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ORIGINAL ARTICLE

Transcriptome of *Geobacter uraniireducens* growing in uranium-contaminated subsurface sediments

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To learn more about the physiological state of *Geobacter* species living in subsurface sediments, heat-sterilized sediments from a uranium-contaminated aquifer in Rifle, Colorado, were inoculated with *Geobacter uraniireducens*, a pure culture representative of the *Geobacter* species that predominates during *in situ* uranium bioremediation at this site. Whole-genome microarray analysis comparing sediment-grown *G. uraniireducens* with cells grown in defined culture medium indicated that there were 1084 genes that had higher transcript levels during growth in sediments. Thirty-four *c*-type cytochrome genes were upregulated in the sediment-grown cells, including several genes that are homologous to cytochromes that are required for optimal Fe(III) and U(VI) reduction by *G. sulfurreducens*. Sediment-grown cells also had higher levels of transcripts, indicative of such physiological states as nitrogen limitation, phosphate limitation and heavy metal stress. Quantitative reverse transcription PCR showed that many of the metabolic indicator genes that appeared to be upregulated in sediment-grown *G. uraniireducens* also showed an increase in expression in the natural community of *Geobacter* species present during an *in situ* uranium bioremediation field experiment at the Rifle site. These results demonstrate that it is feasible to monitor gene expression of a microorganism growing in sediments on a genome scale and that analysis of the physiological status of a pure culture growing in subsurface sediments can provide insights into the factors controlling the physiology of natural subsurface communities.
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Introduction

Contrary to the often-repeated statement that the most environmentally relevant microorganisms cannot be studied in pure culture, it has become increasingly apparent that pure cultures of some of the most environmentally relevant organisms can be recovered (Chisholm *et al.*, 1992; Button *et al.*, 1993; Rappe *et al.*, 2002; Konneke *et al.*, 2005; Nevin *et al.*, 2005; Giovannoni and Stingl, 2007; Shelobolina *et al.*, 2008; Holmes *et al.*, 2008a). When these organisms are available in pure culture, it is possible to combine genome sequence information with physiological, transcriptomic and proteomic studies. This offers the possibility of rapidly developing an understanding of the physiology of microorganisms catalyzing important environmental processes (Lovley, 2003). However, for these physiological studies to be meaningful, the physiology of isolates should be evaluated under conditions that have relevance to the environments of interest.

Geobacter species are a group of organisms from which it has been possible to obtain pure culture isolates of microorganisms known to be environmentally relevant (Nevin *et al.*, 2005; Shelobolina *et al.*, 2008; Holmes *et al.*, 2008a). Molecular and culturing studies have demonstrated that *Geobacter* species are the predominant microorganisms in a wide diversity of subsurface environments in which dissimilatory Fe(III) reduction is an important process for the degradation of both natural organic matter and organic contaminants or for the stimulation of *in situ* bioremediation of metal-contaminated subsurface environments (Rooney-Varga *et al.*, 1999; Anderson *et al.*, 2003; Lovley *et al.*, 2004; North *et al.*, 2004; Coates *et al.*, 2005; Lin *et al.*, 2005; Sleep *et al.*, 2006; Holmes *et al.*, 2007; Winderl *et al.*,

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2007). By designing an isolation medium that mimics subsurface conditions, it has become possible to isolate *Geobacter* species that have 16S rRNA gene sequences that are identical or highly similar to the 16S rRNA sequences that predominate in Fe(III)-reducing subsurface environments (Nevin *et al.*, 2005; Shelobolina *et al.*, 2008; Holmes *et al.*, 2008a).

To date, genome-scale analysis of *Geobacter* physiology has primarily focused on *Geobacter sulfurreducens* (Lovley *et al.*, 2004; Lovley, 2006; Mahadevan *et al.*, 2006). This was the first *Geobacter* species whose genome was sequenced (Methe *et al.*, 2003) and for which a genetic system was developed (Coppi *et al.*, 2001). Although a substantial number of genome-scale gene expression and proteomic studies have been conducted with *G. sulfurreducens* (Esteve-Nunez *et al.*, 2004; Leang and Lovley, 2005; Methe *et al.*, 2005; DiDonato *et al.*, 2006; Giometti, 2006; Khare *et al.*, 2006; Nunez *et al.*, 2006; Coppi *et al.*, 2007; Mahadevan *et al.*, 2008; Postier *et al.*, 2008), with few exceptions (Holmes *et al.*, 2006; Nevin *et al.*, 2008), these studies have primarily focused on cultures grown in chemostats with soluble electron acceptors. Although chemostats offer the advantage of providing physiologically consistent cultures that are harvested at steady-state conditions, conditions in chemostats are likely to be significantly different from those that *Geobacter* species face in subsurface environments. In chemostat studies, cells are grown in nutrient-rich, buffered medium with soluble electron acceptors that would not be found in the subsurface in abundance, such as fumarate. Furthermore, it is not clear whether results from physiological studies of *G. sulfurreducens* are necessarily applicable to the phylogenetic clade of *Geobacter* species that predominates in subsurface environments (Holmes *et al.*, 2007).

Here we report on whole-genome-scale gene expression studies of an environmentally relevant *Geobacter* species, *G. uraniireducens*, grown in subsurface aquifer sediments. The results suggest that the physiology of *Geobacter* cells growing in sediments is significantly different from that of cells grown in defined laboratory media and that gene expression patterns of pure cultures grown in sediments may mimic patterns associated with natural populations of *Geobacter* species in the subsurface.

Materials and methods

Subsurface site and field experiment description

In 2005, a small-scale *in situ* bioremediation experiment was conducted on the grounds of a former uranium ore-processing facility in Rifle, Colorado, during the months of August and September (Holmes *et al.*, 2007). This site, designated the Old Rifle site, is part of the Uranium Mill Tailings Remedial Action program of the US Department of Energy. During the field experiment, a concentrated acetate/bromide solution (100:10 mM) mixed with native groundwater was injected into the subsurface to provide <10 mM acetate to the groundwater over the course of 28 days as described earlier (Holmes *et al.*, 2007).

Subsurface sediments for culture studies were collected near the acetate-injection test plot. These background sediments were collected with a backhoe, placed in sealed mason jars, and stored at 16 °C until use. Unfiltered background groundwater for sediment incubations was pumped to the surface into 5-gallon plastic carboys with a peristaltic pump and stored at 4 °C until use. Before use, the plastic carboys were washed with 10% bleach, rinsed with deionized water and autoclaved for 30 min.

Culturing conditions

Geobacter uraniireducens strain RF4 (ATCC BAA-1134) was obtained from our laboratory culture collection. For sediment incubations, 40 g of the subsurface sediments described above, 6 ml groundwater and acetate (5 mM) were added to 60 ml serum bottles in an anaerobic chamber under an N_2 atmosphere (Nevin and Lovley, 2000). Before inoculation with *G. uraniireducens*, sediments were heat sterilized as described earlier (Nevin and Lovley, 2000). Cells that served as an inoculum (2%) for sediment incubations were first grown in a previously described bicarbonate-buffered, defined medium referred to as FWA-Fe(III) medium (Lovley and Phillips, 1988), with acetate (5 mM) provided as an electron donor and amorphous Fe(III) oxyhydroxide (100 mM) provided as an electron acceptor. The amorphous Fe(III) oxyhydroxide was synthesized in the laboratory as described earlier (Lovley and Phillips, 1986). Un-inoculated sediment controls were monitored for Fe(III) reduction and acetate consumption.

Cells grown with fumarate (20 mM) as the electron acceptor were also grown with acetate (5 mM) as the electron donor in the same bicarbonate-buffered, defined medium (Lovley and Phillips, 1988) and incubated under N_2:CO_2 (80:20). All incubations were performed at 30 °C in the dark.

Analytical techniques

Acetate concentrations were determined with an HP series 1100 high-pressure liquid chromatograph (Hewlett Packard, Palo Alto, CA, USA) with a Fast Acid Analysis column (Bio-Rad laboratories, Hercules, CA, USA) with an eluent of 8 mM H_2SO_4 and absorbance detection at 210 nm as described earlier (Anderson *et al.*, 2003).

Fe(III) reduction in the sediments was monitored by first measuring Fe(II) in the sediments that could be extracted in 0.5 M HCl after a 1 h incubation as described earlier (Lovley and Phillips, 1987; Phillips and Lovley, 1987) with a ferrozine assay in a split-beam dual-detector spectrophotometer

(Spectronic Genosys2; Thermo Electron Corp., Mountain View, CA, USA) at an absorbance of 562 nm. The remaining Fe(III) in the sediments that was not HCl extractable was then converted to Fe(II) by the addition of 0.25 M hydroxylamine as described earlier (Lovley and Phillips, 1987). After the addition of hydroxylamine, samples were incubated for an additional hour and then measured with a ferrozine assay. The percentage of Fe(II) in the sediments was then determined by dividing the HCl-extractable Fe(II) by the hydroxylamine extractable Fe(II).

RNA extraction

For extraction of RNA from batch cultures grown with fumarate as an electron acceptor (100 ml), cells were first split into four separate 50 ml conical tubes (BD Biosciences, San Jose, CA, USA), mixed with RNA Protect (Qiagen, Germantown, MD, USA) in a 1:1 ratio and pelleted by centrifugation at 5000 r.p.m. for 15 min. Pellets were then immediately frozen in a dry ice/ethanol bath and stored at −80 °C. RNA was extracted from these pellets as described earlier (Holmes *et al*., 2006).

RNA was extracted from sediment incubations when 75% of the total iron present in the sediments was reduced. The percentage of Fe(II) in the sediments was determined by dividing the HCl-extractable Fe(II) by the hydroxylamine-extractable Fe(III) as described earlier (Lovley and Phillips, 1987). Groundwater and sediments from the incubations were transferred into 50 ml conical tubes (BD Biosciences), mixed with RNA Protect (Qiagen) in a 1:1 ratio, and cells and sediments were pelleted by centrifugation at 5000 r.p.m. for 15 min. Pellets were then immediately frozen in a dry ice/ethanol bath and stored at −80 °C. RNA was extracted from these pellets as described earlier (Holmes *et al*., 2004).

RNA was also extracted from groundwater collected from the U(VI)-contaminated aquifer during the bioremediation field experiment. To obtain sufficient biomass from the groundwater for RNA extraction, it was necessary to concentrate 15 l of groundwater pumped to the surface with a peristaltic pump by impact filtration on 293 mm diameter Supor membrane disc filters (Pall Corporation, East Hills, NY, USA), which took about 3 min. Once the groundwater was concentrated on the filters, they were placed into whirl-pack bags and immediately flash frozen in a dry ice/ethanol bath. Samples were then shipped back to the laboratory where they were stored at −80 °C. RNA was extracted from filters as described earlier (Holmes *et al*., 2005).

All RNA samples had A_{260}/A_{280} ratios of 1.8–2.0, indicating that they were of high purity (Ausubel *et al*., 1997). To ensure that RNA samples were not contaminated with DNA, PCR amplification with primers targeting the 16S rRNA gene was conducted on RNA samples that had not undergone reverse transcription.

Microarray analysis

RNA was prepared for microarray studies as described earlier (Postier *et al*., 2008). Total RNA (0.5 μg) was amplified using the MessageAmp II-Bacteria Kit (Applied Biosystems/Ambion, Austin, TX, USA) according to the manufacturer's instructions. Ten micrograms of amplified RNA was chemically labeled with Cy3 (for the control fumarate condition) or Cy5 (for the experimental sediment condition) dye using the MicroMax ASAP RNA Labeling Kit (Perkin Elmer, Wellesley, MA, USA) according to the manufacturer's instructions.

The oligonucleotide microarrays used in this study were CustomarrayTM 12K arrays (Combimatrix, Mukilteo, WA, USA) and were designed using the genomic sequence of *G. uraniireducens* (accession number NZ_AAON00000000). A complete record of all oligonucleotide sequences used and raw and statistically treated data files is available in the NCBI Gene Expression Omnibus database (GEO data series number GSE10920).

Results from microarray hybridizations were analyzed by LIMMA mixed model algorithms as described earlier (Benjamini and Hochberg, 1995; Smyth and Speed, 2003; Smyth, 2004; Postier *et al*., 2008). Multiple oligonucleotide probes were analyzed from each gene (three or four probes per gene), and a gene was considered differentially expressed only if at least half of the probes had *P*-values less than or equal to 0.01.

Testing and design of primers for quantitative reverse transcription PCR

To verify results obtained from the microarray experiments, quantitative reverse transcription PCR (qRT-PCR) analyses were performed with RNA extracted from *G. uraniireducens* cells grown with either sediments or fumarate serving as the electron acceptor. All 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 genes were selected for quantitative RT-PCR analyses: *hyaL*, *cusA*, *phoU*, *pstB*, *nifD*, *sodA* and *ppcS*. The housekeeping gene, *proC*, which appears to be constitutively expressed in pure cultures and the environment (Holmes *et al*., 2005, 2006, 2008b; O'Neil *et al*., 2008) and was not differentially expressed in the microarray, was selected as an external control for normalization. The gene, *proC*, encodes pyrroline-5-carboxylate reductase, which is involved in arginine and proline metabolism.

Geobacter hyaL, *cusA*, *phoU*, *nifD*, *ppcS* and *proC* mRNA transcripts in groundwater samples collected during the *in situ* uranium bioremediation experiment were also monitored by quantitative RT-PCR. Before primers could be designed to quantify mRNA transcript abundance *in situ*, cDNA libraries were constructed with products amplified from the

environment with degenerate PCR primer sets that targeted 400–800 bp regions of each Geobacteraceae gene of interest (Table 1). These degenerate primers were designed from nucleotide and amino-acid sequences extracted from the following Geobacteraceae genomes: *G. sulfurreducens* (Methe *et al.*, 2003), *G. metallireducens,* strain FRC-32*, G. uraniireducens, Desulfuromonas acetoxidans*, *Pelobacter carbinolicus*, *Pelobacter propionicus* and *G. bemidjiensis*. Preliminary genome sequence data were obtained from the DOE Joint Genome Institute website http://www.jgi.doe.gov.

Clone libraries were constructed with PCR products from the various degenerate primer sets, and 100 clones were selected for analyses. The predominant sequences detected in the clone libraries were then targeted for quantitative RT-PCR primer design. Out of 100 clones, seven unique sequences were detected in the *proC* clone library, and the sequence that was selected for *proC* primer design accounted for 71% of the clone library sequences. For the *hyaL*, *cusA*, *phoU*, *nifD* and *ppcS* clone libraries, five, five, eight, six and six unique sequences were detected, and the sequences selected for primer design accounted for 58%, 44%, 52%, 74% and 71% of their clone library sequences, respectively.

All degenerate and quantitative RT-PCR primer pairs used for pure culture and environmental studies are listed in Table 1.

PCR amplification parameters and clone library construction

A DuraScript enhanced avian RT single-strand synthesis kit (Sigma-Aldrich, St Louis, MO, USA) was used to generate cDNA as described earlier (Holmes *et al.*, 2004). Previously described parameters were used to amplify genes of interest with degenerate primers (Holmes *et al.*, 2007).

For clone library construction, PCR products were purified with the Gel Extraction Kit (Qiagen), and clone libraries were constructed with the TOPO TA cloning kit, version M (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmid inserts from each clone library were sequenced with the M13F primer at the University of Massachusetts Sequencing Facility.

Quantification of gene expression by quantitative RT-PCR

Once the appropriate cDNA fragments were generated by reverse transcription, quantitative RT-PCR amplification and detection were performed with the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Optimal quantitative RT-PCR conditions were determined using the manufacturer's guidelines. Each PCR mixture consisted of a total volume of 25 μl and contained 1.5 μl of the appropriate primers (stock concentrations, 15 μM) and 12.5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems). Standard curves covering eight orders of magnitude were constructed with serial dilutions of known amounts of purified cDNA quantified with a NanoDrop ND-1000 spectrophotometer at an absorbance of 260 nm.

Nucleotide and amino-acid sequence analysis

Nucleotide and amino-acid sequences from *phoU*, *cusA*, *ppcS*, *hyaL*, *nifD* and *proC* genes detected in

Table 1 Primers used to amplify different gene fragments from *Geobacter uraniireducens* and from Geobacteraceae present in the uranium-contaminated aquifer

Organism and primer type	*Gene*	*Primer set*	*Forward primer*	*Reverse primer*
In situ Geobacter (degenerate)	*ppcS*	geo_ppcS350f/1020r	CTDMAGGGGTGCACCGACTG	TCGTGRCASCCSTCGCACAT
In situ Geobacter (degenerate)	*hyaL*	geo_hyaL30f/620r	GTCTTTACGARRAGTACGTCTG	CCGAARATGCTCGACATGAC
In situ Geobacter (degenerate)	*phoU*	geophoU290f/650r	ACCTGGAGCGGATCGGCGAC	TCGTGTGGCGGATGTCCTTC
In situ Geobacter (degenerate)	*nifD*	geonifD225f/560r	ATCGGTGACGATATCAACGCC	TAGTTCATGGAACGGTAGCAGT
In situ Geobacter (degenerate)	*cusA*	geocusA1150f/1400r	GTCASCTCCAACATCATGTCC	TGGAGAAGGTCTTGGTGAAGGC
In situ Geobacter (degenerate)	*proC*	geoproC75f/471r	ATWGGIGGIGGIAATATGGC	TCCCCACCAGGTCGAACA
In situ Geobacter (qPCR)	*ppcS*	M21_ppcS227/309r	GCACCGACTGTCATCAGGTAAC	GCAGGCGTAGCACTCCATC
In situ Geobacter (qPCR)	*hyaL*	M21_hyaB348/478r	CTGTTGCCGTTGCCATCC	GACTTCGTCGAGCAGGTCTACA
In situ Geobacter (qPCR)	*phoU*	M21phoU27f/227r	GCCAATATCGCCAAAAGGGT	CCGCTGGATCTGCTCGTT
In situ Geobacter (qPCR)	*nifD*	M21nifD108f/268r	CCACCATATCAGTAACGACACCA	TGACGTTGAAGCCGCACTC
In situ Geobacter (qPCR)	*czcA*	M21cusA91f/203r	CGTGCCGAGGTTCTTGACAT	CCGAGTTTCTGATCCGTGGT
In situ Geobacter (qPCR)	*proC*	M21proC156f/287r	TTGCGAAATGAGCGACACC	ATCGCGGCACTTTTCACG
G. uraniireducens (qPCR)	*ppcS*	gu_ppcS500f/619r	ACGGCGAAACATACCTGAAAAT	CAACGCACTTTTTTGACGACTT
G. uraniireducens (qPCR)	*hyaL*	gu_hyaL632f/735r	ATCCCCATCCCCAGACTTTT	AAGGCTCCTGATTTCCGCTAT
G. uraniireducens (qPCR)	*phoU*	gu_phoU336f/615r	GCTGGCTTTCCTCATCCTCA	GGCATTTTTTACCACCACGCT
G. uraniireducens (qPCR)	*pstB*	gu_pstb199f/227r	ATCCTTGACGACACCGACATC	TTGTCGTAGATGTCGGTGTCGT
G. uraniireducens (qPCR)	*nifD*	gu_nifD328f/430r	CACGAGGCACTGGAAAACG	TGAAGGCGGTCAGGTCGT
G. uraniireducens (qPCR)	*cusA*	gu_cusA503f/635r	GGATTGTCACCCCCCAGTT	ACATCGTCCACAGTTATCGCAT
G. uraniireducens (qPCR)	*proC*	gu_proC263f/367r	TCCTGCTCGTGAACCTCTCC	TGTCTGCTTCTCCGATCACCT
G. uraniireducens (qPCR)	*sodA*	gu_sodA324f/430r	AAAGGATTTCAGGGCAACGG	CGTCGTGCTCATTGATCCAG

Abbreviations: qPCR, quantitative PCR.

the environment were compared with the GenBank nucleotide and protein databases using the blastn, blastx and blastp algorithms (Altschul *et al.*, 1990). The subcellular location of *c*-type cytochrome genes differentially expressed in the microarray experiments and *ppcS* sequences detected in groundwater collected from the uranium-contaminated aquifer were predicted with PSORT-B (Gardy *et al.*, 2003), Tmpred (Hofmann and Stoffel, 1993) and SignalP 3.0 Server (Emanuelsson *et al.*, 2007).

Nucleotide sequence accession numbers

The nucleotide sequences of *ppcS*, *hyaL*, *proC*, *phoU*, *cusA* and *nifD* genes amplified from the uranium-contaminated aquifer have been deposited in the GenBank database under accession numbers EU676389–EU676398 and EU519198–EU519202.

Results and discussion

Comparison of the G. uraniireducens *transcriptome in cells grown in sediments vs defined medium*

Geobacter uraniireducens was studied, because it was isolated from the uranium-contaminated site in Rifle, Colorado, and its 16S rRNA gene sequence matched one of the sequences that predominated during *in situ* uranium bioremediation (Shelobolina *et al.*, 2008). Microarray comparisons between *G. uraniireducens* cells grown in sediments vs the soluble electron acceptor, fumarate, showed that 1964 genes (1084 upregulated, 882 downregulated) had at least a twofold difference in transcript levels (Table 2; Supplementary Tables S1 and S2). This accounts for approximately 45% of the genes in the *G. uraniireducens* genome, indicating that gene expression patterns in *G. uraniireducens* were significantly different during growth in sediments than during growth with fumarate provided as an electron acceptor.

The majority of genes that showed significant differences in transcript levels under the two conditions were annotated as coding for proteins involved in energy metabolism, proteins of unknown function and hypothetical proteins (Table 2; Supplementary Tables S1 and S2). There were significantly more genes involved in energy metabolism that were upregulated during growth on sediments (152 genes) than during growth in fumarate (97 genes). Further analysis revealed that the majority of energy metabolism genes with higher transcript levels during growth on sediments coded for proteins predicted to be involved in electron transfer and included many *c*-type cytochromes, hydrogenases and iron-sulfur cluster-binding proteins (Supplementary Table S1).

In general, transcript levels for genes encoding proteins involved in amino-acid and protein biosynthesis were significantly lower during growth on sediments than on fumarate medium (Table 2; Supplementary Table S2). For example, transcript levels for genes for a number of ribosomal proteins, such as *rplL* and *rpsC*, were approximately 20-fold lower during growth on sediments. Transcript levels for protein synthesis genes are typically lower in microorganisms growing at slower growth rates (Gonzalez *et al.*, 2002; Hua *et al.*, 2004; Boccazzi *et al.*, 2005). Thus, these results are consistent with the fact that *G. uraniireducens* grows sevenfold slower on sediments relative to fumarate (DE Holmes, manuscript in preparation). Genes encoding proteins involved in the citric acid cycle, such as malate dehydrogenase (*mdh*) and citrate synthase (*gltA*), also had lower transcript levels in sediment-grown cells (Supplementary Table S2). Expression of these genes was previously shown to be linked to rates of metabolism in both *G. sulfurreducens* and the natural community of *Geobacter* species predominating during *in situ* uranium bioremediation (Holmes *et al.*, 2005, 2008b).

Table 2 The number of genes from different TIGR functional categories that showed at least a twofold difference in expression according to microarray experiments comparing *Geobacter uraniireducens* cells grown in sediments with cells grown in laboratory medium with fumarate serving as an electron acceptor (*P*-value cutoff $\leqslant 0.01$)

Functional category	*Number higher on sediments*[a]	*Number higher on fumarate*[b]
Amino-acid biosynthesis	15	41
Biosynthesis of cofactors, prosthetic groups and carriers	37	32
Cell envelope	69	52
Cellular processes	66	47
Central intermediary metabolism[c]	25	5
DNA metabolism	29	25
Energy metabolism	152	97
Fatty acid and phospholipid metabolism	17	20
Hypothetical proteins	289	167
Mobile and extrachromosomal element functions	14	12
Protein fate	31	43
Protein synthesis	20	77
Purines, pyrimidines, nucleosides and nucleotides	12	24
Regulatory functions	87	53
Signal transduction	19	9
Transcription	9	19
Transport and binding proteins	72	50
Unknown function	121	109
Total	1084	882

[a]Sediment-grown cells were considered the experimental condition.
[b]Fumarate-grown cells were considered the control condition.
[c]Central intermediary metabolism genes include those involved in nitrogen fixation, amino sugar metabolism, one-carbon metabolism, phosphorous compounds, polyamine biosynthesis and sulfur metabolism.

Increased expression of c*-type cytochrome genes in sediment-grown cells*

The majority of differentially expressed genes encoding electron transport proteins were annotated

as *c*-type cytochromes (Supplementary Table S1 and S2). Thirty-four different genes encoding *c*-type cytochromes had at least twofold higher mRNA transcript levels in sediment-grown cells (Table 3). Eleven of these cytochromes are predicted to be located in the periplasm or inner membrane, 11 are predicted to be outer-membrane proteins and 12 of these putative *c*-type cytochromes have an unknown subcellular location.

The most significantly upregulated genes in sediment-grown cells encode homologs of *G. sulfurreducens*' periplasmic *c*-type cytochrome proteins, MacA and PpcS. Homologs of *macA* have also been detected in five additional Geobacteraceae genomes (*D. acetoxidans*, *G. bemidjiensis*, *G. metallireducens*, *G. sulfurreducens* and strain M21). Deletion of *macA* in *G. sulfurreducens* inhibits Fe(III) (Butler *et al.*, 2004) and U(VI) (Shelobolina *et al.*, 2007) reduction. However, this appears to be an indirect effect caused by the fact that the outer-membrane *c*-type cytochrome, OmcB, is not expressed in the *macA*-deficient mutant (Kim and Lovley, 2008). PpcS-like proteins have been found in seven of the additional Geobacteraceae genomes that have been sequenced thus far (*D. acetoxidans*, *G. bemidjiensis*, strain FRC-32, *G. lovleyi*, *G. metallireducens*, *G. sulfurreducens* and strain M21), but the function of this *c*-type cytochrome has yet to be investigated.

A gene that was homologous to *omcB* in *G. sulfurreducens* also had higher transcript levels in sediment-grown cells (Table 3). Gene deletion studies in *G. sulfurreducens* have shown that *OmcB* is critical for electron transfer to Fe(III) (Leang *et al.*, 2003; Leang and Lovley, 2005) and that its expression can be directly linked to rates of Fe(III) reduction (Chin *et al.*, 2004). Another gene that was upregulated in sediment-grown cells was the homolog of *omcG*, which encodes an outer-membrane *c*-type cytochrome in *G. sulfurreducens* that plays an important role in the proper expression of *omcB* (Kim *et al.*, 2006). In addition, the increased expression of a gene that is homologous to the outer-membrane *c*-type cytochrome gene of *G. sulfurreducens*, *omcT*, was observed in sediment-grown cells. The function of this cytochrome in *G. sulfurreducens* is still unclear, but its paralog, OmcS, is required for extracellular electron transfer (Mehta *et al.*, 2005; Holmes *et al.*, 2006).

Table 3 Putative *c*-type cytochrome genes that were upregulated at least twofold in microarray experiments comparing sediment-grown *Geobacter uraniireducens* with cells grown in laboratory media (*P*-value cutoff $\leqslant 0.01$)

Locus ID	*Gene annotation*	*Gene name*	*Cellular location*	*M (log_2)*	*Fold change*	P-*value*
Gura_1316	Cytochrome *c*, 2 heme-binding sites	*macA*	Periplasmic	3.44	10.82	0.000644377
Gura_0456	Cytochrome *c* family protein, 10 heme-binding sites	*ppcS*	Periplasmic	3.42	10.70	3.06E−06
Gura_0397	Cytochrome *c* family protein, 4 heme-binding sites	—	Unknown	2.52	5.72	0.001306348
Gura_0919	Cytochrome *c* family protein, 7 heme-binding sites	—	Outer membrane	2.48	5.59	0.000352109
Gura_0521	Cytochrome *c* family protein, 6 heme-binding sites	—	Unknown	2.32	4.99	0.000634935
Gura_3748	Cytochrome *c* family protein, 3 heme-binding sites	—	Unknown	2.23	4.70	0.000226566
Gura_4255	Cytochrome *c* family protein, 3 heme-binding sites	—	Unknown	2.13	4.39	0.004336673
Gura_0862	Cytochrome *c* family protein, 4 heme-binding sites	—	Unknown	1.91	3.75	1.63E−05
Gura_2035	Cytochrome *c* family protein, 29 heme-binding sites	*omcG*	Outer membrane	1.90	3.72	0.010589166
Gura_2381	Cytochrome *c* family protein, 5 heme-binding sites	*macC*	Inner membrane	1.69	3.24	0.005332236
Gura_2004	Cytochrome *c* family protein, 6 heme-binding sites	*omcT*	Outer membrane	1.63	3.09	0.009042988
Gura_4004	Cytochrome *c* family protein, 7 heme-binding sites	—	Periplasmic	1.62	3.07	3.41E−05
Gura_0453	Cytochrome *c* family protein, 5 heme-binding sites	—	Unknown	1.60	3.03	0.000640646
Gura_1995	Cytochrome *c* family protein, 26 heme-binding sites	—	Periplasmic	1.50	2.84	0.00011356
Gura_0331	Cytochrome *c*, 1 heme-binding site	—	Unknown	1.48	2.79	0.000416866
Gura_0077	Cytochrome *c* family protein, 59 heme-binding sites	—	Outer membrane	1.46	2.75	0.001112771
Gura_0447	Cytochrome *c* family protein, 5 heme-binding sites	—	Unknown	1.45	2.72	0.000583543
Gura_0706	Cytochrome *c* family protein, 3 heme-binding sites	—	Unknown	1.43	2.69	0.000865347
Gura_3655	Cytochrome *c* family protein, 9 heme-binding sites	—	Outer membrane	1.37	2.58	0.000332412
Gura_4069	Cytochrome *c* family protein, 2 heme-binding sites	*hsc*	Periplasmic	1.33	2.52	0.000277139
Gura_3284	Cytochrome *c*, 10 heme-binding sites	—	Outer membrane	1.32	2.49	0.00036512
Gura_0664	Cytochrome *c*, 4 heme-binding sites	*nrfH*	Periplasmic	1.27	2.41	0.000224069
Gura_0994	Cytochrome *c*, 8 heme-binding sites	*omcB*	Outer membrane	1.24	2.36	0.000450211
Gura_0120	Cytochrome *c* family protein, 44 heme-binding sites	—	Outer membrane	1.22	2.33	0.000174918
Gura_3652	Cytochrome *c* family protein, 5 heme-binding sites	—	Periplasmic	1.21	2.31	0.001258448
Gura_0922	Cytochrome *c* family protein, 1 heme-binding site	—	Periplasmic	1.20	2.30	0.001386412
Gura_2615	Cytochrome *c*, 1 heme-binding site	—	Unknown	1.18	2.26	0.000736394
Gura_1837	Cytochrome *c* family protein, 7 heme-binding sites	—	Unknown	1.10	2.14	0.001322768
Gura_0934	Cytochrome *c* family protein, 11 heme-binding sites	—	Unknown	1.07	2.10	0.000297431
Gura_0408	Cytochrome *c* oxidase, subunit I	—	Inner membrane	1.05	2.07	0.000391652
Gura_0398	Cytochrome *c* family protein, 11 heme-binding sites	*omcG*	Outer membrane	1.04	2.05	0.001363666
Gura_0665	Cytochrome *c*, 4 heme-binding sites	*nrfA*	Inner membrane	1.04	2.05	0.000587339
Gura_3417	Cytochrome *c* family protein, 16 heme-binding sites	*omcN*	Outer membrane	1.03	2.04	0.001540045
Gura_0989	Cytochrome *c* family protein, 9 heme-binding sites	—	Outer membrane	0.98	1.97	0.000559325

Protein cellular location was predicted with PSORT-B (Gardy *et al.*, 2003), Tmpred (Hofmann and Stoffel, 1993) and SignalP 3.0 Server (Emanuelsson *et al.*, 2007).

Additional *c*-type cytochrome genes with increased expression in sediment-grown cells included genes for the putative nitrite reductase proteins, NrfA and NrfH. These proteins are generally thought to be involved in the reduction of nitrite to ammonia in other organisms (Simon, 2002). However, extremely low levels of nitrite ($<6\,\mu M$) were detected in the sediments, and *G. uraniireducens* is not capable of nitrite reduction (Shelobolina *et al.*, 2008). A more plausible function for NrfA and NrfH in *G. uraniireducens* grown under these conditions is that they are involved in electron transfer to Fe(III) oxides in the sediment. It has been proposed that NrfA is important for dissimilatory metal reduction in two other metal-reducing species, *Desulfovibrio desulfuricans* and *Shewanella oneidensis* (Barton *et al.*, 2007; Shi *et al.*, 2007).

The genomes of *Geobacter* species sequenced to date contain 100 or more *c*-type cytochrome genes (Methe *et al.*, 2003) (http://www.jgi.doe.gov). The function of $\sim$35 different *c*-type cytochromes has been evaluated in *G. sulfurreducens*. Although further verification is clearly required, it is assumed that the *c*-type cytochromes in *G. uraniireducens* function in a manner similar to that described earlier for *G. sulfurreducens* (Lovley *et al.*, 2004). This assumption is based on the fact that the majority of genes encoding putative *c*-type cytochromes found in the *G. uraniireducens* genome have homologs in the *G. sulfurreducens* genome. For example, out of 105 putative *c*-type cytochrome genes that have been detected in the *G. uraniireducens* genome, 97 have homologs in *G. sulfurreducens* (Supplementary Table S3). The majority of these *c*-type cytochrome proteins are also highly similar to their homologous protein in *G. sulfurreducens*; the average similarity value being 59.75%.

In general, *c*-type cytochromes exposed on the outer surface of the cells of *G. sulfurreducens* appear to play a role in electron transfer to extracellular electron acceptors such as Fe(III) oxides (Mehta *et al.*, 2005), electrodes (Holmes *et al.*, 2006; Nevin *et al.*, 2008) or to U(VI) (Shelobolina *et al.*, 2007). Some of the cytochromes embedded in the outer membrane, such as OmcB (Qian *et al.*, 2007), may also be important for extracellular electron transfer (Leang *et al.*, 2003; Leang and Lovley, 2005), whereas others, such as OmcF, OmcG and OmcH, appear to participate in Fe(III) reduction indirectly by influencing the expression of other outer-surface cytochromes (Kim *et al.*, 2005, 2006). Studies have also suggested that some of the periplasmic and inner-membrane *c*-type cytochromes are involved in the transfer of electrons derived from central metabolism to the outer membrane (Lovley *et al.*, 2004). In addition, some of the abundant periplasmic and outer-surface cytochromes appear to function as capacitors, permitting continued respiration of *Geobacter* species when they are not in direct contact with Fe(III) oxides (Esteve-Núñez *et al.*, 2008).

Increased expression of genes encoding electron transport proteins other than c*-type cytochromes in sediment-grown cells*

A gene for a putative multicopper protein homologous to OmpB of *G. sulfurreducens* was also significantly upregulated in *G. uraniireducens* cells during growth in sediments (Supplementary Table S1). OmpB is important for the reduction of insoluble Fe(III) oxides by *G. sulfurreducens* (Mehta *et al.*, 2006), and *ompB* transcripts were detected in the natural community of *Geobacter* species in the subsurface during a field experiment in which acetate was added to the subsurface to promote *in situ* uranium bioremediation (Holmes *et al.*, 2008b).

Transcripts from a number of hydrogenase genes were also significantly more abundant in *G. uraniireducens* cells grown in sediments (Table 4). The most highly upregulated hydrogenase gene (*hybL*) encodes a subunit of Hyb, which has been shown to be involved in hydrogen uptake in *G. sulfurreducens* (Coppi *et al.*, 2004). Hydrogen uptake in the sediments may have been associated with the

Table 4 Genes encoding hydrogenase proteins that were upregulated at least twofold in microarray experiments comparing sediment-grown *Geobacter uraniireducens* with cells grown in laboratory media (*P*-value cutoff $\leqslant 0.01$)

Locus ID	*Gene annotation*	*Gene name*	*M (log_2)*	*Fold change*	P-*value*
Gura_1945	Membrane-bound [NiFe]-hydrogenase, integral membrane subunit	*hybB*	2.94	7.66	1.92E−05
Gura_0804	Ech hydrogenase, subunit EchD, putative	—	2.08	4.22	0.001014131
Gura_1953	Hydrogenase expression/formation protein HypE	*hypE*	1.88	3.69	0.003698994
Gura_1951	Hydrogenase assembly chaperone hypC/hupF	*hypC*	1.88	3.68	0.000133397
Gura_1123	Cytosolic NiFe-hydrogenase, δ subunit	—	1.77	3.42	0.008961958
Gura_0873	Membrane-bound [NiFe]-hydrogenase large subunit	*hyaL*	1.52	2.87	0.000254351
Gura_1122	Methyl viologen-reducing hydrogenase, large subunit	*mvhL*	1.51	2.84	0.000825733
Gura_0545	Membrane-bound [NiFe]-hydrogenase, small subunit	*hyaS*	1.3	2.47	0.000234908
Gura_1944	Membrane-bound [NiFe]-hydrogenase iron-sulfur cluster binding	*hybA*	1.25	2.38	0.000575456
Gura_0543	Membrane-bound [NiFe]-hydrogenase b-type cytochrome subunit	*hyaB*	1.21	2.31	0.000153132
Gura_3653	Membrane-bound [NiFe]-hydrogenase iron-sulfur cluster binding	*hybA*	1.1	2.15	0.001112771
Gura_0890	Ech-hydrogenase-related complex HyfE-like subunit	*ehrC*	1.04	2.06	0.000350526
Gura_0544	Membrane-bound [NiFe]-hydrogenase, large subunit	*hyaL*	1.00	2.00	0.000631598

recycling of hydrogen evolved as a by-product of nitrogen fixation (Coppi, 2005; Methe *et al.*, 2005), which, as detailed below, appeared to be taking place in the sediments. The function of other hydrogenases in *G. sulfurreducens* is poorly understood (Coppi *et al.*, 2004; Coppi, 2005).

Fixed nitrogen and phosphorous limitation during growth in sediments

Chemical analyses of sediments and groundwater used for the laboratory incubations indicated that sediment-grown *G. uraniireducens* cells were limited for nitrogen and phosphorous. Carbon concentrations in the acetate-amended sediment incubations, on the other hand, were not limiting. Earlier studies have shown that *Geobacter* species are limited for fixed nitrogen at levels below 100 μM (Holmes *et al.*, 2004), and total nitrogen concentrations in the sediment incubations were well below that concentration. For example, the average ammonium concentration in the sediment incubations was 31 μM and combined nitrite and nitrate concentrations were only approximately 6 μM. Studies have also shown that *Geobacter* species are phosphate limited at concentrations below 100 μM (N'Guessan *et al.*, 2008). The average concentration of phosphate detected in the sediment incubations was 10 μM, which is significantly lower than the phosphate-limiting concentration. *Geobacter* is limited for carbon at concentrations below 1 mM (Elifantz *et al.*, 2008), and the concentration of acetate in the sediment incubations was 5 mM. Therefore, sediment-grown *G. uraniireducens* cells were not carbon limited. Further support for this comes from the fact that genes indicative of carbon limitation, such as *cstA* and *csrA* (Dubey *et al.*, 2003), were not upregulated in sediment-grown *G. uraniireducens* cells.

According to the microarray analysis, cells growing in sediments had a greater abundance of transcripts for a number of genes coding for proteins involved in nitrogen fixation (Table 5). Increased expression of the gene for the α-subunit of the nitrogenase protein, *nifD*, has been associated with nitrogen fixation in *G. sulfurreducens* and was detected in the natural community of *Geobacter* species living in petroleum-contaminated subsurface sediments (Holmes *et al.*, 2004; Methe *et al.*, 2005). Studies have shown that *G. uraniireducens* is able to grow with dinitrogen as a nitrogen source (DE Holmes, manuscript in preparation), and nitrogen fixation by *G. uraniireducens* in sediment incubations was consistent with low concentrations of fixed nitrogen present in the sediments from the Rifle site; the total nitrogen concentration in the groundwater was approximately 37 μM. This contrasts with high concentrations of fixed nitrogen sources (3.85 mM) present in the culture medium.

Although increased expression of proteins involved in nitrogen fixation is frequently associated with nitrogen limitation (Bulen and LeComte, 1966; Hageman and Burris, 1978), studies have also shown that under conditions of nitrogen limitation, nitrite reductases can be involved in the anabolic incorporation of nitrogen into biomolecules (Hoffmann *et al.*, 1998; Nakano *et al.*, 1998). Therefore, it is possible that the increased expression of the nitrite reductase genes, *nrfA* and *nrfH*, in sediment-grown cells is the result of nitrogen limitation. However, further investigation into this possibility is necessary.

A number of genes from the *phoU* regulon had higher transcript levels in sediment-grown *G. uraniireducens* (Table 6). Earlier studies have shown that the *phoU* regulon is critical for phosphate homeostasis in many bacteria (Lamarche *et al.*, 2008), and the growth of *G. sulfurreducens* under phosphate-limiting conditions in chemostats resulted in an elevated expression of genes in the *phoU* regulon (N'Guessan *et al.*, 2008). These results indicate that *G. uraniireducens* is limited for phosphate during growth on sediments and are consistent with the finding that phosphate is tightly adsorbed to the subsurface sediments used in this study, making much of it unavailable for uptake by the microorganisms (N'Guessan *et al.*, 2008).

Increased expression of genes involved in heavy metal and oxidative stress responses in sediment-grown cells

Geobacter uraniireducens expressed a number of genes indicative of heavy metal transport during

Table 5 Genes encoding proteins involved in nitrogen fixation that were upregulated at least twofold in microarray experiments comparing sediment-grown *Geobacter uraniireducens* with cells grown in laboratory media (*P*-value cutoff ⩽0.01)

Locus ID	*Gene annotation*	*Gene name*	*M (log_2)*	*Fold change*	P-*value*
Gura_1204	Nitrogenase molybdenum-iron cofactor biosynthesis protein	*nifB*	2.97	7.84	6.67E−06
Gura_3366	Nitrogen regulatory protein P-II, putative	—	2.81	7.00	0.008684882
Gura_1175	Nitrogenase iron protein	*nifH*	1.98	3.94	0.000258176
Gura_1205	NAD(+)—dinitrogen-reductase ADP-D-ribosyltransferase	*dRAT*	1.89	3.71	1.39E−05
Gura_0996	Dinitrogenase iron-molybdenum cofactor biosynthesis	—	1.79	3.45	4.13E−05
Gura_1177	Nitrogenase molybdenum-iron protein, β-subunit	*nifK*	1.32	2.50	0.000357264
Gura_1208	ADP-ribosyl-(dinitrogenase reductase)-activating glycohydrolase	*draG*	1.31	2.48	5.04E−05
Gura_1833	Nitrogen fixation iron-sulfur cluster assembly protein NifS	*nifS*	1.22	2.32	0.001233356
Gura_1176	Nitrogenase molybdenum-iron protein, α-chain	*nifD*	1.01	2.01	0.000788192

Table 6 Genes encoding proteins involved in phosphate limitation that were upregulated at least twofold in microarray experiments comparing sediment-grown *Geobacter uraniireducens* with cells grown in laboratory media (*P*-value cutoff ⩽0.01)

Locus ID	*Gene annotation*	*Gene name*	*M (log_2)*	*Fold change*	P-*value*
Gura_2558	Phosphate ABC transporter, periplasmic phosphate binding	*pstS*	2.51	5.71	4.54E−06
Gura_2560	Phosphate ABC transporter, membrane protein	*pstA*	1.34	2.52	0.013559
Gura_1507	Phosphate ABC transporter, periplasmic phosphate binding	*pstS*	1.18	2.27	0.000977466
Gura_1509	Phosphate ABC transporter, periplasmic phosphate binding	*pstS*	1.13	2.19	0.001309443
Gura_2559	Phosphate ABC transporter, membrane protein	*pstC*	1.11	2.16	0.027016159
Gura_2562	Phosphate transport system regulatory protein PhoU	*phoU*	1.09	2.13	0.012386155
Gura_2561	Phosphate ABC transporter, ATP-binding protein	*pstB*	1.01	2.01	0.01607155
Gura_3329	Phosphate-selective porin	—	0.97	1.96	0.000367369

Table 7 Genes encoding proteins involved in heavy metal resistance that were upregulated in microarray experiments comparing sediment-grown *Geobacter uraniireducens* with cells grown in laboratory media (*P*-value cutoff ⩽0.01)

Locus ID	*Gene annotation*	*Gene name*	*M (log_2)*	*Fold change*	P-*value*
Gura_3321	Molybdenum ABC transporter, ATP-binding protein	*modC*	2.62	6.13	1.04E−05
Gura_0048	Mn/Zn ABC transporter, membrane protein	—	2.46	5.49	2.06E−05
Gura_3460	Zinc transporter	—	2.35	5.09	1.73E−05
Gura_0533	Efflux pump outer membrane protein	—	2.25	4.76	7.08E−05
Gura_3362	Metal ion efflux outer membrane protein family protein	—	2.21	4.62	4.04E−05
Gura_3463	Co/Zn/Cd efflux system component	—	1.70	3.26	0.001860472
Gura_0087	Metal ion efflux outer membrane protein	—	1.64	3.12	0.001127681
Gura_3322	Molybdenum ABC transporter, permease protein	*modB*	1.50	2.82	3.13E−05
Gura_1382	Arsenical resistance operon repressor	—	1.35	2.54	0.000454707
Gura_1365	Tellurite resistance protein-related protein	—	1.21	2.31	0.002288875
Gura_0467	Arsenite efflux pump	*arsB*	1.20	2.30	0.007025268
Gura_0047	Fe/Mn/Zn ABC transporter, ATP-binding protein	—	1.11	2.16	0.001552217
Gura_3360	Heavy metal efflux pump, CusA family	*cusA*	1.11	2.16	0.003089329
Gura_3361	Efflux pump membrane fusion protein	—	1.06	2.09	0.0010951
Gura_3323	Molybdenum ABC transporter, periplasmic molybdenum-binding protein	*modA*	0.97	1.96	0.000791157
Gura_1297	Outer-membrane efflux protein	—	0.97	1.95	0.000866021
Gura_1042	Efflux pump outer-membrane protein	—	0.82	1.76	0.011471135
Gura_2663	Outer-membrane efflux protein	—	0.71	1.63	0.008395762
Gura_4180	Cobalt ABC transporter, membrane protein	*cbiQ-1*	0.71	1.63	0.015297739
Gura_1532	Copper-translocating P-type ATPase	—	0.66	1.58	0.005154452

growth in sediments collected from the uranium-contaminated site.

For example, a number of genes encoding proteins have been shown to be involved in bacterial resistance to such heavy metals as cobalt, zinc, cadmium, arsenic, copper, molybdenum and silver (Nies, 1995, 1999; Grunden and Shanmugam, 1997; Rensing *et al.*, 1997, 1999; Brown *et al.*, 2002; Parro and Moreno-Paz, 2003; Zahalak *et al.*, 2004; Basim *et al.*, 2005; Bencheikh-Latmani *et al.*, 2005; Braz and Marques, 2005; Hu *et al.*, 2005; Methe *et al.*, 2005; Permina *et al.*, 2006) and had higher transcript levels in sediment-grown cells (Table 7).

Transcript levels for a number of genes associated with oxidative stress resistance were also higher in sediment-grown cells (Table 8), although our studies were conducted under anaerobic conditions. A number of studies indicate that increased expression of oxidative stress genes is frequently associated with heavy metal exposure in bacteria (Geslin *et al.*, 2001; Hu *et al.*, 2005; Choudhary *et al.*, 2007).

Increased expression of these heavy metal stress genes can be explained by the fact that relatively high levels of arsenic, barium, molybdenum, radium, selenium and uranium have been detected in both sediment and groundwater collected from the Rifle site (US Department of Energy, 1999). However, increased expression of the molybdate ABC type transporter genes, *modA*, *modB* and *modC* (Table 7), might also be explained by the fact that there is an increased demand for molybdenum under nitrogen-fixing conditions. Earlier studies have shown that increased expression of molybdenum transporter genes is frequently associated with nitrogen-fixing conditions, because molybdenum is an essential component of nitrogenase proteins involved in nitrogen fixation (Grunden and Shanmugam, 1997; Parro and Moreno-Paz, 2003; Zahalak *et al.*, 2004).

Quantification of selected gene transcripts with qRT-PCR

To further evaluate the microarray results and to obtain more quantitative data for comparison with transcript abundance in the *Geobacter* species associated with *in situ* uranium bioremediation,

Table 8 Genes indicative of oxidative stress that were upregulated in microarray experiments comparing sediment-grown *Geobacter uraniireducens* with cells grown in laboratory media (*P*-value cutoff $\leqslant 0.01$)

Locus ID	*Gene annotation*	*Gene name*	*M* (log_2)	*Fold change*	P-*value*
Gura_1969	Glutaredoxin family protein	—	3.32	10.02	1.18251E−06
Gura_2162	Cytochrome d ubiquinol oxidase, subunit II	*cydB*	1.88	3.68	3.827E−05
Gura_1453	Superoxide dismutase	*sodA*	1.67	3.19	0.000429466
Gura_0435	Thioredoxin family protein	—	1.67	3.18	2.26979E−05
Gura_1228	Putative peroxiredoxin	—	1.65	3.15	0.00012007
Gura_0408	Cytochrome *c* oxidase, subunit I	—	1.05	2.07	0.000391652
Gura_0869	Rubredoxin	—	1.04	2.06	0.001364612
Gura_2163	Cytochrome d ubiquinol oxidase, subunit I	*cydA*	0.86	1.82	0.000926513
Gura_4153	Rubrerythrin	—	0.74	1.67	0.010733318

transcript levels of seven different genes encoding proteins involved in electron transport, nitrogen fixation, phosphate homeostasis, heavy metal resistance and oxidative stress response were determined by quantitative RT-PCR analyses. These genes included the putative periplasmic *c*-type cytochrome, *ppcS*, the NiFe hydrogenase subunit, *hyaL*, the heavy metal efflux pump, *cusA*, superoxide dismutase, *sodA*, the α-subunit of the nitrogenase protein, *nifD*, the phosphate uptake regulatory protein, *phoU*, and the phosphate transport protein, *pstB*. The number of transcripts for these key genes was normalized against transcripts from *proC*, which earlier studies have shown to be constitutively expressed under a wide diversity of conditions in *Geobacter* species (Holmes *et al.*, 2005, 2006, 2008b; O'Neil *et al.*, 2008).

In all the instances tested, the genes that microarray comparisons showed to be more highly expressed in sediment-grown cells also had higher transcript levels in sediment-grown cells than in fumarate-grown cells according to quantitative RT-PCR (Figure 1). For example, the number of *ppcS*, *hyaL*, *cusA*, *sodA*, *nifD*, *pstB* and *phoU* mRNA transcripts normalized against the number of *proC* mRNA transcripts was 42.1, 13.4, 6.32, 15.4, 1967.2, 100.9 and 59.5 times greater in sediment-grown cells than in fumarate-grown cells.

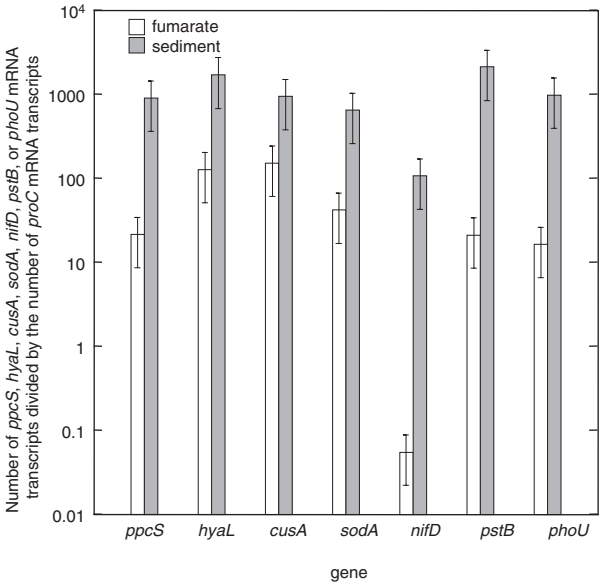

Figure 1 Number of *ppcS*, *hyaL*, *cusA*, *sodA*, *nifD*, *pstB* and *phoU* mRNA transcripts normalized against the number of *proC* transcripts from *G. uraniireducens* cells grown in sediments or a defined medium. Bars represent the mean of five replicates from three separate incubations for each treatment.

Comparison with gene expression in the subsurface

Earlier studies have demonstrated that the addition of acetate to the uranium-contaminated groundwater at the Rifle, Colorado site greatly stimulates the growth of *Geobacter* species, which can account for over 90% of the microorganisms recovered from the groundwater during the height of *in situ* uranium bioremediation (Anderson *et al.*, 2003; Vrionis *et al.*, 2005; Holmes *et al.*, 2007). The expression of several genes by this natural community of *Geobacter* species was examined to determine whether the gene expression patterns in sediment-grown cells of *G. uraniireducens* correlated with those present in natural communities during *in situ* uranium bioremediation.

A number of genes that were highly expressed during growth of *G. uraniireducens* in laboratory sediment incubations had similar transcript abundances relative to *proC* in the natural community of *Geobacter* during the most active period of acetate-stimulated *Geobacter* metabolism (Figures 1 and 2, days 8–20). For example, transcripts for the putative periplasmic *c*-type cytochrome, *ppcS*, normalized against the housekeeping gene, *proC*, were 43 times more abundant in sediment-grown than in fumarate-grown *G. uraniireducens* cells (Figure 1). Quantitative RT-PCR analysis of normalized *ppcS* homologs present in the groundwater during the *in situ* uranium bioremediation field experiment indicated that *ppcS* mRNA transcripts were most abundant when *Geobacter* species were expected to be most actively growing in the subsurface (days 6–16) (Figure 2) (Holmes *et al.*, 2007). Further examination of *ppcS* expression patterns in the groundwater

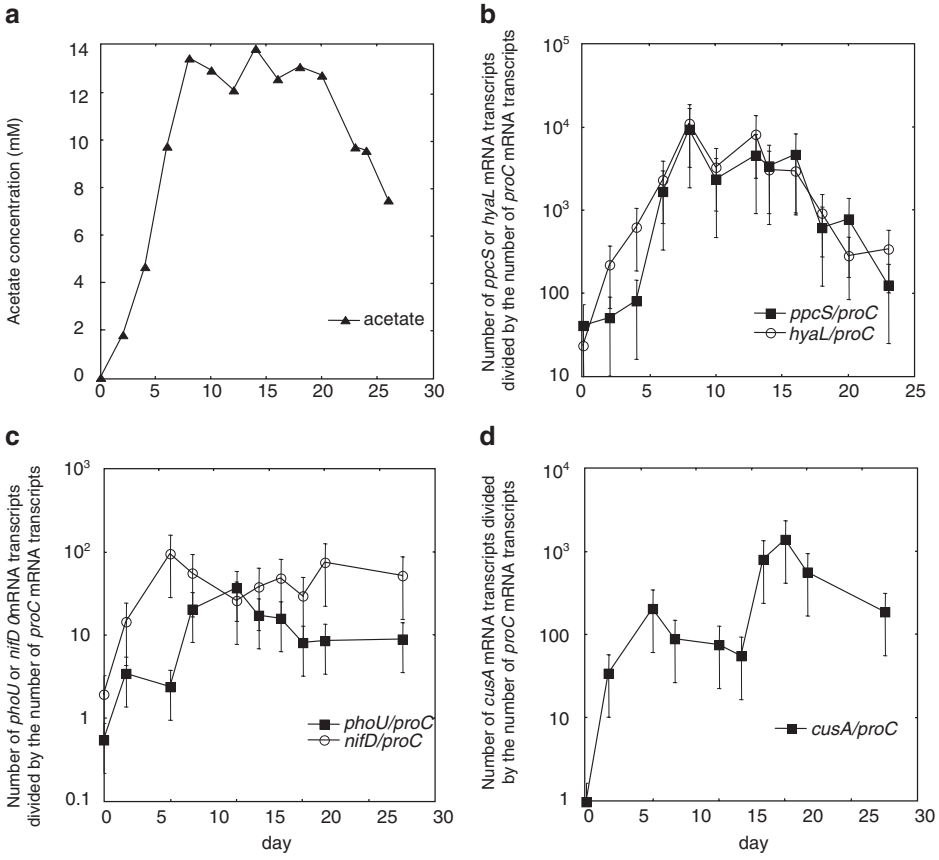

Figure 2 (**a**) Acetate concentrations detected in the groundwater during the U(VI) bioremediation field experiment. (**b**) Number of Geobacteraceae *ppcS* and *hyaL* mRNA transcripts normalized against the number of *proC* mRNA transcripts detected in the groundwater. (**c**) Number of Geobacteraceae *nifD* and *phoU* mRNA transcripts normalized against the number of *proC* mRNA transcripts detected in the groundwater. (**d**) Number of Geobacteraceae *cusA* mRNA transcripts normalized against the number of *proC* mRNA transcripts detected in the groundwater. Each point is an average of triplicate determinations.

showed that the relative expression of *ppcS* compared with *proC* increased as acetate concentrations in the groundwater went up and remained high until groundwater acetate concentrations began to decline (Figure 2b). The gene encoding the Ni-Fe hydrogenase protein, HyaL, showed a similar *in situ* expression pattern (Figure 2b) and, like *ppcS*, had relative transcript abundances similar to those observed in *G. uraniireducens* growing in laboratory sediment incubations when groundwater acetate concentrations were high (days 6–16; Figure 2b).

The gene encoding NifD, which was highly expressed in sediment-grown *G. uraniireducens*, also appeared to be highly expressed during the growth of *Geobacter* species *in situ* (Figures 1 and 2c). These results suggest that it is necessary for *Geobacter* species to fix atmospheric nitrogen during *in situ* uranium bioremediation at this site. Similar results were also observed for *in situ Geobacter* growing in petroleum-contaminated subsurface sediments (Holmes *et al*., 2004).

Relative transcript abundance of the gene encoding the heavy metal efflux pump, *cusA*, in the subsurface community of *Geobacter* species was initially low and more similar to that observed when *G. uraniireducens* was grown with fumarate provided as an electron acceptor (Figures 1 and 2d). However, as the field study continued, the number of *cusA* transcripts increased to levels comparable to those that were observed in *G. uraniireducens* sediment incubations (Figures 1 and 2d). Fe(III) oxides adsorb trace metals, which can be released when the Fe(III) oxides are reduced (Lovley, 1995). Thus, a physiological response to increased release of trace metals might account for the increase in *cusA* transcripts midway through the field experiment.

Additional studies have also indicated that the natural community of *Geobacter* species expressed high levels of transcripts from two genes involved in resistance to oxidative stress, *sodA* and cytochrome d ubiquinol oxidase (*cydA*), during the bioremediation field experiment (Mouser *et al*., 2007). These results are consistent with *sodA* expression patterns observed in sediment-grown *G. uraniireducens* (Figure 1).

Unlike the other genes examined, the number of transcripts for the gene encoding the phosphate uptake regulatory protein, *phoU*, relative to *proC*

transcripts in the subsurface *Geobacter* community was generally lower than that observed when *G. uraniireducens* was grown in sediments (Figure 2c). This suggests that phosphate may not have been a limiting nutrient during the *in situ* uranium bioremediation study. Phosphate dynamics in sedimentary environments are typically complex, and treatments such as heat sterilization might have had an impact on phosphate availability in the laboratory sediment incubations, resulting in this difference in gene expression patterns.

Implications

The results demonstrate that it is possible to assess the gene expression patterns of a microorganism growing in subsurface sediments on a genome scale. The results also suggest that the physiological status of *G. uraniireducens* growing in sediments is significantly different from its status during growth in a defined culture medium with a soluble electron acceptor, a more convenient but less environmentally relevant condition.

Although the microarray analysis was conducted on a pure culture, it was able to provide insights into the physiology of the *Geobacter* species that predominate in the subsurface during *in situ* uranium bioremediation because (1) the abundance of *Geobacter* species during *in situ* uranium bioremediation and their low species diversity (Holmes *et al.*, 2007) result in a subsurface microbial community that is only slightly more diverse than a culture; (2) *G. uraniireducens* was isolated from the site under study with a culture medium that closely mimicked the environment and is very closely related to the *Geobacter* species that predominate during *in situ* uranium bioremediation (Shelobolina *et al.*, 2008); (3) the sediments and associated groundwater used in the laboratory incubations were collected from the study site; and (4) the growth of *G. uraniireducens* in the sediments was stimulated in the same manner as it is during *in situ* uranium bioremediation that is, merely with the addition of acetate.

These considerations and the finding that, in general, the *in situ* expression of genes corresponded with expectations from the pure culture studies further suggest that analysis of the physiological status of subsurface isolates, such as *G. uraniireducens*, growing under different conditions in subsurface sediments may aid in evaluating various strategies for enhancing *in situ* uranium bioremediation. Such studies can clearly help further identify genes whose expression can be used to diagnose the physiological status of *Geobacter* species during attempts to manipulate *in situ* bioremediation in the field. In this way, it may be possible to adjust amendments to the subsurface in a rational manner to optimize the bioremediation process.

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