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A severe reduction in the cytochrome C content of *Geobacter sulfurreducens* eliminates its capacity for extracellular electron transfer

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Summary

The ability of *Geobacter* species to transfer electrons outside the cell enables them to play an important role in a number of biogeochemical and bioenergy processes. Gene deletion studies have implicated periplasmic and outer-surface *c*-type cytochromes in this extracellular electron transfer. However, even when as many as five *c*-type cytochrome genes have been deleted, some capacity for extracellular electron transfer remains. In order to evaluate the role of *c*-type cytochromes in extracellular electron transfer, *Geobacter sulfurreducens* was grown in a low-iron medium that included the iron chelator *(2,2′-bipyridine)* to further sequester iron. Haem-staining revealed that the cytochrome content of cells grown in this manner was 15-fold lower than in cells exposed to a standard iron-containing medium. The low cytochrome abundance was confirmed by in situ nanoparticle-enhanced Raman spectroscopy (NERS). The cytochrome-depleted cells reduced fumarate to succinate as well as the cytochrome-replete cells do, but were unable to reduce Fe(III) citrate or to exchange electrons with a graphite electrode. These results demonstrate that *c*-type cytochromes are essential for extracellular electron transfer by *G. sulfurreducens*. The strategy for growing cytochrome-depleted *G. sulfurreducens* will also greatly aid future physiological studies of *Geobacter* species and other microorganisms capable of extracellular electron transfer.

Introduction

Geobacter sulfurreducens is an intensively studied microorganism that serves as a model system to investigate extracellular electron transfer (EET) in bacteria (Lovley *et al*., 2011). EET is the ability that certain bacteria have for coupling the oxidation of cytoplasmic electron donors with the reduction of insoluble electron acceptors located outside the cell. EET is responsible for biogeochemical processes such as the reduction of Fe-oxides and other metals in soils and sediments (Lovley *et al*., 2004) and for syntrophic electron transfer to methanogens (Rotaru *et al*., 2014). EET is also behind practical applications in the emergent field of electromicrobiology (Lovley *et al*., 2011), where bacteria are directly involved in redox processes with conductive materials (electrodes), which serve as electron acceptors. Microbial electrochemical technologies (MET) for harvesting energy from waste (Logan and Rabaey, 2012) or from soil environments (Domínguez-Garay *et al*., 2013), bioremediating polluted sediments (Lovley *et al*., 2011; Rodrigo *et al*., 2014) or biosensing (Dávila *et al*., 2011) are all based on an effective EET.

The unique ability of *Geobacter* to establish a direct contact with an insoluble electron acceptor is due to the presence of a vast network of cytochromes c that connects the internal cytoplasm with the outermost environment of the cell (Morgado *et al*., 2012; Aklujkar *et al*., 2013). There are about 100 putative *c*-type cytochrome genes encoded in the genome of *G. sulfurreducens* (Methé *et al*., 2003), most of which contain multiple haem groups that can act as electron transfer mediators. Many of these *c*-type cytochromes are exposed on the outermost membrane of the cell (Mehta *et al*., 2005; Ding *et al*., 2006; Qian *et al*., 2007; Leang *et al*., 2010; Inoue *et al*., 2011). Knock-out studies suggest that these *c*-type cytochromes transfer electrons in vivo to a diversity of natural extracelular electron acceptors, such as metals and humic substances (Leang *et al*., 2003; 2005; Mehta *et al*., 2005; Shelobolina *et al*., 2007; Voordeckers *et al*.,

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2010; Orellana *et al.*, 2013). Single gene deletions of *c*-type cytochromes in other iron-reducing bacteria like *Shewanella* showed a similar response for reducing extracellular electron acceptors like uranium (U(VI)) (Marshall *et al.*, 2006). Furthermore, numerous studies have demonstrated that *c*-type cytochromes directly participate in the electrochemical communication with the anode (Holmes *et al.*, 2006; Nevin *et al.*, 2009; Richter *et al.*, 2009; Busalmen *et al.*, 2010; Esteve-Núñez *et al.*, 2011; Jain *et al.*, 2011; Liu *et al.*, 2011; Millo *et al.*, 2011; Strycharz *et al.*, 2011).

The network of cytochromes in *Geobacter* can also function as biocapacitor accepting electrons from acetate metabolism (Esteve-Núñez *et al.*, 2008) when extracellular electron acceptors are not available (Esteve-Núñez *et al.*, 2008; Lovley, 2008). Indeed, the abundant *c*-type cytochromes in current-producing biofilms (Liu *et al.*, 2011; Schrott *et al.*, 2011) provide a capacitance comparable with that of synthetic supercapacitors with low self-discharge rates (Malvankar *et al.*, 2012).

The synthesis of *c*-type cytochromes constitutes a complex process in which iron must be incorporated to the proto*porphyrin* ring to conform each haem group that subsequently will be attached (Stevens *et al.*, 2004). A recent study has explored the iron stimulon, reporting how 24 different *c*-type cytochromes were slightly downregulated with decreasing iron levels (Embree *et al.*, 2014). Interestingly, strategies for promoting transposon insertions in the cytochrome *c* maturation genes *ccmC* and *ccmF1* led to *Shewanella oneidensis* strains unable to perform any kind of anaerobic respiration including the donation of electrons to extracellular electron acceptors like iron, or manganese or intracellular molecules like fumarate or nitrate (Bouhenni *et al.*, 2005).

Although iron is an abundant element in nature, its low solubility forces microorganisms to develop regulatory and transport mechanisms with the purpose of maintaining the iron homeostasis. In *G. sulfurreducens*, two systems belonging to the Feo family have been identified to facilitate the transport of Fe(II) (Cartron *et al.*, 2006). All Feo genes as well as eleven genes encoding components for heavy metal efflux pumps were found to be most downregulated during iron-excess conditions (Embree *et al.*, 2014).

The most important system for regulating the iron metabolism is the ferric-uptake regulator (Fur). Fur acts as a transcriptional repressor, which, in response of the iron availability, controls many genes related to iron acquisition as well as redox-stress resistance, central metabolism and energy production in *G. sulfurreducens* (O'Neil *et al.*, 2008; Embree *et al.*, 2014). Along with Fur, an additional transcriptional regulator called IdeR has been recently suggested to have a role in iron homeostasis for *G. sulfurreducens* (Embree *et al.*, 2014).

In some bacteria, such as the *Rhizobium* genus (Johnston *et al.*, 2007), the Fur-like iron response regulatory protein (Irr) regulates the haem biosynthetic pathway according to the iron availability. Under iron limitation conditions, Irr reduces the haem synthesis in order to avoid porphyrins accumulation that can be highly toxic (Qi *et al.*, 1999; Ishikawa *et al.*, 2011). Although Irr has not yet been found in *Geobacter* species, it is likely that *G. sulfurreducens* has developed a system to limit the synthesis of cytochromes under iron-limiting conditions based on either Fur or IdeR regulators (Embree *et al.*, 2014).

In the present study, we demonstrate that limiting the availability of iron to *G. sulfurreducens* resulted in a decreased cytochrome abundance and a concomitant loss of its capacity for EET while keeping the cell viability.

Results and discussion

High iron requirement for the optimal growth of G. sulfurreducens

The standard freshwater medium for *Geobacter* growth contains approximately 2 μM Fe as part of its trace element cocktail (Lovley and Phillips, 1986). This concentration has been reported to be sufficient to satisfy the Fe requirement of the bacteria (Fukushima *et al.*, 2012). However, it might be expected that the synthesis of the abundant cytochromes in *Geobacter* might impose a need for additional iron. In order to evaluate this, *G. sulfurreducens* was grown in chemostats under continuous culture conditions. Iron was supplied in the ferrous form because the presence of ferric iron results in transcriptional repression of the fumarate respiration (Esteve-Núñez *et al.*, 2004).

With 2 μM ferrous iron, typically used in *G. sulfurreducens* medium, the steady-state acetate concentration and the biomass concentrations stabilized at 1.5 mM and 42.6 mgprot/l respectively. Increasing the ferrous iron concentration to 150 μM led to a reduction of the residual concentration of acetate by a factor of 10 (150 μM). The biomass concentration increased to 51.7 mgprot/l culture (Fig. S1). Adding a pulse of ferrous iron had a similar impact (Fig. S1).

These results suggest that the iron availability limits the growth in typical *G. sulfurreducens*-growing medium. The higher assimilation of acetate in the presence of iron could be explained by the lower K_s obtained in chemostats with Fe(III) rather than with fumarate as Terminal Electron Acceptor (TEA), which leads to a higher affinity for acetate (Esteve-Núñez *et al.*, 2005) when the iron supply is abundant.

When cultured with 2 μM ferrous iron, *G. sulfurreducens* cells contain 1.9×10^{-6} ng iron/cell. This

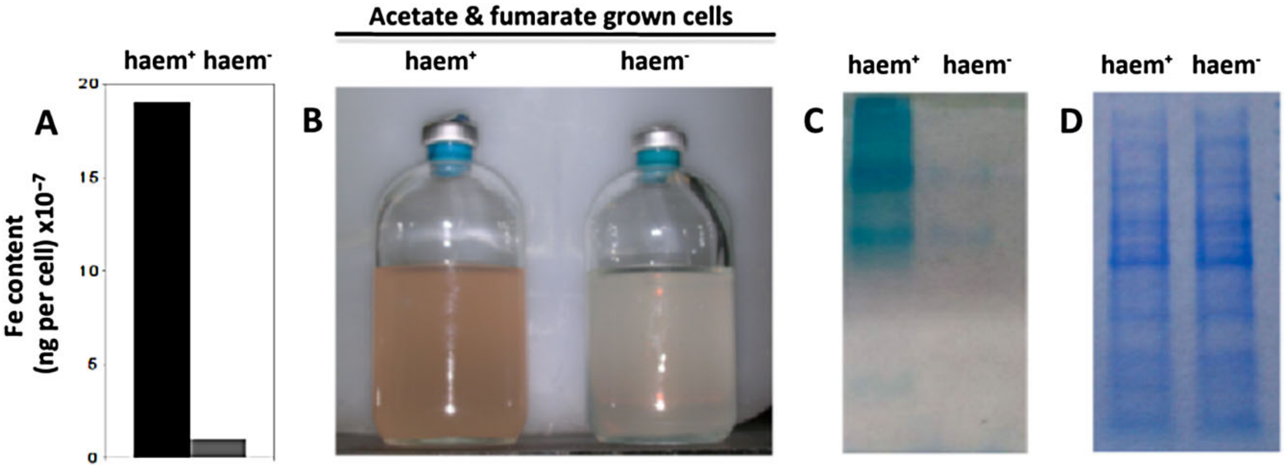

Fig. 1. Analysis of *Geobacter sulfurreducens* cultured under iron-sufficient conditions (haem$^+$) and iron-deficient conditions in presence of the iron chelator bypiridine (haem$^-$). (A) Total cellular iron content analysed by inductively coupled plasma mass spectrometry (ICP-MS); haem$^+$ (black) and haem$^-$ (grey) cells of *G. sulfurreducens.* (B) Photo of the haem$^+$ and the haem$^-$ batch cultures of *G. sulfurreducens.* The analysis of the SDS-PAGE for the protein fraction of both the haem$^+$ and the haem$^-$ *Geobacter cells* after the haem staining (C) and Comassie staining (D).

order of magnitude is higher than the average iron content of other bacteria such as *E. coli* (10^{-8} to 10^{-7} ng/cell, as derived by Andrews *et al*., 2003). *Escherichia coli* is probably the best bacteria studied in terms of microbial iron assimilation (McHugh *et al*., 2003; Kumar and Shimizu, 2011), and it is usually used as a reference model. *Escherichia coli* and *G. sulfurreducens* were cultured both under fumarate-respiring conditions in freshwater medium supplemented with ^{55}Fe to quantify the iron content incorporated to the biomass. The detection of radioactivity in the samples showed a higher content (threefold) of ^{55}Fe in *G. sulfurreducens* cells as compared with *E. coli* (Fig. S2).

The iron content in the cells was also analysed by inductively coupled plasma mass spectrometry and led to results consistent with the radiotracer measurements, approximately 1008 ppm of iron for *G. sulfurreducens* and approximately 297 ppm for *E. coli*. One reason for the difference in iron content between *G. sulfurreducens* and *E. coli* is that the *G. sulfurreducens* genome encodes more than 100 *c*-type cytochromes, whereas only five genes encoding cytochromes are present in *E. coli* (Grove *et al*., 1996; Reid *et al*., 2001). Many of the *G. sulfurreducens* cytochromes are constitutively expressed, regardless of the culture conditions (Ding *et al*., 2006), including during growth in the absence of extracellular electron acceptor, e.g. under fumarate-reducing conditions (Holmes *et al*., 2006; Esteve-Núñez *et al*., 2008). There is remarkably little conservation of *c*-type cytochromes genes across the six *Geobacter* species whose genomes have been sequenced. This suggests that there has not been evolutionary pressure to maintain specific structures that might promote interactions of the cytochromes with the electron acceptors (Lovley, 2008). However, there has been evolutionary pressure for the *Geobacter* species to maintain an abundance of haem. The energetic investment that *Geobacter* species make in the *c*-type cytochrome production could be very adaptive in providing an electron storage capacity that permits electron transfer in the temporary absence of Fe(III) oxides (Esteve-Núñez *et al*., 2008; Lovley, 2008). The hypothesis of the cytochrome network acting as capacitor, where multi-haem could store charge (Esteve-Núñez *et al*., 2008; Schrott *et al*., 2011; Robuschi *et al*., 2013), may be the key to understand this biosynthetic pathway. The electron-storage capacity of the cytochrome network would be useful in the absence of an electron acceptor while conferring *Geobacter* the ability to satisfy maintenance energy requirements to develop motility and search for the nearest available electron acceptor (Childers *et al*., 2002).

Haem⁻ Geobacter *cells*

The high requirement of *G. sulfurreducens* for iron suggests that it might be possible to limit the cytochrome production by limiting the iron availability. In order to further lower the iron availability, the iron non-supplemented medium was amended with bypiridine, an iron chelator. The iron content of cells grown in this manner was 15-fold less (1.2 ng × 10^{-7}/cell) than in cells grown in a typical iron-containing medium (Fig. 1A). Haem staining of whole-cell lysate proteins separated with SDS-PAGE demonstrated that the cytochrome content of cells grown in the low-iron medium was much lower than in cells grown in standard iron-containing medium

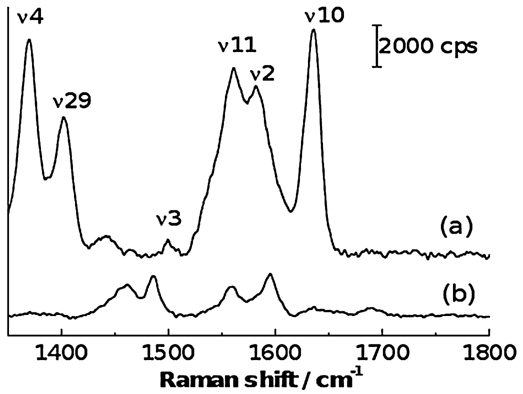

Fig. 2. In-situ nanoparticle-enhanced Raman (NER) spectra of (A) haem^{+} *G. sulfurreducens* and (B) haem^{-} *G. sulfurreducens*, both mixed with Ag nanoparticles. The Raman scattering was enhanced by the plasmonic Ag nanoparticles by several orders of magnitude allowing the selective probing of the vibrational signature of cytochromes.

(Fig. 1B–C). The cultures grown in the low-iron medium were much less red than cells grown in typical iron-containing medium (Fig. 1D).

Scanning electron microscopy (SEM) revealed no difference in cell morphology between cells grown with limited iron concentration versus the standard culture medium, indicating that the cells do not suffer from any major morphological damage due to the absence of the cytochrome network (Fig. S3).

The growth rate of *G. sulfurreducens* declined from 0.050 h^{-1} to 0.035 h^{-1} when iron limited the growth. In contrast, the rates of fumarate reduction per cell in haem^{+} (2.0×10^{-10} mmol/h cell) and haem^{-} (1.9×10^{-10} mmol/h cell) growing cells were similar demonstrating that this key central metabolism reaction was not affected by the absence of cytochromes. This is consistent with the fact that fumarate is reduced at the inner membrane by a membrane-bound fumarate reductase/succinate dehydrogenase that does not involve cytochromes (Butler *et al.*, 2006). These results demonstrate that the low-iron culture conditions provide enough iron for cells to perform central metabolism reactions and assure viable cells.

Cytochromes *c* were shown for the first time to release electron in vivo on electrodes in spectroelectrochemical studies of the outermost membrane of *Geobacter* cells upon reduction on a gold electrode (Busalmen *et al.*, 2008b). Since then, a number of techniques involving infrared (Busalmen *et al.*, 2010; Esteve-Núñez *et al.*, 2011) and Raman spectroscopy (Millo *et al.*, 2011; Virdis *et al.*, 2012; Kuzume *et al.*, 2013; Robuschi *et al.*, 2013) were applied successfully to explore the surface of the bacteria. In order to analyse the outermost membrane of the haem^{-} cells, we used in this study a nanoparticle-enhanced Raman spectroscopy (NERS), a powerful technique that can detect and further provide structure information of haem, which are vicinal to the coinage metal nanoparticle surface. For the NERS measurement in this work, Ag nanoparticles, which act as optical antennas to enhance the Raman response, were deposited onto a submonolayer of bacteria. A SEM/Energy Dispersive X-ray analysis revealed that the Ag nanoparticles located vicinal to the bacterial cells are sufficiently close to enhance the Raman signals of the outermost domains (Kuzume *et al.*, 2013). Figure 2A displays a nanoparticle enhanced Raman (NER) spectrum of *G. sulfurreducens* cells mixed with Ag nanoparticles in a Ar atmosphere. We observed (Table S1) only the haem-related bands (ν_{10}, ν_2, ν_{11}, ν_3, ν_{29} and ν_4) that are known to have a large Raman scattering cross-section due to a significant resonance effect with the 532 nm laser excitation line (Eng *et al.*, 1996; Oellerich *et al.*, 2002; Biju *et al.*, 2007; Yeo *et al.*, 2008). The key haem-related bands as found in the NER spectra are summarized and assigned in Table S1. Figure 2B shows a typical NER spectrum of the haem^{-} *G. sulfurreducens* cells. No specific Raman signals from haem-related domains were found, which represents a direct proof of the absence of haem groups in the haem^{-} sample prepared in this work. The four signals between 1400 and 1600 cm^{-1} can be assigned to the amino acid adenine (Papadopoulou and Bell, 2010) and to citrate-stabilized Ag NanoParticle (NP) (Kuzume *et al.*, 2013), that not related to haem domains.

EET assays

Gene deletion studies have implicated a number of *c*-type cytochromes in EET, but even when multiple cytochrome genes are deleted in the same strain, some EET capability remains (Voordeckers *et al.*, 2010; Orellana *et al.*, 2013). However, the number of cytochrome genes that can be deleted in a single strain is limited. To determine if the lack of cytochromes associated with the growth in a low-iron medium could completely remove the capacity for EET, cells growing with fumarate as electron acceptor were pulsed with 10 mM Fe(III) citrate. No Fe(III) was reduced (Fig. 3), and the rate of fumarate reduction to succinate (1.9×10^{-10} mmol/h per cell) was unaltered. In contrast, when Fe(III) was added to cells growing in a medium with the standard iron content, Fe(III) was rapidly reduced (5×10^{-10} mmol/h per cell) and the fumarate reduction was inhibited.

Another EET process, where cytochromes have been reported to participate, is the electrode reduction in MET. By using electrochemical approaches, such as cyclic voltammetry (Busalmen *et al.*, 2008a), the bioelectrochemical response for the extracellular electron transport was monitored. *Geobacter sulfurreducens* was resuspended in phosphate buffer in the presence of an electron donor, but in the absence of a soluble electron acceptor. Consequently, when *G. sulfurreducens* cells were incubated in a three-electrode cell, just the electrode could act as TEA. A typical voltammogram shows two redox peaks with current maxima at 0.2 and −0.2 V versus Ag/AgCl (Busalmen *et al.*, 2008a; Fricke *et al.*, 2008; Richter *et al.*, 2008), which represents the corresponding oxidation and reduction processes respectively. In contrast to the wild type, *G. sulfurreducens* haem$^-$ cells did not display any redox peak demonstrating that the presence of cytochromes is required for performing a sufficient redox communication with an exocellular electron acceptor, such as a polarized electrode (Fig. 4). The absence of additional current peaks confirms that the cytochrome-related redox reactions comprise the major active compound in *Geobacter* redox activity on polarized electrodes in Bioelectrochemical systems (BES). This conclusion is confirmed by recent findings of several groups (Busalmen *et al.*, 2008b; Millo *et al.*, 2011; Kuzume *et al.*, 2013).

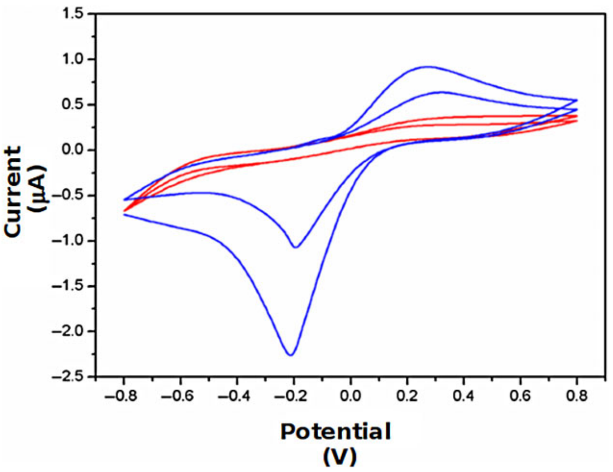

Fig. 4. Cyclic voltammograms of *G. sulfurreducens* cells deposited on a carbon electrode. Blue line: haem$^+$ *G. sulfurreducens*; red line: haem$^-$ *G. sulfurreducens.* The potential refers to an Ag/AgCl reference electrode.

Conclusions

These results demonstrate dramatic impact of available iron on the growth and activity of *G. sulfurreducens*. Adjusting laboratory media to provide a higher iron concentration than that *Geobacter* species experience in a natural environment may promote important applications, such as MET that rely on optimized extracellular electron exchange.

Alternatively, making iron less available yielded cells unable to produce haem groups and studies with these cells confirmed the key role of the vast cytochrome network in EET. Our bioelectrochemical results confirm that cytochromes are essential for direct electron transfer to electrodes. Although we have focused on getting haem$^-$ cells, our methodology allows controlling the level of cytochrome production by varying the doses of the chelator. In consequence, we could generate *Geobacter* cells with different levels of haem content in contrast with previous strategies performed in bacteria for erasing all *c*-type cytochromes through transposon insertions that led to unviable cells under anaerobic conditions (Bouhenni *et al.*, 2005). Furthermore, we believe that haem$^-$ cells reported in this work will also be relevant for other researchers targeting investigations on the physiology of *Geobacter* under EET-free background conditions.

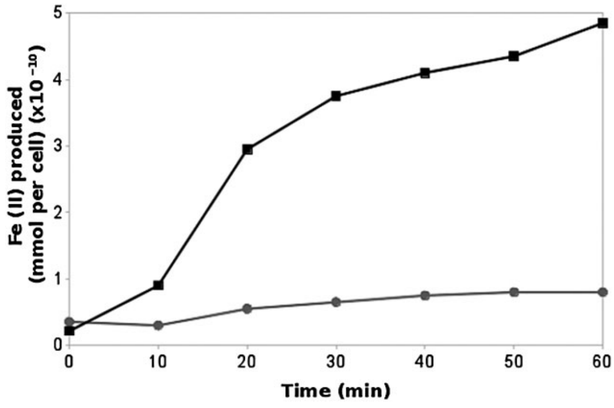

Fig. 3. Fe (III) reduction after addition of 10 mM ferric citrate to haem$^+$ *G. sulfurreducens* cells (black line) and to haem$^-$ *G. sulfurreducens* cells (grey line).

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References

Aklujkar, M., Coppi, M.V., Leang, C., Kim, B.C., Chavan, M.A., Perpetua, L.A., *et al.* (2013) Proteins involved in electron transfer to Fe(III) and Mn(IV) oxides by *Geobacter sulfurreducens* and *Geobacter uraniireducens. Microbiology* **159:** 515–535.

Andrews, S.C., Robinson, A.K., and Rodríguez-Quiñones, F. (2003) Bacterial iron homeostasis. *FEMS Microbiol Rev* **27:** 215–237.

Biju, V., Pan, D., Gorby, Y.A., Fredrickson, J.J., McLean, J., Saffarini, D., and Lu, H.P. (2007) Correlated spectroscopic and topographic characterization of nanoscale domains and their distributions of a redox protein on bacterial cell surfaces. *Langmuir* **23:** 1333–1338.

Bouhenni, R., Gehrke, A., and Saffarini, D. (2005) Identification of genes involved in cytochrome c biogenesis in *Shewanella oneidensis*, using a modified mariner transposon. *Appl Environ Microbiol* **71:** 4935–4937.

Busalmen, J.P., Esteve-Nuñez, A., and Feliu, J.M. (2008a) Whole cell electrochemistry of electricity-producing microorganisms evidence an adaptation for optimal exocellular electron transport. *Environ Sci Technol* **42:** 2445–2450.

Busalmen, J.P., Esteve-Nunez, A., Berna, A., and Feliu, J.M. (2008b) C-type cytochromes wire electricity-producing bacteria to electrodes. *Agnew Chem Int* **47:** 4874–4877.

Busalmen, J.P., Esteve-Nuñez, A., Berná, A., and Feliu, J.M. (2010) ATR-SEIRAs characterization of surface redox processes in *G. sulfurreducens*. *Bioelectrochemistry* **78:** 25–29.

Butler, J.E., Glaven, R.H., Esteve-Núñez, A., Núñez, C., Shelobolina, E.S., Bond, D.R., and Lovley, D.R. (2006) Genetic characterization of a single bifunctional enzyme for fumarate reduction and succinateoxidation in *Geobacter sulfurreducens* and engineering of fumarate reduction in *Geobacter metallireducens*. *J Bacteriol* **188:** 450–455.

Cartron, M., Maddocks, S., Gillingham, P., Craven, C., and Andrews, S. (2006) Feo – transport of ferrous iron into bacteria. *Biometals* **19:** 143–157.

Childers, S.E., Ciufo, S., and Lovley, D.R. (2002) *Geobacter metallireducens* accesses insoluble Fe(III) oxide by chemotaxis. *Nature* **416:** 767–769.

Dávila, D., Esquivel, J.P., Sabaté, N., and Mas, J. (2011) Silicon-based microfabricated microbial fuel cell toxicity sensor. *Biosens Bioelectron* **26:** 2426–2430.

Ding, Y.H., Hixson, K.K., Giometti, C.S., Stanley, A., Esteve-Núñez, A., Khare, T., *et al*. (2006) The proteome of dissimilatory metal-reducing microorganism *Geobacter sulfurreducens* under various growth conditions. *Biochim Biophys Acta* **1764:** 1198–1206.

Domínguez-Garay, A., Berná, A., Ortiz-Bernad, I., and Esteve-Núñez, A. (2013) Silica colloid formation enhances performance of sediment microbial fuel cells in a low conductivity soil. *Environ Sci Technol* **47:** 2117–2122.

Embree, M., Qiu, Y., Shieu, W., Nagarajan, H., O'Neil, R., Lovley, D., and Zengler, K. (2014) The iron stimulon and fur regulon of *Geobacter sulfurreducens* and their role in energy metabolism. *Appl Environ Microbiol* **80:** 2918–2927.

Eng, L.H., Schlegel, V., Wang, D.L., Neujahr, H.Y., Stankovich, M.T., and Cotton, T. (1996) Resonance Raman scattering and surface-enhanced resonance Raman scattering studies of oxido-reduction of cytochrome c3. *Langmuir* **12:** 3055–3059.

Esteve-Núñez, A., Núñez, C., and Lovley, D.R. (2004) Preferential reduction of FeIII over fumarate by *Geobacter sulfurreducens*. *J Bacteriol* **86:** 2897–2899.

Esteve-Núñez, A., Rothermich, A., Sharma, M., and Lovley, D.R. (2005) Growth of *Geobacter sulfurreducens* under nutrient-limiting conditions in continuous culture. *Environ Microbiol* **7:** 641–648.

Esteve-Núñez, A., Sosnik, J., Visconti, P., and Lovley, D.R. (2008) Fluorescent properties of c-type cytochromes reveal their potential role as an extracytoplasmic electron sink in *Geobacter sulfurreducens*. *Environ Microbiol* **10:** 497–505.

Esteve-Núñez, A., Busalmen, J.P., Berná, A., Gutierrez-Garrán, C., and Feliu, J.M. (2011) Opportunities behind the unusual ability of *Geobacter sulfurreducens* for exocellular respiration and electricity production. *Energy Environ Sci* **4:** 2066–2069.

Fricke, K., Harnisch, F., and Schröder, U. (2008) On the use of cyclic voltammetry for the study of anodic electron transfer in microbial fuel cells. *Energy Environ Sci* **1:** 144–147.

Fukushima, T., Sia, A.K., Allred, B.E., Nichiporuk, R., Zhou, Z., Andersen, U.N., and Raymond, K.N. (2012) Bacillus cereus iron uptake protein fishes out an unstable ferric citrate trimer. *Proc Natl Acad Sci USA* **109:** 16829–16834.

Grove, J., Tanapongpipat, S., Thomas, G., Griffiths, L., Crooke, H., and Cole, H. (1996) *Escherichia coli* K-12 genes essential for the synthesis of c-type cytochromes and a third nitrate reductase located in the periplasm. *J Mol Microbiol* **19:** 467–481.

Holmes, D.E., Chaudhuri, S.K., Nevin, K.P., Mehta, T., Methé, B.A., Liu, A., *et al*. (2006) Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. *Environ Microbiol* **8:** 1805–1815.

Inoue, K., Leang, C., Franks, A.E., Woodard, T.L., Nevin, K.P., and Lovley, D.R. (2011) Specific localization of the c-type cytochrome OmcZ at the anode surface in current-producing biofilms of *Geobacter sulfurreducens*. *Environmental Microbiol Rep* **3:** 211–217.

Ishikawa, H., Nakagaki, M., Bamba, A., Uchida, T., Hori, H., O'Brian, M.R., *et al*. (2011) Unusual heme binding in the bacterial iron response regulator protein: spectral characterization of heme binding to the heme regulatory motif. *Biochemistry* **50:** 1016–1022.

Jain, A., Gazzola, G., Panzera, A., Zanoni, M., and Marsili, E. (2011) Visible spectroelectrochemical characterization of *Geobacter sulfurreducens* biofilms on optically transparent indium tin oxide electrode. *Electrochim Acta* **47:** 12530–12532.

Johnston, A.W., Todd, J.D., Curson, A.R., Lei, S., Nikolaidou-Katsaridou, N., Gelfand, M.S., and Rodionov, D.A. (2007) Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium Rhizobium and other alpha-proteobacteria. *Biometals* **20:** 501–511.

Kumar, R., and Shimizu, K. (2011) Transcriptional regulation of main metabolic pathways of cyoA,cydB, fnr, and fur gene knockout Escherichia coli in C-limited and N-limited aerobic continuous cultures. *Microb Cell Fact* **10:** 3.

Kuzume, A., Zhumaev, U., Li, J., Fu, Y., Füeg, M., Esteve-Nuñez, A., and Wandlowski, T. (2013) An in-situ surface electrochemistry approach toward whole-cell studies: charge transfer between *Geobacter sulfurreducens* and electrified metal/electrolyte interfaces through linker molecules. *Electrochim Acta* **112:** 933–942.

Leang, C., Coppi, M.V., and Lovley, D.R. (2003) OmcB, a c-type polyheme cytochrome, involved in Fe(III) reduction in *Geobacter sulfurreducens*. *J Bacteriol* **185:** 2096–2103.

Leang, C., Adams, L.A., Chin, K.J., Nevin, K.P., Meth'e, B.A., Webster, J., *et al*. (2005) Adaption to disruption of electron transfer pathway for Fe(III) reduction in *Geobacter sulfurreducens*. *J Bacteriol* **187:** 5918–59126.

Leang, C., Qian, X.L., Mester, T., and Lovley, D.R. (2010) Alignment of the c-type cytochrome OmcS along pili of *Geobacter sulfurreducens*. *Appl Environ Microbiol* **76:** 4080–4084.

Liu, Y., Kim, H., Franklin, R.R., and Bond, D.R. (2011) Linking spectral and electrochemical analysis to monitor c-type cytochrome redox status in living *Geobacter sulfurreducens* biofilms. *Chemphyschem* **12:** 2235–2241.

Logan, B.E., and Rabaey, K. (2012) Conversion of wastes into bioelectricity and chemicals by using microbial electrochemical technologies. *Science* **337:** 686–690.

Lovley, D.R. (2008) Extracellular electron transfer: wires, capacitors, iron lungs, and more. *Geobiology* **6:** 225–231.

Lovley, D.R., and Phillips, E.J.P. (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Appl Environ Microbiol* **51:** 683–689.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. *Adv Microb Physiol* **49:** 219–286.

Lovley, D.R., Ueki, T., Zhang, T., Malvankar, N.S., Shrestha, P.M., Flanagan, K.A., *et al*. (2011) *Geobacter*: the microbe electric's physiology, ecology, and practical applications. *Adv Microb Physiol* **59:** 1–100.

McHugh, J.P., Rodríguez-Quinoñes, F., Abdul-Tehrani, H., Svistunenko, D.A., Poole, R.K., Cooper, C.E., and Andrews, S.C. (2003) Global iron-dependent gene regulation in *Escherichia coli*. A new mechanism for iron homeostasis. *J Biol Chem* **278:** 29478–29486.

Malvankar, N.S., Mester, T., Tuominen, M.T., and Lovley, D.R. (2012) Supercapacitors based on c-type cytochromes using conductive nanostructured networks of living bacteria. *Chemphyschem* **13:** 463–468.

Marshall, M.J., Beliaev, A.S., Dohnalkova, A.C., Kennedy, D.W., Shi, L., *et al*. (2006) *c*-type cytochrome-dependent formation of U(IV) nanoparticles by *Shewanella oneidensis*. *PLoS Biol* **4:** e268.

Mehta, T., Coppi, M.V., Childers, S.E., and Lovley, D.R. (2005) Outer membrane c-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. *Appl Environ Microbiol* **71:** 8634–8641.

Methé, B.A., Nelson, K.E., Eisen, J.A., Paulsen, I.T., Nelson, W., Heidelberg, J.F., *et al*. (2003) Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. *Science* **302:** 1967–1969.

Millo, D., Harnisch, F., Patil, S.A., Ly, H.K., Schröder, U., and Hildebrandt, P. (2011) In situ spectroelectrochemical investigation of electrocatalytic microbial biofilms by surface-enhanced resonance Raman spectroscopy. *Angew Chem Int Ed Engl* **50:** 2625–2627.

Morgado, L., Dantas, J.M., Simões, T., Londer, Y.Y., Pokkuluri, P.R., and Salgueiro, C.A. (2012) Role of Met(58) in the regulation of electron/proton transfer in trihaem cytochrome PpcA from *Geobacter sulfurreducens*. *Biosci Rep* **33:** 11–22.

Nevin, K.P., Kim, B.C., Glaven, R.H., Johnson, J.P., Woodard, T.L., Methe, B.A., *et al*. (2009) Anode biofilm transcriptomics reveals outer surface components essential for high density current production in *Geobacter sulfurreducens* fuel cells. *PLoS ONE* **4:** e5628.

Oellerich, S., Wackerbarth, H., and Hildebrandt, P. (2002) Spectroscopic characterization of nonnative conformational states of cytochrome c. *J Phys Chem B* **106:** 6566–6580.

O'Neil, R.A., Holmes, D.E., Coppi, M.V., Adams, L.A., Larrahondo, M.J., Ward, J.E., *et al*. (2008) Gene transcript analysis of assimilatory iron limitation in *Geobacteraceae* during groundwater bioremediation. *Environ Microbiol* **10:** 1218–1230.

Orellana, R., Leavitt, J.J., Comolli, L.R., Csencsits, R., Janot, N., Flanagan, K.A., *et al*. (2013) U(VI) reduction by diverse outer surface c-type cytochromes of *Geobacter sulfurreducens*. *Appl Environ Microbiol* **79:** 6369–6374.

Papadopoulou, E., and Bell, S.E.J. (2010) Structure of adenine on metal nanoparticles: pH equilibria and formation of Ag+ complexes detected by surface-enhanced Raman spectroscopy. *J Phys Chem C* **114:** 22644–22651.

Qi, Z., Hamza, I., and O'Brian, M.R. (1999) Heme is an effector molecule for iron-dependent degradation of the bacterial iron response regulator (Irr) protein. *Proc Natl Acad Sci USA* **96:** 13056–13061.

Qian, X., Reguera, G., Mester, T., and Lovley, D.R. (2007) Evidence that OmcB and OmpB of *Geobacter sulfurreducens* are outer membrane surface proteins. *FEMS Microbiol Lett* **277:** 21–27.

Reid, E., Cole, J., and Eaves, D.J. (2001) The *Escherichia coli* CcmG protein fulfils a specific role in cytochrome c assembly. *Biochem J* **355:** 51–358.

Richter, H., McCarthy, K., Nevin, K.P., Johnson, J.P., Rotello, V.M., and Lovley, D.R. (2008) Electricity generation by *Geobacter sulfurreducens* attached to gold electrodes. *Langmuir* **24:** 4376–4379.

Richter, H., Nevin, K.P., Jia, H., Lowy, D.A., Lovley, D.R., and Tender, L.M. (2009) Cyclic voltammetry of biofilms of wild type and mutant *Geobacter sulfurreducens* on fuel cell anodes indicates possible roles of OmcB,OmcZ, type IV pili, and protons in extracellular electron transfer. *Energ Environ Sci* **2:** 506–516.

Robuschi, L., Tomba, J.P., Schrott, G.D., Bonanni, P.S., Desimone, P.M., and Busalmen, J.P. (2013) Spectroscopic slicing to reveal internal redox gradients in electricity-producing biofilms. *Angew Chem Int Ed Engl* **52:** 925–928.

Rodrigo, J., Boltes, K., and Esteve-núñez, A. (2014) Microbial-electrochemical bioremediation and detoxification of dibenzothiophene-polluted soil. *Chemosphere* **101:** 61–65.

Rotaru, A.E., Shrestha, P.M., Liuab, F., Shresthaa, M., Shresthaa, D., Embreec, M., *et al*. (2014) A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. *Energy Environ Sci* **7:** 408–415.

Schrott, G.D., Bonanni, P.S., Robuschi, L., Esteve-Núñez, A., and Busalmen, J.P. (2011) Electrochemical insight into the mechanism of electron transport in biofilms of *Geobacter sulfurreducens*. *Electrochim Acta* **56:** 10791–10795.

Shelobolina, E.S., Coppi, M.V., Korenevsky, A.A., DiDonato, L.N., Sullivan, S.A., *et al*. (2007) Importance of c-type cytochromes for U(VI) reduction by *Geobacter sulfurreducens*. *BMC Microbiol* **7:** 16.

Stevens, J.M., Daltrop, O., Allen, J.W., and Ferguson, S.J. (2004) C-type cytochrome formation: chemical and biological enigmas. *Acc Chem Res* **37:** 999–1007.

Strycharz, S.M., Malanoski, A.P., Snider, R.M., Yi, H., Lovley, D.R., and Tender, L.M. (2011) Application of cyclic voltammetry to investigate enhanced catalytic current generation by biofilm-modified anodes of *Geobacter sulfurreducens* strain DL1 versus variant strain KN400. *Energy Environ Sci* **4:** 896–913.

Virdis, B., Harnisch, F., Batstone, D.J., Rabaey, K., and Donose, B.C. (2012) Non-invasive characterization of electrochemically active microbial biofilms using confocal Raman microscopy. *Energy Environ Sci* **5:** 7017–7024.

Voordeckers, J.W., Izallalen, M., Kim, B.C., and Lovley, D.R. (2010) Role of *Geobacter sulfurreducens* outer surface c-type cytochromes in the reduction of soil humic acid and the humics analog anthraquinone-2,6-disulfonate. *Appl Environ Microbiol* **76:** 2371–2375.

Yeo, B.S., Mädler, S., Schmid, T.H., Zhang, W., and Zenobi, R. (2008) Tip-enhanced raman spectroscopy can see more: the case of cytochrome c. *J Phys Chem* **112:** 4867–4873.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Residual acetate concentration under acetate-limiting conditions with (A) a culture growing in a standard freshwater medium (orange line), (B) growing in Fe(II)-supplemented freshwater medium (blue line), and (C) growing in a standard freshwater medium, but spiked with Fe(II) as indicated by the arrow (purple line).

Fig. S2. ^{55}Fe-content of a filtered cell suspension of (A) *E. coli* and (B) *Geobacter sulfurreducens*.

Fig. S3. SEM images of heme^{+} *G. sulfurreducens* (A) and heme^{-} *G. sulfurreducens* (B).

Table S1. Assignment and frequencies (cm^{-1}) of the heme-related bands of *G. sulfurreducens* (Fig. 2A) in the NER spectrum.