers (stock concentration was 5 μM), 5 μl 10× SYBR green PCR buffer (PE Biosystems, Foster City, CA), 6 μl $MgCl_2$ solution (25 mM; PE Biosystems), 4 μl dNTP mix (2.5 mM; PE Biosystems), 0.5 U AmpErase uracil-*N*-glycosylase (PE Biosystems) and 0.25 U AmpliTaq Gold (PE Biosystems). The thermal cycling parameters consisted of a uracil N-glycosylase (UNG) activation step at 50°C for 2 min, a denaturation step at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 63°C for 1 min. Standard curves were constructed as previously described (Holmes *et al*., 2004c), and covered a range of approximately eight orders of magnitude. To verify amplification and correct amplicon size, aliquots from real-time PCR were examined on an ethidium bromide-stained 1% agarose gel.

Northern hybridization analysis

Probes targeting *omcS* and *omcT* genes from *G. sulfurreducens* were constructed using gene products from the following primer sets: a 382 bp fragment from the *omcS* gene was amplified with 4140-1f (5′-CCAACCAGTT CAGCTGCATC-3′) and 4140-1r (5′-GAAGGGGCCAAGGTT CTGATC-3′), and a 775 bp fragment from the *omcT* gene was amplified with 4142f (5′-ACGTTCGTGGTCTCAACAC-3′) and 4142r (5′-GATGGTCGTGAACTCGTATG-3′). Amplified fragments from *G. sulfurreducens omcS* and *omcT* genes were first gel-purified with the Qiagen gel extraction kit (Qiagen) according to the manufacturer's instructions. In total, 25 ng of these amplicons was used as template for construction of [^{32}P]-dCTP-labelled probes with the NEBlot Kit (New England Biolabs, Beverly, MA), and probe hybridizations were performed at 68°C with QuikHyb Hybridization Solution (Stratagene) according to the manufacturer's instructions. Hybridization products were visualized on a Typhoon 9210 variable mode imager (Amersham Biosciences, Piscataway, NJ), and spot intensities were quantified and compared with ImageQuant software (Amersham Biosciences).

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Genes that were significantly upregulated in the

microarray comparing *G. sulfurreducens* cells grown on poised electrodes harvested at 0.5 mA to cells grown with Fe(III)-citrate provided as the electron acceptor. The values represent the fold-difference in expression between the two conditions according to LIMMA analysis.

Table S2. Genes that were significantly downregulated in the microarray comparing *G. sulfurreducens* cells grown on poised electrodes harvested at 0.5 mA to cells grown with Fe(III)-citrate provided as the electron acceptor. The values represent the fold-difference in expression between the two conditions according to LIMMA analysis.

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