The ISME Journal (2009) 3, 454–465

www.nature.com/ismej

ORIGINAL ARTICLE

Quantifying expression of *Geobacter* spp. oxidative stress genes in pure culture and during *in situ* uranium bioremediation

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As part of an effort to diagnose the physiological status of *Geobacter* species during *in situ* bioremediation of uranium-contaminated groundwater, transcript levels for two genes potentially associated with oxidative stress, *cydA* and *sodA*, were quantified throughout a bioremediation field study in Rifle, CO, USA. Despite the accumulation of Fe(II) in the groundwater, which is inconsistent with the presence of dissolved oxygen, both genes were highly expressed during the bioremediation process. Therefore, the response to oxidative stress was further evaluated with *Geobacter uraniireducens*, an isolate from the Rifle site. When *G. uraniireducens* cultured with fumarate as the electron acceptor was exposed to 5% oxygen for 8 h, there was a significant increase in *cydA* and *sodA* transcripts as well as other genes associated with oxygen respiration or oxidative stress. Oxygen-exposed cells had lower transcript abundance for genes associated with anaerobic respiration, metabolism and motility. Short-term oxygen exposure had little impact on *cydA* transcript levels, as more than 1 h was required for increases to levels comparable to the subsurface. Abundance of *cydA* and *sodA* transcripts for the isolate *G. sulfurreducens* were always higher in cells cultured with Fe(III) compared with fumarate as an electron acceptor, even when fumarate-grown cells were exposed to oxygen, and Fe(III)-grown cells were grown anaerobically. These results suggest that the apparently high *Geobacter cydA* and *sodA* expression during bioremediation cannot necessarily be attributed to oxidative stress and demonstrate that diagnosis of the metabolic status of subsurface microorganisms through transcript analysis should be coupled with appropriate geochemical analyses.

The ISME Journal (2009) **3,** 454–465; doi:10.1038/ismej.2008.126; published online 8 January 2009
Subject Category: integrated genomics and post-genomics approaches in microbial ecology
Keywords: oxygen; *Geobacter*; *cydA*; *sodA*; bioremediation; groundwater

Introduction

In order to improve bioremediation strategies for contaminated subsurface environments, it is necessary to understand the physiological requirements of dominant bacteria at these sites, to monitor their metabolic condition *in situ* and to relate this metabolic state to factors controlling the rate and extent of bioremediation processes (Lovley, 2003). The activity of soil microorganisms can be assessed using a number of different techniques (Lovley, 2003; Saleh-Lakha *et al.*, 2005). Previous research in remediation applications has characterized physiological response by relating the number of key mRNA gene transcripts to metabolic rates (Chin *et al.*, 2004; Holmes *et al.*, 2005), iron availability (O'Neil *et al.*, 2008), naphthalene concentrations (Fleming *et al.*, 1993), dechlorination (Johnson *et al.*, 2005; Lee *et al.*, 2006; Rahm and Richardson, 2008) and limitations on fixed nitrogen (Holmes *et al.*, 2004). Results from these studies have suggested that transcript levels for genes related to central metabolism or nutrient acquisition may be useful for monitoring the *in situ* metabolic state of key microorganisms during bioremediation. However, there are many other environmental conditions that are likely to influence the metabolic status of bacteria during bioremediation (Anderson and Lovley, 1997; Röling and Van Verseveld, 2002;

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Received 2 September 2008; revised 24 November 2008; accepted 24 November 2008; published online 8 January 2009

Saleh-Lakha *et al.*, 2005; Edwards *et al.*, 2007). Most notably, the microbial response to a variety of environmental stresses must be understood and monitored (Hazen and Stahl, 2006).

One environmental stress likely to be encountered by bacteria in anaerobic zones of near-surface contaminated aquifers is exposure to oxygen. This may result from soil moisture infiltration, seasonal shifting of groundwater levels or perturbations during well sampling activities. Anaerobes are thought to be sensitive to intercellular oxygen because it inhibits key enzymes and reacts with biomolecules to produce more reactive radicals, such as superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxide ($HO^{\bullet}$), that can damage or destroy nucleic acids, proteins and enzymes (Keyer and Imlay, 1996; Imlay, 2003). In addition, the introduction of oxygen to iron-reduced soils and groundwater has the potential to produce $HO^{\bullet}$ in Fenton-type reactions that may directly damage outer cellular membranes (Imlay, 2003). Microbial response to oxygen stress has been studied in a diversity of pure cultures. For example, *Dehalococcoides* sp. were found to be strict anaerobes (He *et al.*, 2005), whereas *Desulfovibrio* spp., *Shewanella* spp. and *Geobacter sulfurreducens* are able to tolerate low oxygen concentrations or even grow under aerobic conditions (Moser and Nealson, 1996; Krekeler *et al.*, 1998; Cypionka, 2000; Lin *et al.*, 2004; Beliaev *et al.*, 2005; Lobo *et al.*, 2007). In bioremediation schemes that employ oxidative stress-sensitive species, such as bacteria that predominate during stimulated trichloroethylene (Ellis *et al.*, 2000) or uranium bioremediation (Anderson *et al.*, 2003), efficiency may be drastically reduced if the anaerobic bacteria are exposed to oxygen, resulting in increased biostimulation costs and remediation times. To date, limited research has been conducted on the specific response of *Geobacter* spp. to oxygen, and studies relating mRNA gene expression response to oxidative stress for other bacteria in subsurface environments remain few and difficult to decipher (Parro *et al.*, 2007). This may be due to the fact that anaerobic bacteria have several different mechanisms for dealing with varying levels of intercellular oxygen (Mukhopadhyay *et al.*, 2007).

The purpose of the study summarized here was to evaluate whether oxidative stress is an important factor influencing *in situ* bioremediation of uranium. Previous studies have demonstrated that *Geobacter* species are the primary microorganisms that respond to additions of acetate designed to stimulate *in situ* bioremediation of uranium-contaminated groundwater, and they are involved in the reduction of U(VI) to U(IV) (Anderson *et al.*, 2003; Istok *et al.*, 2004; North *et al.*, 2004; Holmes *et al.*, 2005). *Geobacter uraniireducens* was isolated from uranium-contaminated subsurface sediments in Rifle, CO, USA, undergoing *in situ* uranium bioremediation (Shelobolina *et al.*, 2008). The 16S rRNA gene sequence for *G. uraniireducens* is an exact match to sequences that predominated in groundwater samples taken during field-scale bioremediation studies. Thus, this organism provides the opportunity to study the physiology of microorganisms likely to be involved in uranium bioremediation in the subsurface. The genome sequence of *G. uraniireducens* has recently been determined (www.jgi.doe.gov), and a search of this and other more intensively studied *Geobacter* genomes indicates that they possess genes with high similarities to genes found in other organisms that encode proteins that are known to be involved in an oxidative stress response (Methe *et al.*, 2003). We report here that natural communities of *Geobacter* species do express genes associated with oxidative stress, but as evidenced from pure culture studies, this is not a definitive indication that oxidative stress has a significant impact on *in situ* uranium bioremediation.

Materials and methods

Uranium bioremediation field study

In 2005, a small-scale *in situ* bioremediation experiment was conducted on the grounds of a former uranium ore-processing facility in Rifle, CO, USA, during the months of August and September. This site, designated Old Rifle, is part of the Uranium Mill Tailings Remedial Action (UMTRA) program of the US Department of Energy. The test plot was adjacent to a previously studied experimental plot (Anderson *et al.*, 2003) with similar design but approximately one quarter the size. During the field experiment, an acetate–bromide solution (100:10 mM) mixed with site groundwater was injected into the subsurface to provide ~10 mM acetate as an electron donor to the groundwater over the course of 28 days as described earlier (Holmes *et al.*, 2007).

Prior to sampling, the first 20 l of groundwater was purged through dedicated monitoring well tubing connected to a peristaltic pump. After purge, samples were collected for geochemical analysis, then the pump tubing was connected to a pressurized filtration apparatus containing a series of two filters: a course (1.2 μm) filter followed by a fine (0.2 μm) 263-mm diameter Supor membrane disc filter (Pall Corporation, East Hills, NY, USA). Approximately 10 l of groundwater was concentrated on filters at a rate of 1 l min^{-1}, after which time filters were immediately removed, separately bagged into sterile whirl pak bags (Nasco, Fort Atkinson, WI, USA), flash frozen using an ethanol-dry ice bath and stored at −80 °C until extraction. Collection, preservation, and analysis of acetate and Fe(II) was conducted using ion chromatography and an HCl extractable ferrozine assay as described earlier (Lovley and Phillips, 1986; Anderson *et al.*, 2003).

Chemostat culture
G. uraniireducens RF4 (ATCC BAA-1134) was obtained from our laboratory culture collection and grown in continuous culture under anaerobic conditions with acetate (5 mM) as the electron donor and fumarate (30 mM) as the electron acceptor at a dilution rate of 0.1 ml min^{-1} (0.03 h^{-1}). *G. sulfurreducens* PCA (DSM 12127) was obtained from our laboratory culture collection and grown continuously under anaerobic conditions with acetate (5 mM) as the electron donor and either fumarate (30 mM) or Fe(III) citrate (50 mM) as the electron acceptor as described earlier (Esteve-Nunez *et al.*, 2005). For both cultures, cells were grown in a bicarbonate-buffered medium (Lovley and Phillips, 1986) that was supplemented with a trace mineral and vitamin solution (Balch and Wolfe, 1979). Chemostat cultures of *G. uraniireducens* and *G. sulfurreducens* (200 ml) were kept at a constant temperature of 30 °C, stirred at 150 r.p.m., and the headspace was continuously gassed with a mixture of N_2/CO_2 (80:20) at a rate of 50 ml min^{-1}. Steady-state conditions were maintained for four vessel volumes, and cells were either harvested, or stressed with oxygen by switching to a gas mixture containing 5% or 10% oxygen ($N_2/CO_2/O_2$, 75:20:5 or 70:20:10) for a period of 8 h.

Batch culture
Time course experiments with *G. uraniireducens* were conducted in batch culture with 15 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor. Salts, mineral and vitamin solutions were added in the same concentrations described in continuous culture and cells were grown at a constant temperature of 30 °C. During oxidative stress, cultures were shaken horizontally at 150 r.p.m. to maximize the mixing of oxygen into media. Judging by the rapid and uniform change in color for cultures exposed to oxygen as compared with controls, we believe the mixing rate was sufficient for introducing oxygen into media from a headspace gas. Time course fumarate cultures were stressed with 10% headspace oxygen (vol/vol%) during mid-log-phase growth. Cells were harvested at six different time points: just before stress (T_o), after 10 min, 30 min, 60 min, 6 h and 24 h of exposure. Headspace oxygen concentration was measured with a Hewlett-Packard series HP6890 gas chromatograph equipped with a thermal conductivity detector (Agilent Technologies Inc., Albany, NY, USA) and a Carboxen 1010 PLOT capillary column (Supelco, Bellefonte, PA, USA) with helium as a carrier gas as described earlier (Lin *et al.*, 2004).

RNA extraction
Nucleic acids were extracted from groundwater filters using a modified phenol–chloroform extraction as described earlier (Holmes *et al.*, 2004, 2005). In brief, filters were crushed using liquid nitrogen, separated into eight 2-ml screw top tubes using a sterile RNAse-free spatula, and re-suspended in 800 μl of TPE buffer (100 mM Tris-HCl, 100 mM KH_2PO_4 and 10 mM EDTA; pH 8.0). Cell cultures were harvested by transferring to pre-chilled 50-ml conical tubes and centrifuging at 4000 r.p.m. for 15 min at 4 °C. The supernatant was discarded and the pellet was flash-frozen in a liquid N_2 bath and stored at −80 °C until extraction. Pellets were resuspended in 4 ml of TPE buffer, aliquoted into six separate 2-ml tubes, extracted as described above. The total RNA for groundwater samples and cell cultures were separated from other nucleic acids using the RNeasy RNA cleanup kit (Qiagen Corp., Valencia, CA, USA) and subsequently treated with DNA-free DNase (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA quality was visualized on a 1% agarose gel electrophoresis, and the concentration and purity were quantified using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). All RNA samples were of high purity, with 260/280 ratios of 1.8–2.1. The absence of DNA contamination was verified by performing gene-specific PCR on all samples before RNA was subjected to reverse transcriptase with visualization of PCR products on a 2% agarose gel stained with ethidium bromide.

Microarray analysis
Total RNA (0.5 μg) was amplified using the Bacterial MessageAmp II-Bacteria Prokaryotic RNA amplification kit (Ambion) according to the manufacturer's instructions. Microarray analysis was performed at the University of Massachusetts, Amherst Environmental Technology Center using Customarray 12K microarray chips (Combimatrix, Mukilteo, WA, USA). Oligonucleotide probes were designed from the draft genome of *G. uraniireducens* (accession number NZ AAON00000000). A randomized block configuration was used as the statistical design, consisting of two experimental conditions: a control with anoxic (0% oxygen) growth versus a treatment with 5% oxygen stress. Each condition had three biological replicates and two technical replicates. The oligonucleotide array design, along with the raw and statistically treated results, is available on the NCBI Gene Expression Omnibus database (GSE12455).

The amplified RNA (10 μg) was used to generate fluorescently labeled cDNA and hybridized to array slides as described earlier (Postier *et al.*, 2008). Arrays were immediately scanned using a Genepix 4000B scanner (Molecular Devices Inc., Sunnyvale, CA, USA), analyzed using GenePix and Acuity 4.0 software, and exported for statistical analysis. Background effects during scanning were minimized by utilizing square outlines during spot finding. Data were preprocessed by subtracting the array background signal, calculated as the mean ± 2s.d. for the

lowest 30% of negative controls within the array, from total spot intensity. Oligonucleotide probes with signals less than zero after adjusting to background intensity were omitted from further analysis. For the remaining data, the mean $\log_2$ ratio [$M = \log_2$ (treatment/control)] was calculated, and differentially expressed genes were identified for biological and technical replicates using the linear model for microarray analysis (LIMMA) (Smyth and Speed, 2003; Smyth *et al.*, 2005). Statistical significance was corrected for multiple comparisons. When multiple oligonucleotide probes were available for a given gene (this occurred for 99.4% of the genes), at least 50% of the probes needed to be statistically significant ($P < 0.01$) to be considered differentially expressed. Microarray results for three genes (*cydA*, *sodA* and *proC*) that represented positive and no significant difference in array expression ratios were confirmed using reverse transcription (RT)-PCR. *t*-test analysis between control and oxygen-exposed cells were conducted and tested for significant differences at an $\alpha = 0.01$ level.

Primer design and quantification of mRNA gene transcripts

Gene-specific primers were designed for quantitative reverse transcription PCR (qRT)-PCR analysis from the *G. uraniireducens* and *G. sulfurreducens* genomes available on the DOE Joint Genome Institute website (www.jgi.doe.gov) using the Primer Express software (ABI). Optimal primer pairs were checked for dimers and hairpins using the NetPrimer software (PREMIER Biosoft, Palo Alto, CA, USA) and optimized using PCR gradient analysis. PCR products were cloned using the TOPO p2.1 kit (Invitrogen Corp., Carlsbad, CA, USA) and sequenced to verify gene specificity at the University of Massachusetts, Amherst Sequencing Facility. Table 1 shows the primers used to amplify ~100–150-bp fragments of putative oxidative stress genes. In addition, two housekeeping genes that were not significantly up- or downregulated between oxygen exposed and control cells in the microarray experimental results, *proC* and *rpoB*, were used as controls in qRT-PCR analysis using the primer pairs shown in Table 1. Before qRT-PCR primers targeting *sodA*, *cydA* and *proC* transcripts in the environment could be designed, it was necessary to construct cDNA with products amplified from the environment with degenerate PCR primer sets that targeted 400- to 800-bp regions of each gene of interest. These degenerate primer sets were designed from gene alignments of available genomes, including *G. metallireducens*, *G. sulfurreducens* (Methe *et al.*, 2003), strain *FRC-32*, *Pelobacter carbinolicus* and *P. propionicus.* Preliminary sequence data was obtained from the DOE Joint Genome Institute website (www.jgi.doe.gov). DNA sequences from at least 50 screened clones were used to design more specific qPCR primers for environmental quantification as described earlier (Holmes *et al.*, 2005). All degenerate and quantitative RT-PCR primer pairs used for pure culture and environmental studies are outlined in Table 1. Sample cDNA was generated from mRNA transcripts using the DuraScript Enhanced Avian RT single-strand synthesis kit (Sigma Aldrich) according to the manufacturer's instructions. Positive RT reactions were verified using PCR and visualized on a 2% agarose gel stained with ethidium bromide.

The number of mRNA transcripts was quantified using the Applied Biosystems 7500 Real-Time PCR system (PE Biosystems (Applied Biosystems), Foster City, CA, USA). Reactions (25 μl total volume) consisted of 12.5 μl 2 × POWR SYBR green master mix (Applied Biosystems), 5 μl of 1:10 diluted

Table 1 Forward and reverse primer pairs used for amplifying fragments from *G. uraniireducens* (Gura), *G. sulfurreducens* (Gsu), Geobacteraceae from the Rifle groundwater (Geo), and for quantifying Geobacteraceae with qRT-PCR (Geo qPCR)

Gene	*Forward primer 5′–3′*	*Reverse primer 5′–3′*
Gura cydA 386F/489R	TGGTAGCCATCGCAACAAATC	CATCTCTGCCCTGCCGTTT
Gura macA 656F/758R	GCTCTGCCTGTCACAACGGT	CCGAGGTCTTCCGTGGGT
Gura sodA 141F/248R	GAAAGAGGATAAGACCGCCACA	CCCCCGAGGTTTTCAAAATAGT
Gura rbr 346F/465R	ATCGCCGCTGTTTTCACG	CCAGACCACAGGCGCTTC
Gura proC 263F/367R	TCCTGCTCGTGAACCTCTCC	TGTCTGCTTCTCCGATCACCT
Gura rpoB 3201F/3308R	CGACGATCTTCCTCCTGGTG	ACCCCCTTGTTCCCGTGT
Gsu cydA 610F/710R	CTGCTCAGGAAGAACCGTATCG	AAGTCACCGGAGAGGAATACCA
Gsu sodA 311F/425R	ATGAGGCGTGGGAGAAGGAT	TGCTCGTTGACCCAGAAGTTAA
Gsu Rub 885F/993R	GAGCGTCGCCATCAAGGA	CCGCAGGTGGGTCAAGAA
Gsu Rbr 377F/494R	CGGAAAAGCAGCACGAGAA	GTGTGGAGGTAGCCGCAGTT
Gsu proC 412F/494R	Holmes *et al.* (2008)	
Geo sodA 200F/550R	TGGGARTTTAACGGCATGCG	TCGGCRGCCTTCCAGTCGAT
Geo cydA 80F/590R	TCATYTTCGTGCCSCTCACC	ATCACRAAGAARGCCGCCAC
Geo proC 75F/471R	ATWGGIGGIGGIAATATGGC	TCCCCACCAGGTCGAACA
Geo qPCR sodA 139F/270R	TGCTCGTTGATCCAGAAGTTGA	CCAGGATTTCGGCAGCTTC
Geo qPCR cydA 277F/424R	GGGTGCATCTTCTTTCCATCTG	ACCACCGCCATGAAATCG
Geo qPCR proC 156F/287R	TTGCGAAATGAGCGACACC	ATCGCGGCACTTTTCACG

Abbreviation: qRT-PCR, quantitative reverse transcription-PCR.

template cDNA and 200 pmol of the appropriate primer pair. The thermal cycling parameters consisted of an activation step of 50 °C for 2 min, a denaturation step of 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60–66 °C for 1 min. A standard curve and two types of negative controls were run concurrent with cDNA samples. Standard curves covered a range of approximately eight orders of magnitude (between 10^1 and 10^9), and were constructed as described earlier (Holmes *et al.*, 2004). The first negative controls contained RNA template from each sample that had not been subjected to RT, and the second controls contained reaction solution without template. Real-time PCR product size and amplification were verified on a 2% agarose gel stained with ethidium bromide.

Results and discussion

Expression of putative oxygen stress genes during in situ *uranium bioremediation*

The genes *sodA* and *cydA* are highly conserved in the genome sequences of *Geobacter* species and, based on homology to characterized proteins in *Desulfovibrio*, *Moorella* and *Shewanella* species are predicted to be involved in oxidative stress responses (Methe *et al.*, 2003; Heidelberg *et al.*, 2004; Das *et al.*, 2005; Machado *et al.*, 2006). Therefore, transcript levels for these genes were quantified using primers designed to amplify the *sodA* and *cydA* sequences of the *Geobacter* species that predominated during *in situ* uranium bioremediation at the field study site in Rifle. During the first 2 days of acetate injection, the number of *sodA*, *cydA* and *proC* mRNA transcripts were not detected or approaching the detection limit of the RT-PCR system (approximately 100 molecules for *sodA* and *proC* transcripts, and 500 molecules for *cydA* transcripts) (Figure 1). As acetate concentrations increased in the groundwater following the initiation of acetate injection, transcript levels for *sodA* and *cydA*, normalized to transcript levels of the housekeeping gene, *proC*, also increased (Figures 1a and b).

Transcript levels for *sodA* and *cydA* remained 1–3 orders of magnitude higher than *proC* transcripts during the stimulation experiment, then began to decrease as amendments were discontinued and acetate concentrations declined. Geobacteraceae *sodA* and *cydA* transcripts were recovered despite the fact that considerable Fe(II) was detected throughout the experiment, indicating the presence of an anaerobic environment (Figure 1a). Although both the *sodA* and *cydA* gene expression patterns followed the same general trend with respect to acetate fluctuations, levels of transcripts were not significantly correlated with each other ($R^2 = 0.45$). The *in situ* trends are consistent with batch growth trends of *Geobacter* species grown in uranium-contaminated sediments with acetate amendment, where the number of organisms and their metabolic processes increase to some plateau level, representing a quasi-steady state, and begin to drop off as amendment resources are depleted (Finneran *et al.*, 2002).

Gene expression patterns in G. uraniireducens *in response to oxidative stress*

To further interpret the results obtained from field studies, the transcriptional response of *G. uraniireducens* to oxygen stress was investigated using whole-genome DNA microarray comparisons. Transcript abundance of steady-state chemostat cultures grown with acetate as the electron donor (5 mM) and fumarate as the electron acceptor (30 mM) was compared with transcript abundance in chemostat cultures grown under the same conditions, but exposed to 5% oxygen for 8 h. Exposure to oxygen resulted in differential expression of 469 genes, 270 upregulated and 199 downregulated (Supplementary Table).

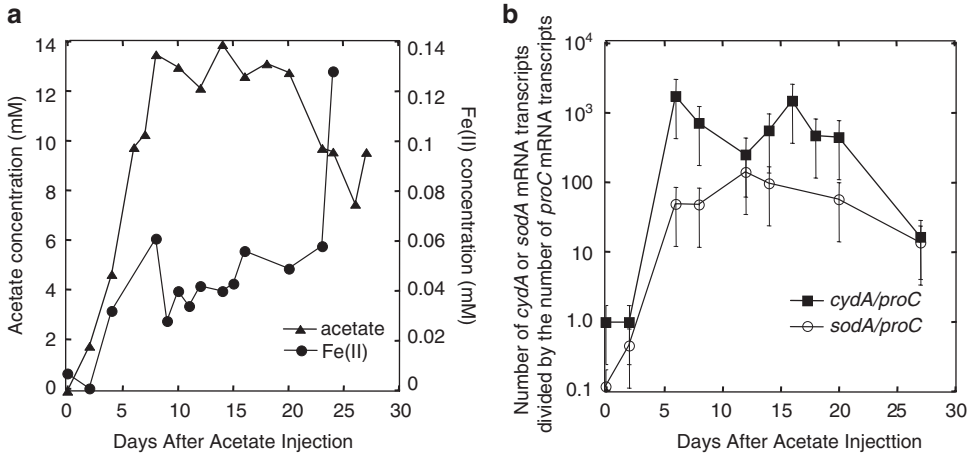

Figure 1 (**a**) Acetate and Fe(II) concentrations in groundwater during biostimulation of a uranium-contaminated aquifer and (**b**) the number of *cydA* and *sodA* mRNA gene transcripts relative to the number of *proC* transcripts expressed *in situ* by Geobacteraceae.

Genes encoding proteins involved in energy metabolism and electron transport had the largest increases in transcript abundance when cells were exposed to oxygen. These included cytochrome *bd*-type quinol oxidases *cydA* and *cydB*, and the cytochrome *c* peroxidase (*macA*) (Table 2). Other significantly upregulated genes included Mn–Fe superoxide dismutase (*sodA*), desulfoferrodoxin (*dfx*), rubredoxin (*rub*) and rubrerythrin (*rbr*) (Table 2). The proteins encoded by these genes are thought to be involved in radical detoxification using superoxide dismutase and superoxide reductase systems (Brumlik and Voordouw, 1989; Voordouw and Voordouw, 1998; Jenney *et al.*, 1999; Silva *et al.*, 1999; Kurtz, 2006).

To further evaluate the expression patterns from the three genes that were monitored in the field studies, and to confirm microarray studies, qRT-PCR was used to quantify *cydA*, *sodA* and *proC* transcripts. In accordance with the microarray results, *cydA* transcripts were 23-fold higher in cells exposed to oxygen and *sodA* transcripts were

Table 2 Genes involved in energy production and metabolism that were differentially expressed ($P>0.01$) in a microarray analysis for *G. uraniireducens* exposed to 5% oxygen

Gene ID	*Annotation*	*Mean* $\log_2$	*Fold change*	*Gene name*
Gura2162	Cytochrome *d* ubiquinol oxidase, subunit II	3.39	10.50	*cydB*
Gura1316	Cytochrome *c*551 peroxidase, 2 heme-binding sites	3.18	9.03	*macA*
Gura2163	Cytochrome *d* ubiquinol oxidase, subunit I	3.01	8.04	*cydA*
Gura2514	Cytochrome *c* biogenesis protein, CcmF/CcyK/CcsA family	2.49	5.60	—
Gura0429	4Fe-4S ferredoxin, iron–sulfur-binding domain protein	2.27	4.82	—
Gura2379	Cytochrome *b*/*b*6 complex, iron–sulfur subunit	2.24	4.71	—
Gura2207	Desulfoferrodoxin ferrous iron-binding domain	2.00	4.01	*dfx*
Gura1837	Cytochrome *c* family protein, 7 heme-binding sites	1.89	3.69	—
Gura2513	Desulfoferrodoxin	1.88	3.67	—
Gura2004	Cytochrome *c* family protein, 6 heme-binding sites	1.68	3.21	*omcT*
Gura1364	Iron–sulfur–oxygen hybrid cluster protein (prismane)	1.62	3.06	*hcp*
Gura3909	Cytochrome *c*, 3 heme-binding sites	1.44	2.72	—
Gura1453	Manganese and iron superoxide dismutase	1.41	2.65	*sodA*
Gura1838	Cytochrome *c*, 5 heme-binding sites	1.30	2.47	—
Gura0864	Oxidoreductase, membrane subunit	1.22	2.33	—
Gura0869	Rubredoxin	1.14	2.21	*rub*
Gura2789	D-Isomer-specific 2-hydroxyacid dehydrogenase, NAD binding	1.12	2.18	*serA*
Gura1344	Nitroreductase 3 family protein	1.09	2.12	—
Gura4153	Rubrerythrin	1.05	2.08	*rbr*
Gura0326	NADH dehydrogenase I, G subunit	1.05	2.07	*nuoG*
Gura0863	Oxidoreductase, iron–sulfur cluster-binding subunit	1.04	2.06	—
Gura0363	Thioredoxin	0.93	1.90	—
Gura0331	Cytochrome *c*, 1 heme-binding site	0.93	1.90	—
Gura3749	Pyridine nucleotide-disulphide oxidoreductase dimerization region	0.71	1.63	—
Gura2277	Rubrerythrin	0.63	1.55	—
Gura3554	Electron transfer flavoprotein, Etf beta-subunit/FixA family	−0.63	−1.55	—
Gura3652	Cytochrome *c* family protein, 5 heme-binding sites	−0.67	−1.59	—
Gura3997	Cytochrome *c*, 6 heme-binding sites	−0.70	−1.63	*omcQ*
Gura0989	Cytochrome *c* family protein, 9 heme-binding sites	−0.71	−1.63	—
Gura4232	NADH dehydrogenase I, M subunit	−0.74	−1.67	*nuoM*
Gura3257	Fumarate reductase, cytochrome *b* subunit, putative	−0.74	−1.67	—
Gura4238	NADH dehydrogenase I, G subunit, putative	−0.75	−1.68	—
Gura0621	Cytochrome *c*, 3 heme-binding sites	−0.77	−1.71	—
Gura3639	Molybdopterin oxidoreductase	−0.80	−1.74	—
Gura4233	NADH dehydrogenase I, L subunit	−0.82	−1.76	*nuoL*
Gura4243	NADH dehydrogenase I, B subunit	−0.84	−1.79	—
Gura4231	NADH dehydrogenase I, N subunit	−0.84	−1.79	*nuoN*
Gura2822	Cytochrome *c*, 12 heme-binding sites	−0.84	−1.79	—
Gura4236	NADH dehydrogenase I, I subunit	−0.85	−1.80	*nuoI*
Gura4242	NADH dehydrogenase I, C subunit	−0.88	−1.84	*nuoC*
Gura3996	Cytochrome *c*, 6 heme-binding sites	−0.89	−1.85	—
Gura3555	4Fe–4S ferredoxin, iron–sulfur-binding domain protein	−0.89	−1.85	—
Gura4241	NADH dehydrogenase I, D subunit	−0.92	−1.89	—
Gura4237	NADH dehydrogenase I, H subunit	−0.96	−1.94	*nuoH*
Gura0500	Cytochrome *c* family protein, 16 heme binding sites	−0.96	−1.95	—
Gura4234	NADH dehydrogenase I, K subunit	−1.03	−2.04	*nuoK*
Gura4240	NADH dehydrogenase I, E subunit	−1.07	−2.09	*nuoE*
Gura0573	Cytochrome *c*, 29 heme-binding sites	−1.13	−2.18	—
Gura4239	NADH dehydrogenase I, F subunit	−1.27	−2.42	*nuoF*
Gura0497	Cytochrome *c*, 23 heme-binding sites	−1.60	−3.03	—
Gura0498	Cytochrome *c*, 24 heme-binding sites	−1.88	−3.68	—

2.7-fold higher. These differences were statistically significant (*t*-test, $P<0.01$). In contrast, transcripts for *proC* were not statistically different between treatments ($P=0.11$); the number of *proC* transcripts in oxygen-exposed cells was only 1.1-fold more abundant.

Genes that were most significantly downregulated were also involved in energy production and metabolism, and encoded nine NADH dehydrogenase (*nuoE-nuoM*) and 10 *c*-type cytochrome family proteins (Table 2). Similar changes in expression patterns have been observed in other bacteria upon changes in redox or energy condition. For example, fumarate reductase and NADH dehydrogenases were downregulated in *Shewanella oneidensis* when the redox potential of the terminal electron acceptor was increased (Beliaev *et al.*, 2005). In *Geobacter sulfurreducens*, downregulation of several different metabolism genes, including *c*-type cytochromes and a NADH dehydrogenase, was observed in nitrogen-fixing cells as opposed to cells grown in the presence of ammonium (Methe *et al.*, 2005). Furthermore, studies with *E. coli* indicated that NADH dehydrogenases were inactivated in the presence of H_2O_2 to limit reactivity with NADH-metal reductants and the production of intercellular radicals (Imlay and Linn, 1988). *G. uraniireducens* demonstrated significant downregulation of genes encoding proteins involved in the formation of pili and flagella as well as chemotaxis sensory regulators under conditions of oxidative stress (Table 3). Evaluation of transcript levels from the gene encoding the pilus assembly protein, *PilO* with quantitative RT-PCR indicated that transcript levels were more than three-fold lower in oxygen-exposed cells (*t*-test, $P<0.01$), consistent with the microarray results. Although cells grown anaerobically had flagella and numerous pili (Figures 2a and b), the number of pili was greatly diminished and flagella were not observed in cells exposed to oxygen (Figures 2c and d). Differential expression of motility and chemotaxis proteins in response to changes in redox conditions, and observations of aerotactic responses upon oxygen exposure have previously been described for facultative iron reducers (Beliaev *et al.*, 2005) and sulfate-reducing bacteria (Krekeler *et al.*, 1998; Cypionka, 2000; Fareleira *et al.*, 2003; Mukhopadhyay *et al.*, 2007). In the case of *G. uraniireducens*, downregulation of motility proteins and observations of reduced pili and flagella suggest that shedding of motility functions may be a stress response mechanism and mode of energy conservation during exposure to oxygen.

Time required for increase in transcript abundance following oxygen exposure

A possible explanation for the detection of relatively high proportions of *sodA* and *cydA* transcripts during *in situ* uranium bioremediation was that the cells being sampled were exposed to oxygen during the sampling procedure. It is possible that cells were exposed to trace amounts of oxygen diffusing into anaerobic groundwater during the short period of filtration ($\sim$10 min), or to atmospheric oxygen levels just after filtration when the filter was removed from the filtration apparatus, placed in sterile bags and flash frozen in an ethanol-dry ice bath. In total, we expect this amounted to no more than 2 min outside the anaerobic groundwater environment before freezing.

To determine the likely time frame of the response to oxygen exposure, gene transcript levels for key genes identified in the microarray experiments were quantified with qRT-PCR before oxygen exposure, immediately after oxidative stress (10 min, 30 min and 60 min), and after a longer duration of oxygen exposure (6 h and 24 h). To insure that a significant oxidative stress existed after 24 h (minimum 5% headspace oxygen concentration (vol/vol%)), cells were initially stressed with 10% oxygen during the mid-log growth phase. Immediately after oxygen injection, *G. uraniireducens* ceased growth and biomass concentrations decreased slightly over the 24-h period (Figure 3). The headspace oxygen concentration decreased with time for both viable and heat-sterilized cells as it reacted with media and biomolecules; however, the rate of decrease was

Table 3 Genes thought to be involved in chemotaxis and cell motility that were differentially expressed ($P<0.01$) in *G. uraniireducens* cultures exposed to 5% oxygen

Gene ID	*Annotation*	*Mean $\log_2$*	*Fold change*	*Gene name*
Gura1811	Fimbrial assembly family protein	−1.08	−2.12	*pilN*
Gura1812	Pilus assembly protein	−1.01	−2.01	*pilO*
Gura1813	Hypothetical protein	−0.99	−1.99	—
Gura4106	Flagellar basal-body rod protein	−0.91	−1.88	*flgG*
Gura4207	Flagellar hook-length control protein	−0.86	−1.81	*fliK*
Gura4199	Flagellar biosynthetic protein	−0.82	−1.77	*fliP*
Gura4201	Flagellar motor switch protein	−0.73	−1.66	*fliN*
Gura3136	Chemotaxis protein	−0.71	−1.63	*cheY*
Gura4109	Flagellar biosynthetic protein (ATPase)	−0.69	−1.61	*flhG*
Gura4209	Flagellar protein FliJ, putative	−0.66	−1.58	*fliJ*
Gura4093	Flagellin export facilitator protein	−0.61	−1.52	*fliS*
Gura2168	Chemotaxis protein	−0.60	−1.52	*cheW*

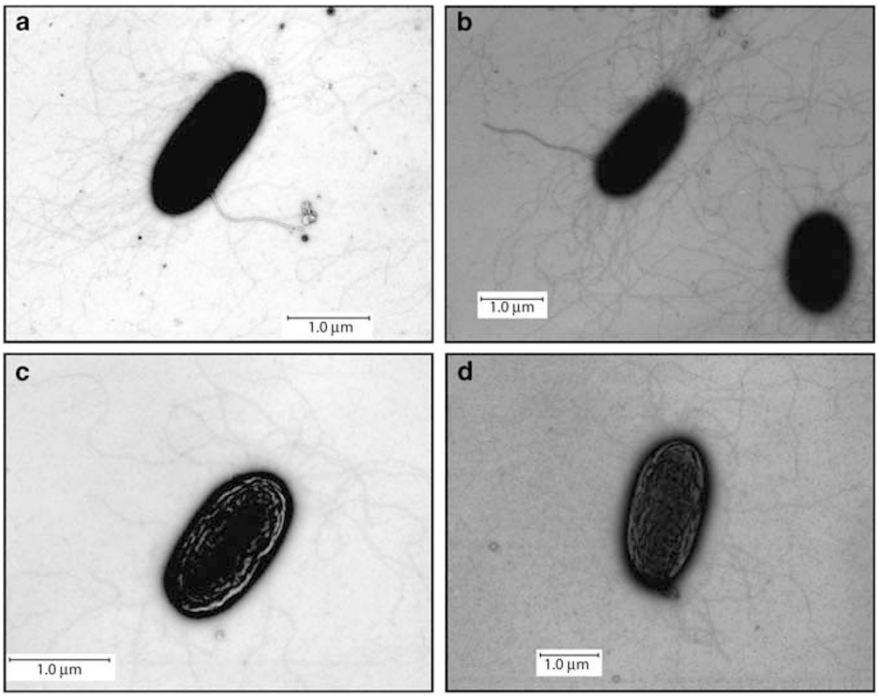

Figure 2 (**a**, **b**) Transmission electron microscope (TEM) images of *G. uraniireducens* grown anaerobically in fumarate as an electron acceptor, expressing pili in the majority of cells (>95%) and flagella in some cells (>10%) using a survey of 100 images on two different slides. (**c**, **d**) Cells grown anaerobically then stressed for 6 h under 10% oxygen results in a reduced number of pili and no observed flagella in a survey of 100 images on two different slides.

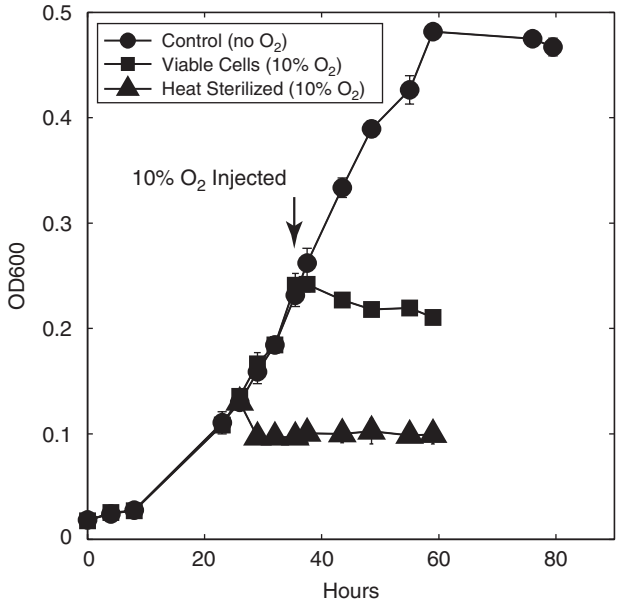

Figure 3 Optical density (absorbance at 600 nm) of *G. uraniireducens* cultured in batch with acetate as the electron donor and fumarate as the electron acceptor under anoxic conditions (circles), during a 24-h period of exposure to 10% oxygen (squares), and after cells were killed with heat during 24-h period exposure to 10% oxygen oxidative stress after heat sterilization (triangles).

more rapid in viable cells, suggesting limited oxygen uptake by *G. uraniireducens* (Figure 4a).

For genes with significant increases in expression upon exposure to oxygen, somewhere between 1 and 6 h was required for transcript levels to increase over twofold (Figures 4c and d). This response was fastest for the gene encoding the superoxide scavenger *SodA* (~1 h) and longer for genes encoding energy metabolism and ion transport proteins such as CydA and MacA, which remained elevated for 6–24 h after initial exposure (Figures 4c and d). In contrast, the levels of housekeeping genes *proC* and *rpoB* decreased slightly over this period as a result of cell death (Figure 4b). Significant differences ($P<0.05$) in the number of transcripts for *cydA*, *macA*, *dfx* and *rbr* in cells grown in batch and exposed to oxygen for 6 h was consistent with the microarray results from chemostat-grown cells exposed to oxygen for 8 h. Although not statistically significant due to large biological replicate variability, transcript levels for *sodA* increased almost twofold after 1 h of oxygen exposure but decreased thereafter (Figure 4d).

Combined results from microarray and time course oxygen studies suggest that monitoring for genes encoding proteins involved in oxidative stress response, including SodA, Dfx and Rbr, may be technically challenging in the environment because fold changes are relatively small and short-lived, as compared with genes encoding CydA or MacA energy metabolism proteins, which have fold changes 5- to 10-fold larger, at levels that remain elevated 24 h after initial oxygen exposure (Table 2; Figures 4c and d).

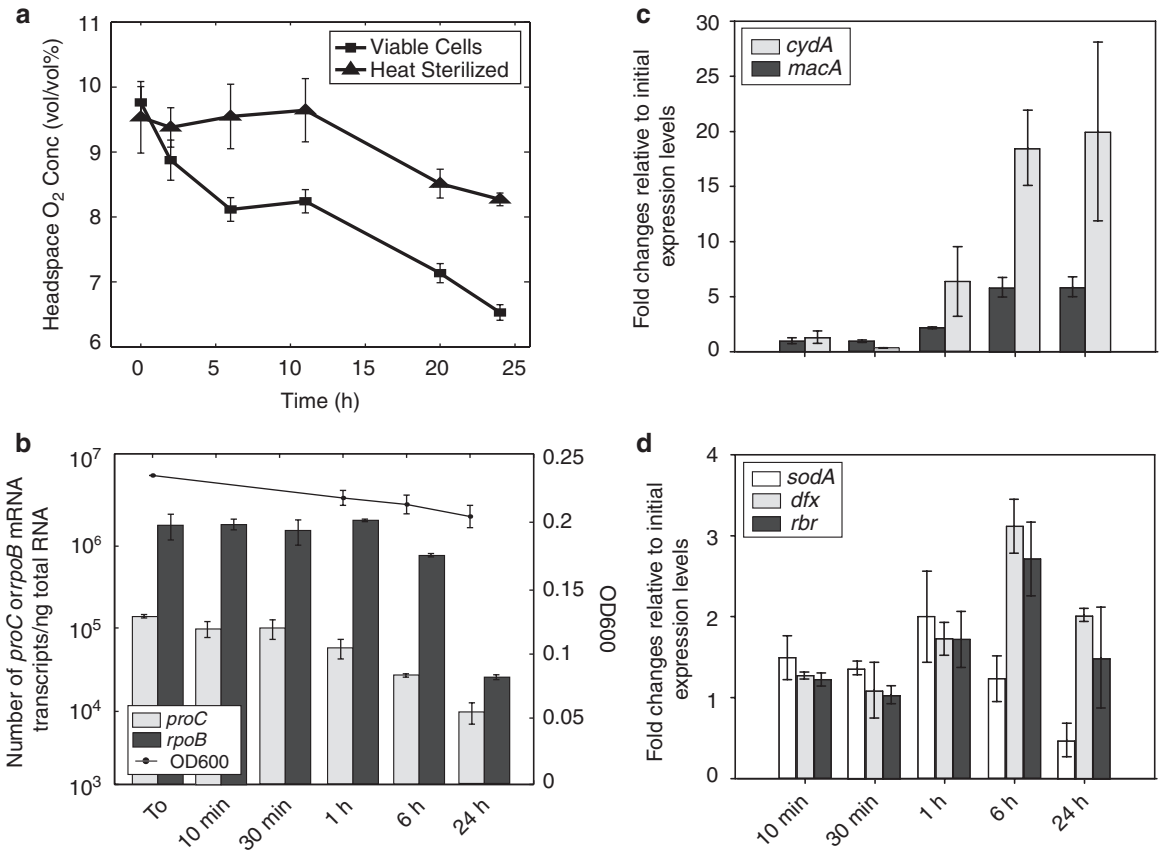

Figure 4 Headspace oxygen concentrations, biomass measurements and transcript abundance levels according to quantitative reverse transcription (qRT)-PCR for seven genes of interest for *G. uraniireducens* cells grown in batch and stressed over 24 h with 10% headspace vol/vol% oxygen. (**a**) Headspace oxygen for viable and heat-sterilized cells. (**b**) The number of pyrroline-5-carboxylate reductase (*proC*), DNA-directed RNA polymerase beta subunit (*rpoB*) mRNA transcripts and biomass measurement (absorbance at 600 nm). (**c**) Fold changes in the number of *cydA* and *macA* mRNA transcripts. (**d**) Fold changes in the number of *sodA*, *dfx* and *rbr* mRNA transcripts. Headspace oxygen concentrations and OD600 results are reported as the mean (line) and standard deviation (error bars) of triplicate biological replicates, whereas expression results are reported as the mean (bars) and range (error bars) of two biological replicates and four technical replicates.

Impact of growth on Fe(III) on expression of oxidative stress genes

The cells in the studies described above were grown with fumarate as the electron acceptor, because it is necessary to use soluble electron acceptors for growth in chemostats and *G. uraniireducens* does not grow on Fe(III) citrate (Shelobolina *et al.*, 2008), the only convenient form of soluble Fe(III) that can be supplied at high concentrations for chemostat growth. However, analysis of a previous whole-genome microarray comparison of *G. sulfurreducens* grown on fumarate and Fe(III) citrate suggested that *cydA* transcript levels were higher in Fe(III)-grown cells (Methe *et al.*, 2005). Transcript abundance for both *cydA* and *sodA* was also higher in *G. sulfurreducens* cells grown on Fe(III) as compared with growth on a current-harvesting electrode (Holmes *et al.*, 2006).

To evaluate the effect of growth on Fe(III) might have on expression of oxidative stress genes compared with the impact that oxygen exposure alone might have on these genes, *G. sulfurreducens* was grown in chemostats with Fe(III) or fumarate as the electron acceptor and cells were exposed to oxygen. Transcript levels of *cydA* and *sodA* were 60 and 12-fold higher, respectively, when cells were grown anaerobically with Fe(III), and were five and eight-fold higher in Fe(III)-grown cells than cells grown on fumarate and exposed to 10% oxygen (Figure 5). Exposure of Fe(III)-grown cells to oxygen increased transcript abundance of *cydA* and *sodA* even further, from more than fourfold with 5% oxygen to greater than 90-fold with 10% oxygen (Figure 5). These results demonstrate that growth on Fe(III) may result in relatively high transcript abundance of *cydA* and *sodA* in the absence of oxygen and that the presence of oxygen in addition to growth on Fe(III) results in even greater increases in transcripts for these genes.

Implications

These results suggest that the initially surprising finding that the natural community of Geobacteraceae

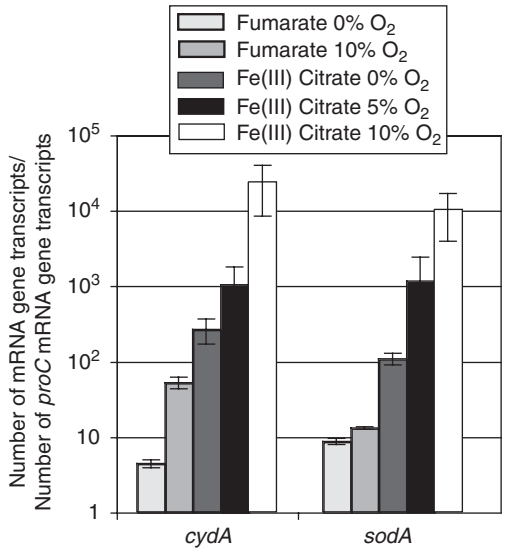

Figure 5 Relative expression of two oxidative stress genes, *cydA* and *sodA*, relative to *proC*, in *G. sulfurreducens* grown in continuous culture with either fumarate or Fe(III) citrate as the electron acceptor under different headspace oxygen concentrations. Results are reported as the mean of three biological replicates and three technical replicates.

involved in *in situ* uranium bioremediation at the Rifle site expressed *sodA* and *cydA* under what would generally be considered anoxic environmental conditions, does not necessarily indicate that these cells were being exposed to oxygen *in situ*. Appreciable dissolved oxygen would not typically be expected in groundwater with substantial dissolved Fe(II) because Fe(II) rapidly reacts with oxygen at circumneutral pH (Lowson, 1982; Morgan and Lahav, 2007). Furthermore, relative expression levels for *cydA* and *sodA* observed in subsurface sediments during uranium biostimulation were between 10^2 and 10^3 during the plateau stage of growth. These ratios are similar to levels observed in *G. sulfurreducens* grown in chemostat culture in the presence of Fe(III) under anaerobic conditions (Figure 5). A potential alternative explanation was that the expression of the relatively high expression of *sodA* and *cydA* was an artifact of the cells being exposed to oxygen during collection. However, this explanation is not consistent with the time course experiments that clearly showed that long-term exposure to oxygen was required for significant increases in relative transcript levels. In addition, this would not explain the low levels of transcripts in *sodA* and *cydA* in the early stages of the field experiment or the decline in *cydA* in the latter stages of the bioremediation, which we believe to be related to *in situ* growth trends.

As the results from growth of *G. sulfurreducens* on Fe(III) demonstrate, factors other than the presence of oxygen may result in high expression of these genes. For example, previous whole-genome microarray studies of *G. sulfurreducens* found elevated expression of these genes during growth with Fe(III) citrate versus growth with fumarate or an electrode serving as the electron acceptor (Methe *et al.*, 2005; Holmes *et al.*, 2006). Furthermore, transcript abundance for a number of genes potentially associated with oxidative stress was also higher when *G. uraniireducens* was grown in sediments as compared with fumarate medium (Holmes *et al.*, 2008). These considerations suggest that when diagnosing metabolic status of subsurface microorganisms through analysis of *in situ* gene transcript levels, it is important to interpret these in context with the appropriate environmental data and a complete understanding of all of the environmental factors that may influence the expression of the genes of interest.

Acknowledgements

This research was supported by the Office of Science Biological and Environmental Research (BER), US Department of Energy, Grant no. DE-FG02-07ER64377.

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