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.

**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; Winderl et al., 2007) and can serve as agents for the bioremediation of metal contamination (Anderson et al., 2003; Chang et al., 2005; Iotok 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...
uranium-contaminated subsurface environment (Holmes et al., 2005).

Attempts to identify key genes whose expression can be specifically linked to metal reduction in Geo bacter 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 Geo bacter-dominated environments would not provide an accurate indication of rates of Fe(III) reduction (Chin et al., 2004). Furthermore, as more Geo bacter genomes have been sequenced, it has become apparent that cytochromes required for Fe(III) reduction by G. sulfurreducens are not necessarily conserved in other Geo bacter 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 (Afkar et al., 2005; Childers et al., 2006; 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 Geo bacter species in environments of interest, levels of Geo bacter 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 Geo bacter species.

**METHODS**

**Source of organisms and culture conditions.** Geo bacter sulfurreducens (DSM 12127T), Pelobacter carbinolicus (DSM 2380T), Pelobacter propionicus (DSM 2379T), Pelobacter acidigallii (ATCC 49970T), Pelobacter acetylenicus (DSM 3246T), Pelobacter massiliensis (DSM 6233T) and Pelobacter venetianus (DSM 2394T) 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 electrode acceptor provided for growth in the anode chamber consisted of an anode poised with a potentiosat (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 potentiosat 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 pBRMCS-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 theompC 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

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<tr>
<td>2657rgUp5’/2657rgUp3’</td>
<td>CCATTTCGCCAGTGACAGCGCGGACAGTGC</td>
<td>GCTATGAAATCCATGGCGATAGCCTCCTTTCC</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>ATGGGTGCTGTCGCAATATGTCG</td>
</tr>
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<td>gs_mcpA5f/305r</td>
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<td>GGGCTGCGAATCCACCAAGT</td>
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</table>

*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., 2004, 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* genes were designed from the genomes of *G. sulfurreducens* (Methé et al., 2003), *Geobacter metallireducens*, ‘Geobacter uraniumireducens’, *Geobacter* sp. FRC-32, *Geobacter bemidiensis*, and ‘Geobacter lovleyi’ genomes. Preliminary sequence data from *G. metallireducens*, ‘G. uraniumireducens’, *Geobacter* sp. FRC-32, *G. bemidiensis* 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_gltA1000/850r, Geo_mdh2606/610r 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*, *gltA* and *proC* mRNA transcripts in the groundwater via quantitative RT-PCR: Rifle_ompB4775/627r, Rifle_ompC31f/193r, Rifle_gltA3388/453r, Rifle_mdh75f/227r and Rifle_proC156f/287r.

The number of *ompB*, *ompC*, *gltA* and *proC* 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’Neill et al., 2008). In addition, when *G. sulfurreducens* and ‘G.
"ura\textit{nireducens}" were grown in chemostats at four different dilution rates, microarray and quantitative RT-PCR analyses showed that \textit{proC} expression levels were relatively constant at the different rates of growth (Barrett et al., 2007; Riso et al., 2007).

Primers targeting the \textit{ompB}, \textit{ompC}, \textit{recA}, \textit{proC} and \textit{rpoD} genes for pure culture studies were designed from the \textit{G. sulfurreducens} genome sequence (Methe et al., 2003). The following primer pairs targeted \textit{ompB}, \textit{ompC}, \textit{recA}, \textit{proC} and \textit{rpoD} genes in \textit{G. sulfurreducens}: Gsulf\textit{ompB}228f/2395r, Gsulf\textit{ompC}408f/518r, Gsulf\textit{recA}660f/737r, Gsulf\textit{proC}2f/777r and Gsulf\textit{rpoD}1132f/1210r.

Probes targeting \textit{ompB} and \textit{ompC} genes from \textit{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 \textit{ompB} gene was amplified with gs\textit{ompB}2206f/2407r, and a 300 bp fragment from the \textit{ompC} gene was amplified with gs\textit{ompC}5f/305r. RT-PCR was done with primers \textit{ompB}-1f/2r and \textit{ompB}-3f/3r to confirm \textit{ompB} operon structure.

### Quantification of gene abundance with most-probable-number (MPN)-PCR.

Optimal amplification conditions for primers designed for MPN-PCR of \textit{Geobacteraceae} \textit{ompB}, \textit{gltA} and \textit{rpoD} genes were determined in a gradient thermal cycler (MJ Research). Primer pairs used to amplify \textit{Geobacter} \textit{ompB}, \textit{rpoD} and \textit{gltA} genes are outlined in Table 1; Geo\textit{ompB}576f/1380r, Geo\textit{rpoD}225f/560r and Geo\textit{gltA}100f/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 \textit{Geobacteraceae} \textit{ompB}, \textit{gltA} and \textit{rpoD} 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 \textit{ompB}, \textit{ompC}, \textit{gltA}, \textit{mdh}, \textit{recA}, \textit{proC} and \textit{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 gel (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 (Stratagen), 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 stability, 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 \textit{ompB} and \textit{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 CLUSTALW (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 CLUSTALW (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 [HK85 (Hasegawa et al., 1985) and Jukes–Cantor (Jukes & Cantor, 1969)]. Bootstrap values were obtained from 100 replicates.

The software programs, FASTA-B (Gardy et al., 2003), TMAPred (Hofmann & Stoeffl, 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 isolectric point was calculated with the EMBOS 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 (TR1)-like Fe(III)-binding domains were identified in the GCG sequence editor by the FINDPATTERN program (Wisconsin Package version 10) with the EXXE motif. PGENSEB, BPROM and FindTerm programs, available through Softberry (www.softberry.com), were used for operon and gene predictions.

### RESULTS

### Prevalence of \textit{ompB} and \textit{ompC} within the \textit{Geobacteraceae}.

Further analysis of the \textit{G. sulfurreducens} genome revealed the presence of an \textit{OmpB} homologue, OmpC, which was 26 % identical and 36 % similar to \textit{OmpB} (Fig. 1). The most similar protein to OmpC that has been characterized thus far is the CotA protein from \textit{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 \textit{B. subtilis}, Mn\textsuperscript{2+} oxidizing activity has been reported in similar laccase proteins isolated from other \textit{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. uranireducens’, ‘G. lovleyi’, Geobacter sp. FRC-32 and G. bismiadiens. 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%
identical/ 53 % similar). Genes with homology to \textit{ompB} and \textit{ompC} were also detected in the genomes of \textit{Anaeromyxobacter dehalogenans}, \textit{Leptothrix discophora}, \textit{Nitrosospira multiformis}, \textit{Oceanobacillus iheyensis}, \textit{Salinispora tropica} and \textit{Thiobacillus denitrificans}.

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

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Protein clade} & \textbf{Organism name and gene} & \textbf{Fibronectin domains} & \textbf{Copper-binding domains} & \textbf{Iron-binding motifs} & \textbf{Predicted cellular location of protein} & \textbf{Presence of signal peptide} & \textbf{Similarity (\%)/ identity (\%)} to \textit{OmpB} or \textit{OmpC} in \textit{G. sulfurreducens} \\
\hline
\textit{ompC} & \textit{Anaeromyxobacter dehalogenans} & 0 & 4 & 3 & Periplasm or outer membrane & Present & 37/26 \\
\hline
\textit{ompB} & \textit{Anaeromyxobacter dehalogenans} & 2 & 4 & 2 & Cytoplasm & Absent & 53/43 \\
\hline
\textit{ompC} & \textit{Desulfuromonas acetoxidans} & 0 & 4 & 0 & Periplasm or outer membrane & Present & 45/29 \\
\hline
\textit{ompC} & \textit{Geobacter bemidjiensis mcpA} & 0 & 4 & 2 & Inner membrane & Present & 38/26 \\
\textit{ompB} & \textit{Geobacter bemidjiensis ompB-1} & 0 & 4 & 2 & Inner membrane & Present & 54/43 \\
\textit{ompB} & \textit{Geobacter bemidjiensis ompB-2} & 0 & 4 & 0 & Periplasm or outer membrane & Present & 54/45 \\
\hline
\textit{ompB} & ‘\textit{Geobacter lovleyi}’ \textit{ompB} & 1 & 4 & 3 & Periplasm or outer membrane & Present & 74/65 \\
\hline
\textit{ompC} & \textit{Geobacter metallireducens mcpA-1} & 0 & 4 & 2 & Peripheral & Present & 60/51 \\
\hline
\textit{ompC} & \textit{Geobacter metallireducens mcpA-2} & 0 & 4 & 1 & Inner membrane & Present & 40/31 \\
\hline
\textit{ompB} & \textit{Geobacter metallireducens ompB-1} & 0 & 4 & 2 & Periplasm or outer membrane & Present & 40/31 \\
\hline
\textit{ompB} & \textit{Geobacter metallireducens ompB-2} & 0 & 4 & 0 & Periplasm or outer membrane & Present & 49/40 \\
\hline
\textit{ompC} & \textit{Geobacter sp. FRC-32 mcpA-1} & 0 & 4 & 2 & Periplasm or outer membrane & Present & 71/61 \\
\hline
\textit{ompB} & \textit{Geobacter sp. FRC-32 ompB} & 0 & 4 & 4 & Inner membrane & Present & 58/47 \\
\hline
\textit{ompC} & \textit{Geobacter sp. FRC-32} & 0 & 4 & 1 & Inner membrane & Present & 46/30 \\
\hline
\textit{ompC} & \textit{Geobacter sulfurreducens mcpA} & 0 & 4 & 3 & Periplasm or outer membrane & Present & 100/100 \\
\hline
\textit{ompB} & \textit{Geobacter sulfurreducens ompB} & 1 & 4 & 1 & Outer membrane & Present & 100/100 \\
\textit{ompB} & ‘\textit{Geobacter uraniireducens}’ \textit{ompB} & 0 & 4 & 1 & Periplasm or outer membrane & Present & 56/49 \\
\hline
\textit{ompB} & \textit{Leptothrix discophora mofA} & 0 & 4 & 2 & Periplasm or outer membrane & Present & 50/37 \\
\hline
\textit{ompC} & \textit{Nitrosospira multiformis} & 0 & 4 & 2 & Cytoplasm & Absent & 52/41 \\
\textit{ompC} & \textit{Oceanobacillus iheyensis} & 0 & 4 & 4 & Cytoplasm & Absent & 41/29 \\
\textit{ompC} & \textit{Salinispora tropica} & 0 & 4 & 3 & Periplasm or outer membrane & Present & 46/36 \\
\hline
\textit{ompC} & \textit{Thiobacillus denitrificans} & 0 & 4 & 3 & Cytoplasm & Absent & 51/43 \\
\hline
\end{tabular}
\caption{Characteristics of multicopper proteins found in \textit{Geobacter} species and other organisms with \textit{OmpB}- or \textit{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 \textit{OmpB} or \textit{OmpC} amino acid sequences from \textit{G. sulfurreducens} (730 aa sequences considered).}
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 32P-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 monicistronic 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 (35 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 ± 0.029 mg protein ml⁻¹ at 0.03 h⁻¹; 0.171 ± 0.014 mg protein ml⁻¹ at 0.07 h⁻¹ (mean ± se, n=3)] and Fe(II) concentrations (47.03 ± 8.48 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 μM.

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

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**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.
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

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**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.
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
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; Hulio 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 ompC 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.

ACKNOWLEDGEMENTS

We would like to thank the DOE Joint Genome Institute (JGI) for providing us with preliminary sequence data from G. metallireducens, ‘G. uranireducens’, G. bemidjiensis, Geobacter sp. FRC-32, ‘G. lovleyi’, P. carbinolicus, P. propionicus and D. acetoxidans. This research was supported by the Office of Science (BER), US Department of Energy with funds from the Environmental Remediation Science Program (grants DE-FG0207ER64377 and DE-FG02-02ER63423) and the Genomes to Life Program (co-operative agreement No. DE-FG02-02ER63446).

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Edited by: M. Tien