Aromatic and Polyaromatic Hydrocarbon Degradation under Fe(III)-Reducing Conditions

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ABSTRACT

Investigations of the Bemidji aquifer have demonstrated that important ground water contaminants such as benzene and naphthalene can be degraded under Fe(III)-reducing conditions found in situ. Mineralization of both compounds occurred without a lag period suggesting that Fe(III)-reducers found in the sediment were oxidizing these contaminants in situ. The area of greatest mineralization activity was confined to a narrow region at the downgradient edge of the Fe(III)-reducing zone. Microbial community analysis using 16S rRNA-based techniques indicated that members of the *Geobacter* family were enriched in sediments collected from this active zone. Furthermore, phylogenetic analyses of DNA sequences recovered from benzene-degrading sediments and enrichment cultures clustered with *Geobacteraceae* known to degrade aromatic compounds while sequences from uncontaminated, background sediments did not. These results could lead to the development of a rapid assay for assessing anaerobic benzene degradation potential at petroleum-contaminated sites.

INTRODUCTION

Petroleum hydrocarbon contamination of aquifers presents a serious threat to ground water resources. While most hydrocarbon compounds are known to be degraded under aerobic conditions many petroleum-contaminated aquifers contain large areas where anaerobic processes are dominant (Anderson and Lovley, 1997; Lovley, 1997b). Specifically, microbial Fe(III) reduction is predicted to be a dominant anaerobic process because Fe(III) is generally the most abundant potential electron acceptor found in aquifer sediments (Lovley, 1997a; Lovley, 1997b). Large decreases in ground water contaminant concentrations are observed in many aquifers where large areas of Fe(III) reduction are observed (Borden and others, 1995; Cozzarelli and others, 1990).

Benzene, a known carcinogen, is relatively mobile in ground water and is often observed to persist in anaerobic environments (Barbaro and others, 1992; Flyvbjerg and others, 1993). Laboratory incubations of sediment have indicated a potential for anaerobic benzene degradation under a variety of anaerobic conditions but the onset of degradation activity is often preceded by long lag periods suggesting that benzene degradation was not occurring in situ (Edwards and Grbic-Galic, 1992; Kazumi and others, 1997; Lovley and others, 1996). Evidence of in situ benzene degradation has been demonstrated in contaminated harbor sediments under sulfate reducing conditions (Coates and others, 1996) and recently in contaminated aquifer sediments under Fe(III)-reducing conditions (Anderson and others, 1998).

Polyaromatic hydrocarbons (PAHs) are also potential ground water contaminants found in petroleum contaminated environments. Many PAHs are known to be carcinogenic and when present in aquifers tend to be associated with the sediment. However some PAHs such as naphthalene do migrate with the ground water and can pose a threat to downgradient water resources (Goerlitz and others, 1985). Most low molecular weight PAH compounds are known to be degraded under aerobic conditions and until recently PAHs were generally thought to persist under anaerobic conditions (Cerniglia, 1992).

Anaerobic PAH degradation has been observed under a variety of electron-accepting conditions. Naphthalene degradation has been observed in laboratory sediment incubations where nitrate was supplied as the electron acceptor (Langenhoff and others, 1996; Thierry and others, 1996) and in contaminated harbor sediments under sulfate reducing conditions (Coates and others, 1996). While naphthalene degradation has also been observed in enrichment cultures established from aquifer sediment under sulfate reducing conditions (Bedessem and others, 1997) and in sulfate reducing ground water downgradient from a leaking underground storage tank (Thierrin and others, 1993), PAH degradation has not been previously observed under Fe(III)-reducing conditions.

This paper describes the results of a study to further examine anaerobic aromatic and polyaromatic degradation within petroleum-contaminated aquifers. The results demonstrate that benzene and naphthalene can be degraded under Fe(III)-reducing conditions found in some but not all petroleum-contaminated aquifers. When this activity is found it may be largely restricted to narrow regions of the aquifer at the downgradient edge of the Fe(III) reduction zone. Furthermore, the detection of specific Fe(III)-reducing microorganisms could lead to the development of a rapid assay to assess benzene degradation potential in petroleum-contaminated aquifers.

MATERIALS AND METHODS

Sediment Collection and TEAP Determination

Sediment samples were collected from a variety of petroleum-contaminated aquifers and evaluated for the potential to anaerobically degrade aromatic and polyaromatic compounds as previously described (Anderson and Lovley, 1999; Anderson and others, 1998; Murphy and Herkelrath, 1996). All sediment samples were collected using either a truck mounted drill rig or hand augers. Sediments collected at the Bemidji aquifer in 1997 are from sites shown in Figure 1.
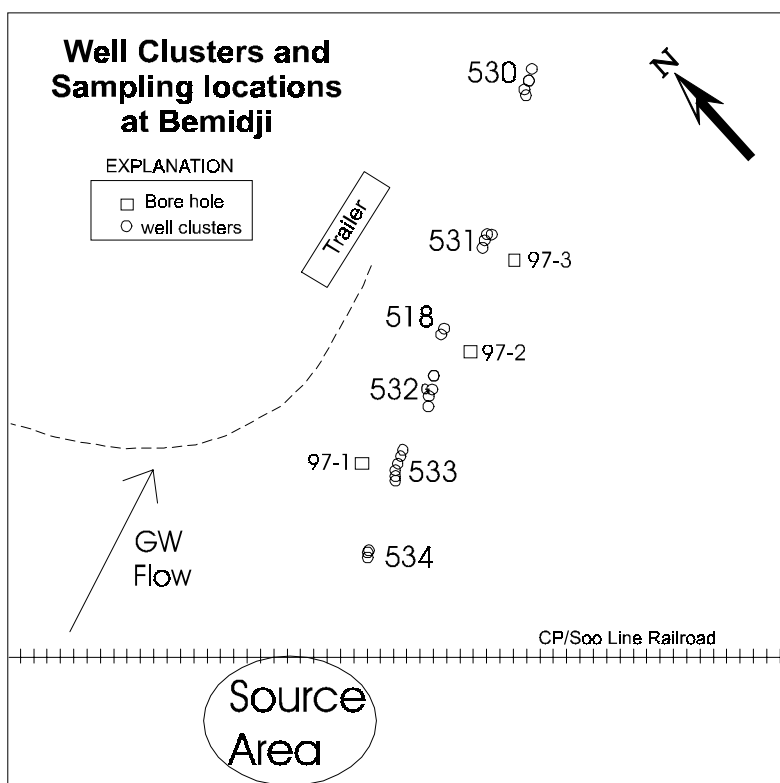
Figure 1. Well clusters and sediment sampling locations at the Bemidji site (adapted from Anderson and others, 1998).

The dominant terminal electron accepting process (TEAP) in collected sediments was assessed using [2-^{14}C]acetate as previously described (Anderson and others, 1998). Fe(III)-reducing conditions are confirmed as the dominant anaerobic process in sampled sediments if the following criteria are met: 1) ground water is depleted of nitrate, 2) Fe(II) is present in the sediments, 3) mineralization of [2-^{14}C]acetate is not inhibited by molybdate, 4) no ^{14}CH$_4$ production observed when sediments are incubated with [2-^{14}C]acetate. The Fe(II) content of the sediments was evaulated using a 0.5N HCl extraction followed by measurement with ferrozine.

Ground Water Analysis

Ground water samples for anion analysis were collected in 40 mL vials or 58 mL serum bottles, chilled on ice and sent via overnight carrier to the laboratory. Once in the laboratory, the samples were immediately analyzed for nitrate and sulfate by ion chromatography (Dionex, DX-100, Sunnyvale, CA). Ground water samples for benzene analysis were placed into no-headspace 40 mL vials and preserved with HCl prior to overnight shipment to the laboratory. Benzene samples were analyzed by gas chromatography (HP6890, Wilmington, DE) coupled with a purge and trap autosampler (OI-Analytical, DPM-16, College Station, TX) according to a modification of EPA method 8015/8020.

Mineralization of Radiolabeled Substrates

The potential for benzene, toluene, naphthalene and phenanthrene degradation in sediments was assessed using [^{14}C]-labeled substrates as previously described (Anderson and Lovley, 1999; Anderson and others, 1998). Sediments (30g) were dispensed into triplicate sets of serum bottles inside an N$_2$-filled glove bag and stoppered with thick butyl rubber stoppers. Anaerobic stock solutions of [^{14}C]-labeled benzene and toluene were prepared using an adaptation of the manufacturer's recommended method of transfer and were added to provide 1μCi of labeled substrate to each sediment bottle.

Radiolabeled naphthalene and phenanthrene, supplied in methanol stocks, were first applied to sediment pellets prepared from the tested sediments and the methanol allowed to evaporate (Anderson and Lovley, 1999). The sediment pellets were then added to serum bottles while under a stream of anaerobic N$_2$. The bottles were vigorously shaken to break up and disperse the pellets inside the bottles.

Headspace samples (1ml) of bottles containing radiolabeled substrates were removed over time and analyzed for ^{14}CO$_2$ and ^{14}CH$_4$ using gas chromatography and gas proportional counting detection. Total mineralization percentages were calculated based on the partitioning of H^{14}CO$_2$ between bottle headspaces and the sediment.

Analysis of Microbial Populations in the Sediment

Analyses of the microbial subsurface community was performed using 16S rRNA-based techniques as previously described (Anderson and others, 1998; Rooney-Varga and others, 1999). Since cultivation-based community analyses can selectively bias the results, a more direct approach was applied at the Bemidji site. DNA was extracted directly from sediments and analyzed using both DGGE and MPN-PCR. Briefly, DNA was extracted from triplicate sediment samples using a freeze-thaw method of cell disruption followed by phenol-chloroform extraction or with the FastDNA soil extraction kit (Bio 101, Vista, CA). For bacterial community analyses, portions of the 16S rDNA sequences were amplified using the PCR and primers 338F-GC and 907R. For analysis of the *Geobacteraceae* community within sediments, 16S rDNA sequences were amplified using primers 338F-GC and Geo825R. The resulting PCR products were subsequently separated on a denaturing gradient electrophoresis gel and resolved with ethidium bromide/transillumination. Excised bands were later sequenced for identification. For MPN-PCR analyses, the extracted DNA was first serially diluted 10-fold into sterile Milli-Q water and portions of each dilutions were used as template in the PCR. In these analyses, *Geobacteraceae* sequences were amplified using primers 8F and

Geo825R. Resulting PCR products were analyzed by gel electrophoresis in agarose (1%) containing ethidium bromide and visualized by transillumination.

RESULTS AND DISCUSSION

Anaerobic Aromatic and Polyaromatic Degradation

Of several sites investigated (Anderson and others, 1998), Bemidji aquifer sediments were the only samples collected that demonstrated a potential for benzene degradation under Fe(III)-reducing conditions. While all sediments from all sites demonstrated a potential for toluene degradation only sediments from a narrow region at the downgradient edge of the Fe(III) reduction zone (site 97-3) at Bemidji readily mineralized [14C]benzene to 14CO$_2$ (Figure 2). No lag period was observed prior to the onset of mineralization suggesting that the organisms within the sediment were preadapted for benzene oxidation and therefore must be oxidizing benzene in situ. No benzene mineralization was observed in uncontaminated, background sediments from Bemidji implying that the observed benzene degradation is associated with petroleum contamination.
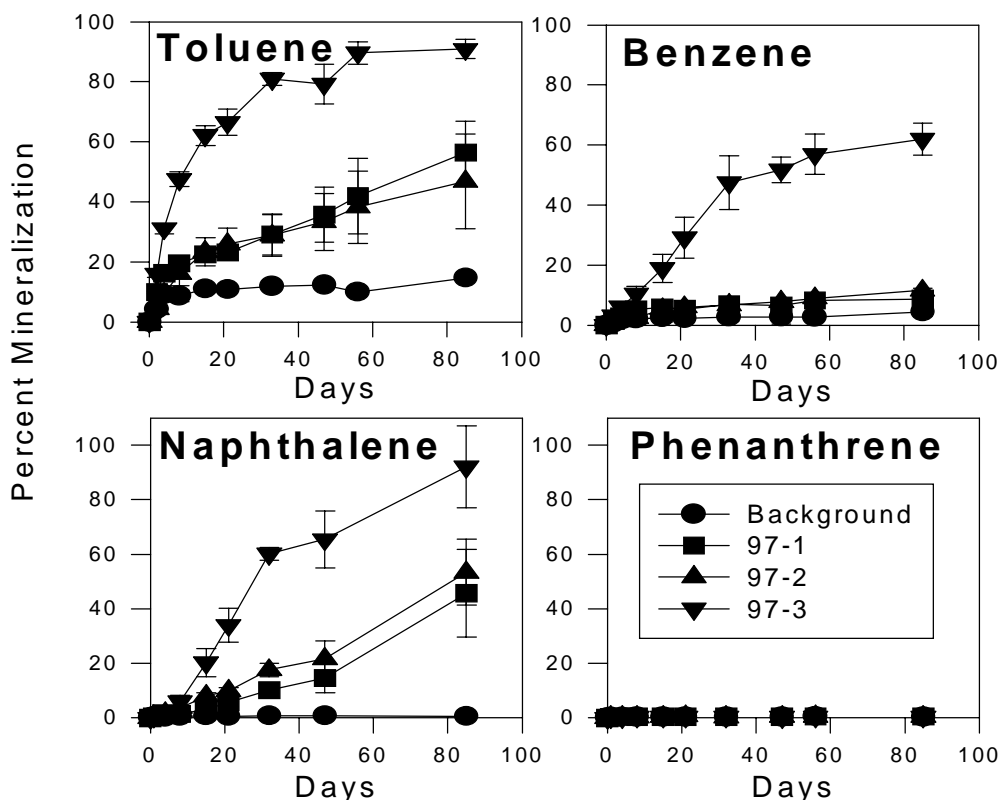
Figure 2. Mineralization [14C] toluene, benzene, naphthalene and phenanthrene in sediments collected from the Bemidji aquifer (adapted from Anderson and Lovley, 1999; Anderson and others, 1998). Results are averages of triplicate analyses.

Bemidji sediments not only rapidly mineralized benzene under Fe(III)-reducing conditions but also readily mineralized [14C]naphthalene to 14CO$_2$. Again, the onset of

mineralization was immediate with no lag period suggesting that organisms present within the subsurface were preadapted for naphthalene degradation and were likely mineralizing this compound in situ. No naphthalene degradation was observed in uncontaminated sediments again indicating that this activity is associated with petroleum contamination. In addition, no mineralization of [^{14}C]phenanthrene was observed in any Bemidji sediments under Fe(III)-reducing conditions demonstrating that oxygen contamination could not account for the observed benzene or naphthalene degradation in contaminated sediments. Phenanthrene is readily oxidized under aerobic conditions but its anaerobic degradation potential may be hindered due to solubility limitations.

These results are the first documented evidence of the potential for *in situ* oxidation of benzene and naphthalene under Fe(III)-reducing conditions found in petroleum-contaminated aquifers. The greatest rates of toluene, benzene and naphthalene degradation were restricted to a narrow region at the downgradient edge of the

Fe(III) reduction zone. These observations indicate that the downgradient edge of Fe(III) reduction zone at Bemidji is an area of intense microbial activity. Detection of similar zones in other petroleum-contaminated aquifers could lead to better understanding of the contribution of anaerobic process to the natural attenuation of ground water contaminants.

Association of *Geobacteraceae* with Anaerobic Benzene Degradation

Investigation of the microbial communities within the Bemidji aquifer focused on organisms involved in the degradation of benzene under Fe(III)-reducing conditions because benzene is frequently the contaminant of greatest concern when present in ground water. DGGE analysis of Bemidji sediments using *Bacteria* primers indicated that relatively few species dominated at each site, particularly the background site (Figure 3).
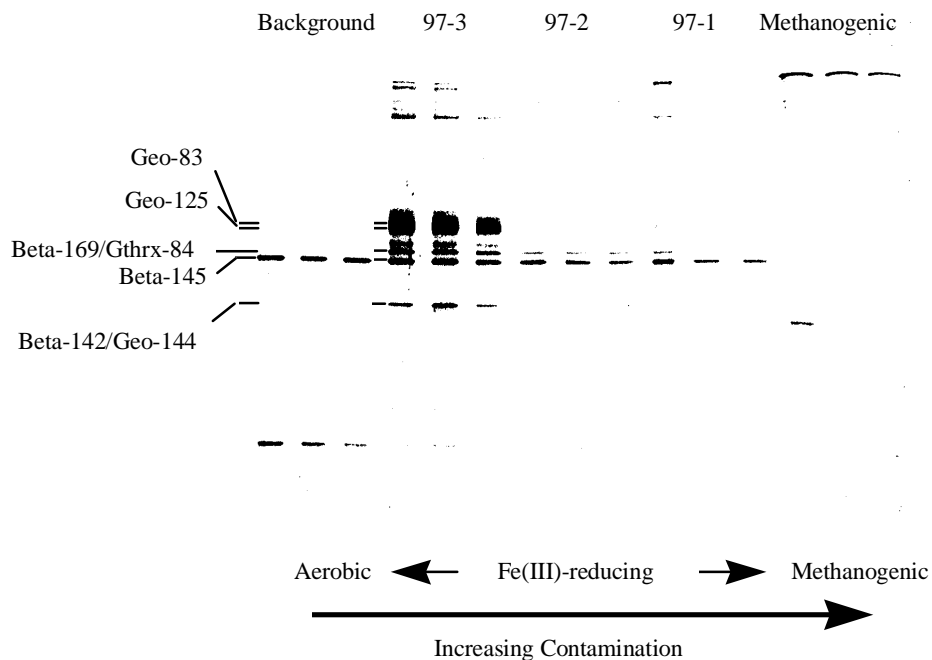
Figure 3. DGGE analysis of DNA extracted from triplicate samples of Bemidji sediment and amplified using *Bacteria* specific primers (adapted from Rooney-Varga and others, 1999). Methanogenic sediments were collected near well cluster 534.

Differences in community composition between background sediments and contaminated sediments were clearly evident as well as differences among the different TEAP zones. More bands were

recovered from sediments within contaminated portions of the aquifer than in uncontaminated portions suggesting that the presence of crude oil in the subsurface stimulated the growth of microorganisms that were not previously dominant in the sediments

Previous cultivation-based results suggested that *Geobacteraceae* were enriched in benzene-degrading sediments collected at site 97-3 (Anderson and others, 1998). While the DGGE results using primers specific for *Bacteria* indicated no dramatic differences among the three Fe(III)-reducing sites investigated, a few bands of much higher intensity corresponding to *Geobacteraceae* (Geo-83, Geo-125) were found in sediments from site 97-3 (Figure 3) suggesting that these organisms were selectively enriched in 97-3 sediments. Members of the *Geobacteraceae* are strict Fe(III)-reducers many of which are known to completely oxidize aromatic compounds. Bands corresponding to *Geothrix* species (Gthrx-84) were found among all Fe(III)-reducing sites while a sequence from the *β*-proteobacteria (Beta-145) was found in both the background and all Fe(III)-

reducing sites. These organisms are not known to oxidize aromatic compounds.

MPN-PCR analysis of DNA extracted from sediments provides an unbiased analysis of the numerical abundance of microbial species relative to cultivation-based techniques. When Fe(III)-reducing sediments collected from the Bemidji aquifer were analyzed by MPN-PCR, *Geobacteraceae* were found to be enriched in sediments known to degrade benzene (Figure 4). *Geobacteraceae* sequences enumerated in benzene-degrading sediments from site 97-3 were more than four orders of magnitude greater than background sediments and more than three orders of magnitude greater than other Fe(III)-reducing sediments that did not degrade benzene (97-1, 97-2). Site 97-3 is located near wells where the concentration of benzene in the ground water sharply decreases with downgradient distance (Figure 5). These results suggest that elevated numbers *Geobacteraceae* detected in benzene-degrading sediment play an important role in removing benzene from the ground water.
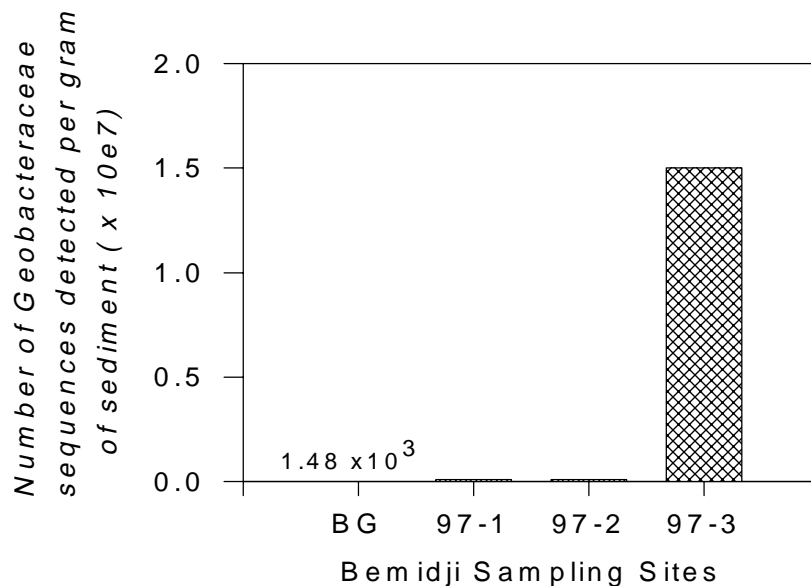
Figure 4. Enumeration of *Geobacteraceae* by MPN-PCR in sediments collected from the Bemidji aquifer (adapted from Anderson and others, 1998). BG is the background site.
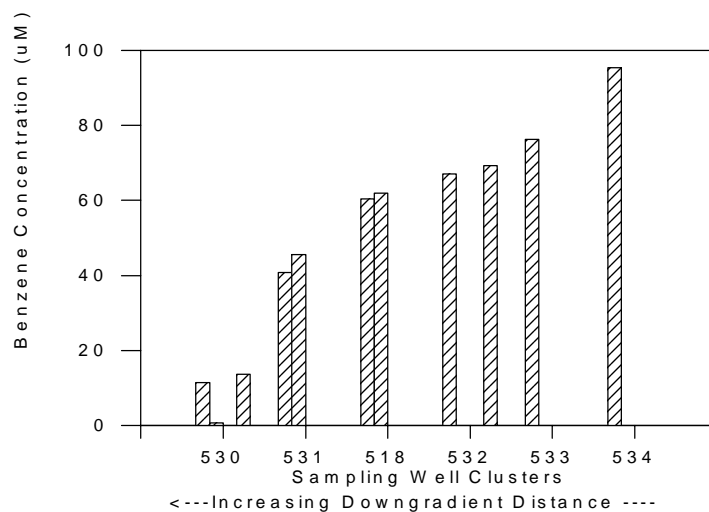
Figure 5. Ground water benzene concentrations in wells at increasing distances downgradient from the source area. Results are averages of triplicate analyses. Sediments sampling site 97-3 is near well cluster 531.
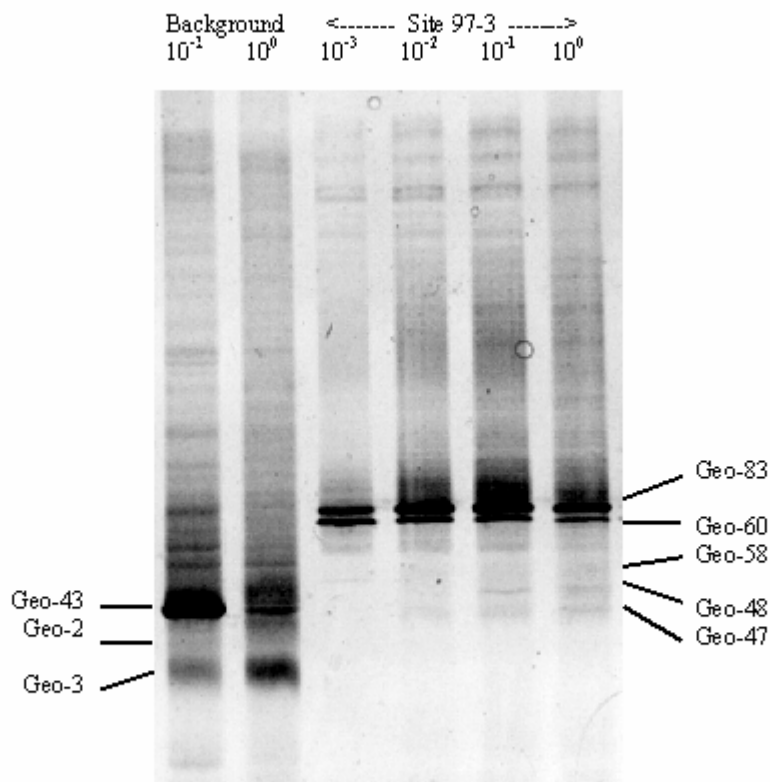
Figure 6. Comparison of *Geobacteraceae* detected in benzene-degrading sediment from site 97-3 and an uncontaminated background sediment (adapted from Rooney-Varga and others, 1999).

More intensive DGGE analysis contrasted the differences between *Geobacteraceae* recovered from site 97-3 and the background sites (Figure 6). Bands from benzene-degrading sediment were clearly different than those from background sediments with no shared bands between the two sites suggesting that different populations of *Geobacteraceae* were found in each sediment. When sequences recovered from both sites were analyzed to infer phylogenetic relationships, two distinct clusters of organisms were observed which correlated with the location of sediment samples. Sequences from the benzene-degrading site (97-3) clustered among *Geobacteraceae* known to degrade aromatic compounds while sequences from the background site did not. Furthermore, DNA extracted from a benzene-degrading enrichment culture established from benzene-degrading sediment collected at Bemidji also contained a sequence (Benz-76, Figure 7) that clustered among the *Geobacteraceae*. These results suggest that a rapid assay could be developed to detect *Geobacteraceae* in contaminated sediments in order to assess the potential for anaerobic benzene degradation in contaminated aquifers.
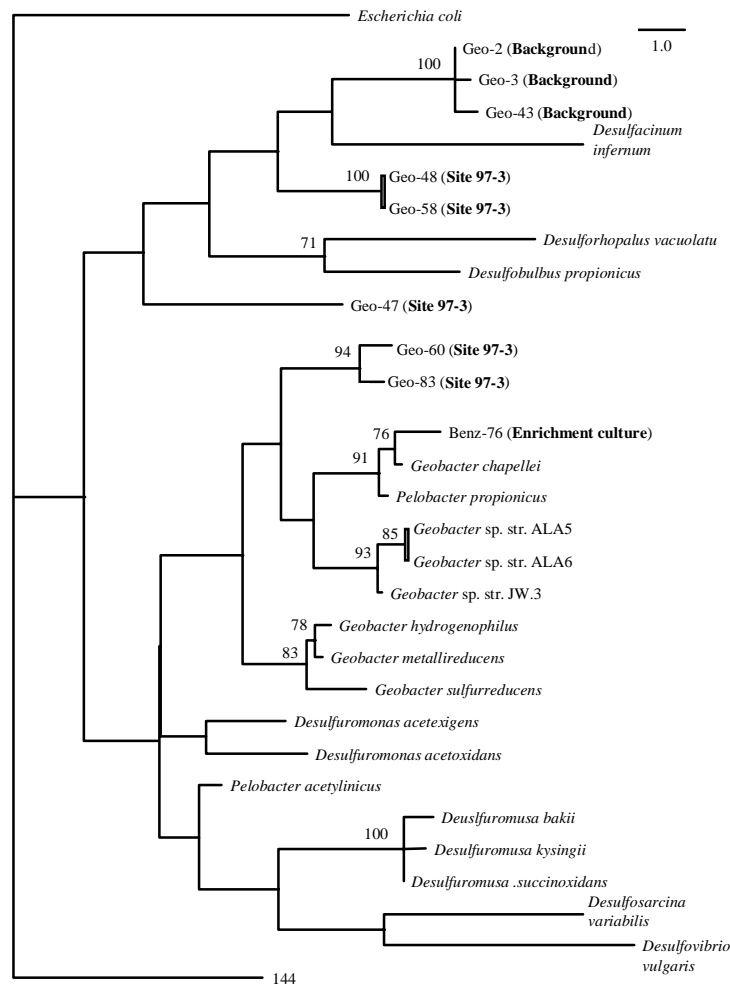
Figure 7. Phylogenetic relationship of sequences detected in benzene-degrading sediment from site 97-3, from an background site and a benzene-degrading enrichment culture developed from benzene degrading sediment collected at the Bemidji site (adapted from Rooney-Varga and others, 1999).

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