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Molecular Analysis of the Metabolic Rates of Discrete Subsurface Populations of Sulfate Reducers

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Elucidating the in situ metabolic activity of phylogenetically diverse populations of sulfate-reducing microorganisms that populate anoxic sedimentary environments is key to understanding subsurface ecology. Previous pure culture studies have demonstrated that the transcript abundance of dissimilatory (bi)sulfite reductase genes is correlated with the sulfate-reducing activity of individual cells. To evaluate whether expression of these genes was diagnostic for subsurface communities, dissimilatory (bi)sulfite reductase gene transcript abundance in phylogenetically distinct sulfate-reducing populations was quantified during a field experiment in which acetate was added to uranium-contaminated groundwater. Analysis of dsrAB sequences prior to the addition of acetate indicated that Desulfobacteraceae, Desulfobulbaceae, and Syntrophaceae-related sulfate reducers were the most abundant. Quantifying dsrB transcripts of the individual populations suggested that Desulfobacteraceae initially had higher dsrB transcripts per cell than Desulfobulbaceae or Syntrophaceae populations and that the activity of Desulfobacteraceae increased further when the metabolism of dissimilatory metal reducers competing for the added acetate declined. In contrast, dsrB transcript abundance in Desulfobulbaceae and Syntrophaceae remained relatively constant, suggesting a lack of stimulation by added acetate. The indication of higher sulfate-reducing activity in the Desulfobacteraceae was consistent with the finding that Desulfobacteraceae became the predominant component of the sulfate-reducing community. Discontinuing acetate additions resulted in a decline in dsrB transcript abundance in the Desulfobacteraceae. These results suggest that monitoring transcripts of dissimilatory (bi)sulfite reductase genes in distinct populations of sulfate reducers can provide insight into the relative rates of metabolism of different components of the sulfate-reducing community and their ability to respond to environmental perturbations.

A major goal of microbial ecology is not only to know what microorganisms are present and the metabolic potential of those organisms as revealed in their genomes but also to understand key in situ physiological characteristics, such as rates of metabolism of individual components of the community. Dissimilatory sulfate reduction has a key role in the global sulfur cycle and represents one of the most important organic matter mineralization processes in a diversity of environments. Sulfate-reducing prokaryotes (SRP) can colonize a variety of niches in marine (11, 13, 26, 55), brackish (27, 32), freshwater (3, 7, 30, 33, 43, 52, 56), and extreme environments (22, 24, 36, 60). SRP are also of interest for their economical relevance in the remediation of naturally or anthropogenically contaminated habitats (1, 9, 16, 18, 23, 25) and for their involvement in the corrosion of metallic oil, gas, or potable water pipelines (37, 48, 50).

Studies on chemostat cultures of Desulfovibrio vulgaris demonstrated that transcript abundance for the gene dsrA, which encodes the α subunit of the dissimilatory (bi)sulfite reductase (DSR) (12), was directly proportional to the sulfate reduction rate in individual cells and that sulfate reduction rates per cell varied significantly depending upon growth rates of the cells and whether the growth of the cells was limited by the availability of electron acceptor or electron donor (57). Thus, abundance of dsrA transcripts in sediments (10) cannot be used to estimate bulk rates of sulfate reduction without additional physiological data not readily obtained with current environmental technologies. However, dsrA transcript abundance can be a guide to the metabolic rate of the individual cells in that environment.

SRP are phylogenetically and physiologically diverse. Although unified by their sulfate-reducing ability, SRP are polyphyletic (i.e., they can be divided in four distinct bacterial phyla and one archaeal phylum), comprising more than 150 cultured species divided into 40 genera (17). Depending on the species, SRP couple the oxidation of H₂ or a variety of carbon substrates to acetate (incomplete oxidizers) or CO₂ (complete oxidizers) to the reduction of sulfate or alternative (in)organic (non)sulfur electron acceptors (47). In the absence of electron acceptors, SRP are also able to perform fermentation (47). Therefore, in order to better understand the in situ physiology of sulfate-reducing microorganisms, it would be beneficial to separately track the metabolism of physiologically distinct populations of sulfate reducers.

One feature in which sulfate reducers differ significantly is their ability to reduce U(VI). Microbial U(VI) reduction is expected to play an important role in the natural cycling of uranium (28). Furthermore, it is an attractive bioremediation tool because reduction of highly soluble U(VI) to poorly soluble U(IV) can be an effective strategy for reducing the mobility of uranium in contaminated subsurface environments (14). Some sulfate reducers such as Desulfovibrio sp. (29),
Desulfotomaculum reducens (54), and Desulfosporosinus sp. (53) are effective U(VI) reducers, whereas others, such as Desulfobacter postgatei, Desulfobulbus propionicus, and Desulfofaba acetoxidans (29), are not. Therefore, information on which populations of sulfate reducers are active under different conditions could greatly aid in the design of strategies for groundwater uranium bioremediation and better constrain the metabolic diversity underlying enzymatic removal processes during uranium bioremediation.

Here, we demonstrate that it is possible to track the activity of different populations of sulfate reducers by individually monitoring transcript abundance for dissimilatory (bi)sulfite reductase genes for each population.

**MATERIALS AND METHODS**

**Site description.** During July to September 2008, a study on bioremediation of uranium-contaminated groundwater was conducted at the Department of Energy (DOE) Rifle Integrated Field Research Challenge (IFRC) site near Rifle, CO (2, 58, 62). Briefly, the site is a floodplain of the Colorado River located in northwestern Colorado. The aquifer is a ~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 was a 12-m by 18-m flow cell comprised of three up-gradient monitoring wells, 10 injection wells, and 12 down-gradient monitoring wells (Fig. 1). Groundwater samples for chemical and molecular analyses were taken from the representative well D04. This is an anoxic site, as demonstrated by the presence of Fe(II) in the samples for chemical and molecular analyses (well D04). (Courtesy of the U.S. Department of Energy.)

Prior to the initiation of the acetate injection reported here, the site had been under natural groundwater flow without amendments for ~11 months, following a previous, short-duration (ca. 21-day) acetate addition study in 2007 (62).

Groundwater samples for geochemical analyses were collected every 2 days after purging 10 liters of groundwater from the well using a peristaltic pump. Sulfide and ferrous iron were measured spectrophotometrically immediately after sampling using the methylene blue method (hydrogen sulfide test; Hach Company, Loveland, CO) for sulfide and the phenanthroline method (AccuVac ampules; Hach Company) for ferrous iron. After filtration through a 0.2-μm-pore-size polytetrafluoroethylene (PTFE [Teflon]) filter (Alltech Associates, Inc., Deerfield, IL), bromide, acetate, and sulfate concentrations were measured using an 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), while U(VI) was measured using a kinetic phosphorescence analyzer (46).

Groundwater samples for molecular analyses were obtained after sampling for geochemical analyses by concentrating 10 liters of groundwater on a 0.2-μm-pore-size, 293-mm-diameter Supor-200 membrane filter ( Pall Life Sciences, Ann Arbor, MI). 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.

**Nucleic acid extraction.** Nucleic acids were extracted from portions of the same filter and crushed with liquid nitrogen (34). Equal volumes of homogenized filter fragments were used for parallel DNA and RNA extractions. Genomic DNA (gDNA) was extracted using a FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH). gDNA was quantified using a NanoDrop spectrophotometer and stored at ~80°C until further analyses.

RNA was extracted using a modified phenol-chloroform method (20). RNA cleanup was performed using an RNasy Mini Kit (Qiagen GmbH, Hilden, Germany), and RNA was treated with DNase (DNA-free Kit, Ambion, Austin, TX). Successful RNA isolation was checked by visualization on a 1% agarose gel. The absence of DNA contamination was confirmed by PCR amplification. RNA was quantified using a NanoDrop spectrophotometer and stored at ~80°C until further analyses.

**dsrAB clone library construction and phylogenetic analysis.** The primers used in this study are listed in Table 1. PCR amplification of an approximately 1.9-kbp dsrAB fragment was performed using the primers DSR1F mix (equimolar mixture of DSR1R, DSR1Fca, DSR1Fb, DSR1Fc, and DSR1Fd) and DSR4Rmix (equimolar mixture of DSR4R, DSR4Ra, DSR4Rb, DSR4Rc, DSR4Rd, and DSR4Re) and the following cycling conditions: initial denaturation at 94°C for 1 min, 35 cycles of 40 s of denaturation at 94°C, 40 s of annealing at 54°C, and 1.5

**FIG. 1.** Aerial view of the uranium bioremediation field site in Rifle, CO. Within the flow cell, the arrow indicates the well sampled for chemical and molecular analyses (well D04). (Courtesy of the U.S. Department of Energy.)
min of elution at 72°C, with a final elution at 72°C for 10 min (30). A positive control of purified dsrAB PCR product from Desulfovibrio vulgaris and a negative control without DNA were always included in PCR amplification experiments. The reaction was carried out in a PTC200 Peltier Thermal Cycler (MJ Research, Waltham, MA). The 50-μl reaction mixture contained 100 ng of DNA, 1× Q-Solution (Qiagen), 1× PCR buffer (Qiagen), 1.5 mM MgCl₂ (Qiagen), a 200 μM concentration of each deoxynucleotide (Sigma), a 0.5 μM concentration of each primer, 0.5% bovine serum albumin (BSA: New England Biolabs, Beverly, MA), and 1.25 U of Taq DNA polymerase (Qiagen). The presence and size of the amplification products were determined by agarose (1%, wt/vol) gel electrophoresis. Bands of the expected sizes were purified from the gel by excision with a sterile surgical blade and purified with a QIAquick Gel Extraction Kit as recommended by the manufacturer (Qiagen).

Clone libraries were constructed from nine representative samples (day 0, 3, 10, 13, 26, 34, 45, 47, and 53 following acetate injection). Four microliters of the reaction mixture, and the samples were incubated at 50°C for 50 min. cDNA was recovered with a total of 1,123 nucleotides; filters were used to exclude transcripts. The reverse transcription (RT) reaction was carried out in two steps. First, the RT master mix contained 2 μl of the appropriate reverse primer (2 μM), 2 μl of deoxynucleoside triphosphate (dNTP) mix (1 mM each dNTP), and 1 μl of diethyl pyrocarbonate (DEPC)-treated water; 5 μl of RNA template (0.01 to 5 μg of RNA) was added for a total reaction mixture volume of 10 μl, and the mixture was incubated at 70°C for 10 min. Then, the PCR master mix (10 μl) consisting of 2 μl of avian myeloblastosis virus (AMV) reverse transcription buffer (1×), 1 μl of RNase inhibitor (1 U/ml), 1 μl of Enhanced AMV reverse transcriptase (1 U/μl), and 6 μl of DEPC-treated water was added to the RT mixture, and the samples were incubated at 50°C for 50 min. cDNA was quantified using a NanoDrop spectrophotometer and stored at −80°C until further analyses.

Quantification of genes and transcripts. The 25-μl qPCR mixture contained 12.5 μl of Power SYBR green PCR Master Mix (Applied Biosystems Inc., Foster City, CA), 1.5 μl of a 150 mM concentration of each primer, and 9.5 μl of a 1:10 dilution of gDNA (dsrB) or cDNA (dsrB transcripts) template. qPCR results were normalized to the total amount of gDNA/cDNA in the 9.5 μl of template solution used to set up the qPCRs. Standard curves were constructed with serial dilutions of known amounts of dsrB amplified with the appropriate primers from environmental gDNA, purified, and quantified with a NanoDrop spectrophotometer. Serial dilutions covered a range of 8 orders of magnitude of template copies per assay (10² to 10⁹). R² values ranged from 0.992 to 0.999. The qPCR efficiency (90% to 95%) was calculated based on the slope of the standard curve. All qPCR assays were run in triplicate. PCR amplification was carried out with a 7500 Real-Time PCR System (Applied Biosystems). Thermal cycling parameters consisted of an activation step at 95°C for 2 min, a denaturation step at 95°C for 10 min, and 50 cycles at 95°C for 15 s and 55°C for 1 min. Amplification and correct amplicon size were verified by running aliquots of qPCRs on an ethidium bromide-stained 1% agarose gel. gDNA extracts were tested for PCR-inhibitory substances by a serial dilution of the template gDNA and subsequent qPCR. Templates were normalized to an equal amount of gDNA/cDNA to enable comparison of different time points.

Nucleotide sequence accession numbers. Representative concatenated partial dsrA and dsrB nucleotide sequences determined in this study have been submitted to the NCBI database under accession numbers HQ690090 to HQ690096.

**RESULTS AND DISCUSSION**

Evidence for acetate additions driving sulfate reduction. As previously reported (62), acetate concentrations in groundwater pumped from well D04 initially increased in response to injection (Fig. 2). As soon as acetate was introduced, there appeared to be an increase in sulfate reduction, as evidenced

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**TABLE 1. Primers targeting SRP used in this study**

<table>
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<tr>
<th>Primer name</th>
<th>5′–3′ Sequence</th>
<th>Target gene</th>
<th>Specificity</th>
<th>Reference or source</th>
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<td>dsrAB</td>
<td>SRP</td>
<td>This study</td>
</tr>
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<td>SRP</td>
<td>This study</td>
</tr>
<tr>
<td>DSr1Fd</td>
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<td>SRP</td>
<td>This study</td>
</tr>
<tr>
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<td>dsrAB</td>
<td>Syntrophaceae cluster</td>
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</tr>
<tr>
<td>DSr4R4Rc</td>
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<td>dsrAB</td>
<td>Desulfobacteraceae cluster, Syntrophaceae cluster</td>
<td>This study</td>
</tr>
</tbody>
</table>

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**Disclosure:** This work was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, through the Geothermal Research Program.
by a decline in sulfate over time and stimulation of dissimilatory metal reduction, as indicated by a decline in U(VI) (Fig. 2). It is assumed that there was also a stimulation of dissimilatory Fe(III) reduction during this same period, but this is difficult to ascertain from groundwater geochemistry. Concentrations of dissolved Fe(II) are not a good proxy for Fe(III) reduction in the subsurface as most of the Fe(II) produced from dissimilatory metal reduction typically remains in solid phases, and Fe(II) concentrations in the groundwater merely reflect geochemical equilibria with multiple Fe(II) phases (38). The simultaneous initiation of sulfate reduction and dissimilatory metal reduction with the addition of acetate can be attributed to the fact that acetate had been added to this site in the previous year and had already begun to enrich for sulfate reducers that could compete with *Geobacter* species for acetate (5). With continued acetate addition, dissolved sulfide began to accumulate in the groundwater, providing additional evidence for sulfate reduction.

Between days 15 and 24 no additions were made to the groundwater, and at day 25 when acetate injections were resumed, acetate concentrations were undetectable (<0.1 mM), and sulfate and uranium concentrations had rebounded (Fig. 2). As previously reported (62), delivery of the injectate to D04 was diminished from day 25, as indicated by low levels of the bromide tracer reaching this location (Fig. 2). However, sulfate reduction rates appeared to accelerate, as evidenced by a more rapid depletion of sulfate over time than observed in the initial phase of acetate additions. The undetectable (<0.01 mM) levels of bromide by day 50, coupled with a rebound in sulfate concentrations, suggested that acetate was no longer being delivered to D04 by this time. U(VI) concentrations remained high following the resumption of acetate additions, which is consistent previous studies that have noted a lack of U(VI) removal during active sulfate reduction (2, 58, 62).

**Sulfate reducers present.** To make a comprehensive inventory of the SRP present at the Rifle site, *dsrAB* clone libraries were constructed from DNA extracted from samples representative of the entire experimental period. Seven groups of *dsrAB* sequences were retrieved, with 99 to 100% sequence identity within each group. One group was in the *Desulfobacteraceae*, three were in the *Desulfobulbaceae*, and three were in the *Syntrophaceae* (Fig. 3). Analysis of *dsrAB* sequences revealed three clades of sulfate reducers at the site: *Desulfobacteraceae* (*dsrAB* clone Rifle08_01), *Desulfobulbaceae* (*dsrAB* clones...
Rifle08_02 to Rifle08_04), and Syntrophaceae (dsrAB clones Rifle08_05 to Rifle08_07) (Fig. 3). The closest cultured relatives to the Desulfobacteraceae and Syntrophaceae sequences are the acetate-oxidizing sulfate reducers Desulfobacter postgatei (96 to 97% sequence identity) and Desulfobacca acetoxidans (69 to 72% sequence identity), respectively. The Desulfovulbaceae sequences were not closely related to known acetate-oxidizing sulfate reducers. Primer DSR1F and DSR4R mixes used in this study were recently implemented with additional variants to improve dsrAB coverage (45). Hence, we do not exclude the possibility that our survey underestimated the dsrAB diversity in the groundwater at Rifle.

Prior to the addition of acetate, Desulfobulbaceae and Desulfobacteraceae were comparable in abundance (Fig. 4). However, following the addition of acetate, Desulfobacteraceae became predominant, and the proportion of Desulfobulbaceae declined significantly. Syntrophaceae had lower abundance prior to acetate additions and remained present at a comparable abundance throughout. This specific response of Desulfobacter to the acetate additions was corroborated with 16S rRNA sequence analysis performed with microarrays (8).

**Expression of dissimilatory (bi)sulfite reductase genes in the three clades.** The activity of the three major clades of sulfate reducers throughout the field study was evaluated by monitoring the abundance of dsrB transcripts in each group. The number of dsrB transcripts in each clade was normalized to the number of copies of dsrB in that clade. SRP that possess multiple dsr operons in their genome have not been reported, and thus this normalization is expected to approximate dsrB transcripts per cell.

The Desulfobacteraceae had a slightly higher abundance of dsrB transcripts than the Desulfobulbaceae or Syntrophaceae prior to the addition of acetate to the subsurface (Fig. 5). Following the resumption of acetate additions on day 25, the abundance of transcripts in the Desulfobacteraceae increased. This coincided with the enhanced rate of sulfate removal noted above, consistent with higher activity of sulfate reducers. When acetate was no longer being delivered to D04, as indicated by diminished bromide and a rebound in sulfate concentrations, dsrB transcript abundance in Desulfobacteraceae declined rapidly, consistent with the expected decline in the activity of sulfate reducers.

In contrast, the abundance of dsrB transcripts remained relatively constant in the Desulfobulbaceae throughout the field experiment. The abundance of dsrB transcripts in the Syntrophaceae increased slightly in the later phases of sulfate reduction but remained low compared to transcript abundance in the Desulfobacteraceae (Fig. 5).

These results suggest that, on a per cell basis, Desulfobacteraceae were much more responsive to the changes in the availability of acetate than the other two groups of sulfate reducers. This interpretation is consistent with the finding that
Desulfobacteraceae became the most dominant group of sulfate reducers following acetate addition.

Implications. These results suggest that, with appropriate design of primers, it is possible to specifically monitor gene expression for the respiratory enzyme dissimilatory (bi)sulfite reductase in different clades of sulfate reducers. Previous studies have analyzed dissimilatory (bi)sulfite gene sequences to describe the distribution of phylogenetically distinct populations of sulfate reducers in a diversity of environments (6, 22, 24, 44, 49). As shown here, when transcript abundance of the dissimilatory (bi)sulfite reductases of different populations is quantified, it is possible to evaluate how the metabolism of competing populations of sulfate reducers shifts in response to changing environmental conditions.

If it is assumed that there is a direct relationship between dsrB transcript abundance and rates of sulfate reduction per cell, as has been described in pure culture studies (57), then the results suggest major differences in the ability of the different clades of sulfate reducers to respond to added acetate.

Whereas members of the Desulfobulbaceae were competitive with Desulfobacteraceae at the Rifle site in the absence of added acetate, Desulfobacteraceae were able to increase their per cell rates of sulfate reduction more effectively and thus outcompete the Desulfobulbaceae once acetate was added. Syntrophaceae were also able to increase respiration rates in the presence of added acetate but not to the levels of the Desulfobacteraceae.

The different responses of the individual clades may be related, at least in part, to which electron donors they are capable of utilizing. Desulfobacter species can effectively metabolize acetate (47), whereas no species of Desulfobulbaceae are known to use acetate (15, 47).

However, other physiological features may also play a role. The Syntrophaceae sequences retrieved were related to the known acetate oxidizer Desulfobacca acetoxidans (41), suggesting that the Syntrophaceae most abundant at the Rifle site were also likely to be capable of acetate consumption. Multiple factors other than the ability to use acetate are likely to determine the outcome of competition for added acetate. For example, genome-scale modeling of the competition between acetate-oxidizing, Fe(III)-reducing Geobacter and Rhodoferax species at the Rifle site demonstrated that the predominance of these two species under different conditions could be attributed to differences in growth yield, specific growth rates, and the capacity for nitrogen fixation (63).

The nutritional requirements for growth on acetate of the cultured Desulfobacca acetoxidans (41) is comparable to that of cultured Desulfobacter species (61), but the mean specific growth rate of Desulfobacter species (maximum growth rate $\mu_{\text{max}}$, 0.8 to 1.1 per day) (42) is approximately twice as fast as that of Desulfobacca acetoxidans ($\mu_{\text{max}}$, 0.3 to 0.4 per day) (41). Higher growth rate is a key factor permitting Geobacter species to outcompete Rhodoferax species when acetate is added at the Rifle site (35, 63). Furthermore, the Syntrophaceae-related dsrAB gene sequences recovered at the Rifle site are only moderately similar to the Desulfobacca acetoxidans sequence,
and thus it is conceivable that the Syntrophaceae-related organisms at Rifle might not have the same ability to metabolize acetate.

Analysis of expression of key genes indicative of the physiological status of Geobacter species within the subsurface community at the Rifle site has provided further insight into the factors controlling the growth of these organisms following acetate addition (19–21, 34, 35, 39, 40, 42, 61). A similar in-depth transcriptional profiling of the sulfate-reducing community is warranted. The approach described here for elucidating the relative activity of different components of the sulfate-reducing community should be applicable to a diversity of environments.

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