Genes for two multicopper proteins required for Fe(III) oxide reduction in *Geobacter sulfurreducens* have different expression patterns both in the subsurface and on energy-harvesting electrodes

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Previous studies have shown that *Geobacter sulfurreducens* requires the outer-membrane, multicopper protein OmpB for Fe(III) oxide reduction. A homologue of OmpB, designated OmpC, which is 36 % similar to OmpB, has been discovered in the *G. sulfurreducens* genome. Deletion of *ompC* inhibited reduction of insoluble, but not soluble Fe(III). Analysis of multiple *Geobacter* and *Pelobacter* genomes, as well as *in situ* *Geobacter*, indicated that genes encoding multicopper proteins are conserved in *Geobacter* species but are not found in *Pelobacter* species. Levels of *ompB* transcripts were similar in *G. sulfurreducens* at different growth rates in chemostats and during growth on a microbial fuel cell anode. In contrast, *ompC* transcript levels increased at higher growth rates in chemostats and with increasing current production in fuel cells. Constant levels of *Geobacter ompB* transcripts were detected in groundwater during a field experiment in which acetate was added to the subsurface to promote *in situ* uranium bioremediation. In contrast, *ompC* transcript levels increased during the rapid phase of growth of *Geobacter* species following addition of acetate to the groundwater and then rapidly declined. These results demonstrate that more than one multicopper protein is required for optimal Fe(III) oxide reduction in *G. sulfurreducens* and suggest that, in environmental studies, quantifying OmpB/OmpC-related genes could help alleviate the problem that *Pelobacter* genes may be inadvertently quantified via quantitative analysis of 16S rRNA genes. Furthermore, comparison of differential expression of *ompB* and *ompC* may provide insight into the *in situ* metabolic state of *Geobacter* species in environments of interest.

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INTRODUCTION

Molecular strategies for monitoring the growth and metabolism of *Geobacter* species are desired because *Geobacter* species play an important role in the degradation of natural and contaminant organic compounds in sedimentary environments (Coates *et al.*, 2005; Lin *et al.*, 2005; Lovley *et al.*, 2004; Rooney-Varga *et al.*, 1999; Sleep *et al.*, 2006; Windler *et al.*, 2007) and can serve as agents for the bioremediation of metal contamination (Anderson *et al.*, 2003; Chang *et al.*, 2005; Istok *et al.*, 2004; North *et al.*, 2004; Petrie *et al.*, 2003). To identify key target genes that can be used to monitor *in situ* *Geobacter* metabolism, the genomes of several *Geobacter* species have been or are in the process of being sequenced (Methe *et al.*, 2003;

www.jgi.doe.gov) and the function of novel genes associated with these genomes is being investigated.

For example, the fact that *nifD*, which encodes the alpha subunit of the dinitrogenase protein involved in nitrogen fixation, is phylogenetically distinct within the *Geobacteraceae*, makes it possible to quantify *Geobacteraceae nifD* transcripts in subsurface sediments (Holmes *et al.*, 2004a). Results from *nifD* expression studies have been shown to be diagnostic of a limitation for fixed nitrogen during growth of *Geobacter* species in subsurface environments (Holmes *et al.*, 2004b). In addition, levels of transcripts for another phylogenetically distinct gene, *gltA*, which encodes a eukaryotic-like citrate synthase in *Geobacter* species, increased as rates of metabolism increased in a pure culture of *Geobacter sulfurreducens*, and *gltA* mRNA transcript levels in groundwater reflected changes in metabolism related to fluctuations in acetate availability during *in situ* bioremediation of a

Abbreviations: MPN-PCR, most-probable-number PCR; NTA, nitrilotriacetic acid.

uranium-contaminated subsurface environment (Holmes *et al.*, 2005).

Attempts to identify key genes whose expression can be specifically linked to metal reduction in *Geobacter* species have been less successful. Genetic studies have identified a number of *c*-type cytochrome genes that are essential for optimal Fe(III) reduction in *G. sulfurreducens*, which has provided insights into the mechanisms for extracellular electron transfer in this organism (Butler *et al.*, 2004; DiDonato *et al.*, 2006; Kim *et al.*, 2005, 2006; Leang *et al.*, 2003; Lloyd *et al.*, 2003; Mehta *et al.*, 2005; Shelobolina *et al.*, 2007). However, none of these genes have proven useful as target genes for environmental studies. For example, the outer-membrane *c*-type cytochrome, *OmcB*, is required for Fe(III) reduction (Kim *et al.*, 2006; Leang *et al.*, 2003; Leang & Lovley, 2005), and levels of *omcB* transcripts were directly related to rates of Fe(III) reduction in chemostat cultures (Chin *et al.*, 2004). However, *omcB* expression patterns were greatly influenced by environmental fluctuations, such as changes in electron acceptor availability, suggesting that tracking *omcB* transcripts in *Geobacter*-dominated environments would not provide an accurate indication of rates of Fe(III) reduction (Chin *et al.*, 2004). Furthermore, as more *Geobacter* genomes have been sequenced, it has become apparent that cytochromes required for Fe(III) reduction by *G. sulfurreducens* are not necessarily conserved in other *Geobacter* species (J. Butler, unpublished data).

It has become apparent recently that outer-surface proteins of *G. sulfurreducens* other than *c*-type cytochromes may be important in Fe(III) oxide reduction (Afkari *et al.*, 2005; Childers *et al.*, 2002; Mehta *et al.*, 2006; Reguera *et al.*, 2005). One of these, designated outer-membrane protein B (*OmpB*), is a putative multicopper protein (Mehta *et al.*, 2006) that is loosely associated with the outer cell surface (Qian *et al.*, 2007), and is required for the reduction of insoluble Fe(III) oxides, but not soluble Fe(III) (Mehta *et al.*, 2006). *OmpB* is predicted to be different from multicopper proteins found in other micro-organisms because it contains sequences indicative of an Fe(III)-binding motif and a fibronectin type III domain.

To determine whether *ompB* expression patterns are diagnostic of physiological responses associated with different metabolic states of *Geobacter* species in environments of interest, levels of *Geobacter ompB* mRNA transcripts were monitored in pure-culture studies and in the environment. In the course of these studies, a homologue of *OmpB*, designated *OmpC*, that is also required for optimal Fe(III) oxide reduction was discovered. These results suggest that the ability to monitor *ompB* and *ompC* expression patterns may provide insights into the physiological status of *Geobacter* species.

METHODS

Source of organisms and culture conditions. *Geobacter sulfurreducens* (DSM 12127^T), *Pelobacter carbinolicus* (DSM 2380^T),

Pelobacter propionicus (DSM 2379^T), *Pelobacter acidigallici* (ATCC 49970^T), *Pelobacter acetylenicus* (DSM 3246^T), *Pelobacter massiliensis* (DSM 6233^T) and *Pelobacter venetianus* (DSM 2394^T) were obtained from our laboratory culture collection and grown under previously described conditions (Holmes *et al.*, 2004a). Standard anaerobic techniques were used throughout (Balch *et al.*, 1979; Nottingham & Hungate, 1969). All media were sterilized by autoclaving and all incubations were at 30 °C. Cells were grown with acetate (10 mM) as electron donor, and fumarate (40 mM), Fe(III) oxide (100 mM), Fe(III) citrate (55 mM), Fe(III)-nitrilotriacetic acid (NTA; 10 mM), Mn(IV) oxide (20 mM), or an electrode poised at +0.52 V (with reference to a standard H₂ electrode) as electron acceptors.

***G. sulfurreducens* grown under batch and chemostat conditions.** *G. sulfurreducens* was grown in electron-donor-limited chemostats with acetate (5 mM) as electron donor and Fe(III) citrate (55 mM) as electron acceptor at 30 °C, as described previously (Esteve-Nunez *et al.*, 2005). *G. sulfurreducens* was grown in chemostats at dilution rates of 0.03 and 0.07 h⁻¹ in triplicate. Analysis of Fe(II), acetate and protein were performed as described previously (Esteve-Nunez *et al.*, 2005).

Growth of *G. sulfurreducens* on an electrode. *G. sulfurreducens* cells were first grown in medium with acetate (10 mM) as electron donor and Fe(III) citrate (55 mM) as electron acceptor. Cells were then pelleted via centrifugation, washed and resuspended in anoxic medium lacking an electron donor or acceptor. This cell suspension served as inoculum for the anaerobic anodic chamber (250 ml medium) of a two-chambered electrode system constructed as described previously (Bond *et al.*, 2002; Bond & Lovley, 2003). The electron acceptor provided for growth in the anode chamber consisted of an anode poised with a potentiostat (AMEL Instruments) at a constant potential of +0.52 V (with reference to a standard H₂ electrode) and acetate (10 mM) was provided as electron donor.

A Power Lab 4SP unit connected to a Power Macintosh computer collected current and voltage measurements directly from potentiostat outputs every 10 s. The data were logged with Chart 4.0 software (ADInstruments) as current (mA) production over time.

Construction of *ompC* deletion mutant. Sequences from all primer pairs used for construction of the *ompC* deletion mutant are outlined in Table 1. Primers 2657rgUp5' and 2657rgUp3' amplified a 500 bp region upstream from the *ompC* gene, while primers 2657rgDn5' and 2657rgDn3' were used to amplify the sequence downstream from *ompC* (500 bp). The kanamycin resistance cassette was amplified from pBBR1MCS-2 (Kovach *et al.*, 1995) with primers KanForR1 and KanRevH3. Recombinant PCR was performed with 2657rgUp5' and 2657rgDn3', which amplified a 2.11 kb fragment, and single-step gene replacement was done as described previously (Leang *et al.*, 2003). Electroporation, mutant isolation and genotype confirmation using Southern hybridization were also performed as described previously (Coppi *et al.*, 2001).

Expression of the *ompC* gene in trans. The *ompC* mutant was complemented with the expression vector pRG5 as described previously (Kim *et al.*, 2005). Primers 2657rg5' and 2657rg3' (Table 1) were used to amplify *ompC* and the upstream native ribosome-binding site from *G. sulfurreducens* chromosomal DNA. The lower-case letters in this primer set represent *EcoRI* restriction sites. The 2.55 kb fragment was digested with restriction enzyme *EcoRI* and cloned into expression vector pRG5 (Kim *et al.*, 2005). The *ompC* gene was sequenced to screen for PCR artefacts. The *ompC* deletion mutant was electroporated with pRG5-*ompC*. Streptomycin was used as the selection marker to screen for the insert.

Table 1. Primer pairs used for this study

Primer pair	Forward primer sequence	Reverse primer sequence
2657rgUp5'/2657rgUp3'	CCACTTTGCAGGTGACGCAGCCGATGTCTG	GCTATGAATTCATGGCGTACGACCTCCTTTCC
2657rgDn5'/2657rgDn3'	GCTATGAAGCTTCGGAAGCCGCACTATGAGGC ATGTG	AAGCACTATCACTTCGGCATTGCCGTGGAGACC
KanForR1/KanRevH3	GCTATGAATTCACCTGGGATGAATGTCAGCTA CTGG	GCTATGAAGCTTTCATAGAAGGCGGCGGTGGAA- TCGAA
2657rg5'/2657rg3'	GCATAGaattcAGGAAAGGAGGTCTGTACGC*	GCATAGaattcTTATCGCGGCGGTCCGAAC*
Geo_gltA100f/850r	TCARTSTATTGGCGGTGC	CGGAGTCTTSAIGCAGAACTC
Geo_mdh260f/610r	RCCTTCATCGCCTGGGAACT	CMRGGWACRCCGACRTAGTA
Geo_ompC680f/1385r	CGKWCAATVCGGCKCCTTACA	AGRGTTCSGAGTTGCAGCC
Geo_proC75f/471r	ATWGGIGGIGGIAATATGGC	TCCCCACCAGGTCTGAACA
Geo_ompB576f/1380r	GATGGIACICCATCAGTGGAT	GGIGTGTCATGAAIGCTTC
Geo_nifD225f/560r	GATGGIACICCATCAGTGGAT	GGIGTGTCATGAAIGCTTC
Gsuf_recA660f/737r	GTGAAGGTGGTCAAGAACAAGGT	GGAAATGCCCTCACCGTAGTAA
Gsulf_proC2f/77r	CATGCTGAAGGGAAGCACTCT	GGCCAGCAGCCCTTTGAT
Gsulf_rpoD1132f/1210r	TCATGAAGGCGGTGGACAA	GCCTGTCTGAATCCACCAAGT
Gsulf_ompC408f/518r	GGAGAACACGTCGGGCAT	GCCCAGTGGAGGGTCTGAT
Gsulf_ompB2281f/2395r	CGCATCAACGACAAGGTCCT	CGCCTTTGGTGGTCTTGGT
Rifle_ompC31f/193r	GTGTTCAGGGCCGGATTCT	TGGCGGTGTCTGAAACTGC
Rifle_ompB477f/627r	GGCGTATGGTCCGTATTCTTG	GGCGATATTGGTGGTGTAGC
Rifle_gltA338f/435r	TCCAAGTTCGCAGCGTTCTA	GGGATACGTGCCACGATGTC
Rifle_mdh75f/227r	CATGGTTCCTTGGTTCG	AGCAGGGCCACAACCTTCG
Rifle_proC156f/287r	TTGCGAAATGAGCGACACC	ATCGCGGCACCTTTTCACG
ompB-1f/2r	CAGTCAGGAGAAGTTGCTC	GTCTTCACCATCACCAAC
ompB-3f/4r	GAATGCATCTGCTCCGAG	CGTGAAGATGCGAAGAAGC
gs_ompB2206f/2407r	ACGGTTCTGGCGTACCCTG	TCTTCAGCAGATCAAGGGCA
gs_mcpA5f/305r	CTTCGCACGCATGTTATTG	TCCGAGTATTGCCGAGTTC

*The lower-case letters in this primer set represent *EcoRI* restriction sites.

Collection of environmental samples. Groundwater samples were collected from a former uranium ore processing facility in Rifle, CO, USA, where a small-scale U(VI) bioremediation experiment was conducted as described previously (Holmes *et al.*, 2005). Acetate was injected into the subsurface to stimulate Fe(III) and U(VI) reduction by *Geobacteraceae*. Groundwater samples were collected every other day over the course of 28 days during the months of August and September 2005 as described previously (Holmes *et al.*, 2007).

Extraction of mRNA from pure cultures and environmental samples. Chemostat cultures (200 ml) at steady state were transferred to pre-chilled 50 ml conical tubes and centrifuged at 4000 r.p.m. for 15 min at 4 °C. Cells were harvested from batch cultures at mid-exponential phase in the same manner. RNA was extracted from these samples as described previously (Holmes *et al.*, 2004b, 2005).

Cells were harvested from current-harvesting electrodes at 0.5, 0.75, 1.0, 1.2, 1.4 and 2.0 mA, as described previously (Holmes *et al.*, 2005). RNA was extracted from these samples as described previously (Holmes *et al.*, 2006). For environmental samples, RNA was extracted with the acetone precipitation protocol described previously (Holmes *et al.*, 2004b).

RNA extracted from all pure culture and environmental RNA samples was purified with the RNA Clean-Up kit (Qiagen) and treated with DNA-free (Ambion), according to the manufacturer's instructions.

Primer design. All of the primer sequences utilized in this study are outlined in Table 1. Degenerate primers targeting *Geobacteraceae* *ompB*, *ompC*, *gltA*, *mdh* and *proC* were designed from the genomes of

G. sulfurreducens (Methe *et al.*, 2003), *Geobacter metallireducens*, 'Geobacter uranireducens', *Geobacter* sp. FRC-32, *Geobacter bemidjiensis*, and 'Geobacter lovleyi' genomes. Preliminary sequence data from *G. metallireducens*, 'G. uranireducens', *Geobacter* sp. FRC-32, *G. bemidjiensis* and 'G. lovleyi' were obtained from the DOE Joint Genome Institute (JGI) website (www.jgi.doe.gov).

The following degenerate primer sets were used to amplify *ompB*, *ompC*, *gltA*, *mdh* and *proC* genes and transcripts from groundwater collected from the uranium-contaminated site: Geo_ompB576f/1380r, Geo_ompC680f/1385r, Geo_gltA100f/850r, Geo_mdh260f/610r and Geo_proC75f/471r.

The predominant sequences detected in cDNA libraries constructed with degenerate primer products were targeted for quantitative RT-PCR primer design. Environmental and pure culture quantitative RT-PCR primers were designed according to the manufacturer's specifications (amplicon size 100–200 bp), and representative products from each of these primer sets were verified by sequencing clone libraries.

The following primer sets were used to quantify the number of *ompB*, *ompC*, *mdh*, *gltA* and *proC* mRNA transcripts in the groundwater via quantitative RT-PCR: Rifle_ompB477f/627r, Rifle_ompC31f/193r, Rifle_gltA338f/435r, Rifle_mdh75f/227r and Rifle_proC156f/287r.

The number of *ompB*, *ompC*, *mdh* and *gltA* mRNA transcripts were normalized against the number of *proC* mRNA transcripts. This gene was selected as an external control because studies have shown that *proC* is constitutively expressed by *Geobacter* species in pure culture studies and in the environment (Holmes *et al.*, 2005, 2006; O'Neil *et al.*, 2008). In addition, when *G. sulfurreducens* and 'G.

uraniireducens were grown in chemostats at four different dilution rates, microarray and quantitative RT-PCR analyses showed that *proC* expression levels were relatively constant at the different rates of growth (Barrett *et al.*, 2007; Risso *et al.*, 2007).

Primers targeting the *ompB*, *ompC*, *recA*, *proC* and *rpoD* genes for pure culture studies were designed from the *G. sulfurreducens* genome sequence (Methe *et al.*, 2003). The following primer pairs targeted *ompB*, *ompC*, *recA*, *proC* and *rpoD* genes in *G. sulfurreducens*; Gsulf_ompB2281f/2395r, Gsulf_ompC408f/518r, Gsulf_recA660f/737r, Gsulf_proC2f/77r and Gsulf_rpoD1132f/1210r.

Probes targeting *ompB* and *ompC* genes from *G. sulfurreducens* for Northern and genomic dot blot analyses were constructed using gene products from the following primer sets; a 201 bp fragment from the *ompB* gene was amplified with gs_ompB2206f/2407r, and a 300 bp fragment from the *ompC* gene was amplified with gs_ompC5f/305r. RT-PCR was done with primers ompB-1f/2r and ompB-3f/3r to confirm *ompB* operon structure.

Quantification of gene abundance with most-probable-number (MPN)-PCR. Optimal amplification conditions for primers designed for MPN-PCR of *Geobacteraceae ompB*, *gltA* and *nifD* genes were determined in a gradient thermal cycler (MJ Research). Primer pairs used to amplify *Geobacter ompB*, *nifD* and *gltA* genes are outlined in Table 1; Geo_ompB576f/1380r, Geo_nifD225f/560r and Geo_gltA100f/850r.

Five-tube MPN-PCR analyses were performed as described previously (Holmes *et al.*, 2002). Serial 10-fold dilutions of DNA template were made, and *Geobacteraceae ompB*, *gltA* and *nifD* genes were amplified by PCR. PCR products were visualized on an ethidium-bromide-stained agarose gel. The highest dilution that yielded product was noted, and a standard five-tube MPN chart was consulted to estimate the number of target genes in each sample.

Quantification of gene expression with quantitative RT-PCR. The DuraScript enhanced avian RT single strand synthesis kit (Sigma) was used to generate cDNA from *ompB*, *ompC*, *gltA*, *mdh*, *recA*, *proC* and *rpoD* transcripts as described previously (Holmes *et al.*, 2004b). Once the appropriate cDNA fragments were generated by RT-PCR, quantitative PCR amplification and detection were performed with the 7500 Real-time PCR System (Applied Biosystems). Optimal quantitative RT-PCR conditions were determined using the manufacturer's guidelines.

Northern hybridization and genomic DNA slot blot analyses. All Northern and slot blot analyses were conducted as described previously (Holmes *et al.*, 2004b, 2006). For Northern blot analysis, RNA was transferred to a NYTRAN SuPerCharge membrane (Schleicher & Schuell) with the TurboBlotter kit (Schleicher & Schuell). For slot blot analyses, genomic DNA was transferred to a Zeta-Probe GT membrane in a slot-blotting manifold (Bio-Rad) as described previously (Holmes *et al.*, 2004b). Northern blot probe hybridizations were performed at 68 °C with QuikHyb Hybridization Solution (Stratagene), according to the manufacturer's instructions, and probes were hybridized to the genomic slot blot membrane as described previously (Holmes *et al.*, 2004b).

PCR amplification parameters and clone library construction. Optimal amplification conditions for all primer sets were determined in a gradient thermal cycler (MJ Research). To ensure sterility, the PCR mixtures were exposed to UV radiation for 8 min prior to addition of DNA or cDNA template and *Taq* polymerase.

For clone library construction, PCR products were purified with the Gel Extraction kit (Qiagen), and clone libraries were constructed with a TOPO TA cloning kit, version M (Invitrogen), according to the

manufacturer's, instructions. One-hundred plasmid inserts from each clone library were then sequenced with the M13F primer at the University of Massachusetts Sequencing Facility.

***ompB* and *ompC* sequence analysis.** Nucleotide and amino acid sequences from *ompB* and *ompC* were compared to the GenBank nucleotide and protein databases using BLASTN and BLASTX algorithms (Altschul *et al.*, 1990). Amino acid sequences for each gene were initially aligned in CLUSTAL_X (Thompson *et al.*, 1997) and imported into the Genetic Computer Group (GCG) sequence editor (Wisconsin Package version 10; Madison, WI, USA). These alignments were then imported into CLUSTAL W (Thompson *et al.*, 1994), MView (Brown *et al.*, 1998) and ALIGN (Pearson, 1990) where identity matrices were generated.

Aligned sequences were imported into PAUP 4.0b10 (Swofford, 1998) where phylogenetic trees were inferred. Distances and branching order were determined and compared using maximum-parsimony and distance-based algorithms [HKY85 (Hasegawa *et al.*, 1985) and Jukes-Cantor (Jukes & Cantor, 1969)]. Bootstrap values were obtained from 100 replicates.

The software programs, PSORT-B (Gardy *et al.*, 2003), TMpred (Hofmann & Stoffel, 1993), and SignalP 3.0 Server (Emanuelsson *et al.*, 2007) were used to identify which proteins had signal peptides, and where these proteins were likely to be localized within the cell. The molecular mass of OmpC was calculated with the SAPS program (Brendel *et al.*, 1992), and the isoelectric point was calculated with the EMBOSS application, IEP (Rice *et al.*, 2000). The RPSBLAST algorithm (Schaffer *et al.*, 2001) was used to identify conserved fibronectin and multicopper domains found in the conserved domain database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Putative Fe(III) transporter (FTR1)-like Fe(III)-binding domains were identified in the GCG sequence editor by the FINDPATTERN program (Wisconsin Package version 10) with the EXXE motif. FGENSEB, BPROM and FindTerm programs, available through SoftBerry (www.softberry.com), were used for operon and gene predictions.

RESULTS

Prevalence of *ompB* and *ompC* within the *Geobacteraceae*

Further analysis of the *G. sulfurreducens* genome revealed the presence of an OmpB homologue, OmpC, which was 26 % identical and 36 % similar to OmpB (Fig. 1). The most similar protein to OmpC that has been characterized thus far is the Cota protein from *Bacillus subtilis* (27 % identical/38 % similar), which is a copper-dependent laccase protein involved in the biosynthesis of a brown spore pigment associated with endospores formed by this organism (Hullo *et al.*, 2001; Martins *et al.*, 2002; Nakamura & Go, 2005). Although metal oxidation has not been detected in the Cota protein from *B. subtilis*, Mn²⁺ oxidizing activity has been reported in similar laccase proteins isolated from other *Bacillus* species (Brouwers *et al.*, 2000; Dick *et al.*, 2006; Francis *et al.*, 2001, 2002).

OmpC consists of 840 aa, with a calculated molecular mass of 90.1 kDa (Brendel *et al.*, 1992), and a predicted isoelectric point (pI) of 4.76 (Rice *et al.*, 2000). Like OmpB, the OmpC sequence contains four deduced copper-binding sites, two near the amino terminus and two near the C terminus. This arrangement is typical of copper oxidases (Brouwers *et al.*,

Fig. 1. Amino acid sequence alignment of OmpB and OmpC from *G. sulfurreducens*. Identical and conservatively substituted residues are highlighted in black and grey, respectively. Double lines identify signal peptides. Numbers indicate amino acids that participate in the formation of type 1, 2 and 3 copper binding, and dots identify potential iron-binding motifs. The fibronectin-like segment of OmpB is indicated with a solid line. Alignment was made with CLUSTAL W.

2000; Quintanar *et al.*, 2007). Unlike OmpB, OmpC does not contain a fibronectin domain (Schaffer *et al.*, 2001). OmpC also contains three potential Fe(III)-binding sites as determined by the FINDPATTERN program (Wisconsin Package version 10) with the EXXE motif, whereas only one predicted Fe(III)-binding site is present in OmpB. A signal peptide with a length of 31 aa was predicted (Emanuelsson *et al.*, 2007), indicating that this protein is secreted from the cytoplasm. The protein is predicted to be located in either the periplasm or the outer membrane (Gardy *et al.*, 2003; Hofmann & Stoffel, 1993).

Genes for OmpB and/or OmpC were detected in all of the available *Geobacter* genomes, including *G. sulfurreducens*, *G. metallireducens*, '*G. uraniireducens*', '*G. lovleyi*', *Geobacter* sp. FRC-32 and *G. bemidjiensis*. These genes form two distinct phylogenetic clades (Fig. 2). A putative multicopper protein was also detected in *Desulfuromonas acetoxidans*; however, it did not cluster with OmpB and OmpC proteins detected in other *Geobacteraceae* species. The multicopper oxidase-like protein found in *D. acetoxidans* was most similar to a Pco-like multicopper oxidase found in the *Bacteroidetes* species '*Gramella forsetii*' (38 %

Fig. 2. Phylogenetic tree comparing multicopper proteins from *Geobacteraceae* isolates and uncultivated *Geobacteraceae* species found in groundwater collected from a uranium-contaminated site with multicopper proteins from other bacterial species. Branching lengths and bootstrap values were determined by Jukes–Cantor analysis with 100 replicates. Multicopper proteins from *Desulfuromonas acetoxidans*, '*Gramella forsetii*' and *Flavobacterium johnsoniae* were used as outgroups for construction of the tree.

identical/ 53% similar). Genes with homology to *ompB* and *ompC* were also detected in the genomes of *Anaeromyxobacter dehalogenans*, *Leptothrix discophora*, *Nitrospira multiformis*, *Oceanobacillus iheyensis*, *Salinispora tropica* and *Thiobacillus denitrificans*.

Signal peptides were detected in all of the *Geobacteraceae* multicopper proteins identified, and the majority of these proteins were predicted to be located in the periplasm or the outer membrane (Table 2). All of the *ompB*-like genes

within the *Geobacter* species contained four predicted copper-binding domains, and each species contained an *ompB*-like gene with iron-binding motifs. In instances in which more than one *ompB*-like gene was found in the genome, the second homologue lacked iron-binding motifs (Table 2). Within the *Geobacter* genus, only the *ompB*-like genes from *G. sulfurreducens* and '*G. lovleyi*' contained fibronectin domains. These differences in *ompB*-like genes may reflect different functions. However, this cannot be further investigated at this time because the genetic system

Table 2. Characteristics of multicopper proteins found in *Geobacter* species and other organisms with OmpB- or OmpC-like multicopper proteins

The similarity and identity values (%) were obtained by comparing amino acid sequences from multicopper proteins associated with the organisms listed below to OmpB or OmpC amino acid sequences from *G. sulfurreducens* (730 aa sequences considered).

Protein clade	Organism name and gene	Fibronectin domains	Copper- binding domains	Iron- binding motifs	Predicted cellular location of protein	Presence of signal peptide	Similarity (%)/ identity (%) to OmpB or OmpC in <i>G. sulfurreducens</i>
<i>ompC</i>	<i>Anaeromyxobacter dehalogenans</i>	0	4	3	Periplasm or outer membrane	Present	37/26
<i>ompB</i>	<i>Anaeromyxobacter dehalogenans</i>	2	4	2	Cytoplasm	Absent	53/43
<i>ompC</i>	<i>Desulfuromonas acetoxidans</i>	0	4	0	Periplasm or outer membrane	Present	45/29
<i>ompC</i>	<i>Geobacter bemidjensis mcpA</i>	0	4	2	Inner membrane	Present	38/26
<i>ompB</i>	<i>Geobacter bemidjensis ompB-1</i>	0	4	2	Inner membrane	Present	54/43
<i>ompB</i>	<i>Geobacter bemidjensis ompB-2</i>	0	4	0	Periplasm or outer membrane	Present	54/45
<i>ompB</i>	' <i>Geobacter lovleyi</i> ' <i>ompB</i>	1	4	3	Periplasm or outer membrane	Present	74/65
<i>ompC</i>	<i>Geobacter metallireducens mcpA-1</i>	0	4	2	Peripheral	Present	60/51
<i>ompC</i>	<i>Geobacter metallireducens mcpA-2</i>	0	4	1	Inner membrane	Present	40/31
<i>ompB</i>	<i>Geobacter metallireducens ompB-1</i>	0	4	2	Periplasm or outer membrane	Present	40/31
<i>ompB</i>	<i>Geobacter metallireducens ompB-2</i>	0	4	0	Periplasm or outer membrane	Present	49/40
<i>ompC</i>	<i>Geobacter</i> sp. FRC-32 <i>mcpA-1</i>	0	4	2	Periplasm or outer membrane	Present	71/61
<i>ompB</i>	<i>Geobacter</i> sp. FRC-32 <i>ompB</i>	0	4	4	Inner membrane	Present	58/47
<i>ompC</i>	<i>Geobacter</i> sp. FRC-32	0	4	1	Inner membrane	Present	46/30
<i>ompC</i>	<i>Geobacter sulfurreducens mcpA</i>	0	4	3	Periplasm or outer membrane	Present	100/100
<i>ompB</i>	<i>Geobacter sulfurreducens ompB</i>	1	4	1	Outer membrane	Present	100/100
<i>ompB</i>	' <i>Geobacter uraniiireducens</i> ' <i>ompB</i>	0	4	1	Periplasm or outer membrane	Present	56/49
<i>ompB</i>	<i>Leptothrix discophora mofA</i>	0	4	2	Periplasm or outer membrane	Present	50/37
<i>ompC</i>	<i>Nitrospira multiformis</i>	0	4	2	Cytoplasm	Absent	52/41
<i>ompC</i>	<i>Oceanobacillus iheyensis</i>	0	4	4	Cytoplasm	Absent	41/29
<i>ompC</i>	<i>Salinispora tropica</i>	0	4	3	Periplasm or outer membrane	Present	46/36
<i>ompC</i>	<i>Thiobacillus denitrificans</i>	0	4	3	Cytoplasm	Absent	51/43

that has been utilized for investigating gene function in *G. sulfurreducens* has not worked in other *Geobacter* species.

Neither *ompB* nor *ompC* were detected in the genomes of the two *Pelobacter* species that have been sequenced, *P. carbinolicus* and *P. propionicus*. Attempts to amplify *ompB* and *ompC* from genomic DNA extracted from various *Pelobacter* species via PCR were unsuccessful. In addition, genomic DNA from *P. carbinolicus*, *P. propionicus*, *P. acidigallici*, *P. massiliensis*, *P. venetianus* and *P. acetylenicus* did not hybridize to ^{32}P -labelled probes specific for the *ompB* and *ompC* genes (data not shown).

Requirement for *ompC* for optimal Fe(III) oxide reduction

Previous studies demonstrated that *G. sulfurreducens* requires *ompB* to reduce insoluble Fe(III) and Mn(IV) oxides, but not for the reduction of soluble, chelated Fe(III) (Mehta *et al.*, 2006). Deletion of *ompC* had no impact on growth with fumarate or Fe(III) citrate as electron acceptor (data not shown). However, similar to the *ompB*-deficient mutant, growth with Fe(III) oxide was significantly impaired (Fig. 3). If the chelator NTA was added to the Fe(III) oxide medium, the *ompC*-deficient mutant was able to reduce Fe(III). Expressing *ompC* *in trans* restored the capacity for Fe(III) oxide reduction. The *ompC*-deficient mutant reduced Mn(IV) oxide and grew on current-harvesting electrodes as well as wild-type cells (data not shown).

Expression of *ompB* and *ompC* in *G. sulfurreducens*

To determine whether the *ompB* and *ompC* genes in *G. sulfurreducens* are monocistronic or part of an operon, *ompB* and *ompC* mRNA transcripts from *G. sulfurreducens* cells grown with acetate (10 mM) as electron donor and fumarate (40 mM) as electron acceptor were analysed (Fig. 4). In Northern hybridizations, the *ompB* probe hybridized with a transcript of ~ 4.5 kb, and the *ompC* probe hybridized with a transcript of ~ 2.5 kb (Fig. 4a). The *ompB* operon results from the Northern hybridization were further confirmed with RT-PCR (data not shown). These results indicated that the *ompB* gene is part of an operon,

and is co-transcribed with a hypothetical protein that consists of about 86 aa, whereas the *ompC* gene is monocistronic. These results are consistent with computational predictions of operon structure (Fig. 4b, c) (www.softberry.com) (Yan *et al.*, 2004).

Further evaluation of *ompB* and *ompC* gene expression patterns demonstrated that *G. sulfurreducens* expresses both of these genes under a variety of growth conditions. For example, both *ompB* and *ompC* transcripts were detected by RT-PCR in RNA extracted from *G. sulfurreducens* cells grown with acetate (10 mM) as electron donor and either fumarate (40 mM), Fe(III) citrate (55 mM), Fe(III) oxide (100 mM), Fe(III) NTA (10 mM), Mn(IV) oxide (20 mM) or an electrode poised at +520mV as electron acceptor (data not shown).

To evaluate whether expression of *ompB* and *ompC* might be affected by changes in the rate of metabolism, as are other genes (Chin *et al.*, 2004; Holmes *et al.*, 2005), *G. sulfurreducens* was grown in acetate-limited chemostats at dilution rates near the lowest (0.03 h^{-1}) and highest (0.07 h^{-1}) rate at which *G. sulfurreducens* can be maintained in such systems (Esteve-Nunez *et al.*, 2005). As expected, the steady-state cell yields [$0.166 \pm 0.029\text{ mg protein ml}^{-1}$ at 0.03 h^{-1} ; $0.171 \pm 0.014\text{ mg protein ml}^{-1}$ at 0.07 h^{-1} (mean $\pm$ SE, $n=3$)] and Fe(II) concentrations ($47.03 \pm 8.48\text{ mM}$ at 0.03 h^{-1} ; 45.92 ± 3.46 at 0.07 h^{-1}) at the two dilution rates were comparable, and steady-state acetate concentrations were below the detection limit of $5\text{ }\mu\text{M}$.

Quantitative RT-PCR analyses showed that mRNA transcript levels of *ompB* normalized against transcripts from the constitutively expressed housekeeping gene *proC* were similar at both dilution rates, whereas normalized transcript levels of *ompC* increased at the higher dilution rate (Fig. 5). Similar to expression patterns observed with the *proC* gene, the number of mRNA transcripts from the housekeeping genes *recA* and *rpoD* was similar at both dilution rates (data not shown).

When *G. sulfurreducens* was grown with acetate provided as electron donor and the anode of a microbial fuel cell as sole electron acceptor, there was an increase in normalized transcript levels of *ompC* as the current increased (Fig. 6). In contrast, transcript levels for the constitutively expressed

Fig. 3. Reduction of insoluble Fe(III) oxide (a) or Fe(III) oxide supplemented with 1 mM NTA (b) by cultures of wild-type (■), the *ompC* deletion mutant (△), or the *ompC* deletion mutant complemented with *ompC* *in trans* (▲). The results are the means of triplicate incubations and error bars represent SD.

Fig. 4. (a) Northern blot analyses of RNA extracted from *G. sulfurreducens* cells at mid-exponential phase, grown with acetate (10 mM) as electron donor and fumarate (55 mM) as electron acceptor. 32 P-labelled probes were specific for the *ompB* and *ompC* genes. (b) The *ompB* operon, including the promoter region and termination site. (c) The *ompC* transcription unit, including the promoter region and termination site.

housekeeping control genes *rpoD*, *recA* and *proC* remained constant. Transcript levels for *ompB* also did not increase with increased current.

In situ expression of *ompB* and *ompC* during uranium bioremediation

Degenerate primers designed to amplify *ompB* or *ompC* from diverse *Geobacter* species yielded PCR products from the groundwater of a previously described (Anderson *et al.*, 2003; Holmes *et al.*, 2005; Vrionis *et al.*, 2005) uranium-contaminated aquifer undergoing *in situ* bioremediation in which *Geobacter* were predominant members of the microbial community (Fig. 2). During the course of a 28-day *in situ* uranium bioremediation field experiment, MPN-PCR estimates of *ompB* tracked with MPN-PCR estimates of *Geobacteraceae nifD* and *gltA* (Fig. 7). As expected from previous analyses of 16S rRNA genes (Holmes *et al.*, 2002, 2005, 2007), the number of transcripts of all three *Geobacteraceae*-specific genes dramatically increased as acetate concentrations rose in the groundwater, and numbers stabilized at the maximum

plateau of acetate and then decreased slightly as acetate concentrations declined towards the end of the experiment.

The *Geobacteraceae* in the subsurface expressed both *ompB* and *ompC* during *in situ* bioremediation (Fig. 8a). Quantitative RT-PCR of *ompB* and *ompC* mRNA transcripts in groundwater collected from this site normalized against the number of *proC* mRNA transcripts indicated that transcription of the *ompB* gene was constitutive and did not appear to be impacted by changes in acetate concentrations in the groundwater (Fig. 8a). In contrast, *ompC* transcript levels initially increased as acetate concentrations increased, and reached peak levels when acetate concentrations were highest. However, after day 12, the number of *ompC* transcripts started to decline, even though acetate concentrations remained high (Fig. 8a).

Inconsistencies between *ompC/proC* transcript ratios in environmental and pure culture studies were apparent. This may have been partly due to the fact that different primer sets were used for the two analyses. It is also possible that cations or other contaminating molecules, such as humic acids, present in the RNA extracted from the

Fig. 5. Number of *ompB* and *ompC* mRNA transcripts normalized against the number of *proC* mRNA transcripts detected in RNA extracted from *G. sulfurreducens* cells grown in chemostats at two different growth rates: 0.03 (grey bars) and 0.07 h⁻¹ (hatched bars). Each chemostat point is the mean of five replicates from three separate chemostat cultures.

Fig. 6. Number of *ompB* (●) and *ompC* (○) mRNA transcripts relative to the number of *proC* mRNA transcripts expressed by *G. sulfurreducens* grown on a current-harvesting electrode poised at +500mV (with reference to a standard H₂ electrode). RNA was extracted from the electrode at 0.5, 0.75, 1.0, 1.2, 1.45 and 2 mA. Each point is the mean of triplicate determinations.

Fig. 7. Groundwater concentrations of *Geobacteraceae* *ompB* (○), *gltA* (●) and *nifD* (■), determined by five-tube MPN-PCR, and acetate concentrations (▼) during the *in situ* uranium bioremediation field experiment at the Rifle site in 2005.

environmental sample had an effect on the results, as it has been shown that these factors can inversely effect quantitative PCR analysis (Stults *et al.*, 2001).

For comparison, transcript levels of genes encoding two TCA-cycle enzymes, citrate synthase (*gltA*) and malate dehydrogenase (*mdh*), were also quantified. As observed in a previous field experiment (Holmes *et al.*, 2005), levels of *gltA* transcripts closely tracked with acetate availability (Fig. 8b). Levels of *mdh* transcripts followed a similar pattern.

DISCUSSION

The results further demonstrate that putative multicopper proteins play an important role in extracellular electron transfer in *G. sulfurreducens* and suggest that monitoring the genes and/or gene transcripts for these proteins may be a useful strategy for evaluating the composition and/or activity of *Geobacter* species in subsurface environments.

Role of putative multicopper proteins in Fe(III) oxide reduction

A previous study illustrated the importance of OmpB in Fe(III) oxide reduction by *G. sulfurreducens* (Mehta *et al.*, 2006), and the results reported here demonstrate that the OmpB homologue, OmpC, is also required for optimal Fe(III) oxide reduction. The fact that genes that encode proteins homologous to OmpB and OmpC are found in all *Geobacter* species investigated suggests that these proteins

Fig. 8. Gene expression patterns and acetate concentrations ($\blacktriangle$) in the groundwater during the *in situ* uranium bioremediation field experiment. (a) Number of *Geobacteraceae ompB* ($\bullet$) and *ompC* ($\blacksquare$) mRNA transcripts normalized against the number of *proC* mRNA transcripts. (b) Number of *Geobacteraceae gltA* ($\bullet$) and *mdh* ($\blacksquare$) mRNA transcripts normalized against the number of *proC* mRNA transcripts. Each point is the mean of triplicate determinations.

are generally important for Fe(III) oxide reduction in species of this genus. Multicopper proteins in other organisms are typically involved in electron transfer reactions (Adams & Ghiorse, 1987; Askwith & Kaplan, 1998; Brouwers *et al.*, 1999; Claus, 2003; Corstjens *et al.*, 1992; Eck *et al.*, 1999; Francis *et al.*, 2001; Ghiorse, 1988; Hullo *et al.*, 2001; Larsen *et al.*, 1999; Miyata *et al.*, 2006; Quintanar *et al.*, 2007; Ridge *et al.*, 2007; Sitthisak *et al.*, 2005), and OmpB and OmpC could have an electron transport function in *G. sulfurreducens*. OmpB is loosely bound to the outer surface (Qian *et al.*, 2007), and thus might directly access Fe(III) oxides. However, computational predictions of OmpC localization are not definitive and its location has not been experimentally investigated. Definitive localization of OmpC, as well as purification and characterization of OmpB and OmpC, are warranted in order to better understand their roles in Fe(III) oxide reduction.

In addition to OmpB and OmpC, *G. sulfurreducens* requires several outer-membrane *c*-type cytochromes (Kim *et al.*, 2006; Leang *et al.*, 2003; Mehta *et al.*, 2005), as well as electrically conductive pili (Reguera *et al.*, 2005) for effective Fe(III) oxide reduction (Lovley *et al.*, 2004; Lovley, 2006). The mechanisms by which these multiple required proteins interact and which protein(s) actually serve as terminal Fe(III) reductases is not yet understood. The finding that *ompB* and *ompC* are not found in *Pelobacter* species, which are phylogenetically intertwined with *Geobacter* species and are capable of Fe(III) oxide reduction, suggests that *Pelobacter* species may utilize a fundamentally different

mechanism for extracellular electron transfer, consistent with a general lack of outer-surface *c*-type cytochromes in these organisms (Haveman *et al.*, 2006; Lovley *et al.*, 1995) and the inability of *P. carbinolicus* to produce electricity (Richter *et al.*, 2007).

Usefulness of *ompB* and *ompC* genes and gene transcripts for environmental studies

The fact that *ompB* and *ompC* genes are not found in *Pelobacter* species but are highly conserved in *Geobacter* species suggests an improved method for quantifying *Geobacter* species in environmental samples. It is difficult to design primers that can specifically quantify *Geobacter* species via quantitative PCR of 16S rRNA genes because *Pelobacter* species within the *Geobacter* phylogenetic cluster are also targeted by such primers. Distinguishing between *Geobacter* and *Pelobacter* species is important. Although both genera are capable of Fe(III) reduction (Lovley *et al.*, 2004), they differ greatly in other physiological characteristics. Most notably, *Geobacter* species are able to completely oxidize organic carbon substrates to carbon dioxide, whereas *Pelobacter* species are not (Lovley *et al.*, 2004).

Quantitative PCR analysis of *ompB* and/or *ompC* genes could provide an alternative estimate of *Geobacter* species. Although this approach could potentially include non-*Geobacter* species which have phylogenetically similar genes (Fig. 2), none of these organisms are expected to thrive in anaerobic environments in which Fe(III) reduction is the

predominant process. Comparison of results from quantitative PCR with primers targeting *Geobacter* 16S rRNA genes with the results from primers targeting *ompB/ompC* genes should provide a good cross-check on molecular estimates of *Geobacter* abundance.

One of the primary justifications for analysing patterns of gene expression in *Geobacter* species is to identify key genes whose expression patterns can be used to diagnose the metabolic state of *Geobacter* species in applications such as groundwater bioremediation and electricity production (Lovley, 2002, 2003, 2006). Surprisingly, the expression patterns of *ompB* and *ompC* are different. Expression of *ompB* appeared to be constitutive in the three environments examined: chemostats, the anodes of microbial fuel cells and the subsurface. In contrast, transcript levels of *ompC* increased with increasing growth rate in chemostats, as current production increased on fuel cell anodes, and in the initial phase of acetate introduction into the groundwater during *in situ* uranium bioremediation. This study also showed that as acetate concentrations increased in the groundwater, there was a rapid increase in the abundance of *Geobacter* species, which eventually stabilized (Fig. 8). Levels of *ompC* transcripts declined in the later phases of acetate addition, once the number of *Geobacter* stabilized, even though *Geobacter* were still metabolically active as indicated by high levels of expression of *gltA* and *mdh*. Previous studies have demonstrated that levels of *gltA* transcripts are diagnostic of rates of metabolic activity (Holmes *et al.*, 2005), and the results presented here suggest that levels of *mdh* transcripts provide similar information.

Thus, these results suggest that increasing levels of *ompC* transcripts relative to *ompB* transcripts over time may be indicative of an actively growing *Geobacter* community. Such information could be useful in designing bioremediation strategies. In some cases it is useful to promote rapid growth to achieve high rates of desirable bioremediation reactions, whereas in other cases, such as *in situ* uranium bioremediation, slower sustained levels of activity without high levels of growth may be desirable.

In summary, the putative multicopper proteins OmpB and OmpC are clearly important in extracellular electron transfer, and monitoring the expression of these genes may provide useful insights into the physiological state of *Geobacter* populations. Further biochemical investigation of these apparently novel proteins seems warranted.

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REFERENCES

- Adams, L. F. & Ghiorse, W. C. (1987). Characterization of extracellular Mn^{2+} -oxidizing activity and isolation of an Mn^{2+} -oxidizing protein from *Leptothrix discophora* SS-1. *J Bacteriol* **169**, 1279–1285.
- Afkar, E., Reguera, G., Schiffer, M. & Lovley, D. R. (2005). A novel *Geobacteraceae*-specific outer membrane protein J (OmpJ) is essential for electron transport to Fe(III) and Mn(IV) oxides in *Geobacter sulfurreducens*. *BMC Microbiol* **5**, 41.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Anderson, R. T., Vrionis, H. A., Ortiz-Bernad, I., Resch, C. T., Long, P. E., Dayvault, R., Karp, K., Marutzky, S., Metzler, D. R. & other authors (2003). Stimulating the *in situ* activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. *Appl Environ Microbiol* **69**, 5884–5891.
- Askwith, C. C. & Kaplan, J. (1998). Site-directed mutagenesis of the yeast multicopper oxidase Fet3p. *J Biol Chem* **273**, 22415–22419.
- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Barrett, E., Holmes, D. E., Mouser, P. J., Chavan, M. A., Larrahondo, M. J., Adams, L. A. & Lovley, D. R. (2007). Monitoring the growth rate of *Geobacter* species in groundwater via analysis of gene transcript levels. In *Abstracts: American Society for Microbiology 107th General Meeting, Toronto, Canada*. Washington, DC: American Society for Microbiology.
- Bond, D. R. & Lovley, D. R. (2003). Electricity production by *Geobacter sulfurreducens* attached to electrodes. *Appl Environ Microbiol* **69**, 1548–1555.
- Bond, D. R., Holmes, D. E., Tender, L. M. & Lovley, D. R. (2002). Electrode-reducing microorganisms that harvest energy from marine sediments. *Science* **295**, 483–485.
- Brendel, V., Bucher, P., Nourbakhsh, I. R., Blaisdell, B. E. & Karlin, S. (1992). Methods and algorithms for statistical analysis of protein sequences. *Proc Natl Acad Sci U S A* **89**, 2002–2006.
- Brouwers, G. J., de Vrind, J. P. M., Corstjens, P. L. A. M., Cornelis, P., Baysse, C. & DeJong, E. W. D. V. (1999). *cumA*, a gene encoding a multicopper oxidase, is involved in Mn^{2+} oxidation in *Pseudomonas putida* GB-1. *Appl Environ Microbiol* **65**, 1762–1768.
- Brouwers, G. J., Vijgenboom, E., Corstjens, P. L. A. M., De Vrind, J. P. M. & de Vrind-de Jong, E. W. (2000). Bacterial Mn^{2+} oxidizing systems and multicopper oxidases: an overview of mechanisms and functions. *Geomicrobiol J* **17**, 1–24.
- Brown, N. P., Leroy, C. & Sander, C. (1998). MView: a web-compatible database search or multiple alignment viewer. *Bioinformatics* **14**, 380–381.
- Butler, J. E., Kaufmann, F., Coppi, M. V., Nunez, C. & Lovley, D. R. (2004). MacA, a diheme *c*-type cytochrome involved in Fe(III) reduction by *Geobacter sulfurreducens*. *J Bacteriol* **186**, 4042–4045.
- Chang, Y. J., Long, P. E., Geyer, R., Peacock, A. D., Resch, C. T., Sublette, K., Pfiffner, S., Smithgall, A., Anderson, R. T. & other authors (2005). Microbial incorporation of ^{13}C -labeled acetate at the field scale: detection of microbes responsible for reduction of U(VI). *Environ Sci Technol* **39**, 9039–9048.
- Childers, S. E., Ciuffo, S. & Lovley, D. R. (2002). *Geobacter metallireducens* accesses insoluble Fe(III) oxide by chemotaxis. *Nature* **416**, 767–769.
- Chin, K. J., Esteve-Nunez, A., Leang, C. & Lovley, D. R. (2004). Direct correlation between rates of anaerobic respiration and levels of mRNA

- for key respiratory genes in *Geobacter sulfurreducens*. *Appl Environ Microbiol* **70**, 5183–5189.
- Claus, H. (2003). Laccases and their occurrence in prokaryotes. *Arch Microbiol* **179**, 145–150.
- Coates, J. D., Cole, K. A., Michaelidou, U., Patrick, J., McInerney, M. J. & Achenbach, L. A. (2005). Biological control of hog waste odor through stimulated microbial Fe(III) reduction. *Appl Environ Microbiol* **71**, 4728–4735.
- Coppi, M. V., Leang, C., Sandler, S. J. & Lovley, D. R. (2001). Development of a genetic system for *Geobacter sulfurreducens*. *Appl Environ Microbiol* **67**, 3180–3187.
- Corstjens, P. L. A. M., Devrind, J. P. M., Westbroek, P. & Devrinddejong, E. W. (1992). Enzymatic iron oxidation by *Leptothrix discophora* – identification of an iron-oxidizing protein. *Appl Environ Microbiol* **58**, 450–454.
- Dick, G. J., Lee, Y. E. & Tebo, B. M. (2006). Manganese(II)-oxidizing *Bacillus* spores in Guaymas Basin hydrothermal sediments and plumes. *Appl Environ Microbiol* **72**, 3184–3190.
- DiDonato, L. N., Sullivan, S. A., Methe, B. A., Nevin, K. P., England, R. & Lovley, D. R. (2006). Role of RelGsu in stress response and Fe(III) reduction in *Geobacter sulfurreducens*. *J Bacteriol* **188**, 8469–8478.
- Eck, R., Hundt, S., Hartl, A., Roemer, E. & Kunkel, W. (1999). A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption. *Microbiology* **145**, 2415–2422.
- Emanuelsson, O., Brunak, S., von Heijne, G. & Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* **2**, 953–971.
- Esteve-Nunez, A., Rothermich, M., Sharma, M. & Lovley, D. (2005). Growth of *Geobacter sulfurreducens* under nutrient-limiting conditions in continuous culture. *Environ Microbiol* **7**, 641–648.
- Francis, C. A., Co, E. M. & Tebo, B. M. (2001). Enzymatic manganese(II) oxidation by a marine alpha-proteobacterium. *Appl Environ Microbiol* **67**, 4024–4029.
- Francis, C. A., Casciotti, K. L. & Tebo, B. M. (2002). Localization of Mn(II)-oxidizing activity and the putative multicopper oxidase, MnxG, to the exosporium of the marine *Bacillus* sp. strain SG-1. *Arch Microbiol* **178**, 450–456.
- Gardy, J. L., Spencer, C., Wang, K., Ester, M., Tusnady, G. E., Simon, I., Hua, S., deFays, K., Lambert, C. & other authors (2003). PSORT-B: improving protein subcellular localization prediction for Gram-negative bacteria. *Nucleic Acids Res* **31**, 3613–3617.
- Ghiorse, W. C. (1988). Microbial reduction of manganese and iron. In *Biology of Anaerobic Microorganisms*, pp. 305–331. Edited by A. J. B. Zehnder. New York: John Wiley & Sons.
- Hasegawa, M., Kiwshino, H. & Yano, T. (1985). Dating the human–ape split by molecular clock of mitochondrial DNA. *J Mol Evol* **22**, 160–174.
- Haveman, S. A., Holmes, D. E., Ding, Y. H., Ward, J. E., Didonato, R. J., Jr & Lovley, D. R. (2006). *c*-Type cytochromes in *Pelobacter carbinolicus*. *Appl Environ Microbiol* **72**, 6980–6985.
- Hofmann, K. & Stoffel, W. (1993). TMbase – a database of membrane-spanning protein segments. *Biol Chem Hoppe Seyler* **374**, 166.
- Holmes, D. E., Finneran, K. T., O'Neil, R. A. & Lovley, D. R. (2002). Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. *Appl Environ Microbiol* **68**, 2300–2306.
- Holmes, D. E., Nevin, K. P. & Lovley, D. R. (2004a). Comparison of 16S rRNA, *nifD*, *recA*, *gyrB*, *rpoB* and *fusA* genes within the family *Geobacteraceae* fam. nov. *Int J Syst Evol Microbiol* **54**, 1591–1599.
- Holmes, D. E., Nevin, K. P. & Lovley, D. R. (2004b). In situ expression of *nifD* in *Geobacteraceae* in subsurface sediments. *Appl Environ Microbiol* **70**, 7251–7259.
- Holmes, D. E., Nevin, K. P., O'Neil, R. A., Ward, J. E., Adams, L. A., Woodard, T. L., Vrionis, H. A. & Lovley, D. R. (2005). Potential for quantifying expression of the *Geobacteraceae* citrate synthase gene to assess the activity of *Geobacteraceae* in the subsurface and on current-harvesting electrodes. *Appl Environ Microbiol* **71**, 6870–6877.
- Holmes, D. E., Chaudhuri, S. K., Nevin, K. P., Mehta, T., Methé, B. A., Liu, A., Ward, J. E., Woodard, T. L., Webster, J. & Lovley, D. R. (2006). Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. *Environ Microbiol* **8**, 1805–1815.
- Holmes, D. E., O'Neil, R. A., Vrionis, H. A., N'guessan, L. A., Ortiz-Bernad, I., Larrahondo, M. J., Adams, L. A., Ward, J. A., Nicoll, J. S. & other authors (2007). Subsurface clade of *Geobacteraceae* that predominates in a diversity of Fe(III)-reducing subsurface environments. *ISME J* **1**, 663–677.
- Hullo, M. F., Moszer, I., Danchin, A. & Martin-Verstraete, I. (2001). CotA of *Bacillus subtilis* is a copper-dependent laccase. *J Bacteriol* **183**, 5426–5430.
- Istok, J. D., Senko, J. M., Krumholz, L. R., Watson, D., Bogle, M. A., Peacock, A., Chang, Y. J. & White, D. C. (2004). *In situ* bioreduction of technetium and uranium in a nitrate-contaminated aquifer. *Environ Sci Technol* **38**, 468–475.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kim, B. C., Leang, C., Ding, Y. H., Glaven, R. H., Coppi, M. V. & Lovley, D. R. (2005). OmcF, a putative *c*-type monoheme outer membrane cytochrome required for the expression of other outer membrane cytochromes in *Geobacter sulfurreducens*. *J Bacteriol* **187**, 4505–4513.
- Kim, B. C., Qian, X., Leang, C., Coppi, M. V. & Lovley, D. R. (2006). Two putative *c*-type multi-heme cytochromes required for the expression of OmcB, an outer membrane protein essential for optimal Fe(III) reduction in *Geobacter sulfurreducens*. *J Bacteriol* **188**, 3138–3142.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Larsen, E. I., Sly, L. I. & McEwan, A. G. (1999). Manganese(II) adsorption and oxidation by whole cells and a membrane fraction of *Pedomicrobium* sp. ACM 3067. *Arch Microbiol* **171**, 257–264.
- Leang, C. & Lovley, D. R. (2005). Regulation of two highly similar genes, *omcB* and *omcC*, in a 10 kb chromosomal duplication in *Geobacter sulfurreducens*. *Microbiology* **151**, 1761–1767.
- Leang, C., Coppi, M. V. & Lovley, D. R. (2003). OmcB, a *c*-type polyheme cytochrome, involved in Fe(III) reduction in *Geobacter sulfurreducens*. *J Bacteriol* **185**, 2096–2103.
- Lin, B., Braster, M., van Breukelen, B. M., van Verseveld, H. W., Westerhoff, H. V. & Roling, W. F. (2005). *Geobacteraceae* community composition is related to hydrochemistry and biodegradation in an iron-reducing aquifer polluted by a neighboring landfill. *Appl Environ Microbiol* **71**, 5983–5991.
- Lloyd, J. R., Leang, C., Hodges Myerson, A. L., Coppi, M. V., Cuifo, S., Methe, B., Sandler, S. J. & Lovley, D. R. (2003). Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*. *Biochem J* **369**, 153–161.
- Lovley, D. R. (2002). Analysis of the genetic potential and gene expression of microbial communities involved in the in situ bioremediation of uranium and harvesting electrical energy from organic matter. *OMICS* **6**, 331–339.
- Lovley, D. R. (2003). Cleaning up with genomics: applying molecular biology to bioremediation. *Nat Rev Microbiol* **1**, 35–44.

- Lovley, D. R. (2006). Bug juice: harvesting electricity with micro-organisms. *Nat Rev Microbiol* 4, 497–508.
- Lovley, D. R., Phillips, E. J. P., Lonergan, D. J. & Widman, P. K. (1995). Fe(III) and S⁰ reduction by *Pelobacter carbinolicus*. *Appl Environ Microbiol* 61, 2132–2138.
- Lovley, D. R., Holmes, D. E. & Nevin, K. P. (2004). Dissimilatory Fe(III) and Mn(IV) reduction. *Adv Microb Physiol* 49, 219–286.
- Martins, L. O., Soares, C. M., Pereira, M. M., Teixeira, M., Costa, T., Jones, G. H. & Henriques, A. O. (2002). Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *J Biol Chem* 277, 18849–18859.
- Mehta, T., Coppi, M. V., Childers, S. E. & 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.
- Mehta, T., Childers, S. E., Glaven, R., Lovley, D. R. & Mester, T. (2006). A putative multicopper protein secreted by an atypical type II secretion system involved in the reduction of insoluble electron acceptors in *Geobacter sulfurreducens*. *Microbiology* 152, 2257–2264.
- Methe, B. A., Nelson, K. E., Eisen, J. A., Paulsen, I. T., Nelson, W., Heidelberg, J. F., Wu, D., Wu, M., Ward, N. & other authors (2003). Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. *Science* 302, 1967–1969.
- Miyata, N., Tani, Y., Maruo, K., Tsuno, H., Sakata, M. & Iwahori, K. (2006). Manganese(IV) oxide production by *Acremonium* sp. strain KR21–2 and extracellular Mn(II) oxidase activity. *Appl Environ Microbiol* 72, 6467–6473.
- Nakamura, K. & Go, N. (2005). Function and molecular evolution of multicopper blue proteins. *Cell Mol Life Sci* 62, 2050–2066.
- North, N. N., Dollhopf, S. L., Petrie, L., Istok, J. D., Balkwill, D. L. & Kostka, J. E. (2004). Change in bacterial community structure during *in situ* biostimulation of subsurface sediment cocontaminated with uranium and nitrate. *Appl Environ Microbiol* 70, 4911–4920.
- Nottingham, P. M. & Hungate, R. E. (1969). Methanogenic fermentation of benzoate. *J Bacteriol* 98, 1170–1172.
- O'Neil, R. A., Holmes, D. E., Coppi, M. V., Adams, L. A., Larrahondo, M. J., Ward, J. E., Nevin, K. P., Woodard, T. L., Vrionis, H. A. & other authors (2008). Gene transcript analysis of assimilatory iron limitation in *Geobacteraceae* during groundwater bioremediation. *Environ Microbiol* (in press).
- Pearson, W. R. (1990). Rapid and sensitive sequence comparisons with FASTP and FASTA. *Methods Enzymol* 183, 63–98.
- Petrie, L., North, N. N., Dollhopf, S. L., Balkwill, D. L. & Kostka, J. E. (2003). Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). *Appl Environ Microbiol* 69, 7467–7479.
- Qian, X., Reguera, G., Mester, T. & Lovley, D. R. (2007). Evidence that OmcB and OmpB of *Geobacter sulfurreducens* are outer membrane surface proteins. *FEMS Microbiol Lett* 277, 21–27.
- Quintanar, L., Stoj, C., Taylor, A. B., Hart, P. J., Kosman, D. J. & Solomon, E. I. (2007). Shall we dance? How a multicopper oxidase chooses its electron transfer partner. *Acc Chem Res* 40, 445–452.
- Reguera, G., McCarthy, K. D., Mehta, T., Nicoll, J. S., Tuominen, M. T. & Lovley, D. R. (2005). Extracellular electron transfer via microbial nanowires. *Nature* 435, 1098–1101.
- Rice, P., Longden, I. & Bleasby, A. (2000). EMBOS: the European Molecular Biology Open Software Suite. *Trends Genet* 16, 276–277.
- Richter, H., Lanthier, M., Nevin, K. P. & Lovley, D. R. (2007). Lack of electricity production by *Pelobacter carbinolicus* indicates that the capacity for Fe(III) oxide reduction does not necessarily confer electron transfer ability to fuel cell anodes. *Appl Environ Microbiol* 73, 5347–5353.
- Ridge, J. P., Lin, M., Larsen, E. I., Fegan, M., McEwan, A. G. & Sly, L. I. (2007). A multicopper oxidase is essential for manganese oxidation and laccase-like activity in *Pedomicrobium* sp. ACM 3067. *Environ Microbiol* 9, 944–953.
- Risso, C., DiDonato, R. J., Postier, B., Valinotto, L. & Lovley, D. R. (2007). Metabolic changes associated with slow versus fast growth in *Geobacter sulfurreducens*. In *Abstracts: American Society for Microbiology 107th General Meeting, Toronto, Canada*. Washington, DC: American Society for Microbiology.
- Rooney-Varga, J. N., Anderson, R. T., Fraga, J. L., Ringelberg, D. & Lovley, D. R. (1999). Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. *Appl Environ Microbiol* 65, 3056–3063.
- Schaffer, A. A., Aravind, L., Madden, T. L., Shavirin, S., Spouge, J. L., Wolf, Y. I., Koonin, E. V. & Altschul, S. F. (2001). Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Res* 29, 2994–3005.
- Shelobolina, E. S., Coppi, M. V., Korenevsky, A. A., DiDonato, L. N., Sullivan, S. A., Konishi, H., Xu, H., Leang, C., Butler, J. E. & other authors (2007). Importance of *c*-type cytochromes for U(VI) reduction by *Geobacter sulfurreducens*. *BMC Microbiol* 7, 16.
- Sitthisak, S., Howieson, K., Amezola, C. & Jayaswal, R. K. (2005). Characterization of a multicopper oxidase gene from *Staphylococcus aureus*. *Appl Environ Microbiol* 71, 5650–5653.
- Sleep, B. E., Seepersad, D. J., Kaiguo, M. O., Heidorn, C. M., Hrapovic, L., Morrill, P. L., McMaster, M. L., Hood, E. D., Lebron, C. & other authors (2006). Biological enhancement of tetrachloroethene dissolution and associated microbial community changes. *Environ Sci Technol* 40, 3623–3633.
- Stults, J. R., Snoeyenbos-West, O. L., Methe, B., Lovley, D. R. & Chandler, D. P. (2001). Application of the 5' fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. *Appl Environ Microbiol* 67, 2781–2789.
- Swofford, D. L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods) Version 4. Sunderland, MA: Sinauer Associates.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22, 4673–4680.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25, 4876–4882.
- Vrionis, H. A., Anderson, R. T., Ortiz-Bernad, I., O'Neill, K. R., Resch, C. T., Peacock, A. D., Dayvault, R., White, D. C., Long, P. E. & Lovley, D. R. (2005). Microbiological and geochemical heterogeneity in an *in situ* uranium bioremediation field site. *Appl Environ Microbiol* 71, 6308–6318.
- Winderl, C., Schaefer, S. & Lueders, T. (2007). Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. *Environ Microbiol* 9, 1035–1046.
- Yan, B., Methe, B. A., Lovley, D. R. & Krushkal, J. (2004). Computational prediction of conserved operons and phylogenetic footprinting of transcription regulatory elements in the metal-reducing bacterial family *Geobacteraceae*. *J Theor Biol* 230, 133–144.

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