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In Situ Expression of \textit{nifD} in \textit{Geobacteraceae} in Subsurface Sediments

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In order to determine whether the metabolic state of \textit{Geobacteraceae} involved in bioremediation of subsurface sediments might be inferred from levels of mRNA for key genes, in situ expression of \textit{nifD}, a highly conserved gene involved in nitrogen fixation, was investigated. When \textit{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 \textit{nifD} transcripts were 4 to 5 orders of magnitude higher than in chemostat cultures provided with ammonium. In contrast, the number of transcripts of \textit{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 \textit{Geobacteraceae} and Fe(III) reduction, as well as the expression of \textit{nifD} in \textit{Geobacteraceae}. Levels of \textit{nifD} transcripts in \textit{Geobacteraceae} decreased more than 100-fold within 2 days after the addition of 100 \textmu M ammonium, while levels of \textit{recA} and total bacterial 16S rRNA in \textit{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 \textit{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 \textit{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 \textit{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 \textit{Geobacteraceae}. For example, \textit{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 \textit{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 \textit{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 \textit{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 \textit{Geobacteraceae} demonstrated that they all contain \textit{nifD}, the gene that encodes the alpha subunit of the dinitrogenase protein (28). Previous physiological studies have also demonstrated that several species of \textit{Geobacteraceae} are able to fix nitrogen (8, 14). In contrast, the genomes of other well-studied metal-reducing microorganisms, such as \textit{Shewanella oneidensis} (24), \textit{Desulfovibrio vulgaris} (25), and \textit{Geothrix fermentans} (www .jgi .doc .gov) do not contain \textit{nifD}, suggesting that the ability to fix nitrogen may be one of the features that permits \textit{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). mRNA levels of \textit{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.
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 ammonia 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 and Toxic Substances 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 N₂ atmosphere and processed as previously described (48). For the ammonium addition experiment, 5 mM acetate and 0.5, 100, 250, or 500 g NH₄Cl 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). 

**Extraction of mRNA from chemostat cultures and sediments.** All sediment and chemostat incubations were made with diethyl pyrocarbonate (DEPC)-treated water. Solutions used during the RNA extraction process were made with diethyl pyrocarbonate (DEPC)-treated water. Tubes were then mixed 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 isoxyme (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 (Barnstead/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 μl 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 RNasea RNA cleanup procedure 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 A₂₆₀/A₂₈₀ 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 genomic DNA, the samples were 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*, *Desulfurobium acetoxidans*, and *Peptococcus carbonicus*. 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). The 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* NIFGE0225F (5′ ATC GTG GAC GAT ATC AAC GCC 3′) and NIFGE060R (5′ TAG TGC ATG GAA CGG TAG CAG T 3′). A 468-bp fragment of the *recA* gene in *Geobacteraceae* was amplified with RECGE020F (5′ ATC TWC GGI CCS GAG TCG TCG GAC AA 3′) and RECGE670R (5′ CCS TCG CCG TAG WAG ATG TCG AA 3′).

**RT-PCR of *nifD*, *recA*, and 16S rRNA transcripts.** The DuraScript enhanced avian RT system (Biorad, Hercules, Calif.) was used to synthesize cDNA from each 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 deoxynucleotide 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 μl of avian RT, 1 μl 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 NIFGE0225F/560R and RECGE0202F/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), 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 (30 s), 60°C (45 s), and 72°C (45 s), 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

<table>
<thead>
<tr>
<th>Environmental condition</th>
<th>nifD cDNA</th>
<th>recA cDNA</th>
<th>16S cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. sulfurreducens</em> in ammonium-free medium</td>
<td>1.3 × 10^7 (±3.2 × 10^6)</td>
<td>4.4 × 10^6 (±7.8 × 10^5)</td>
<td>4.4 × 10^6 (±1.1 × 10^8)</td>
</tr>
<tr>
<td><em>G. sulfurreducens</em> in ammonium-amended medium</td>
<td>1.2 × 10^8 (±3.3 × 10^7)</td>
<td>1.6 × 10^7 (3.4 × 10^6)</td>
<td>6.7 × 10^9 (±1.7 × 10^10)</td>
</tr>
<tr>
<td>Acetate-amended sediments (Fig. 2) day 60</td>
<td>2.2 × 10^7 (±2.4 × 10^6)</td>
<td>9.2 × 10^6 (±4.5 × 10^5)</td>
<td>8.8 × 10^7 (±5.7 × 10^8)</td>
</tr>
<tr>
<td>Control sediments without acetate (Fig. 2) day 60</td>
<td>5.3 × 10^8 (±4.4 × 10^7)</td>
<td>4.7 × 10^7 (±1.1 × 10^6)</td>
<td>6.3 × 10^9 (±1.1 × 10^8)</td>
</tr>
<tr>
<td>Acetate-amended sediments (Fig. 3) day 6</td>
<td>4.2 × 10^8 (±1.7 × 10^7)</td>
<td>5.9 × 10^7 (±4.4 × 10^6)</td>
<td>3.7 × 10^9 (±3.4 × 10^8)</td>
</tr>
<tr>
<td>Control sediments without acetate (Fig. 3) day 6</td>
<td>46.1 (±26.1)</td>
<td>1.4 × 10^8 (±9.2 × 10^6)</td>
<td>1.6 × 10^9 (±3.2 × 10^8)</td>
</tr>
<tr>
<td>Acetate-amended sediments (Fig. 3) day 8 (2 days after ammonium added)</td>
<td>3.2 × 10^8 (±2.1 × 10^7)</td>
<td>1.2 × 10^7 (5.4 × 10^6)</td>
<td>4.5 × 10^8 (±2.7 × 10^7)</td>
</tr>
<tr>
<td>Control sediments without acetate (Fig. 3) day 8 (2 days after ammonium added)</td>
<td>710.2 (±320.2)</td>
<td>9.9 × 10^6 (±6.6 × 10^5)</td>
<td>6.9 × 10^7 (4.4 × 10^6)</td>
</tr>
</tbody>
</table>

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

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 × 10^5 ± 2.34 × 10^5 (three replicates) *nifD* and 1.26 × 10^5 ± 3.0 × 10^4 *recA* transcripts were expressed under nitrogen-fixing conditions compared to 58.5 ± 49.2 *nifD* and 3.86 × 10^5 ± 8.85 × 10^4 *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. sulfurreducens*...
In a similar manner, donor and carbon source, when no fixed nitrogen was added, in response to the addition of a readily utilizable electron donor for Fe(III) reduction.

5), suggesting that the Geobacteraceae were also limited for fixed nitrogen when petroleum constituents were the electron donor.

gen. 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 × 10^6 ± 8.78 × 10^5 recA transcripts were detected in Geobacteraceae in acetate-amended sediments compared to 4.87 × 10^4 ± 2.84 × 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 × 10^7 ± 9.25 × 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.

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, 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 Azorarcus species (Fig. 4). The relative number of Azorarcus 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 Azorarcus species was not expected. Azorarcus 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, Azorarcus species are not known to use Fe(III) as an electron acceptor. The increases in Azorarcus species were similar whether 100 or 250 μM ammonium was added, suggesting...
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 acid sequences considered).
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 were 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 tolune 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).

**ACKNOWLEDGMENTS.**

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