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Diversity of 16S rDNA and Naphthalene Dioxygenase Genes from Coal-Tar-Waste-Contaminated Aquifer Waters

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Abstract

Microbial diversity in four wells along a groundwater flowpath in a coal-tar-waste-contaminated aquifer was examined using RFLP analysis of both 16S rDNA and naphthalene dioxygenase (NDO) genes. Amplified ribosomal DNA restriction analysis (ARDRA) relied upon eubacteria-specific primers to generate four clone libraries. From each library, 100 clones were randomly picked for analysis. Sixty percent of 400 clones contained unique ARDRA patterns. Diversity indices calculated for each community were high (Shannon–Weaver, $H' = 3.53$ to 3.69). Clones representing ARDRA patterns found in the highest abundance were sequenced (31 total). Sequences related to aerobic bacteria (e.g., *Nitrospira, Methylomonas,* and *Gallionella*) predominated among those retrieved from the uncontaminated area of the site, whereas sequences related to facultatively aerobic and anaerobic bacteria (e.g. *Azoarcus, Syntrophus,* and *Desulfotomaculum*) predominated among those retrieved from contaminated areas of the site. Using NDO-specific primers and low-stringency PCR conditions, variability in RFLP patterns was only detected in community-derived DNA (3 of 4 wells) and not in 5 newly isolated naphthalene-degrading pure cultures. The ARDRA patterns of the pure culture isolates were not found in the clone libraries. Polymorphisms in community 16S rDNA and NDO genes found in well-water microorganisms reflected distinctive geochemical conditions across the site. Sequences related to sulfate-reducing bacteria were found in groundwater that contained sulfide, while sequences related to *Gallionella, Syntrophus,* and nitrate-reducing aromatic hydrocarbon-degrading bacteria were found in groundwater that contained ferrous iron, methane, and naphthalene, respectively.

Introduction

The composition of microbial communities reflects physical, chemical, geological, and biological characteristics of their habitats. Thus, information about key community members can be combined with knowledge of ecosystem

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history to develop hypotheses about biogeochemical processes occurring in a given habitat. For example, the composition of bacterial communities present in two deep alkaline, anaerobic aquifers reflected the geochemical signature of the groundwater [16]. Enriched numbers of sulfate-reducing bacteria (SRB) indicated *in situ* oxidation of organic carbon by SRB where concentrations of sulfate, sulfide, and dissolved inorganic carbon (depleted in ^{13}C) were high [16]. Similarly, *in situ* reduction of $^{12}CO_2$ by autotrophic methanogens was inferred in strata containing high numbers of methanogens, low concentration of dissolved inorganic carbon (DIC), and DIC enriched in ^{13}C [16]. More recently, anaerobic methane oxidation in the marine habitat has been shown to involve a close association of SRB and methane-consuming methanogens by stable isotopic analysis of cell components, fluorescent *in situ* hybridization, and related sediment characteristics [3, 30, 54], These examples demonstrate how geochemical and microbiological evidence can be used to document *in situ* biogeochemical processes.

The paradigm of using convergent lines of geochemical and microbiological evidence to understand biogeochemical processes also applies to *in situ* metabolism of organic pollutant compounds [4, 20, 31, 43, 51]. At a diesel-fuel-contaminated aquifer, intrinsic bioremediation was documented by isolating $>10^6$ aerobic and denitrifying petroleum hydrocarbon-degrading organisms per gram; identifying *Azoarcus* species among the isolates; confirming the abundance of *Azoarcus* (1% of the community) by hybridization; demonstrating petroleum hydrocarbon degradation concomitant with electron acceptor loss in microcosms; and measuring depleted oxygen and nitrate and higher levels of dissolved inorganic carbon in the contaminated area [31]. Another recent multidisciplinary study of a petroleum–hydrocarbon contaminated aquifer reported that *Methanosaeta,* an acetoclastic methanogen, predominated where methane and DIC concentrations rose [4]. Furthermore, in a BTEX-contaminated aquifer, *Geobacteracae,* a family that includes iron-reducing benzene degraders, predominated in iron-reducing zones [60].

Current molecular methodologies for describing microbial communities (such as amplified ribosomal DNA restriction analysis (ARDRA), DGGE, and t-RFLP) [12, 15, 32, 50, 56] provide an approach to understanding therelationship between habitat geochemistry and native microorganisms. For example, phylogenetic studies of microorganisms in acid mine drainage communities identified novel, uncultured organisms related to the iron-oxidizing groups "*Ferroplasma,*" *Leptospirillum, Sulfobacillus,* and *Acidimicrobium* predominant in low-pH, pyrite-rich environments [5, 6]. In addition, at a jet-fuel- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation, ARDRA and sequence analysis demonstrated the presence of a diverse microbial community dominated by *Methanosaeta* and *Syntrophus* species, indicating that aceticlastic methanogenesis was the final step in hydrocarbon degradation at this site [11].

Bakermans et al. [1] presented data describing geochemical characteristics of a coal-tar-waste-contaminated study site. In this parallel study, we present complementary information on the site's microbial community. The microbial community in site well waters was characterized by examining (1) naphthalene-degrading pure culture isolates, (2) archetypal naphthalene dioxygenase (*nahAc*) genes, and (3) community structure through ARDRA and sequence analysis. Naphthalene dioxygenase sequence diversity was detected only in community extracted DNA, not in pure culture isolates. ARDRA clone libraries were highly diverse and contained a high number of unique sequences for each groundwater sample examined. Differences in the ARDRA-determined community compositions correlated strongly with anaerobic carbon and energy flow induced by the coal-tar waste.

Materials and Methods

Field Site and Groundwater Sampling

The site, located in South Glens Falls, NY, has been described previously [43, 49, 66, 69, 72, 73]. Groundwater sampling techniques and locations are described in the accompanying article [1].

Isolation of Naphthalene-Degrading Bacteria from Groundwater

On site within 1 h of sampling, a dilution series of groundwater was prepared in phosphate-buffered saline (PBS) (120 mM NaCl, 2.7 mM KCl, 10 mM potassium phosphate buffer pH 7.6) and plated onto Stanier's minimal salts (MSB) plates [65]. Plates were incubated aerobically with exposure to naphthalene vapors at 10°C [33]. After 4 weeks of incubation, colonies were selected for isolation and were purified by multiple streaking of single colonies on MSB plus naphthalene plates. Naphthalene degradation by isolates was verified by mineralization of ^{14}C-naphthalene [41]. The previously described assay was used with the following modifications. Cells were inoculated in 25-mL screw-cap vials (Pierce, Rockland, IL) containing 3 mL LB (10 g/L tryptone, 5 g/L

yeast extract, 10 g/L NaCl, pH 7.2). After incubation for 2 days at room temperature with shaking at 150 rpm, ^{14}C-naphthalene (8628 dpm total) and salicylic acid (to 0.02%) were added to each vial. Labeled naphthalene converted to $^{14}CO_2$ was measured in a scintillation counter (model 5000CE; Beckman Instruments, Inc., Fullerton, CA) after 2 days and compared to negative (uninoculated) and positive (*Pseudomonas putida* NCIB 9816-4) controls.

Sample Collection

Cells were concentrated from groundwater onto 0.22 μm pore size, 142 mm diameter Durapore membranes (Millipore Corp., Bedford, MA). Approximately 5 L of water (17 min at 300 mL/min) was filtered from each well (except MW8, from which approximately 3.5 L of water was filtered). Immediately following concentration, filters were placed into sterile Whirlpak bags (NASCO, Modesto, CA) and immersed in a dry ice/ethanol bath for transport back to the laboratory where they were removed and stored at −80°C.

Nucleic Acid Extraction

The extraction protocol developed by Wilson et al. [72] was used through the first precipitation step with the following modifications. Filter pieces were extracted a total of three times (once with lysis buffer and twice with pH 8.0 buffer). Samples were extracted with an equal volume of phenol equilibrated with pH 8.0 buffer, followed by extraction with an equal volume of 1:1 phenol (pH 8.0): chloroform. Following overnight precipitation, nucleic acids were collected by centrifugation at 12,000 × *g* and 4°C for 30 min. Nucleic acid pellets were washed with 5 mL cold 70% ethanol and recollected by centrifugation at 12,000 × *g* and 4°C for 5 min. The supernatant was removed and the pellet dried by inverting the tube in a sterile hood for 15 min. Pellets were resuspended in 1.4 mL TE buffer (10 mM Tris-HC1, pH 7.5, 1 mM EDTA), incubated overnight at 4°C, mixed by gentle pipetting, aliquoted into three 1.5-mL microcentrifuge tubes, then reprecipitated by addition of 0.1 volumes 4 M sodium acetate pH 4.1, 1.5 μL 20 mg/mL glycogen, and 2 volumes 100% ethanol. The samples were incubated overnight at −20°C and precipitated nucleic acids collected by centrifugation at 14,000 rpm in a benchtop microcentrifuge for 30 min. The supernatants were removed and the nucleic acid pellet washed with 0.5 mL cold 70% ethanol. Pellets were dried by inverting tubes in a sterile hood for 15 min, resuspended in 35 μL buffer TE buffer, incubated at room temperature for 30 min, and mixed by gentle pipetting. The three aliquots of each sample were recombined and stored at −20°C and represented approximately 5×10^6, 4×10^6, 5×10^6, and 1.5×10^5 cells/μl for MW36, MW8, MW12, and MW4, respectively (based on acridine orange direct counts).

PCR for nahAc

In a 200-μL thin-walled Eppendorf tube, 10 μL of extracted nucleic acids, 10 μL H_2O inoculated with cells on the tip of a sterile platinum inoculating needle, or 10 μL H_2O (negative control) was denatured for 5 min at 95°C. Following denaturation, samples were mixed with reaction mix to a final volume of 50 μL containing 1× PCR Buffer (Gibco BRL, Gaithersburg, MD), 1.5 mM $MgCl_2$, 1 μM each of primers Ac114f (5′-CTGGCWWTTYCTCACYCAT-3′) and Ac596r (5′-CRGGTGYCTTCCAGTTG-3′) [72], 50 μM each dNTP, and 1 unit *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD). PCR reactions were performed in a PTC-200 thermocycler (MJ Research Inc., Watertown, MA) as follows: 94°C for 5 min; 30 cycles consisting of 94°C for 30 sec, 43°C for 1 min, and 72°C for 1 min followed by 72°C for 5 min. The low annealing temperature was used in an attempt to amplify more diverse genes. Following PCR, 10 μL of each reaction was analyzed on a low-melting-point agarose gel. Because of the high number of amplicons present, the expected 483-base-pair amplicon was excised for reamplification. The gel slices were melted at 80°C for 10 min and diluted 1:1 with water to 10 μL for addition to the PCR tube.

Reamplification was performed as above except the annealing temperature was raised to 55°C. The resulting amplicons were digested with *Hae*III or *Hha*I (Gibco BRL, Gaithersburg, MD) by adding 2 μL React II Buffer and 1 μL restriction endonuclease to 17 μL PCR reaction and incubating at 37°C for 3.5 h. To stop the reaction, 4 μL of 6× loading dye (15% Ficoll, 0.15% bromphenol blue, 0.15% xylene cyanol FF) was added to each digest and samples were analyzed on a 1.5% agarose gel.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

PCR for eubacterial 16S rDNA genes from community DNA was performed using primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) [35] in 50-μL reactions that contained 1× PCR Buffer (Gibco BRI), 3 mM $MgCl_2$, 40 nM of each primer, 0.40 mM of each deoxynucleoside triphosphate (dNTPs), and 1 U *Taq* DNA polymerase (Gibco BRL) using the PTC-200 thermocycler as follows: 94°C for 5 min; 35 cycles consisting of 94°C for 30 s, 55°C for 1 min 72°C for 1 min followed by 72°C for 5 min. Extracted groundwater DNA (2.75 μL) was used as template while 2.75 μL of sterile water was used in negative controls. PCR products were ligated into the vector pCR2.1 from the TA Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's recommended protocol. After transformation of plasmids into host cells and blue/white screening of colonies, white colonies were picked from plates and the presence of inserts verified by PCR with primers 27f and 1492r. [Note: PCR with primers 27f and 1492r will also amplify the *Escherichia coli* rDNA. False positives were seen in 11.7% of the clones and were easily identified during analysis of RFLP patterns.] Digests and analyses were performed as described above, except both *Hae*III and *Hha*I were used in the same reaction. RFLP patterns were analyzed on 3% MetaPhor agarose (BioWhittaker Molecular Applications, Rockland, ME) gels with 100 base pair ladder (Gibco BRI) as marker. Fragment lengths were determined and patterns compared using BIORAD Diversity Database 1.0 software package (Bio-Rad Laboratories, Her-

cules, CA). Shannon–Weaver index of diversity ($H' = -\sum a_i \ln a_i$, where a_i is the proportional abundance of the ith RFLP pattern [52]) was calculated.

Sequencing of Clones and Sequence Analysis

Cultures were grown overnight at 37°C with shaking at 250 rpm in 5 mL of LB and 100 μg/mL ampicillin in Falcon brand 17 × 100 mm polypropylene round-bottom tubes (Fisher Scientific, Pittsburgh, PA). Following incubation, cells were collected by centrifugation in a TJ-6 tabletop centrifuge with a TH4 rotor and buckets (Beckman Instruments, Palo Alto, CA) for 15 min at 1500× *g* and stored at −20°C. Frozen cell pellets were processed by the Cornell DNA Sequencing Facility (Ithaca, NY) for plasmid purification and sequencing with the primers 27f, 533f (5′-GTGCCAGCMGCCGCGG-3′) [35], and 1492r. Raw sequence data was assembled into full-length sequences using the program SeqMan II (DNAStar, Inc.). Following assembly, the consensus sequence was verified manually by referring to the corresponding ABI chromatograms of the sequencing reactions. Sequences were checked for chimeras using the Ribosomal Database Project's (RDP) Chimera Check program and closest relatives identified using the RDP's Sequence Aligner [44]. Sequences were then imported into ARB (Strunk and Ludwig, http://www.biol.chemie.tu-muenchen.de/pub/ARB/) for phylogenetic tree construction [39].

Accession Numbers

The nucleotide sequence data reported here have been submitted to GenBank under accession nos. AF351212 to AF351240.

Results

At the study site, the monitoring wells (MW) fall linearly along approximately 300 meters of the groundwater flow path. MW4 is in a pristine area 90 m upgradient of where the coal tar waste was buried (now excavated), while MW8 is the first well downgradient (80 m) of the former source area. MW36, further downgradient, currently has the highest level of residual contamination [1]. MW12, furthest downgradient, has been the least polluted throughout its contamination history.

Naphthalene-Degrading Isolates

Five isolates were obtained from two of the four monitoring wells. Four isolates (cb1, cb2, cb3, and ek3) were isolated from MW36, while cb8 was isolated from MW12. All five strains shared the same ARDRA pattern and one representative isolate (cb3) was chosen for sequencing. Sequence analysis showed that cb3 was closely related to *Pseudomonas putida* (98.9% similarity). None of the clone libraries (see below) contained the isolates' ARDRA pattern. PCR amplification of *nahAc* genes with primers Acl 14f and Ac596r detected the *nahAc* allele in all of the five isolates. When digested with *Hae*III and *Hha*I, *nahAc* amplicons from these isolates displayed the same banding patterns as *nahAc* from *P. putida* NCIB 9816-4 (data not shown). Because of lack of evidence for anaerobic naphthalene degradation [1, 42], no attempts were made to isolate anaerobic naphthalene-degrading bacteria.

PCR Amplification of nahAc from Nucleic Acids Extracted from Groundwater

PCR amplification with primers for *nahAc* was used to assess the presence of the archetypal naphthalene degradation genes. The expected mixture of 482 bp amplicons was found in all four groundwater communities tested (Fig. 1A). Additional nonspecific bands were present in all cases (except negative controls). The expected 482 bp amplicons were excised from the gel (shown in Fig. 1A; all lanes, including MW12) for reamplification by PCR (data not shown) prior to RFLP characterization. Gel slices from both the negative control (water) and MW12 failed to reamplify. All successful amplifications (MW4, MW8, MW36, *P. putida* G7, and *P. putida* NICB 9816-4) were digested with either *Hae*III or *Hha*I (see Fig. 1B). As expected, from examination of GenBank *nahAc* entries from cultured microorganisms, digestion of *nahAc* from *P. putida* G7 and *P. putida* NICB 9816-4 with *Hae*III resulted in 353- and 111-bp bands. The *Hae*III RFLP patterns from MW4, MW8, and MW36 all contained the expected bands. However, evidence for previously undescribed diversity (polymorphisms) in the community *nahAc* genes was found. The RFLP pattern of MW8's *Hae*III-digested amplicon contained additional bands at 437 and 255 bp, while the RFLP pattern of MW36's amplicon contained additional bands at 437 and 320 bp. Evidence for the community *nahAc* diversity was also obtained from the *Hha*I digests. Although *Hha*I does not digest the *nahAc* gene of archetypal strains, the RFLP patterns of MW4, MW8, and MW36 contained bands at 430; 290 and 166; and 387 and 199 bp, respectively. Interestingly, none of the wells produced identical patterns when digested with either *Hae*III or *Hha*I; *nahAc* amplicans from each well contained unique polymorphisms.

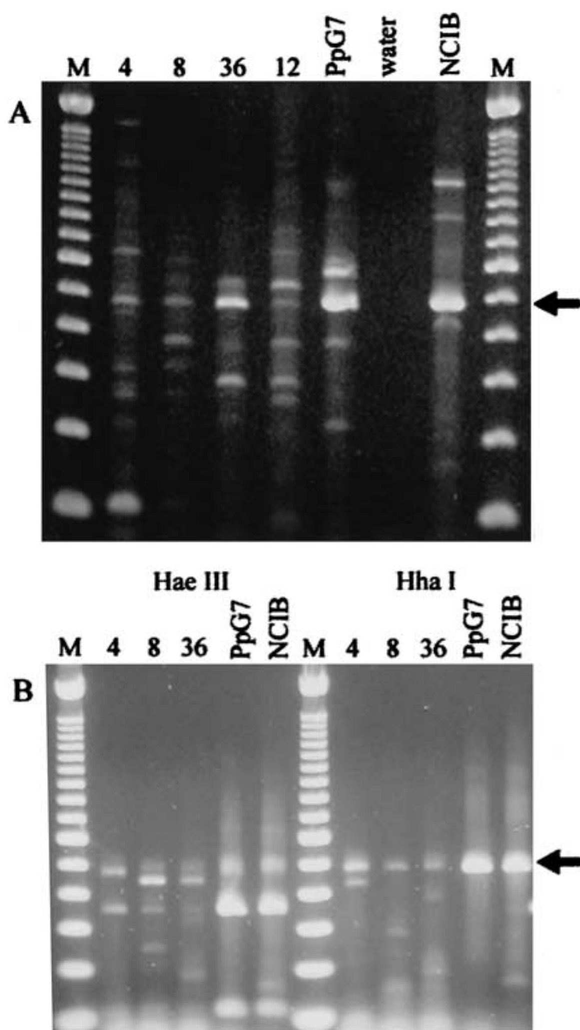

Fig. 1. (A) PCR detection of *nahAc* genes in community DNA extracted from coal-tar-contaminated groundwater. Lanes: M, 100 bp ladder; 4, 8, 36, and 12, DNA from respective monitoring wells; PpG7, *Pseudomonas putida* G7; water, negative control; and NCIB, *Pseudomonas putida* NCIB 9816-4. Arrows show the position of the expected 480-bp amplicons. (B) RFLP analysis of *nahAc* reamplified from bands pictured in A. DNA in lanes 2–6 was digested with *Hae*III; while DNA in lanes 8-12 was digested with *Hha*I. Lanes: M, 100 bp ladder; 4, 8, and 36, DNA from respective monitoring wells; PpG7, *Pseudomonas putida* G7; and NCIB, *Pseudomonas putida* NCIB 9816-4.

16S rDNA Analysis

DNA samples from the four monitoring wells were examined for 16S rDNA genes by PCR with primers 27f and 1492r. The expected amplicon size (~1500 bp) was observed in all samples (except negative controls). Following cloning of the mixture of amplicons into pCR2.1, ARDRA patterns of 400 clones were examined, 100 from each well. Clone nomenclature utilized a hyphenated, paired number system. The first number represents the well from which the DNA originated (4, 8, 36, or 12). The second number represents the clone's number (1 to 160). ARDRA pattern designations were assigned to the first clone from each well found with that representative pattern.

Histograms of the pattern frequencies are presented in Fig. 2. Each clone library contained several ARDRA patterns that occurred repeatedly (left side of each panel) and a predominance of single, unique ARDRA patterns (right side of each panel). Of 100 clones examined in each clone library, 57 to 66% were found to contain unique ARDRA patterns. However, MW4 and MW12 were clearly dominated by one pattern type: 4-11 (23% of clones) and 12-2 (25% of clones), respectively, while MW8 and MW36 had several more abundant members: 36-9 (15%), 36-28 (8%), 36-20 (5%), 8-11 (16%), 8-45 (12%), and 8-4 (7%). The majority of patterns were unique to each clone library, data not shown (90%, 82%, 90%, and 95% for MW4, MW8, MW36, and MW 12, respectively). Of the 242 total patterns, 11 were present in 2 clone libraries and 1 was found in 3 clone libraries. MW4, MW8, and MW36 shared 3 to 4 patterns with each other, while MW12 shared patterns only with MW8. The presence of patterns shared between clone libraries is consistent with hydrologic continuity between wells. The 4 clone libraries shared approximately the same level of diversity (similar number of unique patterns) as determined by the Shannon–Weaver diversity index (H') [45, 52]. H' was equal to 3.58, 3.53, 3.69, and 3.62 for MW4, MW8, MW36, and MW12, respectively. Surprisingly, the currently most contaminated well, MW36, showed the highest diversity ($H' = 3.69$) in its clone library.

Selected 16S rDNA clones were sequenced from each clone libary (31 total). Because simultaneous digestion with 2 restriction enzymes was used to generate ARDRA patterns and can identify 96% of operational taxonomic units [48], clones containing the same ARDRA pattern should be very similar. Therefore, when more than one clone contained the same ARDRA pattern, only one representative clone was sequenced. In MW12, six clones representing ARDRA patterns found in more than one clone were sequenced. Nine, six, and eight clones representing ARDRA patterns found in multiple clones were sequenced from MW4, MW8, and MW36, respectively. In addition, clones found in more than one clone library were sequenced (36-111 and 8-65). As anticipated, sequence analysis revealed the presence of several chimeras (clones 36-31, 36-8, and 8-17). Chimeras were expected to be present in 10% of clones [21]. Clones 12-30, 4-42, and 12-

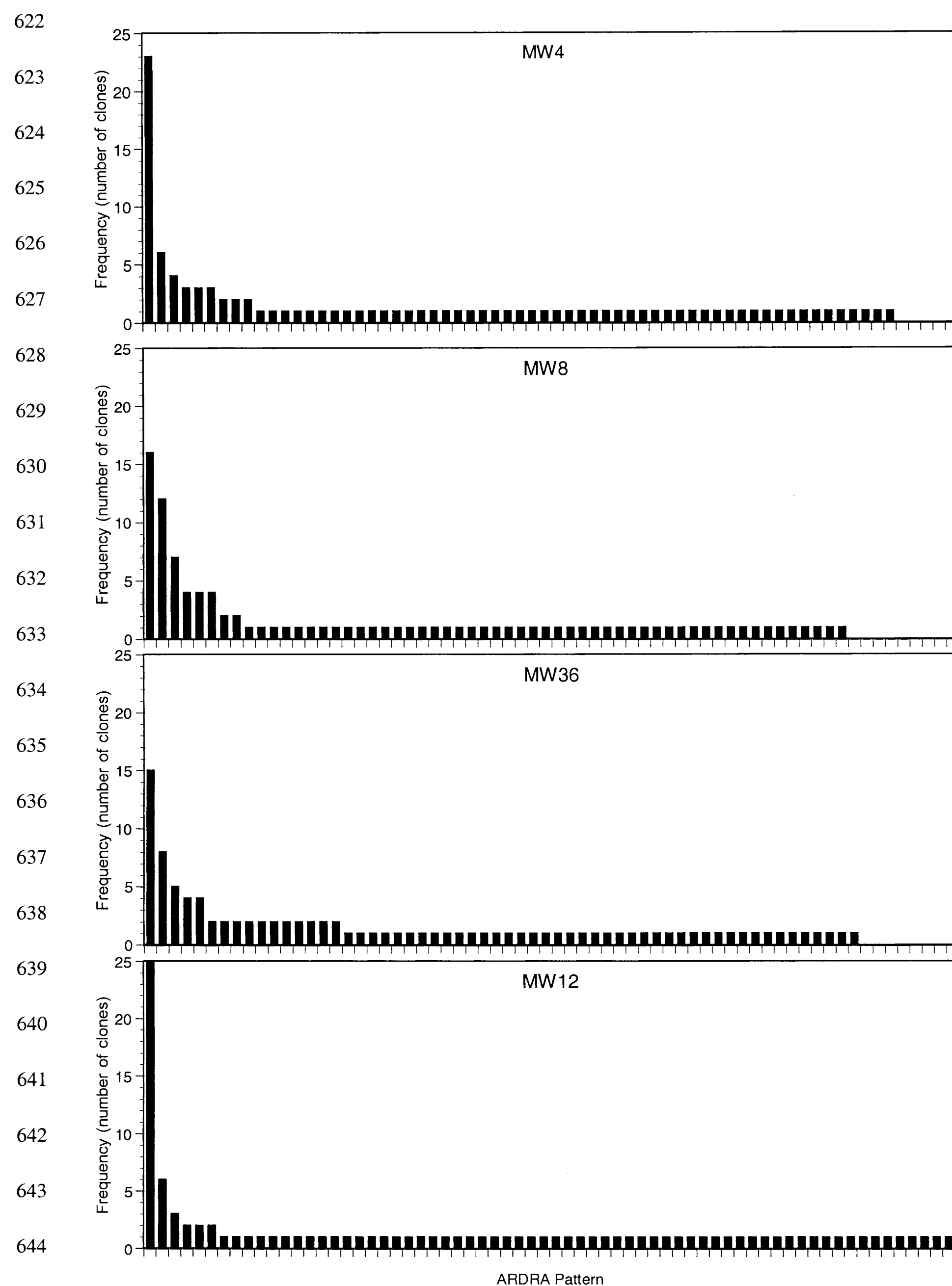

Fig. 2. Histograms of RFLP pattern frequency in four wells.

31 may also be chimeras; however, the diversity of the divisions in which they grouped and the large number of partial sequences from environmental clones that they were similar to precluded the accurate assessment of their status as chimeras. The sequences analyzed represent 47, 39, 41, and 41% of the clones in the clone libraries from MW4, 8, 36, and 12, respectively.

Phylogenetic trees were constructed from sequences obtained from selected clones, pure culture isolates, and reference strains (Fig. 3). The majority of clones from MW4 (pristine, aerobic) grouped with *Nitrospira* (28% of clones); in the γ-proteobacteria (10% of clones) with *Methylomonas* and *Pseudomonas*; or in the β-proteobacteria (8% of clones) with *Comamonas* and *Gallionella.* The majority of clones from MW8 grouped in the β-proteobacteria with *Azoarcus* and *Gallionella* (22% of clones), in the δ-proteobacteria with *Syntrophus gentianae* (13%), or in the γ-proteobacteria with *Methylomonas* (8%). The majority of clones from MW36 grouped with the low-GC gram-positive organisms with *Desulfotomaculum* (19% of clones), in the β-proteobacteria with *Azoarcus* and *Comamonas* (16%), or in the δ-proteobacteria with *Syntro-*

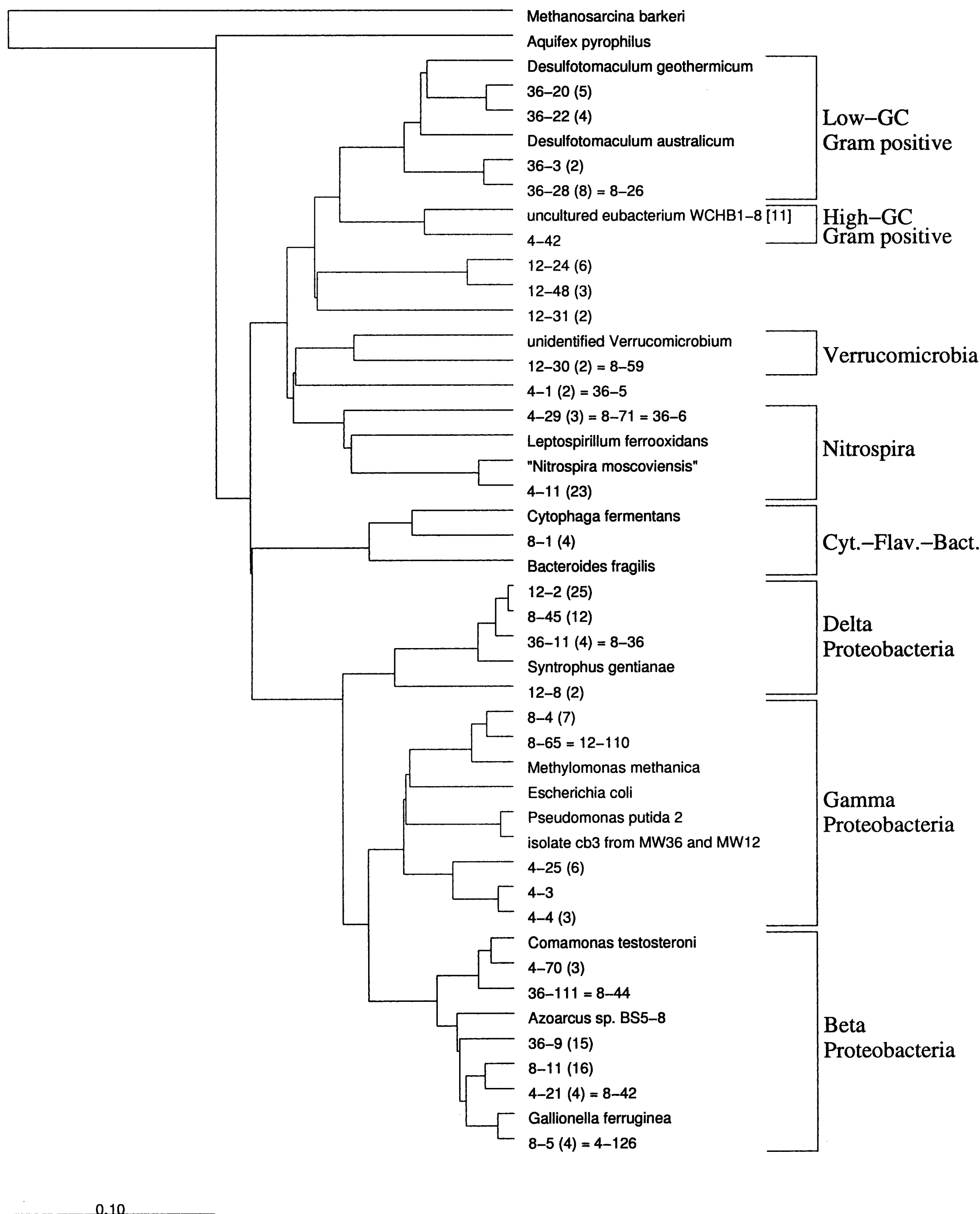

Fig. 3. Phylogenetic tree of 16S rRNA genes derived from 28 clones and 1 isolate from coal-tar-contaminated groundwater. Clones from the four monitoring wells were identified with a hyphenated, paired number system (see text). When a given sequence represented more than one clone, the number of clones with the same ARDRA pattern is in parenthesis following the clone number. *Methanosarcina barkerii* and *Aquifex pyrophilus* served as outgroups. Genera were grouped and assigned lineages [39]. The tree was constructed in ARB using the Neighbor Joining program with Kimura correction and bact_rr5 filter from the Phylip package. The scale bar is equal to 0.10 changes per nucleotide position.

phus gentianae (4%). The majority of clones from MW12 grouped in the δ-proteobacteria with *Syntrophus gentianae* (27% of clones), with no close relatives (11%), or with a new division containing *Verrucomicrobium* (2%).

Discussion

As expected, clear contrasts between culture-based and nonculture-based descriptions of *nahAc* were found in this

study. Growth-based pure culture techniques revealed only *Pseudomonas*-type bacteria and *nahAc* alleles in the contaminated areas, while molecular techniques detected apparent additional naphthalene-degrader diversity as indicated by *nahAc* sequence polymorphisms in three of four wells examined. These results agree with prior investigations elsewhere [25] and at this study site: all naphthalene-degrading bacteria isolated from site surface sediments contained the *nahAc* allele, whereas PCR amplification and RFLP analysis of *nahAc* in DNA extracted from highly contaminated (source) and distant downgradient (seep) sediments revealed additional diversity [27–29]. In contrast, only 45% of naphthalene-degrading isolates from contaminated New Zealand soils contained *nah*-like alleles; 81% of those alleles shared the same RFLP pattern as *nahAc* [38]. DNA extracted from 7 of 9 samples from the New Zealand contaminated soils contained amplifiable *nahAc* alleles; however, amplicon diversity was not analyzed [38]. Diversity is expected from both culture-based and non-culture-based techniques, since at least 13 naphthalene dioxygenase genes have been identified to date [8, 10, 17, 19, 24, 26, 36, 37, 62, 67, 68].

By sequencing representatives of the most abundant ARDRA patterns, we documented changes in community composition along the groundwater flowpath at the study site. We recognize that our data do not approach an accurate census of the organisms in site groundwater because of unavoidable methodological and genetic biases (cell lysis, DNA extraction, primer annealing, multiple PCR cycles, varying gene copy numbers of rRNA, etc. [21, 40, 71]) that distort sequences from their original *in situ* abundances in the sampled microbial community. Thus, we must balance ARDRA-based changes in community composition across the study site against conservative interpretation of data. Several studies have indicated that ARDRA-based community analyses can sometimes correlate well with fluorescent *in situ* hybridization [63] or RNA hybridization analysis of community structure [22, 23].

MW4 is upstream from the contaminated zone and represents the background pristine community present prior to contamination. The predominant genus found in MW4, *Nitrospira* (28%), occurred rarely (0–2%) in the recovered downgradient communities. Members of the genus *Nitrospira* are aerobic, nitrite oxidizers. The γ-proteobacteria, recovered from MW4 and MW8 at approximately the same frequency (10 and 8%, respectively), were barely represented (0 and 1%) in MW36 and MW12, respectively. The γ-proteobacteria found at the site were represented by phylotypes related to *Methylomonas* (aerobic to microaerobic methane oxidizers) and *Pseudomonas* (mesophilic chemoorganotrophs with respiratory metabolism). The β-proteobacteria, detected at 8% in MW4, 22% in MW8, and 16% in MW36, were absent in MW12. Phylotypes related to *Comamonas* (like *Pseudomonas*), *Azoarcus* (facultatively aerobic denitrifiers), and *Gallionella* (iron-oxidizing bacteria that live at oxic–anoxic interfaces and neutral pH) represented the β-proteobacteria. Interestingly, no δ-proteobacteria were found in MW4, which had previously been shown to be methane-free [1]. Conversely, all the contaminated wells contained biogenic methane [1] and sequences closely related to *Syntrophus gentianae* of the δ-proteobacteria. The genus *Syntrophus* comprises bacteria that participate in interspecies hydrogen transfer through the production of hydrogen and are closely associated with hydrogen consumers such as methanogens or sulfate reducers. The low-GC gram-positive organisms, represented by sequences related to *Desulfotomaculum* sp., were rare in MW8 (1%) but significant in MW36 (19%). Species of the genus *Desulfotomaculum* are obligately anaerobic, sulfate reducers that are widespread, form endospores, and are capable of chemolithotrophic growth. The overall trend progressed from communities abundant in sequences related to aerobic bacteria (upstream of the contamination) to communities abundant in sequences related to anaerobic bacteria (in the contaminated zones).

ARDRA patterns of 400 clones (100 clones from each of 4 MW) were predominantly unique to each well, with 60% representing unique patterns. Although less diverse than soil habitats, subsurface microbial communities often support unique microbial species and/or sequences [55]. Thus, diversity and heterogeneity was expected here—and has been seen in other contaminated systems. For example: based on ARDRA, 50% of clones from a hydrocarbon and chlorinated-solvent-contaminated aquifer were unique [11], while analysis of 16S rDNA from petroleum-contaminated sediments also indicated heterogeneity [60]. In highly diverse habitats (e.g., soil), encountering unique, unduplicated ARDRA patterns or sequences is expected [7, 14, 53, 74]. Generally, duplicated patterns or sequences are only found in environments where diversity was reduced by strong selective pressure, such as low pH [14, 45] or hydrologic continuity.

Because of the presence of sulfate and sulfide in site waters [1], the predominance of phylotypes related to

sulfate-reducing bacteria (SRB) in the clone library from MW36 was not unexpected. At another site, aerobic hydrocarbon degradation was hypothesized to stimulate the growth of SRB [13], probably via the consumption of toxic oxygen. SRB closely related to *Desulfosporosinus orientis* (previously named *Desulfotomaculum orientis*) were associated with intrinsic bioremediation of toluene, probably through syntrophic associations [57]. Furthermore, naphthalene degradation under sulfate-reducing conditions by enrichment [9, 47, 59] and pure [18] cultures has been documented. Clearly, potential exists for the direct or indirect involvement of SRB in coal-tar-waste degradation in the vicinity of MW36.

The predominant sequence type obtained from both MW36 and MW8 was highly related to the genus *Azoarcus,* which includes many aromatic hydrocarbon-degrading nitrate reducing bacteria (NRB). *Azoarcus* species have been shown to degrade phenol [61, 70], *m*-xylene [34], and toluene [64] under denitrifying conditions. Naphthalene-degrading NRB have also been isolated [46, 58]. Pure cultures of NAP-3-1, closely related to *Pseudomonas stutzeri,* coupled denitrification to naphthalene oxidation, while NAP-4, closely related to *Vibrio pelagius,* oxidized naphthalene with reduction of nitrate to nitrite [58]. No nitrate was detected in MW36 or MW8 groundwater; however nitrate was found in groundwater both upstream (MW4) and downstream (MW12) of these monitoring wells [1]. Based on the prevalence of phylotypes related to NRB in MW36 and MW8 clone libraries, we hypothesize that any nitrate in recharge waters that reach this depth may be consumed immediately. NRB could potentially be involved in the degradation of the monocyciic aromatic hydrocarbon constituents of the coal tar waste that represent 3.8% of the total mass, of which half is *m*/*p*-xylene [69].

Geochemical data presented in a companion paper [1] firmly established that coal tar waste constituents have induced significant anaerobic carbon and electron flow at the study site. Detection of sequences related to anaerobic bacteria predominantly in the contaminated zones of the site was consistent with the prevalence of *in situ* anaerobic processes. Yet no indication of anaerobic naphthalene biodegradation was found [1]. To reconcile these observations, we hypothesize that low numbers of oxygen-respiring naphthalene-degrading microorganisms are active *in situ* at the anaerobic–aerobic interface that likely surrounds the contaminant plume. In support of this notion, aerobic naphthalene-metabolizing members of the community have been enumerated [1, 2]; furthermore, the present study easily isolated *Pseudomonas* sp. from contaminated groundwater, although their ARDRA patterns were not detected in clone libraries constructed from bulk community DNA. In summary, a mixture of aerobic and anaerobic degradation processes likely prevails at the site.

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