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ORIGINAL ARTICLE

# Molecular analysis of phosphate limitation in *Geobacteraceae* during the bioremediation of a uranium-contaminated aquifer

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**Nutrient limitation is an environmental stress that may reduce the effectiveness of bioremediation strategies, especially when the contaminants are organic compounds or when organic compounds are added to promote microbial activities such as metal reduction. Genes indicative of phosphate-limitation were identified by microarray analysis of chemostat cultures of *Geobacter sulfureducens*. This analysis revealed that genes in the *pst-pho* operon, which is associated with a high-affinity phosphate uptake system in other microorganisms, had significantly higher transcript abundance under phosphate-limiting conditions, with the genes *pstB* and *phoU* upregulated the most. Quantitative PCR analysis of *pstB* and *phoU* transcript levels in *G. sulfurreducens* grown in chemostats demonstrated that the expression of these genes increased when phosphate was removed from the culture medium. Transcripts of *pstB* and *phoU* within the subsurface *Geobacter* species predominating during an *in situ* uranium-bioremediation field experiment were more abundant than in chemostat cultures of *G. sulfurreducens* that were not limited for phosphate. Addition of phosphate to incubations of subsurface sediments did not stimulate dissimilatory metal reduction. The added phosphate was rapidly adsorbed onto the sediments. The results demonstrate that *Geobacter* species can effectively reduce U(VI) even when experiencing suboptimal phosphate concentrations and that increasing phosphate availability with phosphate additions is difficult to achieve because of the high reactivity of this compound. This transcript-based approach developed for diagnosing phosphate limitation should be applicable to assessing the potential need for additional phosphate in other bioremediation processes.**

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## Introduction

The practice of groundwater bioremediation is often highly empirical (Lovley, 2003). For example, a common practice intended to stimulate the activity of subsurface microorganisms involved in bioremediation is to add nutrients, such as nitrogen or phosphate (Martani and Seto, 1991; Norris and Dowd, 1994; Hinchee *et al.*, 1995; Ronen *et al.*, 1996). The rationale is that phosphate is an essential nutrient for microbial metabolism and that phosphate availability may be low in, at least, some contaminated environments. However, without information on the physiological status of the subsurface microorganisms, it is not clear whether such phosphate amendments are warranted or even desirable. Addition of phosphate when the microorganisms are not limited for phosphate or adding more phosphate than is needed to overcome phosphate limitation adds unnecessary costs to bioremediation. Furthermore, adding phosphate beyond what is necessary to support optimal microbial

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activity could have unintended negative consequences. For example, phosphate may form insoluble precipitates with trace metals and other cations that are also important nutrients, making these nutrients less available. Thus, information on the *in situ* phosphate requirements before initiating a bioremediation strategy and during the course of the bioremediation could make it possible to alleviate phosphate limitations impeding optimal bioremediation in the most resource- and cost-effective manner.

One of the most effective strategies for monitoring the metabolic state of microorganisms involved in bioremediation is to quantify *in situ* levels of gene expression (Lovley, 2002, 2003). Geochemical processes in pure cultures as well as in the environment can be linked to increased levels of mRNA for a particular gene (Holmes *et al*., 2004b, 2005). For example, genes involved in naphthalene degradation (nahA) were also detected in sediments where naphthalene mineralization was taking place (Wilson *et al*., 1999). Similarly, high levels of merA were measured when high rates of mercury volatilization occurred (Nazaret *et al*., 1994). In addition, three reductive dehalogenase genes were monitored in samples where trichloroethene bioremediation was applied (Lee *et al*., 2008).

*Geobacter* species are important agents in the bioremediation of organic contaminants (Lovley *et al*., 1989; Rooney-Varga *et al*., 1999; Lin *et al*., 2005), as well as uranium (Anderson *et al*., 2003; North *et al*., 2004; Vrionis *et al*., 2005) and vanadium (Ortiz-Bernad *et al*., 2004b). Analysis of gene transcript abundance within the subsurface *Geobacter* community has been successful in diagnosing rates of metabolism in *Geobacter* species as well as nutrient limitations and stress responses (Holmes *et al*., 2004b, 2005; O'Neil *et al*., 2008; Mouser *et al*., 2009a). Analysis of levels of transcripts for the nitrogen fixation gene, *nifD*, demonstrated that subsurface *Geobacter* species were limited for fixed nitrogen in petroleum-contaminated subsurface sediments and during *in situ* uranium bioremediation (Holmes *et al*., 2004b, 2005). This may not be surprising because in petroleum-contaminated aquifers and in aquifers amended with organic electron donors to promote dissimilatory metal reduction there is likely to be an abundance of organic carbon, but limiting quantities of other nutrients. Although *Geobacter* species can overcome limitations for fixed nitrogen by fixation of abundant nitrogen gas (Bazylinski *et al*., 2000; Coppi *et al*., 2001; Holmes *et al*., 2004a), phosphate sources are likely to be more limited. Until now it has not been possible to assess possible phosphate limitations of *Geobacter* species because of a lack of information on genes, which might be indicative of phosphate limitation.

In other microorganisms, such as *Bacillus subtilis*, *Clostridium acetobutylicum*, *Escherichia coli* or *Pseudomonas aeruginosa,* phosphate-limiting conditions are associated with increased expression of genes in the *pst-pho* operon, which encode genes associated with a high-affinity phosphate uptake system (Nakata *et al*., 1984; Nikata *et al*., 1996; VanBogelen *et al*., 1996; Aguena *et al*., 2002; Allenby *et al*., 2004; Fischer *et al*., 2006). All of the available *Geobacter* species genomes contain at least one copy of the *pst-pho* operon, suggesting that organisms in this genus share this high-affinity uptake system. In the natural environment, the availability of phosphorus often determines microbial growth and activity. For this reason, most bacteria have two phosphate transport systems, which differ in affinity and specificity for phosphate (Bardin and Finan, 1998). The low-affinity phosphate transport system (Pit) transports phosphate into the cells in the presence of high levels of extracellular phosphate (Gachter and Meyer, 1993; Voegele *et al*., 1997; Bardin and Finan, 1998; Van Dien and Keasling, 1999; Botero *et al*., 2000; Harris *et al*., 2001). It is usually constitutively expressed and is dependent on the proton motive force. The high-affinity phosphate specific transport system is induced during phosphate starvation by the Pho regulon and is an ABC transporter (Muda *et al*., 1992; Nikata *et al*., 1996; Aguena *et al*., 2002; Gebhard *et al*., 2006). Therefore, in most environments with low phosphate availability, the high-affinity phosphate transport system is expected to be the primary pathway for phosphate introduction into cells.

The purpose of this study was to identify genes whose expression might be diagnostic of phosphate-limiting conditions in *Geobacter* species and to analyze the expression of these genes during *in situ* uranium bioremediation to determine if the subsurface *Geobacter* species are limited for phosphate under the current strategy of adding only acetate to promote this process. The results suggest that *Geobacter* species are phosphate-limited, but that addition of phosphate may not stimulate metal reduction because of the high capacity of the sediments for phosphate adsorption.

## Materials and methods

*Growth of* Geobacter sulfurreducens *in continuous culture*

*Geobacter sulfurreducens* strain $PCA^T$ (ATCC 51573) was obtained from our laboratory culture collection. *G. sulfurreducens* was grown in electron donor limited chemostats at dilution rate of 0.05 $h^{-1}$ with acetate (5 mM) provided as the electron donor and fumarate (27.5 mM) as the electron acceptor at 30 °C, as previously described (Esteve-Nunez *et al*., 2005). For phosphate-limited chemostats, *G. sulfurreducens* was first grown to steady-state under electron donor limiting conditions as described above. The medium contained 0.217 mM phosphate. The medium was then switched to one that was phosphate free. The chemostats were harvested when the

optical density ($OD_{600}$) had dropped below 0.2. Analyses of acetate, fumarate, succinate, malate and protein were carried out as previously described (Esteve-Nunez *et al*., 2005).

To track the number of mRNA transcripts for *pstB* and *phoU*, *G. sulfurreducens* was grown under donor-limiting conditions, as described above. The medium was switched after approximately 30 h to one that was phosphate free. When the $OD_{600}$ had dropped below 0.1, the medium was again switched to one that contained phosphate. Chemostats were sampled at different time points and the nucleic acids were extracted from the collected biomass as described below.

*Microarray analysis comparing acetate limitation and phosphate limitation*
Deoxyribonucleic acid microarray hybridization and data analyses were carried out as previously described (Methe *et al*., 2005). The expression ratio between acetate and phosphate limiting for three biological replicates and three technical replicates was calculated.

*Site and plot description*
Detailed descriptions of the geology, hydrology and geochemistry of the site in Rifle, CO, USA have been presented elsewhere (Anderson *et al*., 2003; Vrionis *et al*., 2005; Yabusaki *et al*., 2007). The field experimental results described here were obtained as part of the 2007 study of the U.S. Department of Energy's Integrated Field Research Challenge (IFRC) site at Rifle, Co, USA. In 2007, the 10 m × 10 m experimental plot was similar in design as the one from previous field test described in Anderson *et al*. (2003) and Vrionis *et al*. (2005). The experimental plot comprised of an injection gallery, four background-monitoring wells and twelve downgradient monitoring wells. The injection gallery was made of 10 1 m spaced injection wells and was positioned approximately perpendicular to groundwater flow (see Mouser *et al*., 2009b).

Acetate (5 mM-target concentration) was injected into the aquifer for a total period of 30 days from August to September 2007. The injection period was interrupted by a groundwater flush from day 10 to day 17 of the experimental period, to mimic a period of acetate limitation. This study focuses on D02, a well located in the first row of downgradient wells, where some of the lowest U(VI) concentrations ($<0.13$ μM) were achieved by the end of the experimental period. Furthermore, *Geobacteraceae* were the predominant organisms in this well for the majority of the experimental period.

*Groundwater sampling and geochemical analysis*
Before sample collection, 12 l of water were purged from each well at a rate of approximately 2 l min$^{-1}$. About 15 l of groundwater from upgradient and downgradient wells were filtered onto a series of two 293 mm diameter filters, a 1.2 and 0.2 μm Supor membrane filter (Pall Life Sciences, Port Washington, NY, USA) at different time intervals through the course of the field experiment. The filters were flash frozen in an ethanol–dry ice bath, shipped on dry ice to the laboratory and stored at −80 °C until analysis.

Acetate, sulfate, nitrate and bromide were analyzed on site using an ion chromatograph (ICS-1000, Dionex, Sunnyvale, CA, USA) equipped with an autosampler (AS40, Dionex). The samples were separated through an AS22 column (Dionex) with a carbonate/bicarbonate (4.5 mM/1.4 mM) eluent. Phosphate was analyzed by ICP-MS. These samples were run at a 1:5 dilution on a Perkin–Elmer Elan DRC II ICP-MS (Perkin-Elmer, Waltham, MA, USA) using a Micro Mist 0.2 ml min$^{-1}$ nebulizer. The method used a dynamic reaction cell with oxygen as a reaction gas to form the oxide $PO^+$ (phosphorus oxide) and analyzing the oxide at mass 47, using Ga 69 as an internal standard. No other elements were analyzed with PO. Randomly selected samples were spiked with 5 and 20 μg l$^{-1}$ P (Phosphorus), typical recoveries were 99 and 105%, respectively. QC samples and laboratory reagent blanks were also run, showing minimal drift during analysis and no detectable contamination from sample preparation/sampling vessels.

*Extraction of mRNA and DNA from* G. sulfurreducens *chemostat cultures and environmental samples*
Deoxyribonucleic acid was extracted from pure cultures, and Rifle groundwater filters using the Qbiogene soil extraction kit (Qbiogene/MP Biomedicals, Solon, OH, USA) as per the manufacturer's instructions. Nucleic acids were extracted from chemostat samples as previously described, using a trizol:chloroform extraction (Methe *et al*., 2005). Extraction of nucleic acids from groundwater samples were done as described elsewhere (Holmes *et al*., 2004b) with a few modifications, such as: tRNA was not added to the procedure of mRNA extraction from groundwater samples; linear acrylamide was excluded from the precipitation step; finally, additional DNAse treatment was carried out at the end of the extraction using a DNA-free kit (Ambion, Austin, TX, USA) for all samples.

*Design of primers and amplification of* phoU *and* pstB *genes*
Primers targeting *Geobacteraceae phoU*, *pstB*, and *proC* genes were designed from nucleotide sequences from *G. sulfurreducens* (Methe *et al*., 2003), *Geobacter metallireducens*, *D. palmitatis*, *P. carbinolicus* and *P. propionicus* genomes. Preliminary sequence data from *G. metallireducens*, *D. palmitatis*, *P. carbinolicus* and *P. propionicus* genomes was obtained from the DOE Joint Genome

**Table 1** List of *Geobacteraceae* primers used in this study.

| *Primer set* | *Sequence* | *Amplified Geobacteraceae gene* |
|---|---|---|
| phoU-3F<br>phoU-3R | 5′-ATCGTYACSGACCTGGAGCG-3′<br>5′-CCAKRTACTTGGAGATGTAG-3′ | *phoU* from *G. psychrophilus*, *G. bremensis*, *G. uraniireducens*, Rifle environmental DNA |
| phoU-2F<br>phoU-1R | 5′-GASATCGAYGARAAGTGCCT-3′<br>5′-CATGAAGATSACCATYTCGG-3′ | *phoU* from *G. bemidjiensis*, *G. uraniireducens*, *G. psychrophilus*, Rifle environmental DNA |
| phoU-290F<br>phoU-650R | 5′-ACCTGGAGCGGATCGGCGAC-3′<br>5′-TCGTGTGGCGGATGTCCTTC-3′ | *phoU* from Rifle environmental DNA |
| pstB-2F<br>pstB-1R | 5′-TTCCAGAARCCGAACCCSTT-3′<br>5′-CCGAAMCGTCCSGTAATGTA-3′ | *pstB* from *G. psychrophilus*, *G. bemidjiensis*, *G. uraniireducens*, Rifle environmental DNA |
| pstB-3F<br>pstB-1R | 5′-RTCRGCATGGTSTTCCAGAA-3′<br>5′-CCGAAMCGTCCSGTAATGTA-3′ | *pstB* from *G. bremensis* |
| phoU-401F<br>phoU-521R | 5′-TGGTGAAGGAGTCGCTGGA-3′<br>5′-AAGGTGAGCAGTTCCCGTTG-3′ | *phoU* from *G. sulfurreducens* (quantitative PCR (qPCR)) |
| pstB-310F<br>pstB-422R | 5′-CCGTTCCCCAAATCGATCT-3′<br>5′-ATGGCGGCGTTAGTGAGG-3′ | *pstB* from *G. sulfurreducens* (qPCR) |
| phoU-28F<br>phoU-269R | 5′-AATATCGCCAAACGGTCG-3′<br>5′-CGGCTGATGGTGGAGGGA-3′ | *phoU* from Rifle environmental DNA (qPCR) |
| pstB-155F<br>pstB-339R | 5′-CTGAAGACCAACGCCATGAC-3′<br>5′-ACGATGATGATGGTGTAGCG-3′ | *pstB* from Rifle environmental DNA (qPCR) |
| proC-75F<br>proC-471R | Sequences presented elsewhere (Holmes *et al*., 2005) | *proC* from environmental DNA |
| proC-412F<br>proC-496R | Sequences presented elsewhere (Holmes *et al*., in preparation) | *proC* from *G. sulfurreducens* (qPCR) |
| proC-49F<br>proC-143R | 5′-GCGTCCAGCAGTTCTTCA-3′<br>5′-GGCTACAACGCAACCGATG-3′ | *proC* from Rifle environmental DNA (qPCR) |

Institute (JGI) website www.jgi.doe.gov. Table 1 lists all of the primers used in this study. All primers had an optimal annealing temperature of 60 °C. The following PCR parameters were used: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C (45 s), 60 °C (1 min), 72 °C (1 min) and with a final extension step at 72 °C for 10 min (Holmes *et al*., 2004b). The PCR products were resolved on 1% agarose gel and the bands of *phoU* and *pstB* at the expected size were excised and purified with a Gel Extraction Kit (Qiagen, Valencia, CA, USA). Clone libraries were constructed with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmid inserts (48) from each cDNA clone library were then sequenced with the M13F primer using an ABI BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) and ABI DNA Analyzer 3730xl (Applied Biosystems). Sequences were compared with those compiled in GenBank with the BLAST suit of programs to verify the specificity of each primer.

Sequences obtained were aligned and quantitative PCR (qPCR) primers designed (Table 1) to quantify *pstB* and *phoU* as well as *proC* in environmental samples as described below.

*Phylogenetic analysis*

*phoU* and *pstB* gene sequences were compared with NCBI GenBank nucleotide database using the blastn algorithms (Altschul *et al*., 1990). Amino acid and nucleotide sequences for *phoU* and *pstB* genes were initially aligned in MEGA version 4 (Tamura *et al*., 2007) where phylogenetic trees were inferred. Distances and branching order were determined and compared using maximum parsimony and distance-based algorithms (Neighbor-joining and Kimura). Bootstrap values were obtained from 100 replicates.

*Quantification of mRNA transcript levels using reverse transcriptase qPCR*

The reverse transcriptase enhanced avian kit (Sigma, St Louis, MO, USA) was used to reverse complement RNA from either environmental samples or *G. sulfurreducens* cultures in chemostats according to manufacturer's instructions. A 1:10 dilution of the RNA template was made before the reaction to minimize template inhibition. Subsequently, the cDNA was also diluted (1:10) before the qPCR reaction. Each PCR reaction consisted of a

total volume of 25 µl and contained 1.5 µl of primers (stock concentration was 20 µM), 12.5 µl Power SYBR green PCR mix (PE Biosystems, Foster City, CA, USA) and 9.5 µl template. Standard curves were constructed as previously described (Holmes *et al.*, 2004b), and covered a range of 8 orders of magnitude. qPCR reactions were carried out on a 7500 ABI real time (Applied Biosystems, Foster City, CA, USA) according to the ABI guide. The thermal cycling parameter consisted of an activation step at 50 °C for 2 mins, a denaturation step of 95 °C for 10 mins, followed by 45 cycles at 95 °C for 15 secs and 60 °C for 1 min. Triplicate runs of qPCR were done and the fold change was calculated as the ratio between baseline and the change observed. To verify amplification and correct amplicon size, aliquots from real-time PCR were examined on a 1% agarose gel stained with ethidium bromide. The qPCR primers used are listed in Table 1. The housekeeping gene, *proC,* was used for the normalization of chemostat and environmental data.

*Laboratory sediment incubations with phosphate amendment*

Laboratory sediment incubations were conducted, as previously described (N'Guessan *et al.*, 2008), with sediments collected near the acetate-injection test plot. The anoxic sediment-groundwater slurries were amended with 10 mM acetate and 250 µM sodium phosphate (target groundwater concentration). The sediment and groundwater from each set of triplicates was sampled over time under anaerobic conditions. Incubations without phosphate amendments served as controls. Analyses of acetate, sulfate and U(VI) in the groundwater sample was carried out as described above. Iron and uranium concentrations in sediment were determined as previously described (N'Guessan *et al.*, 2008). Phosphate was analyzed by ion chromatography (ICS-1000) as described above.

## Results and discussion

*Expression of* phoU *and* pstB *in response to phosphate limitation in chemostats*

To identify *Geobacter* genes with increased transcript levels during phosphate limitation, chemostats of *G. sulfurreducens* were grown under steady-state conditions with acetate as the electron donor and fumarate as the electron acceptor. Acetate availability was the factor limiting growth. The phosphate concentration was 0.217 mM. Once steady-state conditions (protein, $0.045 \pm 0.0001$ mg ml$^{-1}$; fumarate, $4.054 \pm 0.753$ mM; and acetate $<50$µM) were established for four culture vessel medium turnovers, the medium being added to the chemostat was changed to one that was identical with the exception that phosphate was omitted. As phosphate in the culture vessel was diluted, the cell density started to decrease, indicative of phosphate limitation. Cells were harvested for microarray analysis when the phosphate concentration had decreased to 0.086 mM. At this point cell protein had decreased ($0.029 \pm 0.004$ mg ml$^{-1}$) and (fumarate $6.607 \pm 0.436$ mM) and acetate ($1.106 \pm 0.300$ mM) concentrations were much higher. These changes demonstrate that cell growth was phosphate-limited.

The switch from electron donor-limiting to phosphate-limiting conditions was associated with changes in expression of a variety of genes involved in transport and binding as well as energy metabolism. A total of 35 genes had transcript levels at least twofold higher under the phosphate-limiting conditions (Table 2), whereas 53 genes had transcript levels that were at least twofold lower under this same condition (Table 3).

Decreases in transcript abundance were all less than fivefold (Table 3). Most of the genes with lower transcript levels in phosphate-limited cells were those involved in metabolism and cell growth and included electron transport proteins, various transport and binding proteins, ribosomal proteins and amino acid biosynthesis. The decreased level of transcripts in these genes is consistent with the slower growth of the phosphate-limited cells and the fact that phosphorus is an essential nutrient for the synthesis of nucleic acids and ATP. A similar response was previously noted in phosphate-limited cells of *Sinorhizobium meliloti* (Bardin and Finan, 1998; Krol and Becker, 2004).

The gene with the highest increase in transcript levels in phosphate-limited cells was *pstB* (Table 2). *pstB* has been annotated as the ATP-binding protein of a phosphate ABC transporter (Methe *et al*., 2003; Yan *et al*., 2004). It is part of a predicted operon, which also includes *phoU* (GSU1095), *pstA* (GSU1097), *pstC* (GSU1098) and *pstS* (GSU1099). *pstA*, *pstC* and *pstS* were also upregulated and are annotated to have a role in phosphate uptake, whereas *phoU* is predicted to regulate the expression of the *pst* genes (Table 2), consistent with similar operon organization of related genes in other microorganisms (Nakata *et al*., 1984; Nikata *et al*., 1996; VanBogelen *et al*., 1996; Bardin and Finan, 1998; Aguena *et al*., 2002; Fischer *et al*., 2006). The *pst-pho* operon was previously shown to be the most strongly up-regulated operon under phosphate-limiting conditions in *S. meliloti* (Krol and Becker, 2004) and *Acidothiobacillus ferroxidans* (Vera *et al*., 2003; Krol and Becker, 2004).

Other genes with a greater than fivefold increase in transcript levels in phosphate-limited cells included a putative metal ion efflux outer membrane family protein (GSU1330); an ABC transporter permease protein for an unknown substrate (GSU1340); a protein in the GH3 auxin-responsive promoter family but of unknown function (GSU1092); and two hypothetical proteins (GSU 2700 and GSU 1100). The majority of the remaining proteins with transcript levels that were more than twofold higher in phosphate-limited cells

**Table 2** Reporters whose expression was significantly increased when *Geobacter sulfurreducens* was grown with phosphate as limiting nutrient versus acetate as limiting nutrient

| *Locus ID* | *Common name* | *Main role* | *Subrole* | *Fold change* | *s.d.* |
|---|---|---|---|---|---|
| GSU0074 | elbB, enhancing lycopene biosynthesis protein 2 | Biosynthesis of cofactors, prostethic groups and carriers | Other | 2.622 | 0.251 |
| GSU2078 | rodA, rod shape-determining protein RodA | Cell envelope | Biosynthesis of murein sacculus and peptidoglycan | 2.243 | 0.223 |
| GSU1984 | gumC, polysaccharide chain length determinant protein, putative | | Other | 2.131 | 0.310 |
| GSU1330 | Metal ion efflux outer membrane protein family protein, putative | Cellular Processes | Detoxification | 7.472 | 3.464 |
| GSU1332 | Heavy metal efflux pump, CzcA family | DNA metabolism | | 3.052 | 0.738 |
| GSU0997 | mutM, formamidopyrimidine-DNA glycosylase | Energy metabolism | DNA replication, recombination and repair | 2.048 | 0.270 |
| GSU1538 | Methylamine utilization protein MauG, putative | | Amino acids and amines | 2.955 | 1.003 |
| GSU2732 | orf2-2, cytochrome c family protein | Hypothetical proteins | Electron transport | 2.227 | 0.441 |
| GSU0987 | Conserved hypothetical protein | | Conserved | 3.087 | 0.408 |
| GSU2558 | Conserved domain protein | | Domain | 4.840 | 2.610 |
| GSU2075 | subtilisin | Protein fate | Degradation of proteins | 2.355 | 0.388 |
| GSU1102 | DNA-binding response regulator | Regulatory functions | DNA interactions | 6.496 | 1.525 |
| GSU1095 | phoU, phosphate transport system regulatory protein PhoU | | Other | 9.067 | 2.953 |
| GSU0896 | tldD protein | | | 2.012 | 0.180 |
| GSU1101 | Sensory box histidine kinase | Transport and binding proteins | Protein interactions | 5.652 | 0.999 |
| GSU1096 | pstB, phosphate ABC transporter, ATP-binding protein | | Anions | 12.935 | 4.976 |
| GSU1097 | pstA, phosphate ABC transporter, permease protein | | | 6.650 | 2.484 |
| GSU1098 | pstC, phosphate ABC transporter, permease protein | | | 6.427 | 1.601 |
| GSU1099 | pstS, phosphate ABC transporter, periplasmic phosphate-binding protein | | | 4.974 | 2.073 |
| GSU1340 | ABC transporter, permease protein | | Unknown substrate | 11.121 | 3.052 |
| GSU1331 | Efflux transporter, RND family, MFP subunit | | Metal ion | 2.717 | 0.295 |
| GSU1092 | GH3 auxin-responsive promoter family protein | Unknown function | General | 8.183 | 2.964 |
| GSU1945 | Fibronectin type III domain protein | | | 3.058 | 0.801 |
| GSU3435 | Ankyrin repeat protein | | | 2.849 | 0.776 |
| GSU3464 | gidA, glucose inhibited division protein A | | | 2.543 | 0.551 |
| GSU2427 | Nitrite/sulfite reductase domain protein | | | 2.185 | 0.303 |
| GSU2780 | Hypothetical protein | | | 6.653 | 0.152 |
| GSU1100 | Hypothetical protein | | | 6.017 | 1.412 |
| GSU1944 | Hypothetical protein | | | 3.897 | 1.041 |
| GSU1333 | Hypothetical protein | | | 3.582 | 0.525 |
| GSU1994 | Hypothetical protein | | | 3.299 | 0.882 |
| GSU0715 | Hypothetical protein | | | 2.813 | 1.104 |
| GSU1943 | Hypothetical protein | | | 2.622 | 0.943 |
| GSU0850 | Hypothetical protein | | | 2.415 | 0.084 |
| GSU0790 | Hypothetical protein | | | 2.009 | 0.383 |

Results are sorted first by role category then in descending order by fold change. Fold changes are averages over all the biological and technical replicates.

are hypothetical proteins and proteins involved in transport (Table 2).

### *Changes in* pstB *and* phoU *transcripts in chemostat cultures*

The significant increase in transcript abundance of *phoU* and *pstB* under phosphate-limiting conditions suggested that their abundance might serve as good indicators for phosphate limitation during the growth of *Geobacter* species in the subsurface. This was first evaluated under controlled conditions with *G. sulfurreducens* grown in chemostats. Transcript abundance of *phoU* and *pstB* was normalized to transcript abundance of *proC*, a housekeeping gene that is expressed at consistent levels under many growth conditions (Holmes *et al*., 2005). As expected from the microarray study, introducing

**Table 3** Reporters whose expression was significantly decreased when *Geobacter sulfurreducens* was grown with phosphate as limiting nutrient versus acetate as limiting nutrient

| *Locus ID* | *Common name* | *Main role* | *Subrole* | *Fold change* | *s.d.* |
|---|---|---|---|---|---|
| GSU1906 | leuA, 2-isopropylmalate synthase | Amino acid biosynthesis | Pyruvate family | −4.041 | 0.606 |
| GSU1912 | ilvD, dihydroxy-acid-dehydratase | | | −2.430 | 1.297 |
| GSU1902 | 3-isopropylmalate dehydratase, small subunit | | | −2.357 | 0.286 |
| GSU3068 | murC, UDP-N-acetylmuramate—alanine ligase | Cell envelope | Biosynthesis of murein sacculus and peptidoglycan | −2.014 | 0.420 |
| GSU3072 | murF, UDP-N-acetylmuramoylalanyl-D-glutamyl-2, 6-diaminopimelate-D-alanyl-D-alanyl ligase, frameshifted | | | −2.014 | 0.420 |
| GSU2707 | ackA-1, acetate kinase | Central intermediate metabolism | Other | −2.463 | 0.377 |
| GSU0096 | recR, recombination protein RecR | DNA metabolism | DNA replication, recombination, and repair | −3.393 | 0.651 |
| GSU0346 | nuoI-1, NADH dehydrogenase I, I subunit | Energy metabolism | Electron transport | −4.103 | 0.903 |
| GSU0347 | nuoJ-1, NADH dehydrogenase I, J subunit | | | −3.119 | 0.596 |
| GSU3259 | Cytochrome c family protein | | | −3.083 | 0.639 |
| GSU0339 | nuoB, NADH dehydrogenase I, B subunit | | | −2.882 | 0.150 |
| GSU0348 | nuoK-1, NADH dehydrogenase I, K subunit | | | −2.803 | 0.853 |
| GSU0341 | nuoD, NADH dehydrogenase I, D subunit | | | −2.786 | 0.089 |
| GSU0350 | nuoM-1, NADH dehydrogenase I, M subunit | | | −2.474 | 0.079 |
| GSU0345 | nuoH-1, NADH dehydrogenase I, H subunit | | | −2.204 | 0.255 |
| GSU0349 | nuoL-1, NADH dehydrogenase I, L subunit | | | −2.083 | 0.481 |
| GSU0097 | Pyruvate ferredoxin/flavodoxin oxidoreductase | | Fermentation | −2.872 | 0.348 |
| GSU1738 | Indolepyruvate ferredoxin/flavodoxin oxidoreductase, beta subunit | | | −2.433 | 0.354 |
| GSU1737 | Phenylacetate-CoA ligase | | Other | −3.215 | 0.638 |
| GSU1729 | Phenylacetate-CoA ligase | | | −2.514 | 0.521 |
| GSU1071 | Conserved hypothetical protein | Hypothetical proteins | Conserved | −3.556 | 0.886 |
| GSU0388 | Conserved hypothetical protein | | | −3.505 | 0.482 |
| GSU0317 | Conserved hypothetical protein | | | −2.368 | 0.380 |
| GSU3289 | Conserved hypothetical protein (ferritin-like domain protein) | | | −2.307 | 0.318 |
| GSU0955 | group II intron, maturase | Other categories | Transposon functions | −2.303 | 0.329 |
| GSU1772 | ctpA-2, carboxy-terminal processing protease | Protein fate | Degradation of proteins | −2.466 | 0.530 |
| GSU0276 | Secretion protein, HlyD family | | Protein and peptide | −2.101 | 0.221 |
| GSU2835 | MAP, methionine aminopeptidase, type I | | Protein modification | −2.053 | 0.546 |
| GSU2847 | rplN, ribosomal protein L14 | Protein synthesis | Ribosomal protein | −2.530 | 0.279 |
| GSU2838 | rplO, ribosomal protein L15 | | | −2.413 | 0.355 |
| GSU2865 | rplJ, ribosomal protein L10 | | | −2.351 | 0.256 |
| GSU3235 | rpmA, ribosomal protein L27 | | | −2.278 | 0.875 |
| GSU2852 | rplV, ribosomal protein L22 | | | −2.054 | 0.486 |
| GSU3236 | rplU, ribosomal protein L21 | | | −2.046 | 0.177 |
| GSU2840 | rpsE, ribosomal protein S5 | | | −2.028 | 0.512 |
| GSU3465 | trmE, tRNA modification GTPase TrmE | | tRNA and rRNA base | −2.711 | 0.318 |
| GSU0800 | Amino acid ABC transporter, periplasmic amino acid-binding protein | Transport and binding proteins | Amino acids, peptides | −3.298 | 0.158 |
| GSU1735 | Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein, putative | | | −2.592 | 0.404 |
| GSU1068 | Sodium/solute symporter family protein | | Unknown substrate | −4.411 | 0.395 |
| GSU2352 | Sodium/solute symporter family protein | | | −4.364 | 0.592 |
| GSU1070 | Sodium/solute symporter family protein | | | −4.016 | 0.146 |
| GSU0490 | Acetyl-CoA hydrolase/transferase family protein | Unknown function | Enzymes of unknown specificity | −4.131 | 0.653 |
| GSU3454 | Radical SAM domain protein | | | −2.211 | 0.755 |

**Table 3** (Continued)

| Locus ID | Common name | Main role | Subrole | Fold change | s.d. |
|---|---|---|---|---|---|
| GSU1736 | ACT domain protein | | General | −2.500 | 0.539 |
| GSU3092 | YqeY family protein | | | −2.357 | 0.365 |
| GSU0869 | LysM domain/NLP/P60 family protein | | | −2.047 | 0.573 |
| GSU1932 | Hypothetical protein | | | −2.916 | 0.487 |
| GSU0208 | Hypothetical protein | | | −2.131 | 0.329 |
| GSU2471 | Hypothetical protein | | | −2.340 | 0.487 |

Results are sorted first by role category then in descending order by fold change. Fold changes are averages over all the biological and technical replicates.

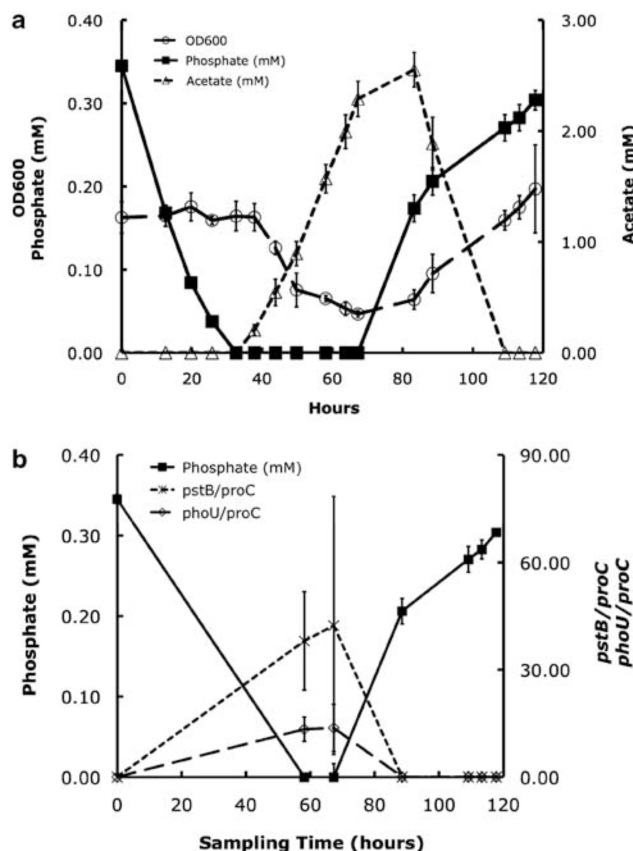


**Figure 1** Phosphate limitation chemostats with *Geobacter sulfurreducens*. (**a**) Changes in cell density and acetate concentration in response to phosphate limitation. (**b**) Transcript abundance of *pstB* and *phoU* normalized to *proC* transcript abundance in various concentrations of phosphate. Results are means of triplicate chemostats.

medium without phosphate into the chemostat resulted in a decrease in cell density and an increase in acetate as growth became phosphate-limited (Figure 1a). When phosphate was reintroduced into the medium, *G. sulfurreducens* was able to grow again and as cell density increased, acetate concentrations decreased (Figure 1a).

Transcripts of *phoU* and *pstB* were not detected during steady-state growth before imposing phosphate limitation (Figure 1b). Levels of *phoU* and *pstB* transcripts increased dramatically under phosphate-limiting conditions. Large s.d. were observed under phosphate limiting conditions when cell density was at its lowest and may have varied between triplicate chemostats. There were more *pstB* transcripts than *phoU* transcripts, consistent with the results from the microarray study. When the chemostat medium reservoir was switched back to phosphate-containing medium, the number of transcripts for both genes rapidly decreased back to baseline levels. These results further suggested that *phoU* and *pstB* expression levels are a sensitive indicator of phosphate availability.

### *Geochemical changes during* in situ *uranium bioremediation*

As expected from previous field studies (Anderson *et al.*, 2003; Vrionis *et al.*, 2005), the addition of acetate to the subsurface resulted in increased acetate concentrations in the downgradient monitoring well (Figure 2a). Acetate concentrations declined after the acetate inputs were stopped temporarily between on day 10 and then began to increase as acetate additions resumed by day 17. Because of the residual acetate in the injection well, the groundwater flush only resulted in a slight decrease in acetate concentration in D02. Increased acetate concentrations were associated with an increase in dissolved Fe(II) (Figure 2b) and a decrease in dissolved U(VI) (Figure 2c), thus suggesting that dissimilatory metal reduction had been stimulated. This was associated with a marked increase in the abundance of *Geobacter* species in the groundwater (Elifantz *et al.*, 2008; Mouser *et al.*, 2009b). *Geobacter* species are known Fe(III)- and U(VI)-reducing microorganisms and are considered to be responsible for most of the dissimilatory metal reduction in these acetate-amended subsurface sediments (Lovley, 1991; Finneran *et al.*, 2002; Holmes *et al.*, 2002, 2005; Anderson *et al.*, 2003; Istok *et al.*, 2004; Vrionis *et al.*, 2005). Within a period of 20 days of acetate addition, the uranium concentration in D02 was reduced by more than 80%, starting at an initial background concentration of about 130 $\mu g\, l^{-1}$ and diminishing to values well below the EPA maximum contaminant level (30 $\mu g\, l^{-1}$) by the end of the study period (Figure 2c).

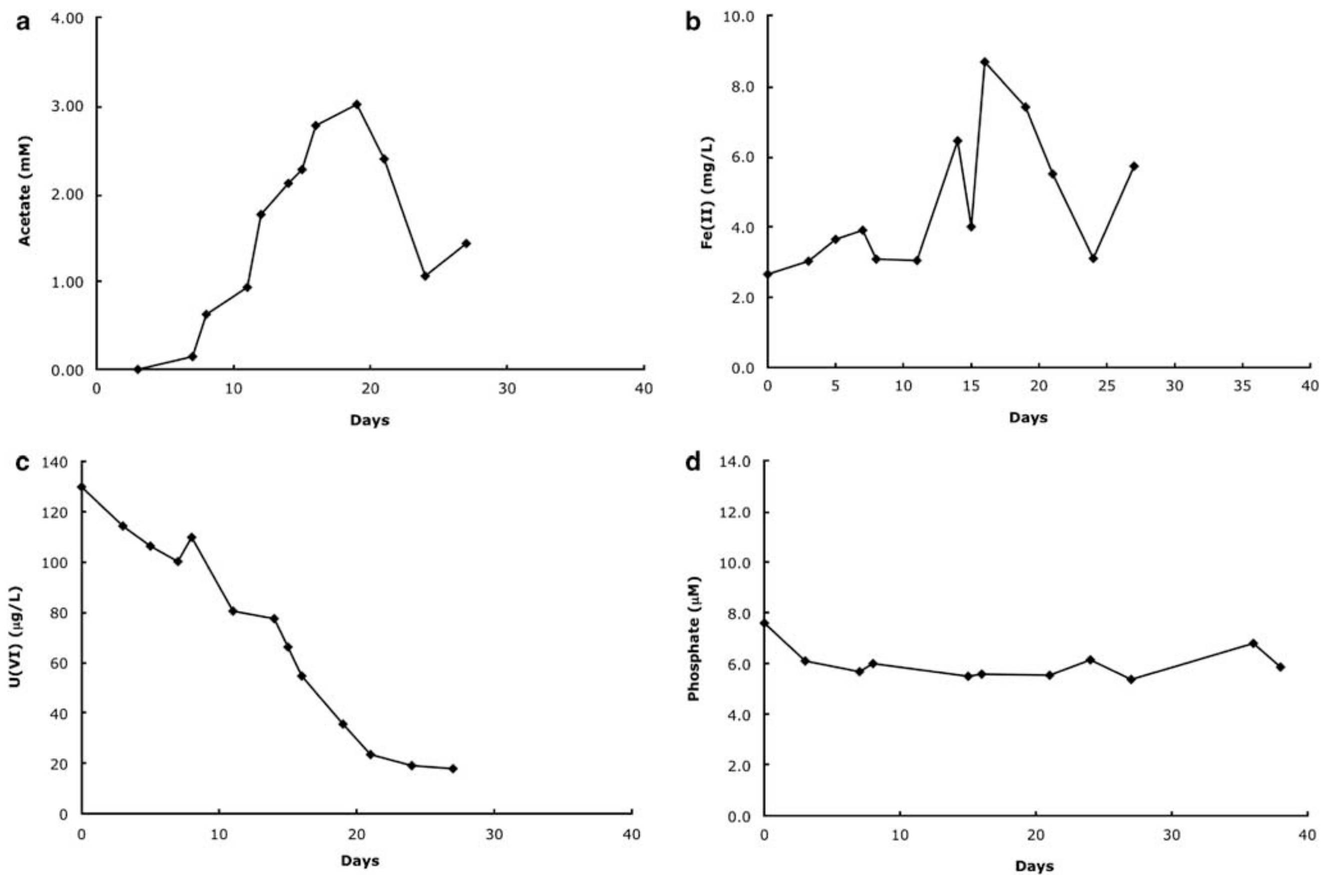


**Figure 2** Geochemical changes associated with the addition of acetate to the subsurface. Groundwater concentrations of acetate (**a**), Fe(II) (**b**), U(VI) (**c**) and phosphate (**d**) following the addition of acetate starting on day 0. Acetate inputs were interrupted from day 10 to day 17 and then resumed.

Phosphate concentrations remained relatively constant during the field experiment (Figure 2d). It was originally hypothesized that phosphate concentrations in the groundwater would increase during biostimulation as a result of the release of iron-bound phosphate species upon reduction of the iron oxides (Johnson and Loeppert, 2006). The constant concentration of phosphate in the groundwater suggests that dissolved phosphate concentrations are in equilibrium with solid phase phosphate. Therefore, as the microorganisms use phosphate, the loss of this nutrient from pore water is replaced by re-equilibration with solid phases.

### *Diversity of the* phoU *and* pstB *genes amplified from the Rifle IFRC site*

To evaluate the diversity of phosphate metabolism genes found in the groundwater at the Rifle IFRC site, *pho*U and *pst*B genes were amplified from genomic DNA extracted from the groundwater on day 7 when dissimilatory metal reduction was very active and *Geobacteraceae* accounted for over 95% of the 16S rRNA gene sequences recovered in libraries constructed from groundwater samples (data not shown). Most (36 of 39) of the *phoU* sequences recovered from the groundwater sample were in a tight gene cluster sharing 96% similarity with each other (Figure 3a). The other three environmental sequences were closely related and shared 87–93% similarity with the other environmental sequences (Figure 3a). The low diversity of *phoU* sequences was reflected in rarefaction curves (data not shown). At and below 98% similarity level, rarefaction curves suggested that the entire diversity of the *phoU* genes were covered in the clone library. The Shannon diversity index at the 98% similarity level was 1.1. This low diversity of *phoU* sequences is consistent with the low diversity of *Geobacter* species in this sample. The environmental samples clustered with the *phoU* from *Geobacter bemidjiensis* and *Geobacter* sp. M21, an isolate from the Rifle site, and were 85–93% similar to these genes (Figure 3a).

The *pstB* genes recovered from groundwater were more diverse (Shannon diversity index 2.1) than *phoU,* but, like *phoU*, were closely related (77–87% similarity) to the *pstB* sequences of Subsurface Clade 1 microorganisms whose genomes are available (Figure 3b). Clusters A, B and C represented 29, 12.5 and 16.7% of the sequences analyzed, respectively (Figure 3b). Sequences within these clusters were at least 98% similar to each other, suggesting that this gene is highly conserved. As with the *phoU*

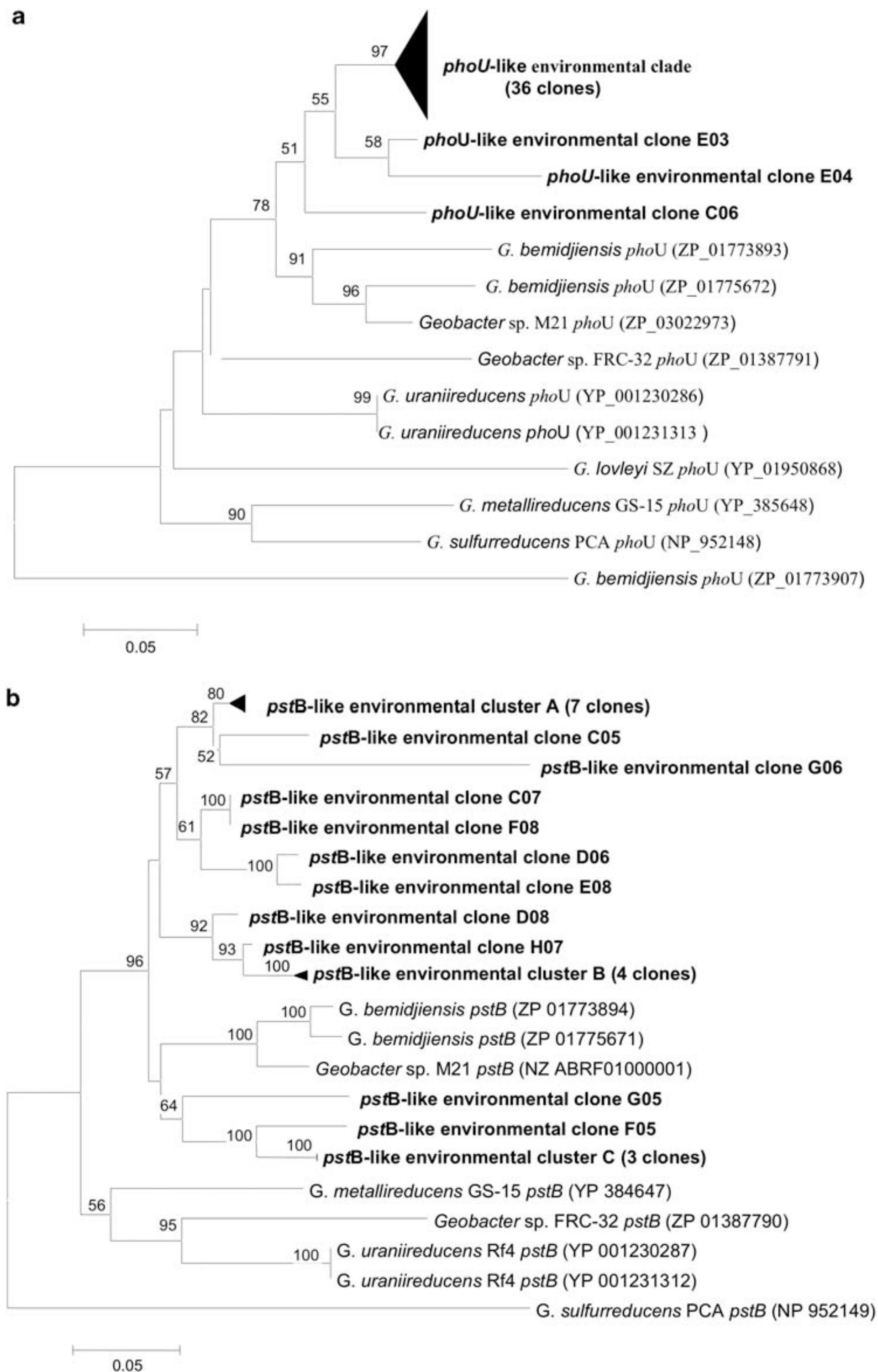


**Figure 3** Phylogenetic trees comparing *Geobacteraceae phoU* genes (**a**) and *pstB* genes (**b**) isolated from the Rifle IFC site with genes from *Geobacteraceae* available in pure culture. For simplicity, branches with more than 98% similarity were collapsed into a cluster. Branch points were supported by the Kimura algorithm and neighbor-joining distance analysis. Bootstrap values were calculated for 100 replicates and values above 50 are shown. The scale bars represent the expected number of changes per nucleotide.

sequences, the *pstB* sequences recovered from the subsurface were most closely related to *pstB* sequences in pure cultures that have been recovered from the Rifle site and other subsurface environments.

### *Abundance of* phoU *and* pstB *transcripts in subsurface* Geobacter

Transcripts of *phoU* and *pstB* in the subsurface *Geobacter* community were not detectable when

acetate amendments were initiated but increased in abundance when acetate availability increased, thereby stimulating the growth and activity of *Geobacter* species (Figure 4). With the exception of a very high abundance of *pstB* transcripts on day 11, the levels of *phoU* transcripts and *pstB* remained within a relatively restricted range, once acetate in the groundwater reached millimolar concentrations (Figure 4). With the exception of the one apparent anomaly in *pstB* abundance on day 11, the relative abundance of *phoU* and *pstB* was higher than that observed under phosphate-replete conditions in chemostats, but somewhat lower than the abundance of these transcripts under phosphate-limiting conditions. These results suggested that phosphate concentrations were less than those required for optimal growth during *in situ* uranium bioremediation or that the form of phosphorus was not as readily available as what might be provided in laboratory studies.

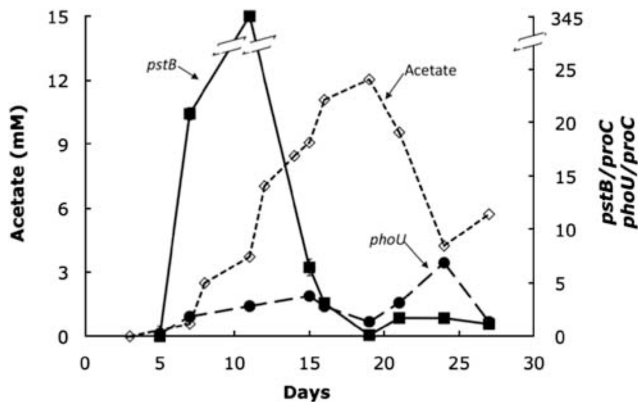


**Figure 4** Transcript abundance of *pstB* and *phoU* normalized to *proC* in D02 compared with acetate concentration during biostimulation of a uranium-contaminated aquifer in Rifle, CO, USA. Error bars represent the propagation of error of triplicate quantitative PCR (qPCR) reactions.

*Addition of phosphate in sediment incubations*

The finding that *Geobacter* species might be phosphate-limited suggested that the addition of phosphate to the subsurface might enhance the growth and activity of the subsurface *Geobacter* species and might stimulate rates of U(VI) reduction. Previous studies have demonstrated that the results

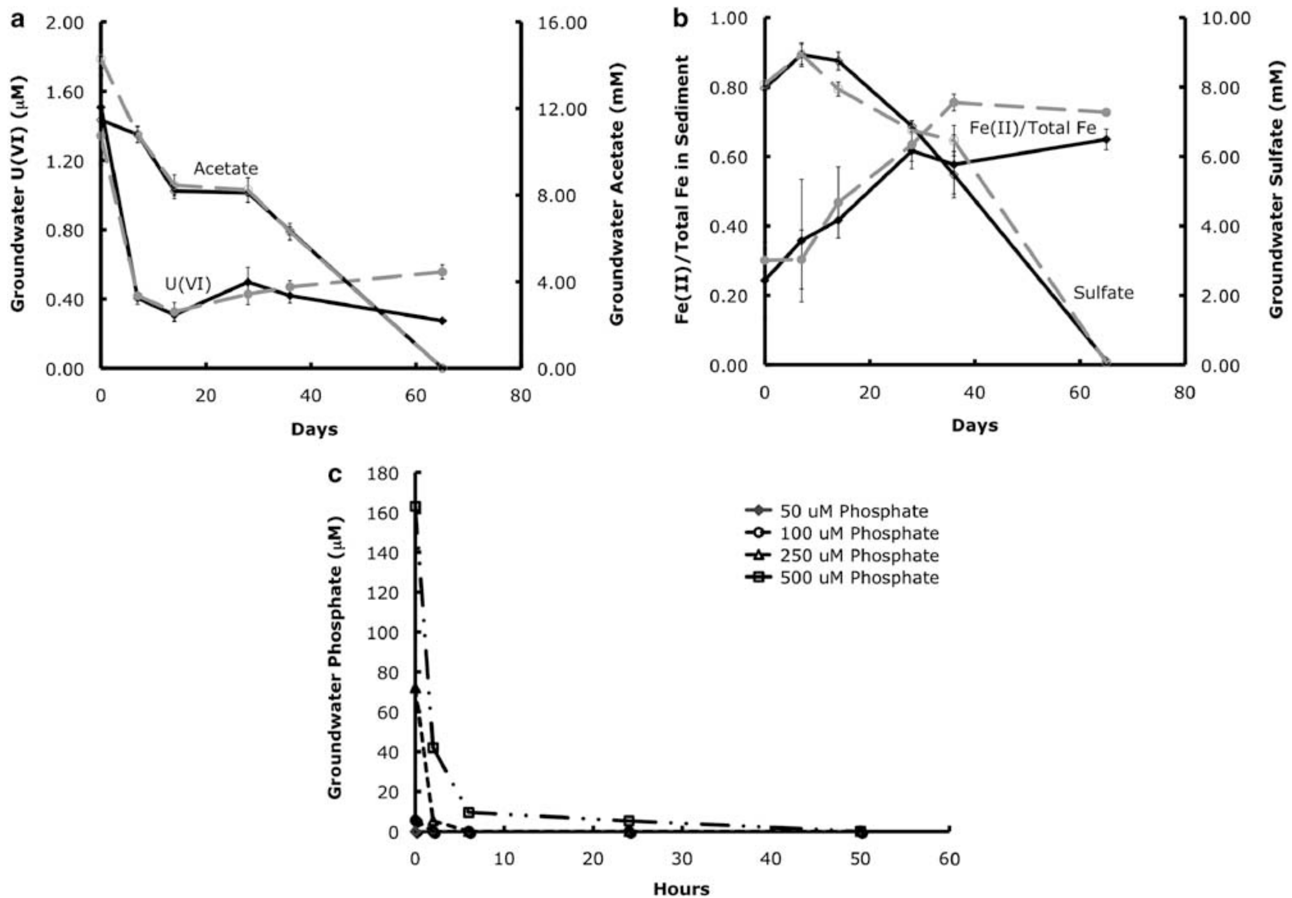


**Figure 5** Changes in geochemical parameters in sediment incubations with no nutrient amendment (solid lines) or amended with 250 μM phosphate (dashed lines). (**a**) Groundwater U(VI) and acetate concentrations; (**b**) fraction of Fe(II) in sediment; and (**c**) removal of phosphate from groundwater. Results are means of duplicate incubations.

from anoxic incubations of subsurface sediment and groundwater can provide an accurate indication of the impact of subsurface amendments (Finneran *et al.*, 2002; Holmes *et al.*, 2002; Ortiz-Bernad *et al.*, 2004a; N'Guessan *et al.*, 2008). Therefore, in order to evaluate whether adding phosphate might stimulate dissimilatory metal reduction at the Rifle site, sediment and associated groundwater, incubated under anoxic conditions, were amended with acetate to simulate *in situ* uranium bioremediation. As expected from previous studies (Finneran *et al.*, 2002; Ortiz-Bernad *et al.*, 2004a; N'Guessan *et al.*, 2008), consumption of the added acetate was associated with the reduction of Fe(III) to Fe(II) and the removal of U(VI) from the groundwater (Figure 5). Adding phosphate, which is calculated to increase the groundwater phosphate concentration to 250 μM, had no impact on the rate of acetate consumption, Fe(III) reduction or U(VI) removal (Figure 5). When phosphate concentrations were increased to potentially provide 500 μM phosphate, there was still no impact (data not shown).

One reason that the added phosphate failed to stimulate rates of acetate metabolism metal reduction may have been the high phosphate adsorption capacity of the sediment. Added phosphate was rapidly removed from the groundwater (Figure 5c). It has been shown that aquifer sediments generally have low phosphorus content and high phosphate sorption capacity (Patrick and Khalid, 1974; Mc Callister and Logan, 1978).

## Implications

These findings suggest that phosphate availability may limit the growth of *Geobacter* species during subsurface bioremediation and that the phosphate limitation cannot readily be alleviated with the direct addition of phosphate because of the high adsorptive capacity of sediments for phosphate. Despite this phosphate limitation U(VI) is effectively reduced, suggesting that the optimal growth of *Geobacter* species in the subsurface is not necessary for the success of this bioremediation strategy.

Phosphate is commonly added empirically as an amendment to stimulate subsurface bioremediation without information on whether the microorganisms involved in bioremediation are, in fact, phosphate-limited. An approach similar to the one described here may be useful for evaluating whether phosphate additions are warranted. For example, a strategy for promoting reductive dechlorination of chlorinated contaminants in subsurface environments is to add electron donors (Major *et al.*, 2002; Christ *et al.*, 2005), which, as with *in situ* uranium bioremediation, may result in phosphate limitations. However, the potentially complex geochemistry of phosphate must also be taken into account because as shown here, simple addition of phosphate may not materially increase the dissolved phosphate available to the microorganisms. With no amendment of phosphate, phosphate limitation does not appear to be an impediment to achieving the goal of U(VI) reduction in field experiments performed to date. Over longer times of bioremediation (years vs months) it is possible that adsorbed phosphorus could be consumed, creating a more serious phosphate limitation. Future research might usefully focus on the time required to deplete bioavailable phosphate in sediments and on approaches to releasing sediment phosphate to make it more bioavailable.

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