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 (Dominguez-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 extracellular electron acceptors, such as metals and humic substances (Leang et al., 2003; 2005; Mehta et al., 2005; Shelobolina et al., 2007; Voordeckers et al.,
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 protoporphyrin 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 Ks 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
order of magnitude is higher than the average iron content of other bacteria such as E. coli \((10^{-8} \text{ to } 10^{-7} \text{ 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 \(^{55}\text{Fe}\) to quantify the iron content incorporated to the biomass. The detection of radioactivity in the samples showed a higher content (threefold) of \(^{55}\text{Fe}\) 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 bipyridine, an iron chelator. The iron content of cells grown in this manner was 15-fold less \((1.2 \text{ ng } \times 10^{-7} / \text{cell})\) 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.
upon reduction on a gold electrode (Busalmen et al. 2008b). Since then, a number of techniques involving studies of the outermost membrane of electron in vivo on electrodes in spectroelectrochemical central metabolism reactions and assure viable cells. Culture conditions provide enough iron for cells to perform hydrogenase that does not involve cytochromes (Butler et al., 2006). These results demonstrate that the low-iron concentration versus the standard culture medium could completely remove the capacity for 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 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 \textit{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 \textit{G. sulfurreducens} cells mixed with Ag nanoparticles in a Ar atmosphere. We observed (Table S1) only the haem-related bands (\(\nu_{10}\), \(\nu_{2}\), \(\nu_{11}\), \(\nu_{29}\) and \(\nu_{4}\)) 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\(^{-}\) \textit{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. \textit{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).

**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.

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**References**


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.