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ORIGINAL ARTICLE

Characterization and transcription of arsenic respiration and resistance genes during *in situ* uranium bioremediation

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The possibility of arsenic release and the potential role of *Geobacter* in arsenic biogeochemistry during *in situ* uranium bioremediation was investigated because increased availability of organic matter has been associated with substantial releases of arsenic in other subsurface environments. In a field experiment conducted at the Rifle, CO study site, groundwater arsenic concentrations increased when acetate was added. The number of transcripts from *arrA*, which codes for the α-subunit of dissimilatory As(V) reductase, and *acr3*, which codes for the arsenic pump protein Acr3, were determined with quantitative reverse transcription-PCR. Most of the *arrA* (>60%) and *acr3-1* (>90%) sequences that were recovered were most similar to *Geobacter* species, while the majority of *acr3-2* (>50%) sequences were most closely related to *Rhodoferax ferrireducens.* Analysis of transcript abundance demonstrated that transcription of *acr3-1* by the subsurface *Geobacter* community was correlated with arsenic concentrations in the groundwater. In contrast, *Geobacter arrA* transcript numbers lagged behind the major arsenic release and remained high even after arsenic concentrations declined. This suggested that factors other than As(V) availability regulated the transcription of *arrA in situ*, even though the presence of As(V) increased the transcription of *arrA* in cultures of *Geobacter lovleyi,* which was capable of As(V) reduction. These results demonstrate that subsurface *Geobacter* species can tightly regulate their physiological response to changes in groundwater arsenic concentrations. The transcriptomic approach developed here should be useful for the study of a diversity of other environments in which *Geobacter* species are considered to have an important influence on arsenic biogeochemistry.

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Introduction

The addition of organic electron donors to promote microbial reduction of soluble U(VI) to less soluble (IV) has shown promise as a strategy to prevent the spread of uranium-contaminated groundwater (Finneran *et al.*, 2002; Holmes *et al.*, 2002; Anderson *et al.*, 2003; Istok *et al.*, 2004; Vrionis *et al.*, 2005; Wall and Krumholz, 2006; Wu *et al.*, 2006, 2007; Luo *et al.*, 2007; Shelobolina *et al.*, 2008; Junier *et al.*, 2010; Prakash *et al.*, 2010; Williams *et al.*, 2011). However, U(VI) reduction is accompanied by significant microbial reduction of Fe(III) oxides (Anderson *et al.*, 2003; Vrionis *et al.*, 2005), and possibly other electron acceptors, that might have undesirable consequences. For example, when cultured in sediments from a uranium-contaminated aquifer, the subsurface isolate *G. uraniireducens* increased the transcription of genes indicative of heavy metal stress, compared with growth in defined medium (Holmes *et al.*, 2009). This might have been a response to the release of trace metals previously adsorbed onto Fe(III) oxides as the sediment Fe(III) oxides were reduced. *G. uraniireducens* also upregulated the transcription of a gene

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encoding an arsenite efflux pump, suggesting a need to respond to increased arsenic levels (Holmes *et al.*, 2009).

Elevated concentrations of arsenic have been noted in some subsurface environments in which organic matter has been introduced (Dowdle *et al.*, 1996; Ahmann *et al.*, 1997; Rowland *et al.*, 2004, 2007; Islam *et al.*, 2004, 2005a; Lear *et al.*, 2007; Héry *et al.*, 2008, 2010). In some instances, these elevated arsenic concentrations could be the result of microbial reduction of less soluble As(V) to more soluble As(III) (Dowdle *et al.*, 1996; Ahmann *et al.*, 1997; Zobrist, 2000; Mukhopadhyay *et al.*, 2002; Oremland and Stolz, 2003, 2005; Stolz *et al.*, 2006). Alternatively, As(V) adsorbed to Fe(III) oxides can be released into solution when Fe(III) oxides are reduced (Devitre *et al.*, 1991; Dixit and Hering, 2003; Campbell *et al.*, 2005; Pedersen *et al.*, 2006), or Fe(II) resulting from Fe(III) reduction can abiotically reduce As(V) (Islam *et al.*, 2004; Tufano and Fendorf, 2008a, b; Amstaetter *et al.*, 2010).

A wide phylogenetic diversity of microorganisms can utilize As(V) as an electron acceptor to support anaerobic growth (Newman *et al.*, 1997a; Oremland *et al.*, 2000; Oremland and Stolz, 2005; Hollibaugh *et al.*, 2006; Stolz *et al.*, 2006). The gene *arrA*, which encodes the α-subunit of the dissimilatory arsenate reductase protein (ArrA), has been used to document the presence of dissimilatory As(V)-reducing bacteria in a diversity of subsurface environments (Kulp *et al.*, 2004, 2006; Malasarn *et al.*, 2004; Hollibaugh *et al.*, 2006; Lear *et al.*, 2007; Pederick *et al.*, 2007). There are a number of previously described dissimilatory As(V)-reducing bacteria, such as *Shewanella trabarsenatis* ANA3 (Saltikov *et al.*, 2003a; Saltikov and Newman, 2003b) and *Sulfurospirillum barnesii, Sulfurospirillum arsenophilus* (Stolz *et al.*, 1999), *Alkaliphilus oremlandii* (Fisher *et al.*, 2008), *Bacillus arseniciselenatis* (Blum *et al.*, 1998), a *Desulfosporosinus* species (Perez–Jimenez *et al.*, 2005) and *Desulfotomaculum auripigmentum* (Newman *et al.*, 1997a, b). The presence of genes for As(V) respiration in several *Geobacter* genomes suggests that certain *Geobacter* species might also have this ability (Lear *et al.*, 2007; Duval *et al.*, 2008), and preliminary studies have shown that *G. uraniireducens*, an organism isolated from the Rifle site, can grow with As(V) provided as an electron acceptor (Héry *et al.*, 2008).

The cells of many bacteria and archaea have arsenic resistance systems that pump toxic arsenite (As(III)) out of the cell. The efflux pumps, ArsB or Acr3, are the most critical of these proteins, as the presence of these transporter proteins alone is sufficient to provide resistance to As(III) (Rosen, 1999, 2002; Oremland and Stolz, 2003). ArsB and Acr3 are unrelated families of arsenic transporters and homologs of each type are widespread throughout bacteria, archaea and fungi (Rosen, 1999; Mukhopadhyay *et al.*, 2002). Although Acr3 is less well characterized, it is present in more phylogenetically distant species than ArsB (Rosen, 1999; Achour *et al.*, 2007; Cai *et al.*, 2009). On the basis of the phylogenetic dissimilarities, Acr3 members can be divided in two subfamilies, Acr3(1) and Acr3(2) (Rosen, 1999; Wysocki *et al.*, 2003; Achour *et al.*, 2007). The presence of arsenic resistance genes can serve as biomarkers of arsenic contamination in environmental samples (Anderson and Cook, 2004; Ford *et al.*, 2005; Jackson *et al.*, 2005; Cai *et al.*, 2009).

More important than documenting the presence of a gene in the environment is quantifying the degree to which the gene is transcribed. Monitoring gene transcript abundance in subsurface microbial populations during *in situ* uranium bioremediation has been a useful strategy for documenting the physiological status of the microorganisms influencing groundwater quality (Holmes *et al.*, 2004a, 2005, 2009; O'Neil *et al.*, 2008; Mouser *et al.*, 2009a, b; N'Guessan *et al.*, 2010). Here we report on the potential for release of arsenic during *in situ* uranium bioremediation and the transcription of genes related to groundwater arsenic biogeochemistry.

Materials and methods

Subsurface site and field experiment description

During August–October 2010, a study on bioremediation of uranium-contaminated groundwater was conducted at the Department of Energy's (DOEs) Integrated Field Research Challenge (IFRC) site near Rifle, CO, USA (Anderson *et al.*, 2003; Vrionis *et al.*, 2005; Wilkins *et al.*, 2010; Williams *et al.*, 2011). The aquifer is an ~6.5-m thick heterogeneous alluvial deposit consisting of unconsolidated clay, silt, sand, gravel and cobbles lying on weathered claystone of the Wasatch formation. The groundwater table is ~3.5 m below surface, and the flow is toward the Colorado river. The experimental plot comprised of a six injection wells gallery, nine downgradient monitoring wells and one background monitoring well (Supplementary Figure S1 in Supplementary material). As previously described (Williams *et al.*, 2011), an acetate–bromide solution (50/20 mM) was prepared by mixing native groundwater pumped from an upgradient portion of the aquifer into a storage tank with sodium acetate (Sigma, St Louis, MO, USA) and sodium bromide (Sigma). This mixture was added to the subsurface via the injection wells to achieve target aquifer concentrations of 5 mM over the course of 30 days, as previously described (Anderson *et al.*, 2003; Williams *et al.*, 2011).

Groundwater sampling and geochemical analysis

Groundwater samples for chemical and molecular analyses were taken from wells CD01 and CD04 (Supplementary Figure S1). Samples for geochemical

analyses were collected after purging 12 liters of groundwater from the wells using a peristaltic pump. Ferrous iron was measured spectrophotometrically, immediately after sampling using the phenanthroline method (AccuVac ampules, Hach Company, Loveland, CO, USA) for ferrous iron. After filtration through a 0.2-μm pore-size polytetrafluoroethylene ((Teflon)) filter (Alltech Associates Inc., Deerfield, IL, USA), acetate concentrations were measured using a Dionex ICS-1000 ion chromatograph equipped with a IonPac AS22 column, an ASRS 300 suppressor, and 4.5 mM carbonate–1.4 mM bicarbonate eluent (Dionex Corporation, Sunnyvale, CA, USA). Dissolved oxygen (DO) values were obtained using a luminescent oxygen sensor (YSI Inc., Yellow Springs, OH, USA).

Samples for arsenic analysis were filtered (polytetrafluoroethylene; 0.45 μm) and preserved with trace metal grade 12 N HNO_3, and concentrations were determined using ion-coupled plasma mass spectrometry (Elan DRCII ICP-MS, PerkinElmer, CA, USA). In the 2010 field experiment, groundwater samples were not properly preserved for speciation analysis. However, samples from the same experimental plot taken in 2011 for arsenic speciation analysis showed that ~95% of the total arsenic in the background wells (CU01) was arsenate (Stucker *et al.*, submitted). It is likely that arsenate concentrations in the background well (CU01) in 2010 were similar to 2011, as other geochemical measurements were comparable.

Groundwater samples for molecular analyses were obtained after sampling for geochemical analyses by concentrating 40 liters of groundwater on a 0.2-μm pore size, 293-mm-diameter Supor-200 membrane filter (Pall Life Sciences, Ann Arbor, MI, USA). Filters were quickly sealed into a sterile whirl pack, flash frozen in an ethanol-dry ice bath, and stored at −80 °C until nucleic acid extraction.

Preparation of G. lovleyi *resting cell suspensions and measurement of arsenate*

G. lovleyi SZ was cultured in an anaerobic basal NB medium (Bond and Lovley, 2003) containing 20 mM acetate as electron donor and 20 mM fumarate as electron acceptor. The previously published protocol (Shelobolina *et al.*, 2007) was used for preparing resting cells and performing cell suspension experiments. Cells were harvested during late exponential phase, washed twice with NB medium and resuspended to an OD600 between 0.15 and 0.20 in NB medium containing acetate (20 mM), and As(V) (5 mM). A heat-killed control was prepared by autoclaving the cell suspension for 30 min before the addition of As(V). A control without acetate was also performed. The concentration of arsenate was monitored with a Dionex ICS-1000 ion chromatograph, fitted with a 4-mm ion exchange column (AS22-SC) and AG22-SC guard column (Dionex Corporation). Samples were eluted in 4 mM Na2CO3/1 mM $NaHCO_3$ (flow rate 1.2 ml min^{-1}; pressure 1.38610 Pa). Anions were detected by suppressed conductivity detection.

DNA, RNA extraction and reverse transcription

Genomic DNA and total RNA from *G. lovleyi* grown in NB medium containing acetate and fumarate, or acetate and fumarate + arsenate were extracted, as previously described (Miletto *et al.*, 2011). RNA from environmental samples was extracted using a modified phenol–chloroform method, as previously described (Holmes *et al.*, 2004a). RNA cleanup was performed using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), and RNA was treated with DNase (DNA-free Kit, Ambion, Austin, TX, USA). Successful RNA isolation was checked by visualization on a 1% (w/v) agarose electrophoresis gel in 1 × TBE buffer. The absence of DNA contamination was confirmed by PCR amplification. RNA was quantified using a NanoDrop spectrophotometer (Thermo SCIENTIFIC, Wilmington, DE, USA) and stored at −80 °C until further analyses. An Enhanced Avian HS reverse transcription-PCR kit (Sigma) was used to generate cDNA as previously described (Miletto *et al.*, 2011) cDNA was quantified using a NanoDrop spectrophotometer and stored at −80 °C until further analyses.

PCR regimens, clone library construction and phylogenetic analysis

PCRs were as follow: 1 × Q buffer (Qiagen), 0.4 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 μM of each primer (Table1), 5 μg of bovine serum albumin, 2.5 U of Taq DNA polymerase (Qiagen) and 5 ng of cDNA matrix in a final volume of 50 μl. Environmental *arrA* genes were amplified using primer sets designed elsewhere (Kulp *et al.*, 2006). Primers and conditions (Achour *et al.*, 2007) to amplify environmental *arsB/Acr3* genes were modified by Fahy *et al.* (submitted) (Table 1). All the PCR products were checked in 1% (w/v) agarose electrophoresis in 1 × TBE buffer and stained with ethidium bromide (0.2 μg ml^{-1}). DNA bands were detected under ultraviolet light. For clone library construction, PCR products were purified with the Gel Extraction Kit (Qiagen), ligated into the pCRTOPO2.1 TA cloning vector (Invitrogen, Bleiswijk, The Netherlands) and transformed into competent *Escherichia coli* TOP 10 F' cells (Invitrogen), according to the manufacturer's instructions. Recombinant clones were analyzed by PCR using primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′). Inserts of clones from the different libraries were directly sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA, USA) with M13 primers, following the manufacturer's procedures. Sequences were assembled by using the software Sequencer v4.1.4 and were compared with sequences deposited in the GenBank

Table 1 Primers used in this study for cDNA clone library construction and real-time PCR quantification of arsenic biotransformation-related genes (*arrA* and *arsB/acr3*) and the housekeeping gene *recA*

Target	*Primer set*	*Forward primer (5′–3′)*	*Reverse primer (5′–3′)*	*Annealing temperature (°C)*	*Amplicon size (bp)*	*Reference or source*
arrA	Clone library	HAArrA-D1F/HAArr-G2R	CCGCTACTACACCGAGGGCWWYT GGGRNTA	CGTGCGGTCCTTGAGCTCNWDRT TCCACC	53.5	~500	Kulp *et al*., 2006
	Real-time PCR	qArr1F/qARR1R	GATCCACGCTTCTCCACCTC	CCCCGGCTTTAAAGAGGTTC	60	~172	This study
arsB	Clone library	AarsB1F/AarsB1R	GAACATCGTCTGGAAYGCNAC	GTACACCACCAGRTACATNCC	55	~750	Achour *et al*., 2007[a]
acr3-1	Clone library	Aacr1F/Aacr2R	GGCCTGATCGTNATGATGTAYCC	GCGATGGCCAGCTCRAARTTRTT	57[b]	~750	Achour *et al*., 2007[a]
	Real-time PCR	qA1-3F/qA1-2R	ATGGCCAGCTCGAAaTTGTT		60	~94	This study
acr3-2	Clone library	dacr5F/dacr4R	TGATCTGGGTCATGATCTTCCCVAT GMTGVT	CGGCCACGGCCAGYTCRAARAARTT	52	~750	Achour *et al*., 2007[a]
	Real-time PCR	qA2-1F/qA2-1R	GGCCAGTTCGAAGAAGTTGG	GCCGATTTTGATCCAGGTGT	60	~127	This study
recA	Clone library	recA-48F/recA-583R	CCAGATHGARAARCAGTT	TTCATACGGATCTGGTTGAT	55	~540	This study
	Real-time PCR	qrecA-2F/qrecA-3R	TTGAGAAGCAGTTCGGCAAA	CGGTCCGAAGATCTCGATG	60	~156	This study

[a]Primers modified by Fahy *et al*., unpublished.
[b]57°–0.5° for 10 cycles, 52° for 30 cycles.

DNA database by using the BLAST algorithm (Altschul *et al*., 1998). Alignments were achieved by using ClustalX v1.83 (Thompson *et al*., 1997) and corrected with ProSeq v2.9 (Filatov, 2002) before the construction of phylogenetic trees with Mega v4 (Tamura *et al*., 2007). The tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method and all positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The confidence level of the phylogenetic tree topology was evaluated by performing 1000 bootstrap replications. Homologous coverage calculation and rarefaction analysis of each library was performed as previously described (Giloteaux *et al*., 2010).

Design of primers for real-time PCR

All quantitative PCR (qPCR) 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.

Primers Glov-arrA1637f (5′-ACCTTTCCATCCAG CAACAG-3′) and Glov-arrA1737r (5′-ACCCCTT GCCTTGAGTTTTT-3′) for quantitative reverse transcription PCR targeting the *arrA* gene from *G. lovleyi* were designed using Primer3 software (http://frodo.wi.mit.edu/). *ArrA* transcripts were normalized against the number of *recA* mRNA transcripts. The gene *recA* codes for DNA repair protein recombinase A, and transcription of this gene appears to be constitutive in pure cultures and in the environment (Holmes *et al*., 2005; O'Neil *et al*., 2008; Mouser *et al*., 2009b), and was selected as an external control for normalization of transcription levels. *G. lovleyi recA* gene was amplified with primers Glov-recA44f (5′-TTGAAAAGCAGTTTGG CAAA-3′) and Glov-recA199r (5′-AGGGCCATAG ACCTCAATT-3′).

The predominant sequences detected in the *arrA* and *acr3-1* cDNA clone libraries clustered with *Geobacter* and accounted for >65 and >85% of the sequences. qPCR primers were designed from these dominant sequences.

The housekeeping gene, *recA*, was used for normalization to correct for potential differences in mRNA extraction or other sampling discrepancies. Before *in situ recA* qPCR primers could be designed, it was necessary to identify the dominant *recA* sequences found in the groundwater during *in situ* bioremediation. Therefore, a degenerate PCR primer set (Table 1) that targeted an ~550-bp region of *recA* was designed from nucleotide sequences extracted from the following genomes: *G. sulfurreducens*, *G. metallireducens*, *Geobacter* sp. FRC-32, *G. uraniireducens* Rf4, *G. lovleyi* SZ, *Geobacter* sp. *M21*, *Geobacter* sp. M18, *G. bemidjiensis*, *Desulfuromonas acetoxidans*, *Pelobacter carbinolicus*, *Pelobacter propionicus*, *Dechloromonas aromatica* and *R. ferrireducens*.

To determine which *recA* transcripts were most predominant in groundwater, cDNA made by reverse transcription of RNA extracted from the groundwater was used as the template for this degenerate primer set. A clone library was then constructed, and 200 clones were selected for analyses. *Geobacter* sequences detected in the *recA* clone library were most similar to the same *Geobacter* species observed in the *arrA* and *acr3*-1 libraries. qPCR primers were designed targeting these *Geobacter* species and sequences obtained from the *arrA*, *acr3-1* and *recA* qPCR primer sets showed that they all targeted the same organisms.

The nucleotide sequences of *arrA*, *acr3-1* and *acr3-2* genes amplified from the uranium-contaminated aquifer have been deposited in the GenBank database under the accession numbers HE974855-HE974887.

Quantification of transcripts with real-time PCR

The 25 µl qPCR mixture contained 12.5 µl of Power SYBR green PCR Master Mix (Applied Biosystems), 1.5 µl of a 2.5 µM concentration of each primer, 5 ng of cDNA template in a final volume of 25 µl. PCR amplification was carried out with a 7500 real-time PCR System (Applied Biosystems). Cloned *arrA*, *acr3-1*, *acr3-2* and *recA* genes from the cDNA libraries were chosen to create a standard curve (Smith *et al.*, 2006). Standard curves covering eight orders of magnitude were constructed with serial dilutions of known amounts of purified DNA quantified with a NanoDrop ND-1000 spectrophotometer at an absorbance of 260 nm. Transcript abundances were calculated from appropriate standard curves. The qPCR efficiency (95–99%) was calculated on the basis of the slope of the standard curve. All qPCR assays were run in triplicate. Thermal cycling parameters consisted of an activation step at 50 °C for 2 min, a denaturation step at 95 °C for 10 min, and 50 cycles at 95 °C for 15 s and 60 °C for 1 min. This was followed by the construction of a dissociation curve by increasing the temperature from 60 to 95 °C at a ramp rate of 2%. A single predominant peak was observed in the dissociation curve of each gene, supporting the specificity of the PCR product.

Results and discussion

Biogeochemistry and arsenic release during acetate amendments

Pre-injection levels of DO in the Rifle aquifer were well below 0.5 mg l^{-1}, with most values <0.1 mg l^{-1}. Low DO levels are a characteristic feature of the Rifle groundwater and inferred to result from a combination of organic (for example, lignitic carbon) and inorganic (for example, Fe(II) or Fe(II)-bearing minerals) reductants present within the aquifer and associated with aquifer sediments. Following acetate amendment, DO values fell to levels at or below the detection limit (<0.01 mg l^{-1}) of the luminescent oxygen sensor used to quantify DO, and remained at these low levels for the duration of the experiment. DO values for locations not impacted by acetate varied from 0.2 to 0.5 mg l^{-1} over the experimental period. pH values were largely stable during the experiment, ranging from 7.1 to 7.2, owing to the well-buffered nature of the groundwater and sediments.

Before acetate amendments, groundwater arsenic concentrations were ca. 1.5 µM and arsenic remained at these levels in background wells not amended with acetate throughout the field experiment (Figure 1). With the addition of acetate, there was a rapid increase in arsenic in well CD01, the well closest to the injection gallery (Figure 1a). The increase in arsenic was associated with an accumulation of Fe(II), which continued accumulating even as arsenic declined. Previous studies have demonstrated a similar coincident release of arsenic and Fe(II) under anaerobic conditions (Rochette *et al.*, 1997, 2000). Arsenic adsorbed to the surface of Fe(III) oxides or incorporated within the Fe(III)-

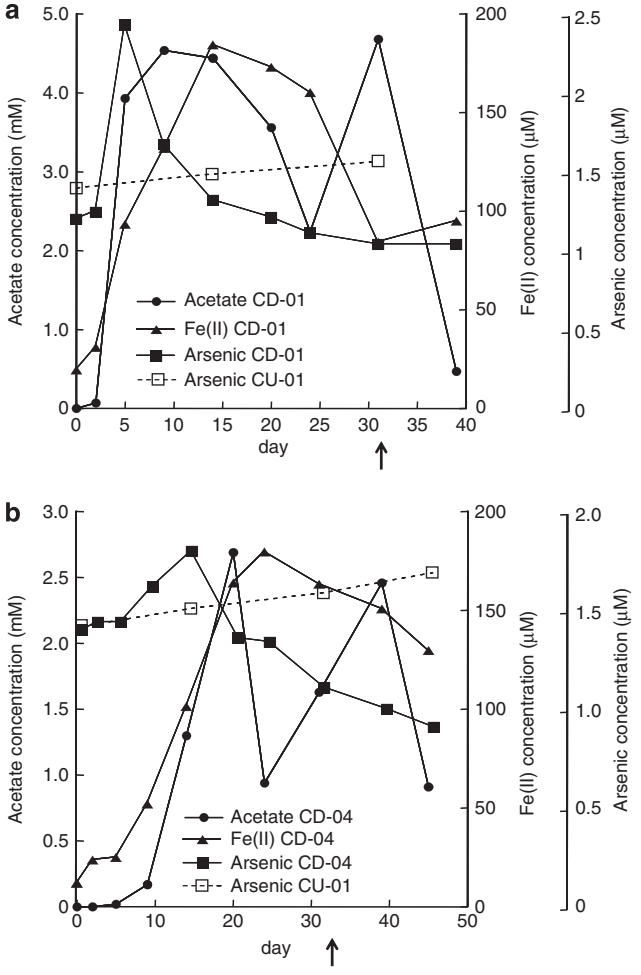

Figure 1 Acetate, Fe(II) and arsenic concentrations in well CD01 (**a**) and CD04 (**b**) and arsenic concentrations in the background well CU-01 (**a**) during acetate amendment at the Rifle site. The arrow indicates the end of acetate injection at day 31.

containing minerals may be released when Fe(III) is reduced (Cummings *et al.*, 1999; Nickson *et al.*, 2000; McArthur *et al.*, 2001; Bose and Sharma, 2002; Harvey *et al.*, 2002; Nicholas *et al.*, 2003; Horneman *et al.*, 2004; Van Geen *et al.*, 2004), and microbial reduction of As(V) to As(III) can enhance mobilization (Nicholas *et al.*, 2003; Oremland and Stolz, 2003; Lloyd and Oremland, 2006).

After the initial arsenic release, arsenic concentrations slowly started to decline after day 5 in CD01 and day 14 in CD04, even though acetate continued to be injected until day 31 (Figure 1). One potential explanation for this decline in arsenic is the fact that the divalent cations Ca^{2+}, Mg^{2+} and Fe^{2+} increased significantly in the groundwater (Supplementary Figure S2), and studies have shown that these ions promote As precipitation (Appelo *et al.*, 2002; Smedley and Kinniburgh, 2002; Smith *et al.*, 2002). Precipitation with sulfide (Newman *et al.*, 1997b) and a change in pH (Smedley and Kinniburgh, 2002) were unlikely to have affected arsenic release as sulfide was not detected, and the pH was neutral throughout this field experiment.

Arsenic concentrations in the downgradient well, CD04, were lower than those observed in CD01 and increased before significant acetate concentration reached the site. The complex dynamics of dissolved arsenic noted above make it difficult to determine which of the arsenic release and precipitation possibilities were responsible for this pattern, but the initial increase in dissolved arsenic in CD04 may have resulted from dissolved arsenic moving downgradient from CD01.

Although the arsenic in the groundwater was not speciated, the following year, acetate addition and the onset of reducing conditions resulted in increased concentrations of As(III) in groundwater collected from the same well (45–65% of the arsenic was As(III)) (Stucker *et al.*, submitted). Although we cannot rule out the formation of other arsenic species during the 2010 amendment experiment, that is, thioarsenate (Stucker *et al.*, submitted), it is likely that arsenic speciation patterns in 2010 were similar.

Phylogenetic analysis of transcribed arsenic functional genes detected from Rifle groundwater

To determine which *arrA*, *arsB* and *acr3* sequences were being transcribed during the *in situ* bioremediation experiment, mRNA was extracted from the groundwater and cDNA libraries were made from *arrA*, *arsB* and *acr3* transcripts. The predicted amino-acid sequences for the arsenic reductase subunit ArrA in the transcribed genes recovered from CD01 and CD04 formed four distinct phylogenetic clusters, sharing 60–98% similarity with each other (139 amino-acid sequences considered) (Figure 2). Sequences in cluster A accounted for 67 and 73% of the ArrA sequences recovered from CD01 and CD04, respectively, and shared 64–78% and 64–82% amino-acid sequence similarity with *G. lovleyi* and *G. uraniireducens* ArrA proteins, respectively. Clusters B and C branched separately in the phylogenetic tree and represented 22% and 33% of the clones from CD04 and CD01, respectively. These sequences could not be affiliated with confidence to any known sequences of previously described As(V)-respiring bacteria. Sequences from cluster D represented 5% of the clones from CD04

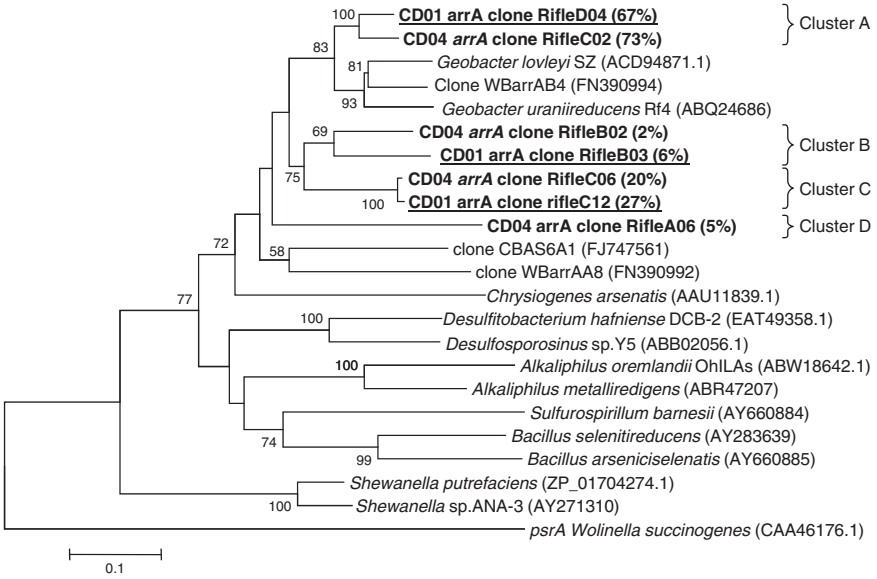

Figure 2 Phylogenetic comparison of ArrA sequences found in the groundwater to ArrA from other known arsenate respiring bacteria (139 amino acids considered). Clones obtained from both CD01 and CD04 wells in this study are in bold, and clones from CD01 are underlined. The percentage of clones is indicated in brackets. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above or near the relevant nodes.

samples, branched separately in the phylogenetic tree, and thus could not be affiliated with any known As(V) respirers.

Overall, the majority (67% and 73% for CD01 and CD04, respectively) of *arrA* sequences were most closely related to sequences previously described in *Geobacter* species, with little nucleotide diversity among the sequences (Figure 2). Rarefaction analysis revealed high diversity coverage values (0.96 and 0.93 for CD01 and CD04, respectively), and the rarefaction curves reached a plateau (see Supplementary data; Figure S3). The most predominant species were most similar to *G. uraniireducens* Rf4, which was isolated from the Rifle site (Shelobolina *et al.*, 2008) and *G. lovleyi*. The high similarity of the *arrA* clones to putative *arrA* genes from *Geobacter* spp. retrieved in this study is of particular interest as *Geobacter* spp. are the dominant Fe(III)-reducing bacteria in Rifle (Holmes *et al.*, 2002, 2007; Anderson *et al.*, 2003), and their presence at relatively high abundance in bacterial communities has previously been correlated with As release in Asian aquifers (Islam *et al.*, 2004; Lear *et al.*, 2007; Rowland *et al.*, 2007; Héry *et al.*, 2008).

Transcripts of the genes *arsB* and *acr3*, which encode membrane subunits from an arsenic oxyanion translocation pump (Rosen, 1999, 2002), were evaluated in order gain insight into arsenic resistance. The ArsB sequences recovered were highly diverse, with clones mainly related to the *Gammaproteobacteria* and *Firmicutes* classes (data not shown). *Gammaproteobacteria* and *Firmicutes* only accounted for 0–5% and 2–9% of the 16S rRNA sequences detected during active uranium bioremediation in this field experiment (Holmes *et al.*, submitted), and thus the *AarsB* genes were not studied further.

Phylogenetic studies have shown that the other arsenic resistance gene, *acr3,* is very diverse and two acr3 subfamilies, *acr3-1* and *acr3-2*, are seen among bacteria (Wysocki *et al.*, 2003; Achour *et al.*, 2007). Sequences from both subfamilies were recovered in groundwater collected from this site (Figure 3). The majority, that is, 90% and 86% for CD01 and CD04, respectively, of *acr3-1* sequences (Figures 3a and 4) were most similar to *Geobacter* species (Figures 3a and 4) and shared 86–96% and 83–91% amino-acid similarity to *G. uraniireducens*, an isolate recovered from the Rifle site (Shelobolina *et al.*, 2008) and *G. lovleyi* ACR3-1 proteins (Figure 3a). These numbers are consistent with results from 16S rRNA clone libraries and FISH analysis showing that up to 89% of the 16S rRNA sequences from well CD04 were most similar to *Geobacter* species during active Fe(III) reduction (Holmes *et al.*, submitted).

Less abundant were other sequences in the *Deltaproteobacteria*, most closely related to *Desulfovibrio* or *Anaeromyxobacteria* sequences. Sequences which shared 97% similarity with the *Betaproteobacteria* member *D. aromatica* were also detected (Figures 3 and 4). The high coverage values calculated for *acr3-1* clone libraries (0.83 and 0.8 for CD01 and CD04, respectively) together with rarefaction analysis suggested that the almost entire diversity of *acr3-1* genes was covered with these clone libraries (Supplementary Figure S3).

Sequences that fell within the ACR3-2 subfamily of arsenic transporters were related to the *Proteobacteria* and *Verrucomicrobia* phyla (Figure 3b). More than 65% of the *acr3-2* sequences were affiliated with members of the *Betaproteobacteria* class, with the majority of clones (>50%) being most closely related to *acr3-2* sequences from the Fe(III)-reducing bacterium, *R. ferrireducens* (Figure 3b). The diversity of *acr3-2* sequences was significantly higher than what was seen in *acr3-1* clone libraries in contrast with previous studies (Achour *et al.*, 2007; Cai *et al.*, 2009) and clone library coverage values from *acr3-2* libraries were 0.61 and 0.65 for CD01 and CD04 samples respectively.

Some bacteria have genes coding for proteins involved in both arsenic reduction and arsenic resistance (that is, *arr* and *ars* operons). This has been observed in *Shewanella* strain ANA-3 (Saltikov and Olson, 2002; Saltikov *et al.*, 2003a, 2005; Saltikov and Newman, 2003b), *G. uraniireducens* and *G. lovleyi.* In many of these organisms, all of these genes are located in an arsenic island within the genome. For example, the locus tag for the *arr* operon in *G. uraniireducens* is Gura_0469-Gura_0471 and the locus tag for the arsenic resistance genes is Gura_0468 to Gura_0467.

Analysis of As(V) respiration in Geobacter

A putative *arr* operon is present in the genomes of both *G. lovleyi* and *G. uraniireducens*. Preliminary analysis of this operon shows that it consists of three functional genes: a gene coding for a hypothetical protein (Glov_1150 and Gura_0469); a gene coding for a protein that has a molybdopterin cofactor binding site and is homologous to the α-subunit of the arsenate respiratory reductase protein ArrA (Glov_1149 and Gura_0470); and a gene coding for a protein that is homologous to the beta subunit of arsenate respiratory reductase ArrB (Glov_1148 and genes 3 and 4 in *G. uraniireducens*). The *G. uraniireducens arr* operon actually has four putative genes; however, only three of them appear to be functional. There are two ferredoxin genes that could potentially encode ArrB; however, one of them (gene 3) contains a stop codon in the 342 basepair (Figure 5b). Further analysis of the *arr* operon in both *G. uraniireducens* and *G. lovleyi* showed that it contains Shine-Delgarno ribosome binding sites upstream from all three start codons, a promoter region, and a *rho*-independent terminator region (Figure 5).

Further evidence of functionality of the Arr protein found in *G. lovleyi* comes from the fact that ArrA and ArrB from *G. lovleyi* are 60.3% and 53%

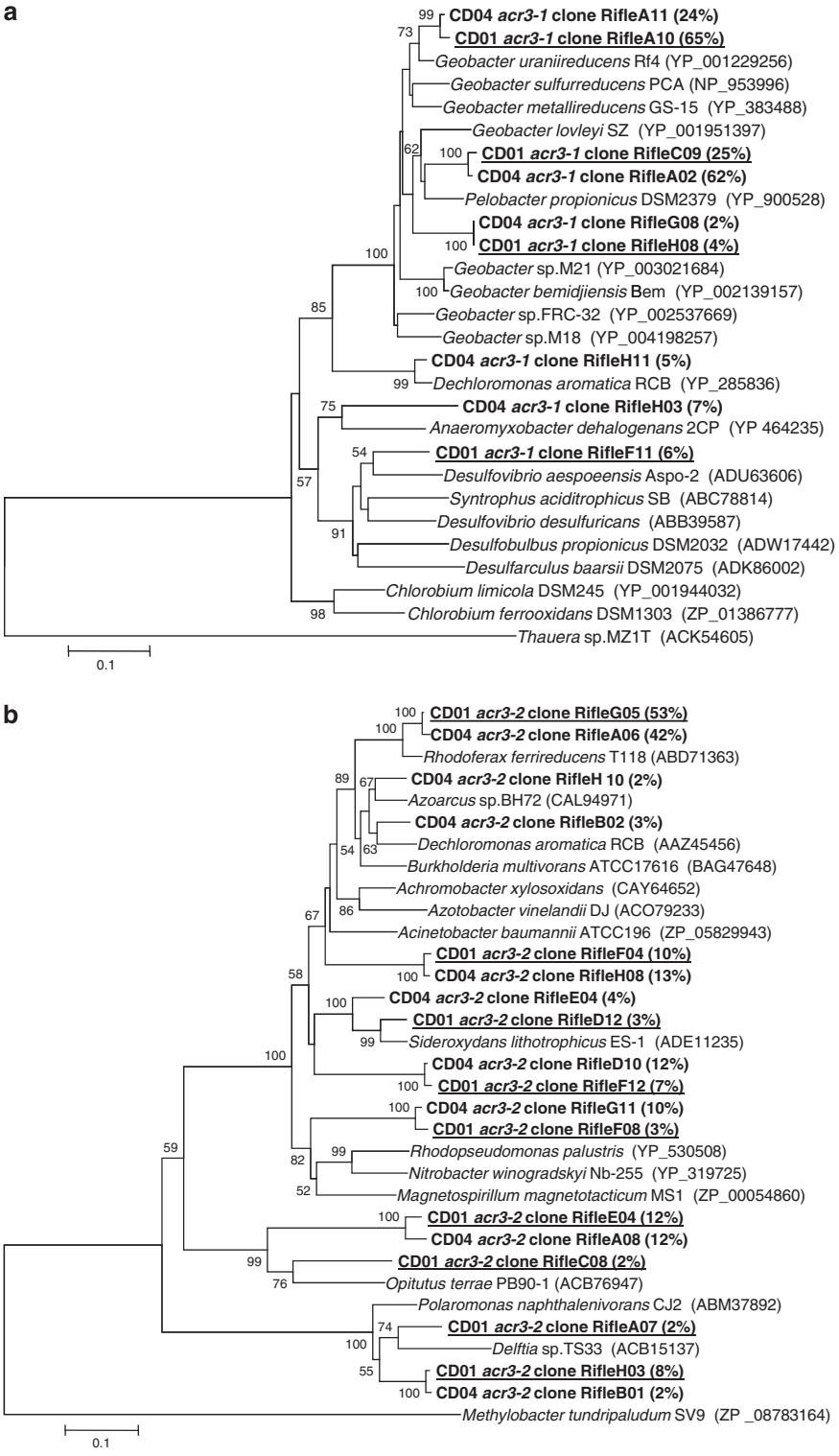

Figure 3 (**a**) Phylogenetic comparison of Acr3-1 sequences found in the groundwater to Acr3-1 from other known arsenic-resistant bacteria (257 amino acids considered) and (**b**) phylogenetic comparison of Acr3-2 sequences found in the groundwater to Acr3-2 from other known arsenic-resistant bacteria (259 amino acids considered). Clones obtained from both CD01 and CD04 wells in this study are in bold, and clones from CD01 are underlined. The percentage of clones is indicated in brackets. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above or near the relevant nodes.

similar to ArrA and ArrB found in *Shewanella* sp. *ANA-3*, a known arsenate respiring bacterium (Saltikov and Newman, 2003b). As shown in Supplementary Figure S4, the *arrA* gene from both organisms codes for a molybdopterin oxidoreductase protein, and contains: (i) a cysteine-rich

motif (C-X_2-C-X_3-C-X_{27}-C) that is predicted to coordinate an iron–sulfur cluster; (ii) a conserved cysteine residue of the molybdenum-binding domain, which is likely to represent the amino acid that coordinates to the molybdenum; (iii) a twin arginine translocation (TAT) signal (RRDFLK); and (iv) a potential cleavage site at Ala-31, which is indicative of localization to the cytoplasmic membrane. Comparative analysis of ArrB from *G. lovleyi* showed that this gene codes for a 4Fe-4 S ferredoxin iron-sulfur binding domain protein similar to ArrB found in the *Shewanella* sp. *ANA-3* (Supplementary figure S4).

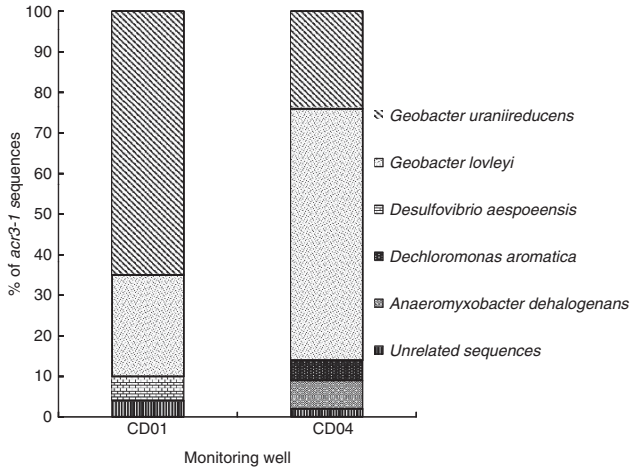

Figure 4 Relative abundance of *acr3-1* sequences from two downgradient monitoring wells CD01 and CD04.

It has also been reported that *G. uraniireducens* can grow with As(V) provided as an electron acceptor (Héry *et al*., 2008), and cell suspensions of *G. lovleyi* reduced As(V) with acetate as the electron donor (Figure 6a). There was no As(V) reduction in acetate-free controls. Attempts to grow *G. lovleyi* with As(V) as the sole electron acceptor were not successful. However, when *G. lovleyi* was grown with fumarate as an electron acceptor in addition to As(V), *arrA* transcripts were 3 fold more abundant than in cultures grown with fumarate as the electron acceptor in the absence of As(V) (Figure 6b), demonstrating a transcriptional response to the presence of As(V).

Transcription and abundance of arsenic functional genes over time

It was not feasible to track gene transcription patterns for the full diversity of *arrA*, *acr3-1* and *acr3-2* genes over time. Therefore, this study focused on transcription of the dominant sequences from the *arrA* and *acr3-1* clone libraries from CD01 and CD04; which were most similar to *Geobacter* species, the predominant members of the microbial community.

The abundance of transcripts for the arsenic resistance *acr3-1* genes associated with *Geobacteraceae* species closely tracked the abundance of arsenic in the groundwater (Figure 7). There was a significant positive correlation between the number of *Geobacter acr3-1* transcripts and arsenic concentrations (Pearson's correlation $r = 0.91$ and 0.84

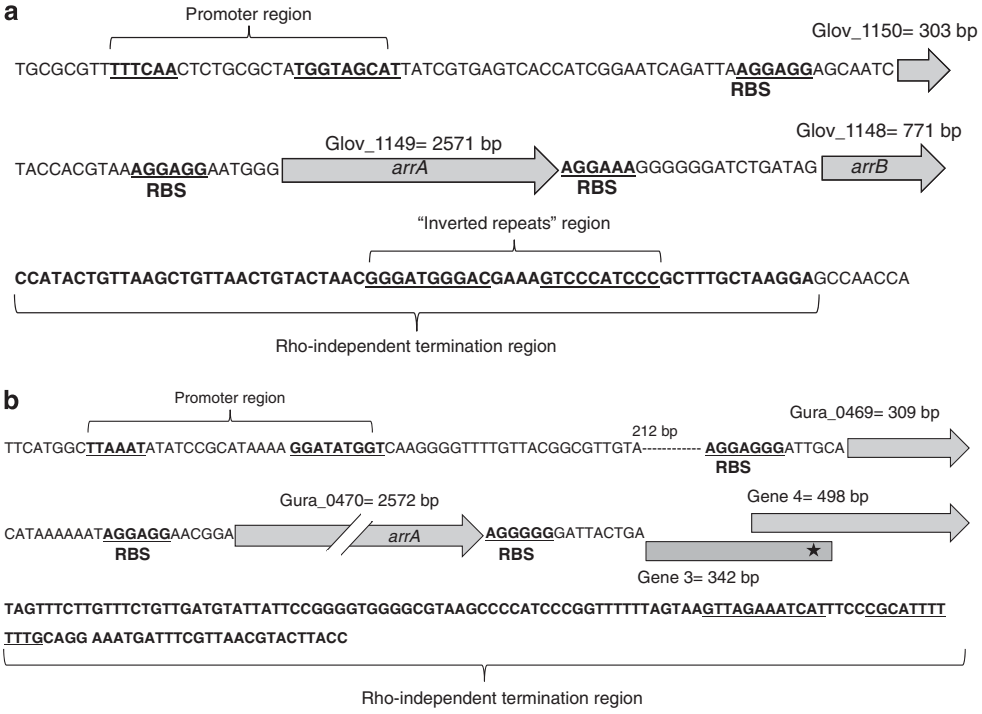

Figure 5 The *arr* operon of *G. lovleyi* (**a**) and *G. uraniireducens* (**b**), including the promoter region and termination site. RBS, ribosome-binding site

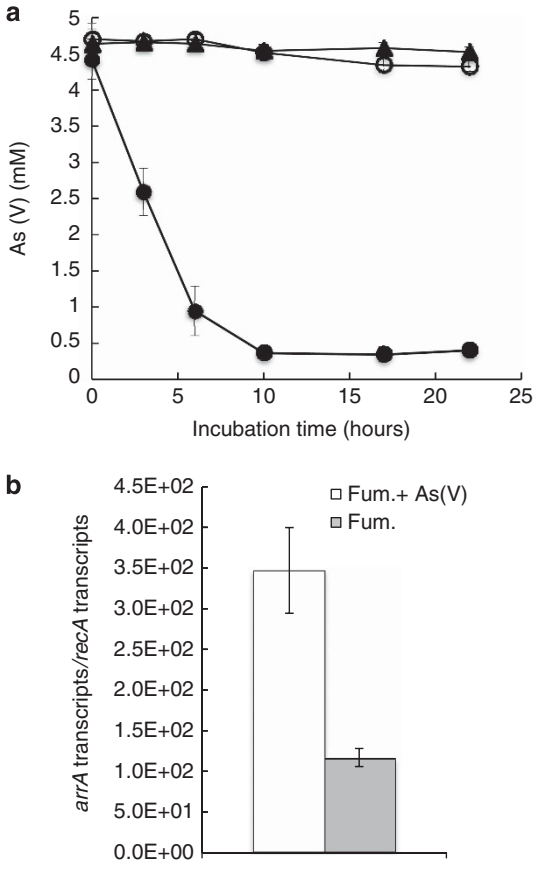

Figure 6 (**a**) Measurement of As(V) concentrations in *G. lovleyi* cell suspensions provided 5 mM As(V). (●) Resting cells with acetate (20 mM) added as an electron donor; (▲) Heat-killed control cells; and (○) Resting cells without acetate. (**b**) Transcription levels of *G. lovleyi arrA* gene, normalized against *G. lovleyi recA*, in cells grown with either fumarate or fumarate and As(V) as potential electron acceptors.

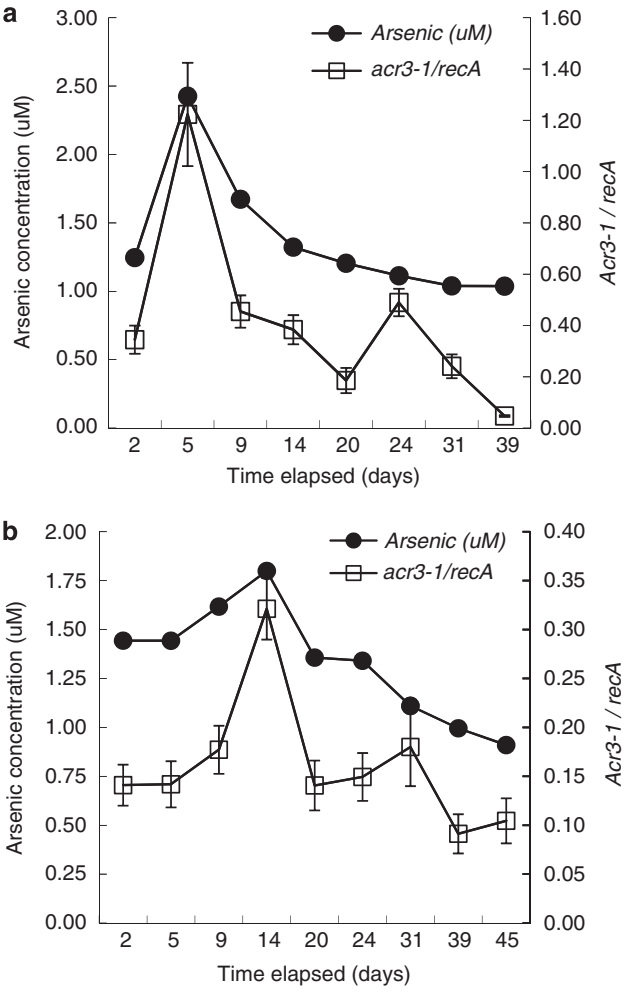

Figure 7 Arsenic concentrations and transcripts levels of *acr3-1* normalized to *recA* in well CD01 (**a**) and CD04 (**b**) over the course of the biostimulation experiment at Rifle, CO, USA. Error bars represent propagation of errors of triplicate real-time PCR reactions. Open squares, *acr3-1* genes; closed circles, arsenic.

($P<0.05$) and Spearman's correlation $R=0.67$ and 0.81 ($P<0.05$) for CD01 and CD04, respectively). Transcript abundance did not correspond well with acetate ($r=0.31$ and -0.11 ($P>0.05$) and $R=0.21$ and -0.33 ($P>0.05$)) or Fe(II) concentrations ($r=-0.08$ and -0.16 ($P>0.05$) and $R=0.04$ and -0.24 ($P>0.05$)).

The abundance of *Geobacter arrA* transcripts in the subsurface community lagged behind the increases in dissolved arsenic and remained high even after groundwater arsenic concentrations declined (Figure 8). There was some correspondence between the number of *arrA* transcripts and acetate ($r=0.63$ and 0.52 ($P<0.05$) and $R=0.52$ and 0.74 ($P<0.05$) for CD01 and CD04) and Fe(II) concentrations ($r=0.8$ and 0.64 ($P<0.05$) and $R=0.62$ and 0.79 ($P<0.05$) for CD01 and CD04).

When acetate concentrations declined after the acetate amendments were stopped on day 31, there was a dramatic decline in the relative transcription of *arrA* (Figure 8). This gene transcription pattern is similar to that previously noted for central metabolic genes (Holmes *et al*., 2004a, 2005, 2007), such as citrate synthase (Holmes *et al*., 2005), and suggests that increased transcription of this respiratory gene could be part of an overall increase in genes involved in energy generation, rather than a specific response to the availability of As(V) as an electron acceptor.

Implications

The results demonstrate that the subsurface *Geobacter* population rapidly responded to changes in arsenic concentrations in the groundwater by modulating the transcription of a gene for arsenic resistance. This result further emphasizes that transcriptome analysis is a powerful method for diagnosing the physiological status of subsurface microbial communities.

The factors controlling the transcription of *arrA*, presumed to encode a subunit of a dissimilatory As(V) reductase, is less clear. *G. lovleyi*, which was shown to be capable of acetate-dependent As(V) reduction, increased transcription of *arrA* in the presence of As(V). However, during uranium

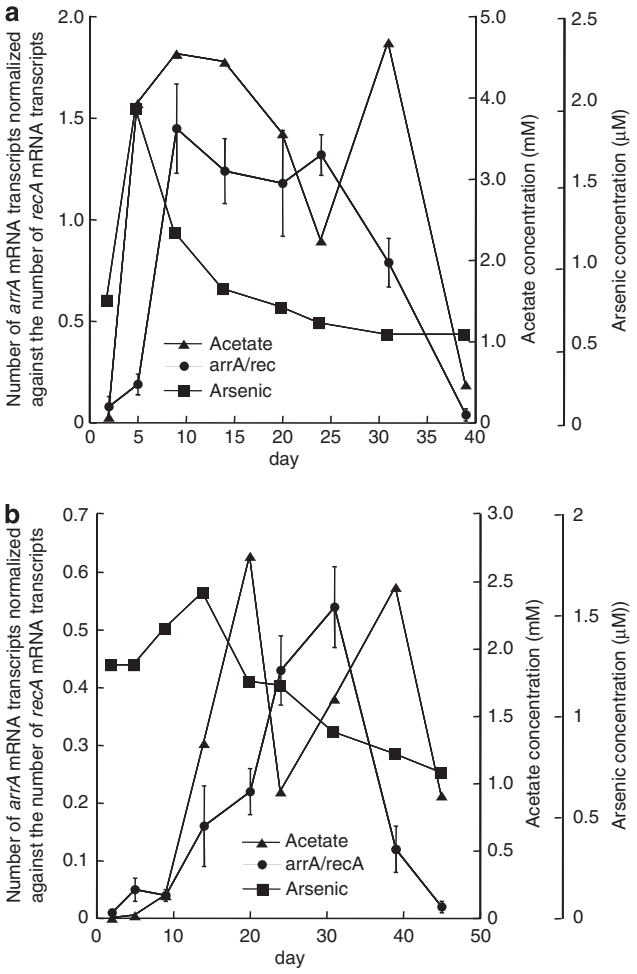

Figure 8 Arsenic and acetate concentrations and transcripts levels of *arrA* normalized to *recA* in well CD01 (**a**) and CD04 (**b**) over the course of the biostimulation experiment at Rifle, CO, USA. Error bars represent propagation of errors of triplicate real-time PCR reactions. Closed circles, *arrA* genes; closed triangles, acetate; closed squares, arsenic.

bioremediation *arrA* transcription patterns appeared to be correlated with the availability of acetate rather than arsenic concentrations in the subsurface. This enhanced transcription of *arrA* when acetate levels were high might be associated with a generalized increase in anaerobic respiratory genes under these conditions. More information on the availability of As(V) as an electron acceptor throughout the bioremediation process will be required to better interpret this data.

Quantifying the abundance of 16S rRNA gene sequences or genes more directly related to arsenic have demonstrated an association of *Geobacter* species with arsenic release in aquifer sediments and arsenic-rich lake sediments (Islam *et al.*, 2004; Héry *et al.*, 2008, 2010). The transcriptomic approach reported here has the potential to provide more in-depth analysis of the metabolic activity associated with arsenic biogeochemistry in such environments because gene transcription is more directly related to physiological activity than gene presence. A similar transcriptomic approach should be applicable to the study of subsurface arsenic biogeochemistry in environments in which organisms other than *Geobacter* species predominate.

Conflict of interest

The authors declare no conflict of interest.

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