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In Situ Expression of *nifD* in *Geobacteraceae* in Subsurface Sediments

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In order to determine whether the metabolic state of *Geobacteraceae* involved in bioremediation of subsurface sediments might be inferred from levels of mRNA for key genes, in situ expression of *nifD*, a highly conserved gene involved in nitrogen fixation, was investigated. When *Geobacter sulfurreducens* was grown without a source of fixed nitrogen in chemostats with acetate provided as the limiting electron donor and Fe(III) as the electron acceptor, levels of *nifD* transcripts were 4 to 5 orders of magnitude higher than in chemostat cultures provided with ammonium. In contrast, the number of transcripts of *recA* and the 16S rRNA gene were slightly lower in the absence of ammonium. The addition of acetate to organic- and nitrogen-poor subsurface sediments stimulated the growth of *Geobacteraceae* and Fe(III) reduction, as well as the expression of *nifD* in *Geobacteraceae*. Levels of *nifD* transcripts in *Geobacteraceae* decreased more than 100-fold within 2 days after the addition of 100 μM ammonium, while levels of *recA* and total bacterial 16S rRNA in *Geobacteraceae* remained relatively constant. Ammonium amendments had no effect on rates of Fe(III) reduction in acetate-amended sediments or toluene degradation in petroleum-contaminated sediments, suggesting that other factors, such as the rate that *Geobacteraceae* could access Fe(III) oxides, limited Fe(III) reduction. These results demonstrate that it is possible to monitor one aspect of the in situ metabolic state of *Geobacteraceae* species in subsurface sediments via analysis of mRNA levels, which is the first step toward a more global analysis of in situ gene expression related to nutrient status and stress response during bioremediation by *Geobacteraceae*.

The addition of nutrients to stimulate microbial metabolism is a common practice in the bioremediation of subsurface environments (20, 26). However, these additions are typically done in an empirical manner with little or no information about the actual nutritional requirements of the subsurface microbial community (36). A more rational approach might be to evaluate the metabolic state of the community prior to making amendments by documenting the expression of genes that respond to the presence or absence of a specific nutrient.

This strategy may be most feasible in environments in which one group of microorganisms predominates during the bioremediation process, because metabolic traits unique to this group of organisms can be monitored. One example of a group of microorganisms that frequently dominate microbial communities that have been associated with the effective bioremediation of organic and metal contaminants in subsurface environments is the *Geobacteraceae*. For example, *Geobacteraceae* were the predominant organisms associated with the anaerobic degradation of petroleum (4, 55, 59) and landfill leachate (54) contaminants coupled with the reduction of Fe(III) in subsurface environments. The addition of acetate to stimulate dissimilatory metal reduction by the *Geobacteraceae* was also an effective strategy for promoting the reductive precipitation of uranium from contaminated groundwater (5, 19), and active U(VI) reduction was associated with an enrichment of 16S rRNA gene sequences of *Geobacteraceae* that accounted for 40 to 90% of the microbial community (5, 27).

It was hypothesized that fixed nitrogen might be one of the nutrients limiting the activity of *Geobacteraceae* during bioremediation (8). Petroleum contamination provides significant quantities of organic carbon but little fixed nitrogen. Furthermore, when U(VI) bioremediation and dissimilatory Fe(III) reduction were stimulated in field trials and laboratory incubations with the addition of acetate, fixed nitrogen was not added to the sediments (5, 19). Evaluation of 30 species of *Geobacteraceae* demonstrated that they all contain *nifD*, the gene that encodes the alpha subunit of the dinitrogenase protein (28). Previous physiological studies have also demonstrated that several species of *Geobacteraceae* are able to fix nitrogen (8, 14). In contrast, the genomes of other well-studied metal-reducing microorganisms, such as *Shewanella oneidensis* (24), *Desulfovibrio vulgari* (25), and *Geothrix fermentans* (www.jgi.doe.gov) do not contain *nifD*, suggesting that the ability to fix nitrogen may be one of the features that permits *Geobacteraceae* to effectively compete in subsurface environments.

Analysis of mRNA levels in subsurface sediments may be the most direct method for specifically assessing the physiological state of microorganisms involved in subsurface bioremediation. Increased levels of mRNA for a particular gene have been linked to specific metabolic and/or geochemical processes in pure-culture studies (10, 13, 18, 43, 45, 51, 57, 65, 66, 68) and in the environment (11, 16, 21, 23, 30, 32, 33, 44, 49–51, 56, 67–70). For example, increased levels of mRNA transcripts from a gene involved in naphthalene degradation (*nahA*) were detected in sediments in which naphthalene was being mineralized (21, 67). Expression of *tfdA*, a gene involved in the degradation of the herbicide 2,4-dichlorophenoxyacetic acid was associated with greater herbicide transformation (16), and higher rates of mercury volatilization were accompanied by higher levels of *merA* expression in some environmental samples (44). However, mRNA analysis does not appear to have been previously employed to assess the in situ metabolic state

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of microorganisms in subsurface environments during bioremediation.

Here we present results from RNA analysis of subsurface sediments that suggest that *Geobacteraceae* living in a petroleum-contaminated aquifer or in subsurface sediments amended with acetate in order to stimulate dissimilatory metal reduction express *nifD* and that *nifD* expression is repressed when ammonium is added to the sediments. These findings represent an important first step in assessing the metabolic status of the *Geobacteraceae* during in situ bioremediation.

MATERIALS AND METHODS

Chemostat culture. *Geobacter sulfurreducens* (ATCC 51573) was obtained from our laboratory collection and cultured under anaerobic conditions in acetate-limited chemostats, with acetate (5 mM) provided as the electron donor and Fe(III) citrate (55 mM) provided as the electron acceptor at 30°C, as described elsewhere (18a). The dilution rate was 0.05 h^{-1}.

Sediment collection and laboratory incubations. Sediments were collected from the U.S. Geological Survey Groundwater Toxics Site in Bemidji, Minn. These aquifer sediments have been contaminated with crude oil for 18 years as a result of a break in an oil pipeline (7, 15, 29, 37). This site contains extensive zones of Fe(III) reduction (3, 4, 55), and studies have suggested that microorganisms within the family *Geobacteraceae* are involved in anaerobic degradation of toluene and benzene at this site (4, 55, 59). Sediments were collected from the Fe(III) reduction zone of the contaminant plume and a nearby pristine site, just outside the contaminant plume in 1999 and in 2003 as previously described (4). The samples collected from the Fe(III) reduction zone of the contaminant plume were used to determine whether *Geobacteraceae* express nitrogen fixation genes in the environment.

For sediment incubations, 40 g of sediment was added to 60-ml serum bottles in an anaerobic chamber under a N_2 atmosphere and processed as previously described (48). For the ammonium addition experiment, 5 mM acetate and 0, 50, 100, 250, or 500 μM NH_4Cl were added from sterile anaerobic stocks, and the remainder of the liquid was added as anaerobic groundwater (3.1 ml), for a total of 4 ml. Fe(II) and total HCl-extractable iron were monitored as previously described (4). In order to monitor toluene degradation, [U-^{14}C]toluene stocks were prepared as previously described (4). [U-^{14}C]toluene (1 μCi) was added to each sediment bottle. Each bottle was also amended with unlabeled toluene from a stock prepared with anaerobic groundwater in order to provide a final concentration of ca. 50 μM. NH_4Cl (0, 100 or 250 μM) was added from sterile anaerobic stocks. Total $^{14}CO_2$ was determined from $^{14}CO_2$ in the headspace and the partitioning of $H^{14}CO_3^-$ added to sediment as previously described (39).

Extraction of mRNA from chemostat cultures and sediments. All sediment and chemostat incubations were conducted in triplicate. Solutions used during the RNA extraction process were made with diethyl pyrocarbonate (DEPC)-treated water (Ambion). Chemostat cultures (200 ml) at steady state were transferred to prechilled 50-ml conical tubes and centrifuged at 3,150 × *g* for 15 min at 4°C. The supernatant was discarded, and pellets were flash frozen in an ethanol-dry ice bath and stored at −80°C. Prior to RNA extraction, the pellet was resuspended in 1.5 ml of TPE buffer (100 mM Tris-HCl, 100 mM KH_2PO4, 10 mM EDTA; pH 8.0) and aliquoted into eight separate 2-ml screw cap tubes.

RNA was extracted from sediment incubations when 60 to 75% of the HCl-extractable iron in the sediments was reduced. Five grams of sediment was divided into 10 separate aliquots of 0.5 g each and dispensed into 2-ml screw cap tubes, and 500 μl of TPE buffer was added to each tube.

Sediments from the field that were used for in situ RNA expression analysis were collected as follows: ca. 5 g of sediment was aliquoted into a 50-ml conical tube containing 10 ml of cold (4°C) TPE buffer and 30 ml of cold (4°C) RNA Protect solution (QIAGEN). Tubes were mixed manually 20 times, frozen on dry ice, and immediately transported to the laboratory, where they were stored at −80°C. Samples were then thawed on ice and pelleted at 4,900 × *g* for 15 min. The pellet was then dispensed into 10 separate 0.5-g aliquots, and 500 μl of TPE buffer was added.

The same RNA extraction protocol was followed for all of the sediment and chemostat culture samples once they were suspended in TPE buffer. First, 100 μl of Plant RNA Isolation Aid (Ambion) and 1 ml of cold acetone (stored at −20°C) were added to the cell or sediment suspensions. Tubes were then mixed manually ~20 times and centrifuged at 16,100 × *g* for 5 min. The supernatant was discarded, and 2 μl of Superase-In (Ambion) was added to the pellet, which was then resuspended in 1 ml of sterile DEPC-treated water (Ambion). Ten microliters of lysozyme (50 mg/ml), 3 μl of proteinase K (20 mg/ml), and 30 μl of 10% sodium dodecyl sulfate solution were added and this mixture was incubated at 37°C for 10 min. Samples were then centrifuged at 16,100 × *g* for 15 min, and the supernatant was transferred to new 2-ml screw cap tubes. Fifty microliters of Plant RNA Isolation Aid, 10 μl of yeast tRNA (10 mg/ml; Ambion), 600 μl of hot acidic (70°C; pH 4.5) phenol (Ambion), and 400 μl of chloroform-isoamyl alcohol (24:1; Sigma) were added to the supernatant. These tubes were then mixed on a Labquake rotator (Barnestead/Thermolyne, Dubuque, Iowa) for 10 min and centrifuged at 16,100 × *g* for 5 min. The aqueous layer was removed and transferred to new 2-ml screw cap tubes, and 600 μl of hot acidic (70°C; pH 4.5) phenol (Ambion) and 400 μl of chloroform-isoamyl alcohol (24:1; Sigma) were added. Tubes were mixed on a rotator for 5 min and centrifuged at 16,100 × *g* for 5 min. The aqueous layer was removed again and transferred to a new tube, and 100 μl of 5 M ammonium acetate (Ambion), 4 μl of linear acrylamide (5 mg/ml; Ambion), and 1 ml of cold (−20°C) isopropanol (Sigma) were added.

Nucleic acids were precipitated at −20°C for 1 h and pelleted by centrifugation at 16,100 × *g* for 0.5 h. The pellet was then cleaned with cold (−20°C) 70% ethanol, dried, and resuspended in sterile DEPC-treated water (Ambion). The resuspended pellets were combined and cleaned with the RNeasy RNA cleanup kit (QIAGEN) according to the manufacturers instructions. The RNA cleanup product was then treated with DNA-free DNase (Ambion) according to the manufacturer's instructions.

The RNA extraction method described here provided high-quality mRNA from natural populations in aquifer sediments. All of the mRNA samples had A_{260}/A_{280} ratios of 1.8 to 2.0, indicating that they were of high purity (6). In order to confirm the fact that PCR products generated from the cDNA template did not result from the amplification of contaminating DNA, all PCR analyses included negative controls with RNA that had not been subjected to reverse transcriptase PCR (RT-PCR).

Testing and design of primers and probes. Degenerate primers targeting *nifD* and *recA* in *Geobacteraceae* were designed from nucleotide sequences from *G. sulfurreducens*, *Geobacter metallireducens*, *Desulfuromonas acetoxidans*, and *Pelobacter carbinolicus*. These sequence data were obtained from The Institute for Genomic Research (TIGR) website (http://www.tigr.org) and the Department of Energy (DOE) Joint Genome Institute (JGI) website (www.jgi.doe.gov). These nucleotide sequences were initially aligned in CLUSTALX and imported into the Genetics Computer Group (Madison, Wis.) sequence editor (Wisconsin Package, version 1.0). This alignment was then examined, and conserved regions were targeted for primer design.

The following primers were used to amplify a 335-bp fragment of the *nifD* gene from *Geobacteraceae* NIFGEO225F (5′ ATC GGT GAC GAT ATC AAC GCC 3′) and NIFGEO560R (5′ TAG TTC ATG GAA CGG TAG CAG T 3′). A 468-bp fragment of the *recA* gene in *Geobacteraceae* was amplified with RECGEO202F (5′ ATC TWC GGI CCS GAG TCG TCG GGC AA 3′) and RECGEO670R (5′ CCS TCG CCG TAG WAG ATG TCG AA 3′).

RT-PCR of *nifD*, *recA*, and 16S rRNA transcripts. The DuraScript enhanced avian RT single-strand synthesis kit (Sigma) was used to generate cDNA from extracted *nifD*, *recA*, and 16S rRNA transcripts in two steps. The first reaction mixture had a total volume of 10 μl and consisted of template RNA (0.5 μg), 40 pmol of the appropriate reverse primer, 2 μl of deoxynucleoside triphosphate solution (2.5 mM), and DEPC-treated water. This mixture was incubated at 70°C for 10 min. Ten microliters of a second solution containing 2 μl of 10× RT buffer, 1 U of avian RT, 1 U of RNase inhibitor, and 6 μl of DEPC-treated water was then added to the first solution and allowed to incubate at 50°C for 50 min.

Amplification of *nifD*, *recA*, and 16S RT-PCR products and construction of cDNA libraries. Optimal amplification conditions for NIFGEO225F/560R and RECGEO202F/670R were determined in a gradient thermal cycler (MJ Research Inc., Waltham, Mass.). The amplification parameters included an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 95°C (30 s), 60°C (45 s), and 72°C (45 s), with a final extension at 72°C for 10 min.

In an attempt to reduce PCR bias and provide the most reliable evaluation of microbial community dynamics, more than one bacterial primer set targeting different regions of 16S rRNA was used. The primer sets included 8F (17) with 519R (34) and 338F (1) with 907R (34). Once the appropriate 16S cDNA fragments were generated by RT-PCR, 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), 50°C (1 min), and 72°C (1 min), with a final extension step at 72°C for 7 min. To ensure sterility, the PCR mixtures were exposed to UV radiation for 8 min prior to the addition of the cDNA template and *Taq* polymerase.

16S rRNA, *recA*, and *nifD* PCR products were purified with a gel extraction kit (QIAGEN), and clone libraries were constructed with a TOPO TA cloning kit, version M (Invitrogen, Carlsbad, Calif.), according to the manufacturers' instruc-

TABLE 1. Concentrations of *Geobacteraceae nifD* and *recA* mRNA and total bacterial 16S rRNA gene expression as determined from slot blot hybridization analysis of cDNA

Environmental condition	Concn[a] of:		
	nifD cDNA	*recA* cDNA	16S cDNA
G. sulfurreducens in ammonium-free medium	1.3×10^7 ($\pm 3.2 \times 10^6$)	4.4×10^6 ($\pm 7.8 \times 10^5$)	4.4×10^9 ($\pm 1.1 \times 10^9$)
G. sulfurreducens in ammonium-amended medium	1.2×10^3 ($\pm 3.3 \times 10^2$)	1.6×10^7 (3.4×10^6)	6.7×10^{10} ($\pm 1.7 \times 10^{10}$)
Acetate-amended sediments (Fig. 2) sampled day 60	2.2×10^7 ($\pm 2.4 \times 10^6$)	9.2×10^7 ($\pm 4.5 \times 10^6$)	8.8×10^9 ($\pm 5.7 \times 10^8$)
Control sediments without acetate (Fig. 2) sampled day 60	5.3×10^3 ($\pm 4.4 \times 10^3$)	4.7×10^5 ($\pm 1.1 \times 10^5$)	6.3×10^7 ($\pm 1.1 \times 10^7$)
Acetate-amended sediments (Fig. 3) day 6	4.2×10^6 ($\pm 1.7 \times 10^6$)	5.9×10^6 ($\pm 4.4 \times 10^6$)	3.7×10^9 ($\pm 3.4 \times 10^8$)
Control sediments without acetate (Fig. 3) day 6	46.1 (± 26.1)	1.4×10^4 ($\pm 9.2 \times 10^3$)	1.6×10^7 ($\pm 3.2 \times 10^6$)
Acetate-amended sediments (Fig. 3) day 8 (2 days after ammonium added)	3.2×10^4 ($\pm 2.1 \times 10^4$)	1.2×10^6 (5.4×10^5)	4.5×10^9 ($\pm 2.7 \times 10^8$)
Control sediments without acetate (Fig. 3) day 8 (2 days after ammonium added)	710.2 (± 320.2)	9.9×10^3 ($\pm 6.6 \times 10^2$)	6.9×10^7 ($\pm 4.4 \times 10^7$)

[a] Units are numbers of mRNA molecules per microgram of total RNA. All values are the means ± the standard deviations of results of triplicate hybridizations.

tions. One hundred plasmid inserts from each cDNA clone library were then sequenced with the M13F primer at the University of Massachusetts Sequencing Facility.

Quantification of *nifD* and *recA* expression with TaqMan PCR. Optimal TaqMan PCR conditions were determined by using the manufacturer's guidelines. Each PCR mixture consisted of a total volume of 50 μl and contained 5 μl of the appropriate primers (5 μM) (NIFGEO225F/560R or RECGEO202F/670R), 5 μl of the RT-PCR product, 5 μl of 10× SYBR green PCR buffer (PE Biosystems, Foster City, Calif.), 6 μl of $MgCl_2$ solution (25 mM; PE Biosystems), 4 μl of the deoxynucleoside triphosphate mix (2.5 mM; PE Biosystems), 10 μl of buffer Q solution (QIAGEN), 2 μl of bovine serum albumin (10 mg/ml; New England Biolabs, Beverly, Mass.), 0.5 U of AmpErase uracil-*N*-glycosylase (PE Biosystems), and 0.5 U of *Taq* polymerase (QIAGEN).

The RT-PCR products used to construct the TaqMan standard curve were purified by the protocol for phenol extraction and ethanol precipitation of nucleic acids outlined in *Current Protocols in Molecular Biology* (6). Standard curves were constructed with serial dilutions of known amounts of purified cDNA quantified with a Shimadzu (Baltimore, Md.) UV2401-PC dual-beam spectrophotometer at an absorbance of 260 nm. Serial dilutions covered a range of ~8 orders of magnitude. PCR amplification and detection were performed with the GeneAmp 5700 sequence detection system (PE Biosystems). To verify amplification and correct amplicon size, aliquots from real-time PCR were examined on an ethidium bromide-stained 1% agarose gel.

Quantification of *nifD*, *recA*, and 16S rRNA expression by slot blot hybridization analysis. cDNA generated from *nifD* and *recA* and from bacterial 16S rRNA transcripts in *Geobacteraceae* were also analyzed by slot blot hybridization. Standard curves were constructed with serial dilutions of known amounts of purified cDNA that covered a range of ~6 orders of magnitude. In order to denature cDNA, samples were incubated at 65°C for 5 min in 3 volumes of a solution containing 500 μl of formamide, 162 μl of formaldehyde (37% solution), and 100 μl of 10× TBE buffer (0.9 M Tris-borate, 0.02 M EDTA). The cDNA was then chilled on ice, and 1 volume of cold (4°C) 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer was added.

Once cDNA was denatured, all samples were immobilized on the Zeta-Probe GT membrane (Bio-Rad, Hercules, Calif.) in the same manner. The membrane was soaked in 10× SSC buffer for 1 h prior to placement in the slot blotting manifold (Bio-Rad). Equal quantities of samples were applied to the slot blotting apparatus according to the manufacturer's instructions. The membrane was then immersed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min, followed by 1 min in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2], 0.001 M EDTA), and the nucleic acids were fixed to the membrane in a XL-1500 UV cross-linker (Spectronics Corporation, Westbury, N.Y.) according to the manufacturer's instructions.

Probes targeting *nifD* and *recA* and bacterial 16S rRNA genes in *Geobacteraceae* were constructed using gene products from the following primer pairs: NIFGEO225F/560R, RECGEO202F/670R, and 8F/519R. Amplified fragments from *G. sulfurreducens nifD, recA*, and 16S rRNA genes were first gel purified with the QIAGEN gel extraction kit (QIAGEN) according to the manufacturer's instructions. Twenty-five nanograms of these amplicons was used as the template for construction of [^{32}P]dCTP-labeled probes with a NEBlot kit (New England Biolabs, Inc.), and probe hybridizations were performed with the NorthernMax Gly kit (Ambion) according to the manufacturer's instructions. Hybridization products were visualized on a Typhoon 9210 variable-mode imager (Amersham Biosciences, Piscataway, N.J.), and spot intensities were quantified and compared with ImageQuant software (Amersham Biosciences).

Phylogenetic analysis. 16S rRNA, *nifD*, and *recA* gene sequences were compared to sequences in the GenBank nucleotide and protein databases using BLASTN and BLASTX algorithms. Nucleotide and amino acid sequences for each gene were initially aligned in CLUSTAL X (63) and imported into the Genetics Computer Group sequence editor (Wisconsin Package version 10), where alignments were checked and hypervariable regions were masked. These alignments were then imported into CLUSTAL W (64) and Mview (9), where similarity and identity matrices were generated.

Aligned sequences were imported into PAUP 4.0b10 (62), where phylogenetic trees were inferred. Distances and branching order were determined and compared using character-based (maximum parsimony and maximum likelihood) and distance-based (HKY85 and Jukes-Cantor) algorithms. Bootstrap values were obtained from 100 replicates. Preliminary sequence data from *G. sulfurreducens*, *G. metallireducens*, *D. acetoxidans*, *P. carbinolicus*, *Pelobacter propionicus*, and *Geothrix fermentans* were obtained from the TIGR website (http://www.tigr.org) and the DOE JGI website (www.jgi.doe.gov).

RESULTS AND DISCUSSION

***nifD*, *recA*, and 16S rRNA gene expression by *G. sulfurreducens* in chemostat cultures.** There have been no previous reports on the regulation of nitrogen fixation genes in the *Geobacteraceae*. In order to determine whether transcription of nitrogen fixation genes was repressed in the presence of ammonium, pure-culture studies were first conducted with *G. sulfurreducens*. *G. sulfurreducens* was grown in chemostats under the electron donor-limiting conditions that might typically be found in subsurface environments, with acetate as the limiting electron donor and an excess of Fe(III) as the electron acceptor. The cultures were either provided with ammonium or no source of fixed nitrogen. TaqMan and slot blot hybridization analyses of RNA extracted from these cultures indicated that the number of *nifD* mRNA transcripts was 4 to 5 orders of magnitude higher in the cultures that were required to fix nitrogen and that levels of *recA*, a gene expected to be constitutively expressed (31), and 16S rRNA transcripts were slightly lower in the absence of ammonium. TaqMan PCR analyses indicated that $4.93 \times 10^6 \pm 2.34 \times 10^6$ (three replicates) *nifD* and $1.26 \times 10^5 \pm 3.0 \times 10^4$ *recA* transcripts were expressed under nitrogen-fixing conditions compared to 58.5 ± 49.2 *nifD* and $3.86 \times 10^6 \pm 8.85 \times 10^5$ *recA* transcripts in ammonium-rich medium. Slot blot cDNA hybridization analyses gave similar results (Table 1). These results demonstrated that the up-regulation of nitrogenase genes in *G. sulfurredu-*

cens, and presumably other closely related organisms, could readily be detected by monitoring *nifD* transcripts.

***nifD* expression in acetate-amended background sediments and petroleum-contaminated sediments.** As previously observed (46), the addition of acetate to pristine, organic-poor subsurface sediments from the background site stimulated Fe(III) reduction in anaerobic incubations. After 60 days of incubation, ca. 75% of the Fe(III) in the acetate-amended sediment had been reduced, whereas only ca. 10% had been reduced in sediments incubated without added acetate (data not shown). As expected from previous results (59), there was a significant enrichment of *Geobacteraceae* in the sediments in which Fe(III) reduction had been stimulated. Seventy percent of the 16S rRNA gene sequences recovered in a clone library from the acetate-amended sediments were most closely related to organisms in the *Geobacteraceae*, whereas less than 2% of the sequences could be assigned to the *Geobacteraceae* in the sediments not amended with acetate. Associated with this enrichment in the proportion of *Geobacteraceae* in the acetate-amended sediments was a 100-fold increase in the level of bacterial 16S rRNA transcripts (Table 1). Furthermore, levels of mRNA for *recA* recovered with primers or probes specific for *recA* in *Geobacteraceae* were also ca. 2 orders of magnitude higher in the acetate-amended sediments. Quantitative analyses with TaqMan PCR indicated that $2.73 \times 10^6 \pm 8.78 \times 10^5$ *recA* transcripts were detected in *Geobacteraceae* in acetate-amended sediments compared to $4.87 \times 10^4 \pm 2.84 \times 10^4$ *recA* transcripts expressed in *Geobacteraceae* in nonamended sediments. Similar results were obtained from slot blot hybridization analyses of these samples (Table 1). These results demonstrate that there was significant growth of *Geobacteraceae* in response to the added acetate and the higher rates of Fe(III) reduction.

Analysis of levels of *nifD* mRNA in *Geobacteraceae* with both the TaqMan and slot blot hybridization approaches demonstrated that *nifD* expression in *Geobacteraceae* was 4 to 5 orders of magnitude greater in acetate-amended sediments than in nonamended controls (Table 1). TaqMan analyses indicated that $1.60 \times 10^7 \pm 9.25 \times 10^6$ *nifD* transcripts were expressed in *Geobacteraceae* in acetate-amended sediments compared to only 77.5 ± 20.8 *nifD* transcripts in nonamended sediments. Similar results were obtained from slot blot analyses (Table 1). These results suggested that the actively metabolizing *Geobacteraceae* in the sediments highly expressed *nifD* in response to the addition of a readily utilizable electron donor and carbon source, when no fixed nitrogen was added. In a similar manner, *nifD* transcripts were detected in *Geobacteraceae* in petroleum-contaminated sediments collected from the Fe(III) reduction zone of the aquifer (see Fig. 5), suggesting that the *Geobacteraceae* were also limited for fixed nitrogen when petroleum constituents were the electron donor for Fe(III) reduction.

Effect of added ammonium on *nifD* expression in Fe(III)-reducing sediments. In order to further evaluate the metabolism of the *Geobacteraceae* in the acetate-amended sediments, it was necessary to provide more sediment Fe(III), which was nearly depleted. Therefore, fresh sediment was mixed with some of the acetate-amended sediment (49:1, vol/vol). Additional acetate (5 mM) was added to ensure that the electron donor supply would be adequate for continued Fe(III) reduction. The sediments that had not been amended with acetate were mixed with fresh sediment in the same manner. Fe(III) reduction continued without a lag in the acetate-amended sediments (Fig. 1A). On day 6, when ca. 60% of the sediment Fe(III) had been reduced in the acetate-amended sediments, mRNA levels for *nifD* in *Geobacteraceae* were high (Fig. 1B and Table 1), suggesting that the *Geobacteraceae* continued to highly express nitrogen fixation genes.

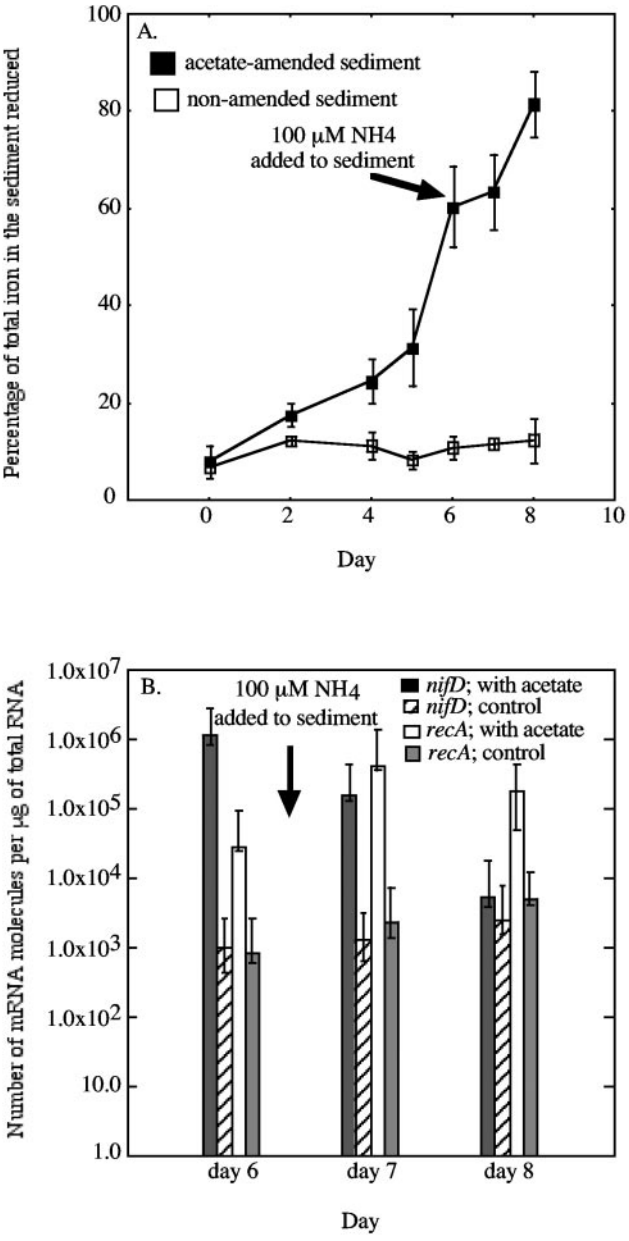

FIG. 1. (A) Fe(II) production before and after the addition of ammonium (100 μM) in acetate-amended and control sediments. Each point is the average of results from triplicate samples from triplicate incubations of each sediment type. (B) *recA* and *nifD* concentrations determined by TaqMan analysis over time. Each point is the average of results of five replicates from the triplicate incubations of the sediments. Error bars represent one standard deviation.

Ammonium was then added to the sediments on day 6. While the addition of ammonium did not appear to effect

Fe(III) reduction (Fig. 1A), a dramatic decrease in *nifD* expression by *Geobacteraceae* was observed over the next 2 days (Fig. 1B and Table 1). In contrast, only slight changes were observed in the number of total bacterial 16S rRNA and *recA* mRNA transcripts in *Geobacteraceae* (Fig. 1 and Table 1). These results suggest that the addition of ammonium to acetate-amended Fe(III)-reducing sediments resulted in a decrease in *nifD* expression by the *Geobacteraceae*. The addition of ammonium to the control sediments that had not been amended with acetate had little detectable impact on the low levels of *nifD* and *recA* in *Geobacteraceae*, reflecting the low activity of *Geobacteraceae* in these sediments (Fig. 1 and Table 1).

Effect of ammonium on microbial activity. The apparent lack of stimulation of Fe(III) reduction following the addition of ammonium in the above studies was further investigated in studies in which ammonium was added at the start of the incubation when Fe(III) reduction was first stimulated with the addition of acetate. The addition of a range of ammonium concentrations did not have a significant effect on Fe(III) reduction (Fig. 2A). This was despite the fact that slot blot hybridization analysis of sediments collected on day 24, when ca. 50% of the HCl-extractable iron had been reduced, indicated that the *Geobacteraceae* in the sediment were highly expressing *nifD* in the sediments not amended with ammonium but that the expression of *nifD* was repressed in *Geobacteraceae* in the ammonium-amended sediments (Fig. 2B). In a similar manner, the addition of 100 or 250 μM ammonium had no effect on the anaerobic degradation of toluene in the Fe(III)-reducing, petroleum-contaminated sediments (Fig. 3).

These results suggest that even though the *Geobacteraceae* were limited for fixed nitrogen in both the acetate-amended sediments and the petroleum-contaminated sediments, this was not the ultimate factor limiting the rate of Fe(III) reduction and toluene degradation. For example, the activity of Fe(III)-reducing microorganisms may be severely limited by their ability to access insoluble Fe(III) oxides (38, 41, 42, 48). The addition of chelators that solubilize Fe(III) (41, 42) or electron shuttles that alleviate the need for direct contact between Fe(III)-reducing organisms and Fe(III) oxides (38, 40) can greatly accelerate both Fe(III) reduction and the degradation of aromatic hydrocarbons in aquifer sediments. Thus, the need for *Geobacteraceae* to continually establish direct contact with fresh Fe(III) oxides over time (12, 47) can kinetically constrain rates of Fe(III) reduction and may be an overriding factor limiting the rate of Fe(III) reduction and contaminant oxidation coupled to Fe(III) reduction.

Effect of added ammonium on community structure. After 24 days of incubation in the presence of added ammonium, the levels of *recA* transcripts in *Geobacteraceae* appeared to be lower than in sediments not amended with ammonium (Fig. 2B). Given the fact that the pure-culture and sediment studies with *Geobacteraceae* summarized above suggested that the absence of ammonium did not substantially influence *recA* expression, these results suggested that long-term exposure to added ammonium might have resulted in a decrease in *Geobacteraceae* in the sediments. Analysis of 16S RNA sequences in the RNA that was extracted on day 24 indicated that *Geobacteraceae* accounted for ca. 70% of the 16S rRNA sequences recovered from sediments amended with acetate,

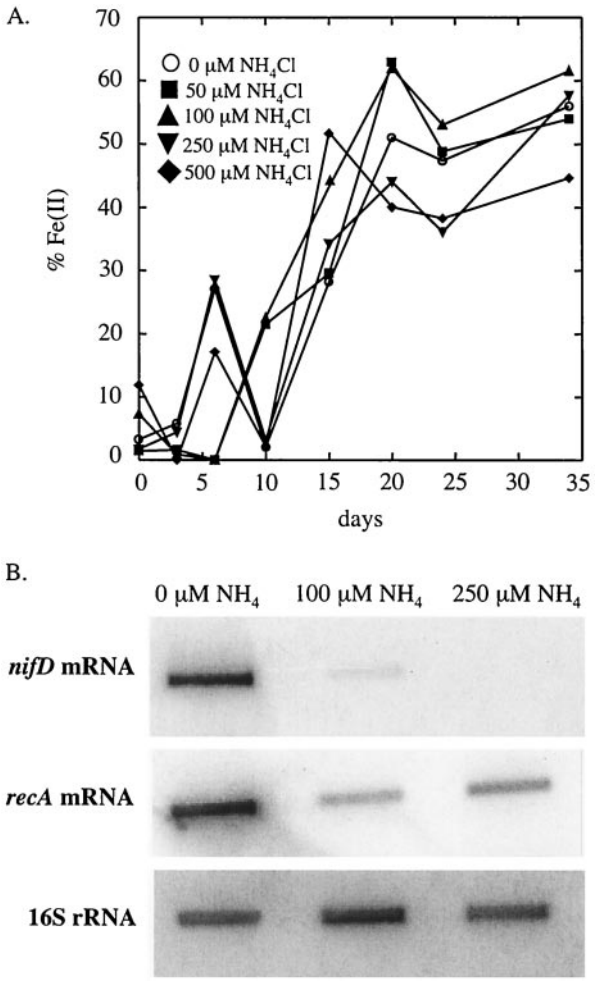

FIG. 2. (A) Fe(II) production in acetate-amended sediments supplemented with various concentrations of ammonium. The results are means of triplicate incubations for each treatment. (B) Slot blot hybridization of the RT-PCR product for *nifD*, *recA*, and bacterial 16S rRNA in *Geobacteraceae* in sediments collected on day 24, when ca. 50% of the HCl-extractable iron had been reduced.

but not those amended with ammonium. In sediments amended with acetate and ammonium, *Geobacteraceae* accounted for only ca. 40% of the sequences (Fig. 4). This decline in the relative number of sequences of *Geobacteraceae* was accompanied by an increase in the percentage of sequences most closely related to organisms in the β-*Proteobacteria*, most notably *Azoarcus* species (Fig. 4). The relative number of *Azoarcus* sequences increased from ca. 15% in sediments not amended with ammonium to ca. 35% of the sequences in clone libraries from the ammonium-amended sediments.

This enrichment of microorganisms with 16S rRNA gene sequences closely related to known *Azoarcus* species was not expected. *Azoarcus* are capable of growing anaerobically with nitrate as the electron acceptor (2, 22, 52, 53, 58, 60, 61, 71), but previous studies have shown that nitrate is not available (i.e., there is <1 μM) in these sediments (3). To our knowledge, *Azoarcus* species are not known to use Fe(III) as an electron acceptor. The increases in *Azoarcus* species were similar whether 100 or 250 μM ammonium was added, suggesting

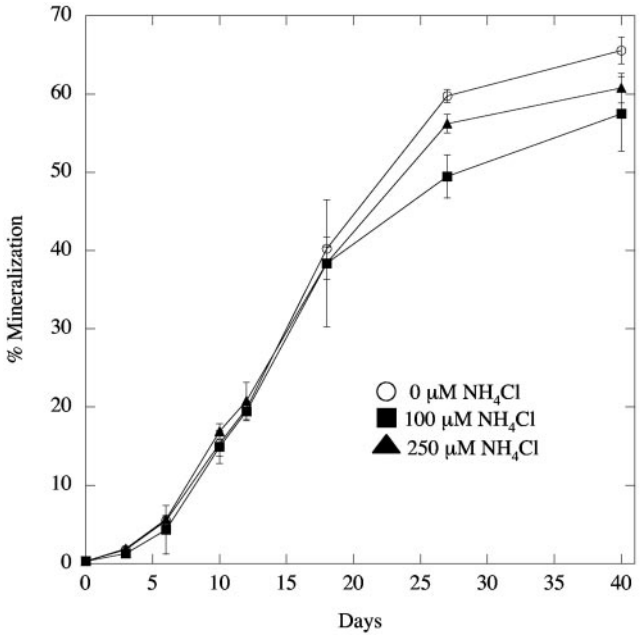

FIG. 3. [U-^{14}C]toluene mineralization in Fe(III)-reducing petroleum-contaminated subsurface sediments amended with various concentration of ammonium. The results are means of results from triplicate incubations for each treatment. Error bars represent one standard deviation.

that the ammonium served as a nutrient rather than as a source of nitrate originating from some form of anaerobic ammonium oxidation. Further evaluation of this unexpected change in community structure was beyond the scope of these studies, but these results emphasize the fact that manipulating environmental conditions in subsurface sediments may not only alter the metabolism of the microorganisms that were predominant prior to the environmental manipulation, but also bring about significant changes in the structure of the microbial community. Thus, it is important to study the effect of nutrient amendments on natural communities rather than single organisms inoculated into sediments as has previously been done (10, 43, 65). There is also a clear need to properly design primers and/or probes if the goal is to monitor the physiological response of a specific population.

Phylogenetic analysis of 16S, *recA*, and *nifD* cDNA clone libraries. Analysis of cDNA clone libraries constructed from mRNA extracted from sediments indicated that the *nifD* and *recA* primer sets used in this study preferentially amplified *nifD* and *recA* mRNA being expressed by species of *Geobacteraceae*. For example, all of the *nifD* clones examined showed at least 83.9% amino acid sequence identity to the *G. sulfurreducens nifD* sequence (105 amino acids considered). In addition, all of the *nifD* amino acid sequences were between 85.6 and 100% identical to each other. Analysis of *recA* cDNA clones showed that all of the *recA* amino acid sequences were at least 80.1% identical to the *G. sulfurreducens recA* sequence (120 amino

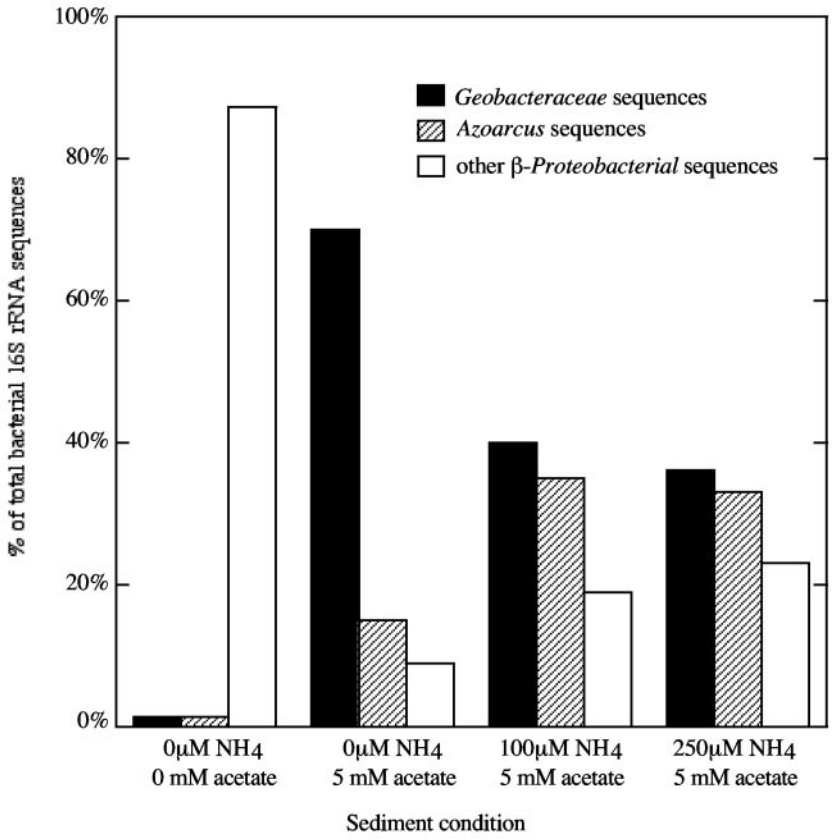

FIG. 4. Relative proportions of sequences in cDNA clone libraries of 16S rRNA gene sequences assembled from acetate-amended sediments collected on day 24, when ca. 50% of the HCl-extractable iron had been reduced.

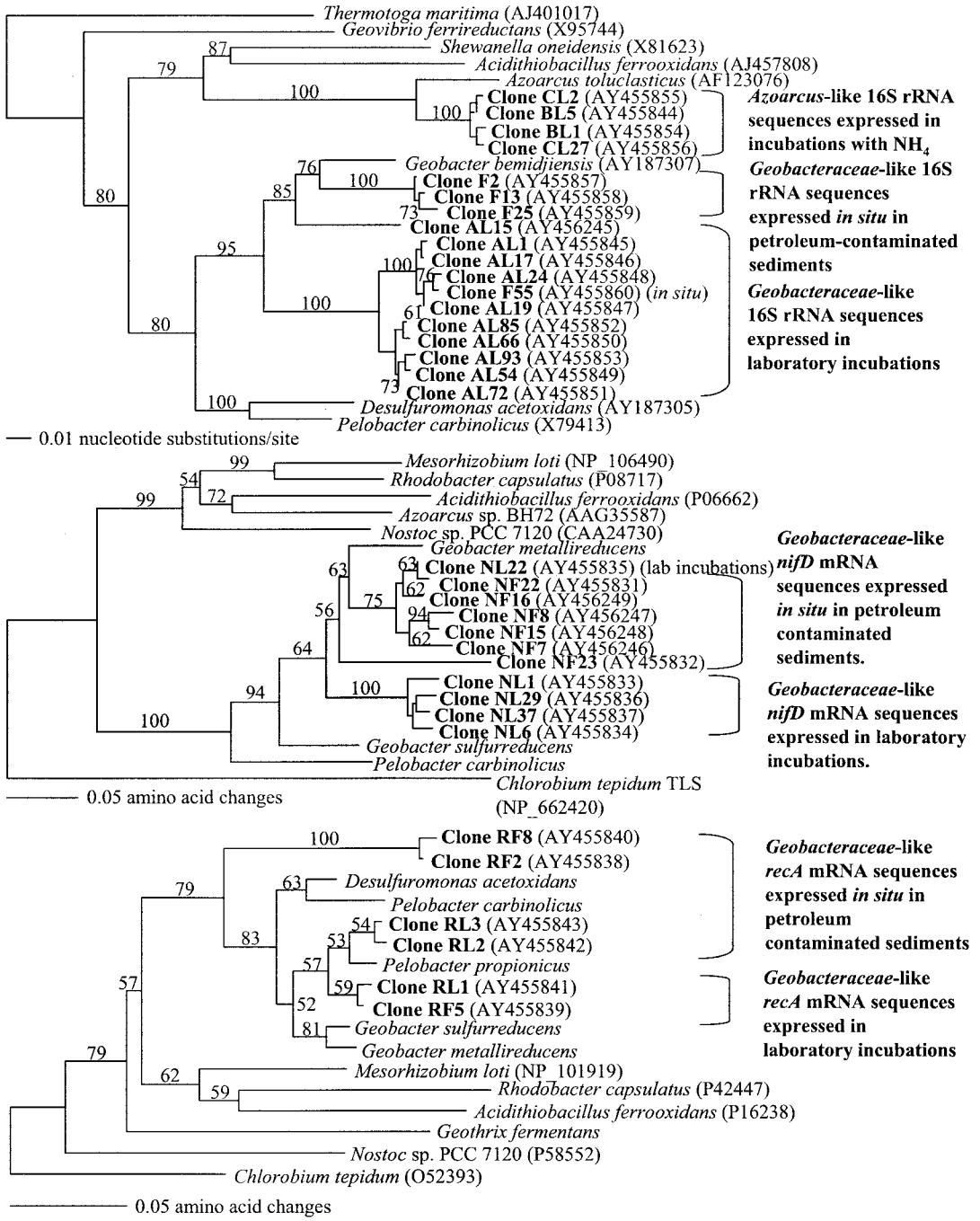

FIG. 5. Phylogenetic analysis of 16S rRNA, *nifD* mRNA, and *recA* mRNA sequences amplified from *Geobacteraceae* in acetate-amended Fe(III)-reducing background sediments and the Fe(III)-reduction zone of a petroleum-contaminated aquifer. (A)16S rRNA nucleotide sequence comparisons made by the Jukes-Cantor algorithm with *Thermotoga maritima* used as the outgroup; (B) *nifD* amino acid sequence comparisons made by maximum parsimony analysis with *Chlorobium tepidum* used as the outgroup; (C) *recA* amino acid sequence comparisons made by maximum parsimony analysis with *C. tepidum* used as the outgroup.

acids considered). The sequence identity of the *recA* gene fragments also ranged from 76.5 to 100%, and the majority (80.1%) of *recA* amino acid sequences were ca. 98% identical.

The 16S rRNA sequences expressed in *Geobacteraceae* in the acetate-amended background sediments or the petroleum-contaminated sediments all clustered within the freshwater *Geobacter* clade of the family (Fig. 5). The sequences were 81.9 to 92.5% similar to the 16S rRNA sequence of "*Geobacter bemidjiensis*," a pure-culture isolate from this site, sharing 88.4 to 100% sequence identity. All of the *nifD* and *recA* sequences from *Geobacteraceae* also clustered with freshwater *Geobacter* species (Fig. 5). These results further suggest that, as has been noted elsewhere (E. Shelobolina, H. Vroinis, and D. R. Lovley, manuscript in preparation), the *Geobacteraceae* that predom-

inate in subsurface environments are related to those that can be recovered in pure culture.

Implications. The results suggest that it is possible to assess the in situ metabolic state of *Geobacteraceae* in the subsurface by quantifying levels of mRNA specific to *Geobacteraceae* in the sediments. *nifD* expression in *Geobacteraceae* was high in sediments amended only with acetate, whereas *nifD* mRNA levels dropped significantly when ammonium was also added. In situ gene expression analyses also indicated that *nifD* was being expressed in the Fe(III) reduction zone of the petroleum-contaminated aquifer. The fact that similar results were obtained from analysis of mRNA with PCR-based and slot blot hybridization techniques provided added assurance of the reliability of these results. Most importantly, rather than studying mRNA extracted from sediments inoculated with pure cultures, these studies focused on gene expression patterns in *Geobacteraceae* within mixed natural microbial communities in the sediments.

The finding that supplying ammonium was not sufficient to stimulate Fe(III) reduction or toluene degradation emphasizes the fact that a multitude of factors are likely to be important in controlling the metabolism and growth of *Geobacteraceae* in the subsurface. Thus, a more global analysis of the in situ metabolic state of the predominant *Geobacteraceae*, encompassing potential stress responses and overall nutrient status, rather than focusing on a single nutrient, is desirable. The ability to effectively extract the high-quality mRNA described here and the increasing availability of genome sequence data from *Geobacteraceae* that predominate in environments of interest are providing the tools that will be required for such a global analysis of gene expression in *Geobacteraceae* (35, 36).

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