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Composition and Diversity of Microbial Communities Recovered from Surrogate Minerals Incubated in an Acidic Uranium-Contaminated Aquifer

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Our understanding of subsurface microbiology is hindered by the inaccessibility of this environment, particularly when the hydrogeologic medium is contaminated with toxic substances. In this study, surrogate geological media contained in a porous receptacle were incubated in a well within the saturated zone of a pristine region of an aquifer to capture populations from the extant communities. After an 8-week incubation, the media were recovered, and the microbial community that developed on each medium was compared to the community recovered from groundwater and native sediments from the same region of the aquifer, using 16S DNA coding for rRNA (rDNA)-based terminal restriction fragment length polymorphism (T-RFLP). The groundwater and sediment communities were highly distinct from one another, and the communities that developed on the various media were more similar to groundwater communities than to sediment communities. 16S rDNA clone libraries of communities that developed on particles of a specular hematite medium incubated in the same well as the media used for T-RFLP analysis were compared with those obtained from an acidic, uranium-contaminated region of the same aquifer. The hematite-associated community formed in the pristine area was highly diverse at the species level, with 25 distinct phylotypes identified, the majority of which (73%) were affiliated with the β-*Proteobacteria*. Similarly, the hematite-associated community formed in the contaminated area was populated in large part by β-*Proteobacteria* (62%); however, only 13 distinct phylotypes were apparent. The three numerically dominant clones from the hematite-associated community from the contaminated site were affiliated with metal- and radionuclide-tolerant or acidophilic taxa, consistent with the environmental conditions. Only two populations were common to both sites.

Effective management and reclamation of contaminated subsurface geological media requires an understanding of the composition of extant microbial communities. To date, characterization of indigenous subsurface microbial populations has mainly focused on microbes collected from sediment or rock samples (3, 6, 10, 22, 41) or groundwater (5, 7, 27). Subsurface sediment-based diversity studies are often impractical because sediment cores are both expensive and difficult to acquire. While groundwater is more readily obtained than sediments, contaminated groundwater poses the added problem of secondary waste generation. Due to these limitations, studies requiring numerous samples (e.g., temporal studies) are unrealistic with current methods. It has been suggested that those microorganisms most likely to be involved in contaminant transformation are often associated with the surfaces of the geological matrix through which the groundwater percolates (15, 18, 20, 21, 28). While water-sampling techniques allow for temporal and large-scale spatial sampling of transient or planktonic communities, they may not capture key populations partitioned on the solid phases.

Recently, investigators have used surrogate solid media contained in a porous receptacle, referred to hereafter as a biofilm coupon, to sample microbial communities in the subsurface with a propensity to associate with surfaces (42). The retrievable coupon can contain particles such as hematite or quartz with surface properties representative of the natural geological matrix. These surfaces serve as sites for colonization by planktonic microbial populations and promote close physical and temporally stable cooperative associations between members of the community. Biofilm coupons thus offer a pragmatic means of recovering populations of microorganisms that form communities optimized to function under a particular set of environmental conditions.

One objective of this study was to compare subsurface microbial communities that developed on media in biofilm coupons to those associated with the sediment and groundwater phases. Intact-biofilm PCR (IB-PCR), a modified PCR method that circumvents the need to separate DNA from other sample components prior to amplification of community 16S rRNA genes, is described for use in situations where recovery of DNA from samples containing certain mineral phases is problematic. A second objective of the study was to characterize and compare the indigenous microbial communities that

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TABLE 1. Groundwater characteristics of uncontaminated Background Area wells[a] and contaminated Area 3 well FW026

Well	Area	pH	ORP[b]	Dissolved components (mg liter^{-1})			
				Total Fe[c]	Nitrate[d]	Uranium[e]	TOC[f]
FW300	Background	6.13	NA[g]	0.05	1.20	0.00	25.96
FW303	Background	7.52	+437	NA	NA	NA	NA
FW026	Area 3	3.43	+354	17.4	8,262	42.74	68.27

[a] Most data have not yet been compiled for well FW303; therefore, data from well FW300, approximately 10.5 m southeast of well FW303, are provided as a proxy.
[b] Oxidation-reduction potential (mV), calibrated to the standard hydrogen electrode.
[c] Total dissolved Fe was measured by inductively coupled plasma-mass spectrometry (ICP-MS).
[d] Dissolved nitrate was measured by ion chromatography.
[e] Dissolved uranium was measured by ICP-MS.
[f] Amount of total organic carbon was determined by combustion.
[g] NA, not available.

developed on media (specular hematite particles) in biofilm coupons incubated at two saturated subsurface sites at a U.S. Department of Energy (DOE) Field Research Center (FRC) near Oak Ridge, Tenn. At one site, the subsurface is contaminated with radionuclides, metals, organics, and nitric acid and is currently under investigation for potential biological remediation. A nearby pristine region of the aquifer possesses similar hydrogeological characteristics and provides for a suitable comparison with the contaminated site. The results suggest that phylogenetically distinct microbial communities inhabit the two FRC sites, pristine and contaminated. Furthermore, microbial diversity at the contaminated site was diminished relative to the pristine community and was dominated by organisms apparently adapted to the harsh conditions.

MATERIALS AND METHODS

Site description. The U.S. DOE's Office of Science recently designated an area of the Oak Ridge Reservation (Oak Ridge, Tenn.) as an FRC in order to bring together scientists from multiple disciplines with the common goal of acquiring fundamental knowledge in support of in situ bioremediation. The FRC lies within the boundaries of the Y-12 National Security Complex near the Oak Ridge National Laboratory. The contaminant plume occupying the underlying aquifer is the result of leakage from two primary sources: the Boneyard/Burnyard and the S-3 Ponds. The Boneyard/Burnyard consisted of unlined trenches in which a variety of wastes including magnesium chips, solvents, and laboratory chemicals were burned from 1943 until 1968. The unlined S-3 Ponds received U-bearing liquids mixed with nitric acid from 1951 until 1983, when they were denitrified and capped. Due to the codisposal of nitric acid with the wastes, nitrate concentrations typically near 8,000 mg liter^{-1} have been measured in the groundwater, and the pH varies between 3.0 and 6.5 (Table 1). The contaminant plume occupies two distinct geological formations, Maynardville limestone and Nolichucky shale, and is enriched in organics (e.g., chloroaliphatics and aromatics), metals (e.g., Ni, Al, and As), and radionuclides (e.g., ^{238}U and ^{99}Tc). ^{238}U has been detected at concentrations as high as 43 pCi g of soil^{-1} and 13,600 pCi liter of groundwater^{-1}. By contrast, radionuclides and organic contaminants are below detection limits in the uncontaminated background area, and metals are at concentrations typical for the region (19, 48). For further information on this field site, we direct the reader to the DOE FRC web page (http://www.esd.ornl.gov/nabirfrc/index.html).

Sampling mineral surface-associated microbial communities with biofilm coupons. To entrain and capture microbial populations from the subsurface, biofilm coupons composed of capped stainless steel mesh cylinders (25.4 cm by 1.27 cm) (45) were filled with various solid substrata, hereafter referred to as coupon-associated media, and lowered by fishing line into the saturated zone of pristine (background area well FW303) and contaminated (Area 3 well FW026) regions of the aquifer, where they were incubated for approximately 8 weeks. Prior to deployment, minerals and coupons were sterilized by combustion (550°C, 6 h). Minerals included micaceous, specular hematite (diameter, 1 to 3 mm) from Minas Gerais, Brazil (kindly provided by K. Rosso, Pacific Northwest National Laboratory), illite shale (catalog no. 46 V 0315; Ward's Natural Science), uncontaminated saprolite from the FRC subsurface (a gift from P. Jardine, Oak Ridge National Laboratory), and coarse quartz sand. The different coupon-associated media were separated within the coupon apparatus by plugs of glass wool. Following retrieval, the coupons were shipped to the Idaho National Engineering and Environmental Laboratory (INEEL) at 4°C by overnight courier, where they were frozen at −80°C until analysis of 16S DNA coding for rRNA (rDNA), as described below. For geochemical characteristics of the background area and Area 3, see Table 1.

Retrieval of subsurface groundwater and sediments. Triplicate 4-liter aliquots of groundwater were pumped from a depth of 5.5 m from well FW303, and planktonic and colloid-associated cells were captured on 0.22-μm-pore-size filters. Sediments were collected within 1 m of well FW303 from depths of 4.9 m (unsaturated), 5.1 m (variably saturated), and 5.4 m (saturated), using a hollow-stem auger fitted with a 5.08-cm-diameter split-spoon sampler. Filters and sediments were frozen immediately and shipped to the INEEL on dry ice within 72 h. Additionally, core material from the original FW303 (background) and FW026 (contaminated) boreholes was acquired from FRC archives and used for 16S rDNA clone library construction (see below); these were shipped and stored at 4°C for approximately 3 weeks prior to analysis.

DNA extraction from colonized coupon-associated media, sediments, and groundwater filters. Community genomic DNA was obtained from hematite (triplicate 1.5-g samples), quartz sand (9.2 g), illite shale (6.9 g), saprolite (3.8 g), glass wool separating saprolite and illite (1.3 g), glass wool separating hematite and sand (0.76 g), sediments (10 g each), and groundwater filters (4 liters each) using bead-beating kits from either MoBio (UltraClean soil DNA kit or UltraClean Mega Prep soil DNA kit, depending on sample size) or Bio101 (FastDNA SPIN kit for soil). Sediments were allowed to thaw at 4°C overnight before DNA extraction. Bead beating was performed in a water bath at 65°C, and shaking was done at 400 rpm (30 min) for the Mega Preps or with a Mini-BeadBeater (BioSpec Products) at room temperature, or 2,500 rpm (1 min) for the other kits; all other steps were carried out as recommended by the manufacturers. DNA from the Mega Prep kit was eluted in 8 ml of water and concentrated to 100 μl by precipitation in NaCl and ethanol. DNA from the smaller kits was eluted in 50 μl of water and not concentrated further.

PCR of 16S rRNA genes. 16S rRNA genes were PCR amplified from each community genome extracted with the bead-beating kits. Each 50-μl reaction contained the following (final concentration): 1× PCR buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 200 μM (each) deoxynucleotide triphosphate (Invitrogen), 0.5 μM (each) forward and reverse primer (Invitrogen), 0.4-μg μl^{-1} molecular-grade bovine serum albumin (Roche), and 1.25 U of *Taq* DNA polymerase (Invitrogen). For clone libraries, conserved regions of the 16S rRNA gene were targeted with eubacterial forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′). For terminal restriction fragment length polymorphism (T-RFLP) analysis, fluorescently labeled 16S rDNA PCR products were generated using primer 8F-FAM (eubacterial primer 8F modified with phosphoramidite fluorochrome 5-carboxyfluorescein; Invitrogen) in conjunction with 907R (5′-CCGTCAATTCMTTTRAGTTT-3′, where M = A + C and R = A + G). Five microliters of genomic DNA was used as a template for 50-μl-volume PCRs. The following cycling conditions were applied: 4 min at 94°C followed by 30 cycles of denaturation at 94°C (1 min), annealing for 1 min across a broad range of temperatures, primer extension at 72°C (1.5 min), and a final extension step at 72°C (4 min). The Mastercycler Gradient thermocycler (Eppendorf) was used to generate a gradient of annealing temperatures from 40 to 60°C. Distinct PCRs were incubated at annealing temperatures of 40.1, 42.7, 46.7, 49.9, 50.0, 52.7, 56.8, and 60.0°C, and the products were combined prior to cloning or T-RFLP.

Intact-biofilm PCR. The DNA extracts from hematite media amplified poorly; therefore, a whole-cell PCR approach was developed to overcome any limitations imposed by the extraction method. Community 16S rRNA genes were directly amplified from hematite media using IB-PCR in which colonized particles were added directly to PCR tubes containing water and buffer and heated at 99°C for 15 min to lyse the attached cells. The temperature was subsequently lowered to 80°C, and the remaining PCR components were added before initiating the same cycling regime as described above.

T-RFLP analysis. T-RFLP was used to compare the microbial communities from the pristine well colonizing the various coupon-associated media with one another and with the communities associated with the groundwater and sediments. Twenty microliters from each replicate PCR (multiple annealing temperatures) was combined and purified with the Wizard PCR Preps DNA purification kit (Promega). Approximately 200 ng of amplicons from each community DNA was digested in triplicate with 10 U of the restriction endonuclease MspI (New

England BioLabs) at 37°C for 3 h. Restriction fragments were purified with 3 μl of sodium acetate (3 M, pH 5.2) and 66 μl of ethanol (70%), air dried, and resuspended in 10 μl of water. Samples were denatured by heating to 95°C for 3 min followed by submersion in an ice bath. The denatured DNA (2 μl), along with the internal standard Rox 1000 (Applied Biosystems), was loaded onto a model 377 DNA sequencer (Applied Biosystems) employing a Cambrex Long Ranger XL 5% polyacrylamide denaturing gel and electrophoresed at 51°C, 3 kV for 4.5 h. The resulting data were analyzed by using Genescan version 2.1 (Applied Biosystems). Peaks with <50 fluorescence units were discarded, as were those that were not present in at least two of the three replicate profiles.

Statistical analysis of T-RFLP data. Replicate T-RFLP profiles were aligned manually, and a consensus profile was generated for each community that consisted of mean fragment lengths and fluorescence intensities. The consensus profiles were aligned manually for comparison. Jaccard similarity coefficients, which make use of only presence/absence data, and Euclidean distances, which represent weighted continuous data (both peak position and peak height), were calculated using the paleo-ecology statistics freeware package PAST (17). Cluster analyses of the T-RFLP patterns were performed, applying the unweighted pair-group average (UPGMA) algorithm to the distance measures in either PAUP (version 4.0b10; Sinauer Associates, Inc.) or PAST.

Construction and screening of 16S rDNA clone libraries. Clone libraries of amplified 16S rRNA genes from the populations colonizing hematite media incubated in both pristine and contaminated regions of the aquifer were constructed from the IB-PCRs. Amplification reactions from the eight different annealing temperatures were combined and purified with the Wizard PCR Preps DNA purification Kit (Promega). Amplicons were ligated overnight at 4°C into the pGEM-T Easy vector (Promega) and transformed into competent *Escherichia coli* JM109 cells. Ligation and transformation were carried out as recommended by the manufacturer. Transformed cells were plated onto S-Gal agar (Sigma) with 100 μg of ampicillin ml^{-1} (sodium salt) and incubated at 37°C for 16 h. Approximately 100 white colonies from each library were chosen at random and used as templates for whole-cell PCR. The whole-cell PCR mix was the same as that described above for IB-PCR with the following exceptions: cells from a single transformed *E. coli* colony were used as the DNA source in place of hematite media, thermocycling employed a single annealing temperature of 50°C, and primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′), flanking the insertion site on the vector, were used to reamplify the insert. Reamplified inserts were digested with the restriction endonucleases MspI and HinP1I (New England BioLabs) in NEB2 buffer (37°C, 5 h) for RFLP analysis and resolved in 3% agarose (NuSieve 3:1 agarose). Clones were conservatively clustered into RFLP types from which a single representative was sequenced in full for a minimum of 2× coverage.

Sequencing and phylogenetic analysis. Purified plasmids (QIAprep Spin Miniprep kit; QIAGEN) or M13 PCR products were sequenced by using primers M13F, M13R, 515F (5′-GTGCCAGCMGCCGCGGTAA-3′, where M = A + C), 519R (5′-ATTACCGCGGCTGCTGG-3′), 1100F (5′-CAACGAGCGCAACCCT-3′), and 1100R (5′-AGGGTTGCGCTCGTTG-3′). Sequencing reactions were performed by using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and an ABI 3700 automated DNA sequencer (Applied Biosystems).

Electropherograms were edited by using Chromas "freeware" (version 1.45; School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia), and sequences were assembled with the BioEdit sequence alignment editor freeware (version 5.0.9; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (16). Sequences were aligned with ClustalW and grouped together based on sequence similarity using Sequence Grouper (Andrew Shewmaker, INEEL). Because of known errors introduced during PCR and cloning, sequences with ≥97% sequence similarity to one another were considered indistinguishable and treated as a single phylotype (49).

All assembled sequences were examined for chimeric artifacts using CHIMERA_CHECK from the Ribosomal Database Project II (RDP) (32); potentially chimeric sequences were not given further consideration. Nonchimeric sequences were aligned to the 16S rDNA sequences of the closest cultured organisms from the GenBank and RDP databases, using the ClustalW alignment tool in BioEdit. Sequences were manually corrected by using MacClade software (version 3.0; Sinauer Associates, Inc., Sunderland, Mass.) to ensure that only homologous nucleotides were compared between sequences. The edited alignments were evaluated with the maximum-parsimony, maximum-likelihood, and distance methods using the PAUP package. Trees generated with all three methods were congruent, with only minor rearrangements in branching order. Phylogenetic inference and evolutionary distance calculations were generated using the distance Jukes-Cantor model (gamma parameter equal to 2.0). Ten thousand bootstrap replicates were used to obtain confidence estimates for the phylogenetic trees.

Calculation of diversity indices. The number and frequency of phylotypes identified in the clone libraries were used to estimate the diversity of the different communities by using PAST (17). The Shannon diversity index (H) (also referred to as the Shannon-Weaver or Shannon-Wiener index) was calculated using the equation $H = -\Sigma p_i(\ln p_i)$, in which p_i is the frequency of the i^{th} phylotype. A higher H value indicates greater diversity. Equitability (J) was calculated as $J = H(\ln S^{-1})$, where S is the total number of clones. Equitability values vary from 0 to 1 and reflect the ratio of the observed diversity (H) to the maximum diversity within a sample, where maximum diversity equals 1. The Simpson dominance index (D) was calculated by using the following equation, where an increase in 1/D is indicative of an increase in diversity: $D = \Sigma p_i^2$.

Nucleotide sequence accession numbers. All clone sequences have been deposited in the GenBank/DDBJ/EMBL databases under accession numbers AY622227 to AY622271, as indicated in Tables 3 to 5.

RESULTS

Comparison of sediment and groundwater communities to those captured on coupon-associated media within biofilm coupons. T-RFLP analysis of PCR-amplified 16S rRNA genes was used to compare microbial communities associated with natural sediments and groundwater with those that developed on coupon-associated media surfaces contained within a biofilm coupon in the background area. Sufficient PCR products for T-RFLP analysis were obtained from all genomic DNA extracts with the exception of the hematite. To overcome this limitation, a method that directly amplified the genomic DNA extracted from the hematite surface-associated community in the presence of other sample components was developed. IB-PCR of hematite particles provided concentrations of PCR products similar to those obtained from the bead-beating extractions. All T-RFLP patterns were aligned manually and used to calculate Jaccard similarity coefficients (based on the presence or absence of a terminal restriction fragment (T-RF) (Table 2).

The Jaccard similarities were further used to perform cluster analysis (Fig. 1). Clusters were formed that reflected, to some degree, community origin. The groundwater and sediment communities were clearly distinct from one another, sharing in common only 11 of their 70 combined T-RFs. The communities from three replicate groundwater samples were most similar to one another, as were the communities from the three sediment samples. A distinct cluster contained all of the communities from the coupon-associated minerals with the exception of the community from the illite shale, all of which were more similar to the groundwater than to the sediments. Nearly identical dendrogram topology was obtained when T-RF fluorescence intensity was considered using Euclidean distances in place of Jaccard similarities (data not shown). Notably, the communities associated with the coupon-associated minerals were again more similar to the groundwater communities than to the sediment communities when fluorescence intensity was considered.

The glass wool- and quartz sand-associated communities were most similar to each other (Jaccard value range of 0.458 to 0.600). With the exception of the relatively high level of similarity of the hematite-associated community to the community from groundwater sample 1 (Jaccard similarity coefficient of 0.400), the quartz-associated community was marginally more similar to the communities recovered from groundwater (Jaccard value range of 0.382 to 0.419) than were

TABLE 2. Jaccard similarity coefficients for comparison of the T-RFLP profiles of communities from natural groundwater and sediments from the background area with those formed on surrogate geological media incubated in the same well

	Jaccard similarity coefficient											
	Hematite	Glass1[a]	Glass2[b]	Illite	Saprolite	Quartz	Sediment1[c]	Sediment2[d]	Sediment3[e]	Water1[f]	Water2[f]	Water3[f]
Hematite	1.000	0.500	0.360	0.250	0.370	0.345	0.154	0.125	0.174	0.400	0.370	0.379
Glass1		1.000	0.458	0.367	0.407	0.600	0.148	0.121	0.100	0.333	0.357	0.323
Glass2			1.000	0.448	0.444	0.464	0.185	0.118	0.120	0.370	0.393	0.355
Illite				1.000	0.406	0.469	0.188	0.189	0.170	0.303	0.286	0.263
Saprolite					1.000	0.419	0.167	0.139	0.135	0.379	0.355	0.324
Quartz						1.000	0.156	0.132	0.109	0.400	0.419	0.382
Sediment1							1.000	0.417	0.182	0.222	0.207	0.188
Sediment2								1.000	0.381	0.182	0.171	0.158
Sediment3									1.000	0.140	0.135	0.148
Water1										1.000	0.905	0.720
Water2											1.000	0.800
Water3												1.000

[a] Glass1, glass wool separating saprolite and illite shale.
[b] Glass2, glass wool separating hematite and sand.
[c] Sediment1, unsaturated sediment.
[d] Sediment2, variably saturated sediment.
[e] Sediment3, saturated sediment.
[f] Water1, Water2, Water3, triplicate 4-liter groundwater samples.

communities associated with the other coupon-associated media (Jaccard coefficient range of 0.263 to 0.393). The hematite-associated community was slightly more similar to the saturated sediment community (sediment sample 3; Jaccard coefficient of 0.174) than were the communities associated with any other coupon-associated medium tested (Jaccard coefficient range of 0.100 to 0.170).

With the exception of quartz sand, each of the coupon-associated media captured populations that were detected in sediment samples but remained undetected by analysis of groundwater (data not shown). For example, the T-RFLPs for illite, saprolite, and one of the two glass wool communities all possessed a T-RF that was also present in sediment sample 3 but was absent from the groundwater samples. Whether samples prepared from larger volumes of groundwater would improve the detection of these populations is unknown. Similarly, the coupon-associated media captured 22 populations (T-RFs) that were not detectable in T-RFLPs of either the sediments or groundwater. Thus, coupon-associated media incubated in the subsurface provided appreciable quantities of a number of native populations that remained indiscernible when sediments or groundwater served as the source of community DNA.

Comparison of microbial community diversity in the saturated zone of pristine and contaminated regions of the FRC aquifer. (i) 16S rDNA clones from biofilm coupon-associated hematite. Biofilm coupons containing specular hematite were used to capture and compare community diversity in the saturated zone of Area 3 well FW026 and background area well FW303. Specular hematite was selected over other media based on the evidence from T-RFLP analysis that of the various media evaluated, the community captured on hematite was most similar to the (saturated) sediment-associated community at the background area. Unfortunately, we have not yet been able to relate community composition based on IB-PCR to that obtained by conventional DNA extraction and PCR methodologies, since the presence of hematite compromised the recovery of DNA when the latter extraction procedure was used.

Thirteen distinct phylotypes were identified from 100 non-chimeric clones generated from IB-PCR-derived products from hematite particles incubated in the contaminated groundwater (Table 3; Fig. 2). Members of the *Proteobacteria* dominated the library in Area 3, with four phylotypes affiliated with the β-*Proteobacteria* (62% of the library), another four affili-

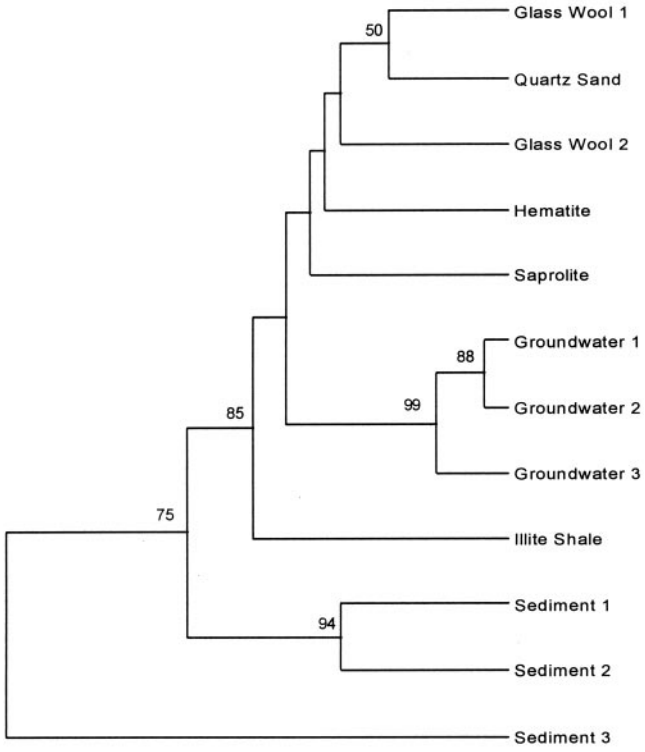

FIG. 1. Cluster analysis of 16S rRNA-based T-RFLP patterns from communities associated with various surrogate geological media and natural sediment and groundwater in a pristine region of the FRC aquifer, Oak Ridge, Tenn. The UPGMA algorithm was used to cluster patterns based on Jaccard similarities. Bootstrap values above 50 are indicated at the appropriate nodes (based on 1,000 bootstrap replicates).

TABLE 3. Bacterial 16S rDNA clones from communities formed on hematite in FRC Area 3 well FW026

Clone ID	GenBank no.	Frequency[a]	Affiliation[b] (% similarity)	Putative division
C-CG17	AY622228	59	*Alcaligenes* sp. strain L6 (95)	β-*Proteobacteria*
C-CS3	AY622233	24	*Frateuria* sp. NO-16 (96) (AF376025)	γ-*Proteobacteria*
C-CF16	AY622227	4	*Methylobacterium radiotolerans* (99)	α-*Proteobacteria*
C-CU62	AY622234	3	*Pseudomonas straminea* (99)	γ-*Proteobacteria*
C-CJ32	AY622229	2	*Beutenbergia cavernosa* (96)	*Actinobacteria*
C-CY80	AY622237	1	*Herbaspirillum seropedicae* (96)	β-*Proteobacteria*
C-DA88	AY622239	1	*Burkholderia* sp. A6.2 (98)	β-*Proteobacteria*
C-CZ82	AY622238	1	*Duganella zoogloeoides* (98)	β-*Proteobacteria*
C-CL42	AY622230	1	*Pseudomonas syringae* (89)	γ-*Proteobacteria*
C-CX74	AY622236	1	*Sphingobacterium antarcticum* (99)	γ-*Proteobacteria*
C-CO51	AY622232	1	*Microbacterium* sp. VKM Ac-2050 (99)	*Actinobacteria*
C-CV63	AY622235	1	*Nocardioides* sp. MWH-CaK6 (98)	*Actinobacteria*
C-CM46	AY622231	1	Clone MTAC17 (92)	Unknown

[a] Frequency of a given RFLP-type out of 100 total clones.
[b] Closest identified match in the GenBank database followed by percent similarity. Clone C-CM46 did not match any identified sequences in the database.

ated with the γ-*Proteobacteria* (29% of the library), and one affiliated with the α-*Proteobacteria* (4% of the library). No δ-*Proteobacteria* were found associated with hematite incubated in the contaminated groundwater. In addition to the proteobacterial clones, three phylotypes affiliated with the *Actinobacteria* (4% of the library) and one unidentified lineage (1% of the library) were also present. The library was dominated by a clone similar to *Alcaligenes* sp. (95% similarity) and another most similar to *Frateuria* sp. (96% similarity), which together contributed 83% of the library. Clone C-CL42 displayed a particularly low level of similarity to its closest known relative, a *Pseudomonas* sp. (89% similarity). Clone C-CM46 was not significantly related to any known lineage (Fig. 2).

Hematite particles incubated in groundwater at the pristine site, when subjected to IB-PCR, yielded 34 distinct phylotypes (of 95 clones screened), 10 of which were potentially chimeric and thus were disregarded (Table 4; Fig. 2). Like the hematite-associated community from the Area 3 contaminated site, members of the *Proteobacteria* dominated the library from the background area, with a particularly high frequency of those belonging to the β subdivision. The background area hematite-associated community was composed of 13 phylotypes affiliated with the β-*Proteobacteria* (69% of the library), six affiliated with the γ-*Proteobacteria* (21% of the library), two affiliated with the α-*Proteobacteria* (3.5% of the library), and one affiliated with the δ-*Proteobacteria* (1.2% of the library). Two nonproteobacterial clones were also present, one affiliated with the *Bacteroidetes* (2.4% of the library) and another affiliated with the *Verrucomicrobia* (1.2% of the library). A clone most similar to the dissimilatory Fe(III)-reducing β-proteobacterium *Rhodoferax ferrireducens* (13) (98% sequence similarity) made up 45% of the background area library. Other clones observed more than once included relatives of *Pseudomonas mandelii*, *Oxalobacter* sp., *Pseudoxanthomonas mexicana*, *Herbaspirillum seropedicae*, *Aquamonas gracilis, Novosphingobium* sp., and *Flavobacterium columnare*. Clone B-AB39, while clearly belonging to the β-*Proteobacteria*, was not highly related to any known microorganism (Fig. 2).

(ii) 16S rDNA clones from sediment extracts. Microbial communities captured on surrogate minerals in biofilm coupons were compared with those recovered from cores of natural geological material retrieved during drilling of background area well FW303 and Area 3 well FW026. A clone library constructed from DNA extracted by bead-beating of core material from well FW303 yielded eight unique phylotypes after screening 100 total clones (Table 5; Fig. 2). No chimeric sequences were detected in this library. The library was dominated by two *Pseudomonas* phylotypes, clones S-A1 and S-H52, together making up 90% of the library. Clone S-A1, most similar to *Pseudomonas agarici* (98% sequence similarity), comprised 54% of the library, while clone S-H52, most similar to *Pseudomonas rhodesiae* (99% sequence similarity), comprised another 36% of the library. These two phylotypes shared 97% similarity with one another but are left separate because two distinct clusters of clones emerged when they were aligned with one another. The next-most-frequent phylotypes, each contributing 3% to the total library, were clones S-E105 and S-D24, most similar to strains of the genera *Aquaspirillum* (97% sequence similarity) and *Arthrobacter* (98% sequence similarity), respectively. Clone S-G30, representing only 1 clone of 100 in the library, shared 98% sequence similarity with *Duganella zoogloeoides*. The remaining three phylotypes, S-B2, S-F26, and S-J147, each encountered only once in the library, branched deeply and did not group with any characterized lineages (Fig. 2).

Attempts to amplify 16S rRNA genes from DNA extracts of contaminated sediments collected from well FW026 in Area 3 were unsuccessful.

Diversity indices. Measures of diversity were calculated from the 16S rRNA gene clone library data (Table 6). By nearly all accounts, the library generated from sediment-extracted DNA suggested the lowest level of diversity. Richness, dominance, and the Shannon index were all lower than those calculated for the two hematite biofilm communities. Only the equitability of the sediment extract library was similar to that of one of the biofilm coupon libraries.

The background area hematite biofilm community displayed the greatest overall diversity. This community was marked by rather high richness and an even distribution of phylotypes, indicated by the dominance index, Shannon index, and equitability. The hematite-associated community from the contaminated Area 3 well was far less diverse than that from the background area well, with only half of the number of unique clones and a more skewed distribution of individuals within a few dominant phylotypes. It should be noted that these indices

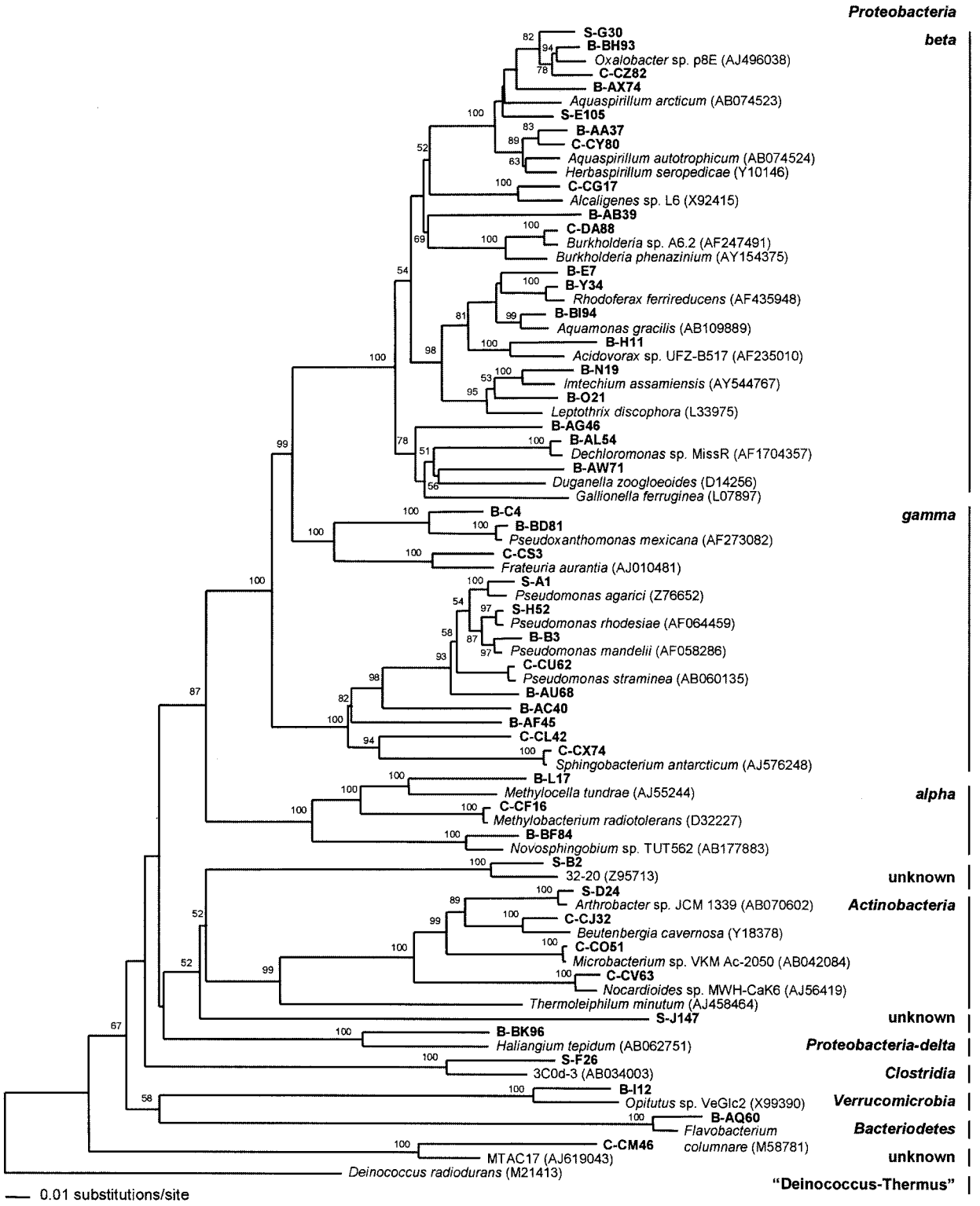

FIG. 2. Phylogenetic relationships of nearly full-length 16S rDNA sequences. The phylogenetic tree was constructed using PAUP based on maximum-distance analysis with Jukes-Cantor correction. The values at the nodes are bootstrap probabilities (percentages) based on 10,000 replicates; only values greater than 60% are shown. The scale bar indicates 0.01 nucleotide substitutions per site. Clone designations beginning with B, C, or S indicate the library of origin: S, sediment core from background area well FW303; B, biofilm coupon from background area well FW303; C, biofilm coupon from contaminated Area 3 well FW026. Sequences with accession numbers were obtained from the RDP or GenBank databases.

TABLE 4. Bacterial 16S rDNA clones from communities formed on hematite in FRC background area well FW303

Clone ID	GenBank no.	Frequency[a]	Affiliation[b] (% similarity)	Putative division
B-Y34	AY622248	38	*Rhodoferax ferrireducens* (98)	β-*Proteobacteria*
B-B3	AY622240	6	*Pseudomonas mandelii* (98)	γ-*Proteobacteria*
B-BH93	AY622261	5	*Oxalobacter* sp. p8E (97)	β-*Proteobacteria*
B-BD81	AY622259	5	*Pseudoxanthomonas mexicana* (98)	γ-*Proteobacteria*
B-C4	AY622241	4	*Pseudoxanthomonas mexicana* (95)	γ-*Proteobacteria*
B-AA37	AY622249	4	*Herbaspirillum seropedicae* (97)	β-*Proteobacteria*
B-E7	AY622242	3	*Aquamonas gracilis* (95)	β-*Proteobacteria*
B-BF84	AY622260	2	*Novosphingobium* sp. TUT562 (96)	α-*Proteobacteria*
B-AQ60	AY622255	2	*Flavobacterium columnare* (96)	*Bacteroidetes*
B-L17	AY622245	1	*Methylocella tundrae* (92)	α-*Proteobacteria*
B-B194	AY622262	1	*Aquamonas gracilis* (97)	β-*Proteobacteria*
B-AL54	AY622254	1	*Dechloromonas* sp. MissR (98)	β-*Proteobacteria*
B-AG46	AY622253	1	*Gallionella ferruginea* (91)	β-*Proteobacteria*
B-AX74	AY622258	1	*Aquaspirillum arcticum* (95)	β-*Proteobacteria*
B-AB39	AY622250	1	*Burkholderia phenazinium* (90)	β-*Proteobacteria*
B-H11	AY622243	1	*Acidovorax* sp. UFZ-B517 (94)	β-*Proteobacteria*
B-AW71	AY622257	1	*Zoogloea* sp. strain DhA-35 (91) (AJ011506)	β-*Proteobacteria*
B-N19	AY622246	1	*Imtechium assamiensis* (97)	β-*Proteobacteria*
B-O21	AY622247	1	*Ideonella* sp. B513 (96) (AB049107)	β-*Proteobacteria*
B-AU68	AY622256	1	*Pseudomonas rhodesiae* (96)	γ-*Proteobacteria*
B-AF45	AY622252	1	*Pseudomonas putida* (90)	γ-*Proteobacteria*
B-AC40	AY622251	1	*Pseudomonas* sp. NZ111 (92)	γ-*Proteobacteria*
B-BK96	AY622263	1	*Haliangium repidum* (92)	δ-*Proteobacteria*
B-112	AY622244	1	*Opitutus* sp. VeGlc2 (93)	*Verrucomicrobia*

[a] Frequency of a given RFLP-type out of 85 total clones.
[b] Closest identified match in the GenBank database followed by % similarity.

do not take into account phylogenetic distances among community members. Thus, while the background area hematite-associated community appears to be the most diverse of those studied here, the observation that nearly all clones belonged to a limited number of lineages in the *Proteobacteria* (Fig. 2) is not taken into account.

DISCUSSION

The limitations of enrichment and isolation methods for uncovering true microbial diversity are well known. While the method described here using coupon-associated media incubated in a well certainly has its own limitations, it provides a new approach to improve our understanding of microbial diversity in subsurface environments. Collection of complex communities on the surfaces of exogenously introduced substrata offers a rapid and inexpensive approach to investigating microbial diversity in the subsurface. Biofilm coupons may be especially beneficial for studying polluted environments where contaminants can interfere with analyses and secondary wastes must be carefully considered. Comparison of the various communities formed on different substrata incubated concurrently in pristine groundwater revealed that care must be exercised when interpreting the results from such studies. The communities that formed on the different substrata were not only distinct from one another, they were also distinct from the groundwater and sediments from the same site (Fig. 1); none of the communities associated with the surrogate media in the biofilm coupons was perfectly congruent with any of the groundwater or sediment communities. However, the communities that colonized coupon-associated media were more similar to groundwater samples than sediments. This result may not be surprising given the fact that the coupon-associated media were exposed to the local groundwater for only 8 weeks, providing a relatively limited inoculum and a short period for

TABLE 5. Bacterial 16S rDNA clones from sediments[a] of the FRC background area borehole FWB303

Clone ID	GenBank no.	Frequency[b]	Affiliation[c] (% similarity)	Putative division
S-A1	AY622264	54	*Pseudomonas agarici* (98)	γ-*Proteobacteria*
S-H52	AY622270	36	*Pseudomonas rhodesiae* (99)	γ-*Proteobacteria*
S-E105	AY622267	3	*Aquaspirillum autotrophicum* (97)	β-*Proteobacteria*
S-D24	AY622266	3	*Arthrobacter* sp. JCM 1339 (98)	*Actinobacteria*
S-G30	AY622269	1	*Duganella zoogloeoides* (98)	β-*Proteobacteria*
S-B2	AY622265	1	Clone 32-20 (95)	Unknown
S-F26	AY622268	1	Clone 3C0d-3 (93)	*Clostridia*
S-J147	AY622271	1	*Thermoleiphilum minutum*[d] (AJ458464)	*Actinobacteria*

[a] Sediments were stored at 4°C for approximately 3 weeks prior to analysis.
[b] Frequency of a given RFLP type out of 100 total clones.
[c] Closest identified match in the GenBank database followed by percent similarity.
[d] The sequence of clone S-J147 did not appear to be chimeric; however, it did not significantly match anything in the GenBank database, cultured or uncultured. Portions of the gene shared significant similarity with that of *Thermoleiphilum minutum*, but across the entire gene similarity was only 76%.

TABLE 6. Diversity of microbial communities in the FRC background area well FW303 and contaminated Area 3 well FW026 based on data from 16S rDNA clone libraries of sediments and coupon-associated hematite

Library source	No. of clones[a]	% Coverage[b]	Richness[c]	Dominance	Shannon[d]	Equitability
Background area sediment	100	96	8	0.42	1.1	0.53
Background area hematite	85	81	25	0.22	2.3	0.71
Area 3 hematite	100	92	13	0.41	1.3	0.52

[a] The number of individual nonchimeric clones that were grouped by RFLP and ≥97% sequence similarity.
[b] The percentage of clones that were at least duplicated in the library (34).
[c] The number of distinct phylotypes in the library based on RFLP analysis.
[d] Shannon-Weaver diversity index (see "Materials and Methods").

successional changes to occur compared to the situation with the native sediments. New studies are needed to determine the significance of coupon incubation time on community diversity and structure associated with surrogate minerals.

Several studies from subsurface (21, 29, 30, 43), freshwater (8, 47), and marine (8, 9, 52) environments have indicated differences in microbial activity and diversity between attached and planktonic communities, and some investigators have concluded that the majority of biomass and activity are dominated by attached microorganisms (2, 15, 18, 20, 43, 51). The sediment and groundwater T-RFLPs at the FRC were highly distinct from one another, sharing fewer than 16% of their T-RFs in common. This calls into question the common practice of using either groundwater or sediments, but not both, to characterize the microbial diversity of a subsurface site. More work is clearly needed to better understand the distribution of populations and their activities between the attached and planktonic phases in the subsurface. Lehman et al. (30) reported that uncontaminated basalt core material recovered from the Snake River Plain Aquifer was nearly devoid of biomass (1-g samples), while the surrounding groundwater boasted nearly 10^5 cells per ml. Conversely, in a region of the same aquifer contaminated with various organic pollutants, the attached cells (aerobic heterotrophs) outnumbered those in the planktonic state. Large-pore-diameter dialysis chambers filled with either crushed basalt or water were used in a manner similar to use of the biofilm coupons in this study. The investigators found that the composition of the community on the crushed basalt was very similar to that in the water but that the metabolic activities of the two were distinct (30).

In recent years, investigators have reported that some microorganisms preferentially attach to different solid substrata. Caccavo and Das (4) demonstrated that the Fe(III)-reducing bacterium *Shewanella alga* attached more rapidly and to a higher cell density to goethite than to either ferrihydrite or hematite. Lower and colleagues (31) used atomic force microscopy with living cells of *Shewanella oneidensis* to demonstrate its preference for goethite over diaspore (an Al-bearing analog of goethite) under anaerobic conditions. Curiously, in the presence of oxygen the cells were more attracted to the diaspore than to the goethite, indicating a change in adhesive/attractive forces between the cell and the mineral due to oxygen tension and a likely change in surface protein expression. The T-RFLPs from the two glass wool media in our study were most similar to that from the quartz sand medium, suggesting that similar physicochemical properties (e.g., SiO_2 content and electrostatic attraction or repulsion) may play a role in selection of the attached communities. By the same token, we cannot be sure that the hematite medium used to compare microbial communities in the pristine and contaminated regions of the FRC aquifer did not select for a disproportionate density of Fe(III)-reducing *R. ferrireducens*-like organisms (Table 4). Mineral phase surface properties clearly influence microbial colonization and activities (39). Use of biofilm coupons containing relevant geological media offers an excellent experimental framework for such studies in the field.

In the present study, DNA extracted directly from sediments of an acidic U-contaminated aquifer was not readily PCR amplifiable, possibly due to coextraction of PCR inhibitors (24, 46, 53) or low biomass in the sample. However, when sterile particles of hematite were incubated in the well for 8 weeks and analyzed without direct DNA extraction (IB-PCR), considerable diversity was uncovered that would have otherwise remained cryptic. Thus, whereas a bead-beating method failed to provide a means for comparing microbial community diversity at these particular subsurface sites, the biofilm coupon combined with IB-PCR enabled such an analysis.

The structure and diversity of a microbial community adapted to a particular environment should reflect conditions of the surrounding milieu. This concept appears to hold across a variety of different microbial habitats, including those contaminated by anthropogenic activities (3, 25, 33, 36, 40). For instance, acid mine drainage harbors acid-tolerant and acidophilic *Bacteria* and *Archaea* (1, 11), and metal-tolerant microbes are readily isolated from metal-contaminated media (37, 38). Many of the hematite-associated *Bacteria* detected in the background area were common soil and water microorganisms. Most were affiliated with aerobic *Bacteria*, consistent with the redox status the aquifer. By contrast, the structure of the microbial community from Area 3 may be the result of strong selective pressures exerted by the contaminants. The three predominant populations in the hematite-associated community in Area 3 appear to be adapted to the high-metal and low-pH conditions of this region of the aquifer. Nearly 60% of the clones were from an organism most similar to the genus *Alcaligenes*, which contains metal-tolerant representatives (26). Another 24% of the clones were affiliated with the genus *Frateuria*, a group of strictly aerobic acidophiles (50). The presence of a phylotype with 99% sequence similarity to *Methylobacterium radiotolerans* (4% of the library) is consistent with the high radionuclide content based on the reported radiotolerance of *M. radiotolerans* and other *Methylobacterium* spp. (23). None of these was found in the background area libraries. The remaining 13% of the clones, like those from the background area, were affiliated with common environmental taxa.

Although β- and γ-*Proteobacteria* dominated all three libraries, most of the specific phylotypes identified were unique to a single library. There were, however, some phylotypes that appear to have been present in more than one library. Dominant sediment extract clones S-A1 and S-H52 (Table 5), highly related to *Pseudomonas* spp., both shared 97% sequence similarity with *Pseudomonas* sp. clone B-B3 (Table 4) from the hematite-associated community in the background area. Two clones most similar to *H. seropedicae*, B-AA37 from the back-

ground area (Table 4) and C-CY80 from Area 3 (Table 3), were 97% similar to one another and 97% similar to clone S-E105 from the sediment extract library (Table 5). Three β-proteobacterial clones related to the genera *Duganella* and *Oxalobacter*, S-G30 from the sediment extract (Table 5), B-BH93 from the hematite-associated community in the background area (Table 4), and C-CZ82 from Area 3 (Table 3), were also 97% similar to one another.

The use of IB-PCR to amplify genes of interest from surface-associated populations eliminates many of the biases inherent in DNA extraction (14, 35). However, it likely favors those populations that lyse readily at 99°C. Notably, no gram-positive organisms, with the exception of the three clones from the community in Area 3 associated with the *Actinobacteria* (Table 3), were identified in IB-PCR-generated libraries. The dominating presence of *Proteobacteria* in the IB-PCR-generated libraries may be a result of a more readily lysed cell wall among organisms in this division. However, it is worth considering that half of the phylotypes identified by bead-beating extraction of sediments in the background area (comprising 94% of the individual clones examined; Table 5) were also *Proteobacteria*, suggesting that the proteobacterial dominance seen in the hematite-associated community by IB-PCR may not be an artifact of the method but may in fact reflect actual community composition.

Peacock and colleagues (42) recently used a similar biofilm coupon apparatus loaded with glass wool or powder activated carbon beads to sample the microbial communities stimulated by electron donor amendment at the FRC. These investigators found that while the type of solid substratum influenced the overall biomass and community composition, the communities were generally dominated by members of the *Proteobacteria*. Similar to our study's findings, rRNA genes were discovered that were affiliated with the genera *Alcaligenes* and *Frateuria*.

Fields et al. (12) reported considerably greater diversity in nearby groundwater at the FRC, possibly because they examined a larger number of clones than were examined in this study. These investigators identified nearly 80 distinct populations in the background area groundwater and between 19 and 34 populations in groundwater from three different wells in Areas 1 and 3 (contaminated area). While diversity and equitability were greater for all samples relative to ours, the authors did observe diminished diversity in the contaminated wells relative to the background area, as we also reported here. Again, *Proteobacteria* were the numerically dominant clones at both sites. Distinct from findings of our study and that of Peacock et al. (42) described above, however, the most abundant populations in the contaminated groundwater were *Azoarcus* and *Pseudomonas* spp. Most notably, Fields did not detect *Alcaligenes* or *Frateuria* spp. in the contaminated groundwater.

Petrie and coworkers (44) found that Fe(III)-reducing communities were also quite distinct at both sites in the FRC and that pH and nitrate played a constraining role. Iron-reducing enrichment cultures from the background area were almost exclusively affiliated with the *Geobacteraceae*, while those from the contaminated sediments were dominated by a population most similar to *Anaeromyxobacter dehalogenans* (44), neither of which was observed in our studies of surface-associated communities.

Ecological theory holds that strong selective pressures decrease microbial diversity. We hypothesized that the high metals content and low pH of the Area 3 aquifer would exert pressure on the microbial community, selecting populations that can tolerate or even take advantage of the contaminants. Based on our analysis of the microbial communities formed on hematite particles in Area 3, diversity of the indigenous community was indeed considerably lower than that in the background area. Furthermore, the predominant populations at the contaminated site were closely related to metal-tolerant (*Alcaligenes* sp.), acidophilic (*Frateuria* sp.), or radiation-resistant (*M. radiotolerans*) microorganisms, consistent with the prevailing conditions. Informed management and remediation efforts should take into account the influence of the environmental conditions on the extant microbial community and attempt to either capitalize on or mitigate the strong selective pressures exerted by the contaminants.

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REFERENCES

1. **Baker, B., and J. F. Banfield.** 2003. Microbial communities in acid mine drainage. FEMS Microbiol. Ecol. **44:**139–152.
2. **Bekins, B. A., E. M. Godsy, and E. Warren.** 1999. Distribution of microbial physiologic types in an aquifer contaminated by crude oil. Microb. Ecol. **37:**263–275.
3. **Bhattacharya, D., P. M. Sarma, S. Krishnan, S. Mishra, and B. Lal.** 2003. Evaluation of genetic diversity among *Pseudomonas citronellolis* strains isolated from oily sludge-contaminated sites. Appl. Environ. Microbiol. **69:** 1435–1441.
4. **Caccavo, F., Jr., and A. Das.** 2002. Adhesion of dissimilatory Fe(III)-reducing bacteria to Fe(III) minerals. Geomicrobiol. J. **19:**161–177.
5. **Chang, Y. J., A. D. Peacock, P. E. Long, J. R. Stephen, J. P. McKinley, S. J. Macnaughton, A. Hussain, A. M. Saxton, and D. C. White.** 2001. Diversity and characterization of sulfate-reducing bacteria in groundwater at a uranium mill tailings site. Appl. Environ. Microbiol. **67:**3149–3160.
6. **Cheung, P.-Y., and B. K. Kinkle.** 2001. Mycobacterium diversity and pyrene mineralization in petroleum-contaminated soils. Appl. Environ. Microbiol. **67:**2222–2229.
7. **Cho, J.-C., and S.-J. Kim.** 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol. **66:**956–965.
8. **Crump, B. C., E. V. Armbrust, and J. A. Baross.** 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol. **65:**3192–3204.
9. **DeLong, E. F., D. G. Franks, and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38:**924–934.
10. **Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace.** 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. **64:**3869–3877.
11. **Edwards, K. J., P. L. Bond, T. M. Gihring, and J. F. Banfield.** 2000. An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. Science **287:**1796–1799.
12. **Fields, M. W., T. Yan, S.-K. Rhee, S. L. Carroll, and J. Zhou.** Microbial community structure and composition from subsurface groundwater with

high levels of nitrate, heavy metals, and uranium. (Submitted for publication.)
13. **Finneran, K. T., C. V. Johnsen, and D. R. Lovley.** 2003. *Rhodoferax ferrireducens* sp. nov.; a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). Int. J. Syst. Evol. Microbiol. **53:**669–673.
14. **Frostegård, Å., S. Courtois, V. Ramisse, S. Clerc, D. Bernillon, F. Le Gall, P. Jeannin, X. Nesme, and P. Simonet.** 1999. Quantification of bias related to the extraction of DNA directly from soils. Appl. Environ. Microbiol. **65:** 5409–5420.
15. **Godsy, E. M., D. F. Goerlitz, and D. Grbic-Galic.** 1992. Methanogenic biodegradation of creosote contaminants in natural and simulated groundwater ecosystems. Ground Water **30:**232–242.
16. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.
17. **Hammer, Ø., D. A. T. Harper, and P. D. Ryan.** 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electronica **4.** [Online.] http://palaeo-electronica.org/2001_1/past/issue1_01.htm.
18. **Harvey, R. W., R. L. Smith, and L. George.** 1984. Effect of organic contamination upon microbial distributions and heterotrophic uptake in a Cape Cod, Mass., aquifer. Appl. Environ. Microbiol. **48:**1197–1202.
19. **Hatcher, R. D. J., P. J. Lemiszki, R. B. Dreier, R. H. Ketelle, R. R. Lee, D. A. Lietzke, W. M. McMaster, J. L. Foreman, and S. Y. Lee.** 1992. Status report on the geology of the Oak Ridge Reservation. Report ORNL/TM-12074. Oak Ridge National Laboratory, Oak Ridge, Tenn.
20. **Hazen, T. C., L. Jimenez, G. L. de Victoria, and C. B. Fliermans.** 1991. Comparison of bacteria from deep subsurface sediment and adjacent groundwater. Microb. Ecol. **22:**293–304.
21. **Holm, P. E., P. H. Nielsen, H. J. Albrechtsen, and T. H. Christensen.** 1992. Importance of unattached bacteria and bacteria attached to sediment in determining potentials for degradation of xenobiotic organic contaminants in an aerobic aquifer. Appl. Environ. Microbiol. **58:**3020–3026.
22. **Holmes, D. E., K. T. Finneran, R. A. O'Neil, and D. R. Lovley.** 2002. Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. Appl. Environ. Microbiol. **68:**2300–2306.
23. **Ito, H., and H. Iizuka.** 1971. Taxonomic studies on a radio-resistant *Pseudomonas*. Agric. Biol. Chem. **35:**1566–1571.
24. **Jacobsen, C. S., and O. F. Rasmussen.** 1992. Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. Appl. Environ. Microbiol. **58:**2458–2462.
25. **Johnsen, K., S. Andersen, and C. S. Jacobsen.** 1996. Phenotypic and genotypic characterization of phenanthrene-degrading fluorescent *Pseudomonas* biovars. Appl. Environ. Microbiol. **62:**3818–3825.
26. **Kanazawa, S., and K. Mori.** 1996. Isolation of cadmium-resistant bacteria and their resistance mechanisms 1. Isolation of Cd-resistant bacteria from soils contaminated with heavy metals. Soil Sci. Plant Nutr. **42:**725–730.
27. **Kleikemper, J., M. H. Schroth, W. V. Sigler, M. Schmucki, S. M. Bernasconi, and J. Zeyer.** 2002. Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. Appl. Environ. Microbiol. **68:** 1516–1523.
28. **Kolbel-Boelke, J., E. Anders, and A. Nehrkorn.** 1998. Microbial communities in the saturated groundwater environment. II. Diversity of bacterial communities in a Pleistocene sand aquifer and their in vitro activities. Microb. Ecol. **16:**31–48.
29. **Lehman, R. M., F. S. Colwell, and G. A. Bala.** 2001. Attached and unattached microbial communities in a simulated basalt aquifer under fracture- and porous-flow conditions. Appl. Environ. Microbiol. **67:**2799–2809.
30. **Lehman, R. M., S. P. O'Connell, A. Banta, J. K. Fredrickson, A.-L. Reysenbach, T. L. Kieft, and F. S. Colwell.** 2004. Microbiological comparison of core and groundwater samples collected from a fractured basalt aquifer with that of dialysis chambers incubated in situ. Geomicrobiol. J. **21:**169–182.
31. **Lower, S. K., M. F. Hochella, Jr., and T. J. Beveridge.** 2001. Bacterial recognition of mineral surfaces: nanoscale interactions between *Shewanella* and α-FeOOH. Science **292:**1360–1363.
32. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, J. M. Stredwick, G. M. Garrity, B. Li, G. J. Olsen, S. Pramanik, T. M. Schmidt, and J. M. Tiedje.** 2000. The RDP (Ribosomal Database Project) continues. Nucleic Acids Res. **28:**173–174.
33. **Mandelbaum, R. T., D. L. Allan, and L. P. Wackett.** 1995. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. Appl. Environ. Microbiol. **61:**1451–1457.
34. **Marchesi, J. R., A. J. Weightman, B. A. Cragg, R. J. Parkes, and J. C. Fry.** 2001. Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis. FEMS Microbiol. Ecol. **34:**221–228.
35. **Martin-Laurent, F., L. Philippot, S. Hallet, R. Chaussod, J. C. Germon, G. Soulas, and G. Catroux.** 2001. DNA extraction from soils: old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. **67:**2354–2359.
36. **McLean, J. S., T. J. Beveridge, and D. Phipps.** 2000. Isolation and characterization of a chromium-reducing bacterium from a chromated copper arsenate-contaminated site. Environ. Microbiol. **2:**611–619.
37. **Mergeay, M., S. Monchy, T. Vallaeys, V. Auquier, A. Benotmane, P. Bertin, S. Taghavi, J. Dunn, D. van der Lelie, and R. Wattiez.** 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. FEMS Microbiol. Rev. **27:**385–410.
38. **Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. van Gijsegem.** 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. **162:**328–334.
39. **Neal, A. L., K. M. Rosso, G. G. Geesey, Y. A. Gorby, and B. J. Little.** 2003. Surface structure effects on direct reduction of iron oxides by *Shewanella oneidensis*. Geochim. Cosmochim. Acta **67:**4489–4503.
40. **Niggemyer, A. M., S. Spring, E. Stackebrandt, and R. F. Rosenzweig.** 2001. Isolation and characterization of a novel As(V)-reducing bacterium: implications for arsenic mobilization and the genus *Desulfitobacterium*. Appl. Environ. Microbiol. **67:**5568–5580.
41. **Ogram, A., W. Sun, F. J. Brockman, and J. K. Fredrickson.** 1995. Isolation and characterization of RNA from low-biomass deep-subsurface sediments. Appl. Environ. Microbiol. **61:**763–768.
42. **Peacock, A. D., Y.-J. Chang, J. D. Istok, L. Krumholtz, R. Geyer, B. Kinsall, D. Watson, K. L. Sublette, and D. C. White.** 2004. Utilization of microbial biofilms as monitors of bioremediation. Microb. Ecol. **47:**284–292.
43. **Pedersen, K., and S. Ekendahl.** 1990. Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. Microb. Ecol. **20:**37–52.
44. **Petrie, L., N. N. North, S. L. Dollhopf, D. L. Balkwill, and J. E. Kostka.** 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl. Environ. Microbiol. **69:**7467–7479.
45. **Peyton, B. M., and M. J. Truex.** June 1997. In situ biofilm coupon device. U.S. patent 5,641,642.
46. **Rossen, L., P. Norskov, K. Holmstrom, and O. F. Rasmussen.** 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int. J. Food Microbiol. **17:**37–45.
47. **Simon, M.** 1985. Specific uptake rates of amino acids by attached and free-living bacteria in a mesotrophic lake. Appl. Environ. Microbiol. **49:** 1254–1259.
48. **Solomon, D. K., G. K. Moore, L. E. Toran, R. B. Dreier, and W. M. McMaster.** 1992. Status report—a hydrologic model of the Oak Ridge Reservation. Report ORNL/TM-12026. Oak Ridge National Laboratory, Oak Ridge, Tenn.
49. **Speksnijder, A. G. C. L., G. A. Kowalchuk, S. De Jong, E. Kline, J. R. Stephen, and H. J. Laanbroek.** 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Appl. Environ. Microbiol. **67:**469–472.
50. **Swings, J., J. De Ley, and M. Gillis.** 1984. Genus *Frateuria*, p. 210–213. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore, Md.
51. **Thomas, R. A. P., K. Lawlor, M. Bailey, and L. E. Macaskie.** 1998. Biodegradation of metal-EDTA complexes by an enriched microbial population. Appl. Environ. Microbiol. **64:**1319–1322.
52. **Unanue, M. B., B. Ayo, I. Azua, I. Barcina, and J. Iriberri.** 1992. Temporal variability of attached and free-living bacteria in coastal waters. Microb. Ecol. **23:**27–39.
53. **Wilson, I. G.** 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63:**3741–3751.