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

Catherine L. Reardon,1 David E. Cummings,2† Lynn M. Petzke,2 Barry L. Kinsall,3‡ David B. Watson,3 Brent M. Peyton,4 and Gill G. Geesey1,2*

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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
TABLE 1. Groundwater characteristics of uncontaminated Background Area wells* and contaminated Area 3 well FW026

<table>
<thead>
<tr>
<th>Well Area</th>
<th>pH</th>
<th>ORP</th>
<th>Total Fe</th>
<th>Nitrate</th>
<th>Uranium</th>
<th>TOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW300 Background</td>
<td>6.13</td>
<td>NA</td>
<td>0.05</td>
<td>1.20</td>
<td>0.00</td>
<td>25.96</td>
</tr>
<tr>
<td>FW303 Background</td>
<td>7.52</td>
<td>437</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FW026 Area 3</td>
<td>3.43</td>
<td>354</td>
<td>17.4</td>
<td>8.26</td>
<td>42.74</td>
<td>68.27</td>
</tr>
</tbody>
</table>

* 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.

** Oxidation-reduction potential (mV), calibrated to the standard hydrogen electrode.

† Total dissolved Fe was measured by inductively coupled plasma-mass spectrometry (ICP-MS).

‡ Dissolved nitrate was measured by ion chromatography.

§ Dissolved uranium was measured by ICP-MS.

∥ Amount of total organic carbon was determined by combustion.

# NA, not available.

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**Notes:**
- **Total Fe** concentration: 0.05 to 1.20 mg liter⁻¹
- **Nitrate** concentration: 0.00 to 8.26 mg liter⁻¹
- **Uranium** concentration: 0.00 to 42.74 mg liter⁻¹
- **TOC** concentration: 25.96 to 68.27 mg liter⁻¹

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**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 Nolichucky shale, and is enriched in organics (e.g., chloroaliphatics and aromatics). The S-3 Ponds received which a variety of wastes including magnesium chips, solvents, and laboratory chemicals were burned from 1943 until 1968. The unlined S-3 Ponds received unlined from hematite media using IB-PCR in which colonized particle...
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 submergence 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 those that were not present in at least two of the three replicate profiles.

Sequences and sequence alignment. T-RFLP profiles were determined 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). Clustering analyses by 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 $H = -\sum_{i=1}^{S} p_i \ln p_i$, in which $p_i$ is the frequency of the $i^{th}$ phylotype. A higher $H$ value indicates greater diversity. Equitability ($I$) was calculated as $I = H/(\ln S)$, 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 $D$ is indicative of an increase in diversity: $D = \frac{S}{1}$.

Nucleotide sequence accession numbers. All clone sequences have been deposited in the GenBank/DDBJ/EMBL databases under accession numbers NY622227 to NY622271, 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 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
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-
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 Fratercula sp. (96% similarity), which together contributed 8% 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 mandelli, 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 zoogloeoidei. 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

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>GenBank no.</th>
<th>Frequency</th>
<th>Affiliation (% similarity)</th>
<th>Putative division</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-CG17</td>
<td>AY622228</td>
<td>59</td>
<td>Alcaligenes sp. strain L6 (95)</td>
<td>β-Proteobacteria</td>
</tr>
<tr>
<td>C-CS3</td>
<td>AY622233</td>
<td>24</td>
<td>Fratercula sp. NO-16 (96) (AF376025)</td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>C-CF16</td>
<td>AY622227</td>
<td>4</td>
<td>Methylbacterium radiotolerans (99)</td>
<td>α-Proteobacteria</td>
</tr>
<tr>
<td>C-CU62</td>
<td>AY622234</td>
<td>3</td>
<td>Pseudomonas strain A6.2 (98)</td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>C-CJ32</td>
<td>AY622229</td>
<td>2</td>
<td>Beutenbergia cavernosa (96)</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>C-CY80</td>
<td>AY622237</td>
<td>1</td>
<td>Herbaspirillum seropedicae (96)</td>
<td>β-Proteobacteria</td>
</tr>
<tr>
<td>C-DA88</td>
<td>AY622239</td>
<td>1</td>
<td>Burkholderia sp. A6.2 (98)</td>
<td>β-Proteobacteria</td>
</tr>
<tr>
<td>C-CS82</td>
<td>AY622238</td>
<td>1</td>
<td>Duganella zoogloeoidei (98)</td>
<td>β-Proteobacteria</td>
</tr>
<tr>
<td>C-CL42</td>
<td>AY622230</td>
<td>1</td>
<td>Pseudomonas syringae (99)</td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>C-CY74</td>
<td>AY622236</td>
<td>1</td>
<td>Sphingobacterium antarcticum (99)</td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>C-CO51</td>
<td>AY622232</td>
<td>1</td>
<td>Microbacterium sp. VKM Ac-2050 (99)</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>C-CV63</td>
<td>AY622235</td>
<td>1</td>
<td>Nocardioides sp. MWH-CaK6 (98)</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>C-CM46</td>
<td>AY622231</td>
<td>1</td>
<td>Clone MTAC17 (92)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* Frequency of a given RFLP-type out of 100 total clones.  
* Closest identified match in the GenBank database followed by percent similarity. Clone C-CM46 did not match any identified sequences in the database.
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.
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 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 communities that colonized coupon-associated media were more similar to 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>
<thead>
<tr>
<th>Clone ID</th>
<th>GenBank no.</th>
<th>Frequency$^a$</th>
<th>Affiliation$^b$ (% similarity)</th>
<th>Putative division</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-A1</td>
<td>AY622264</td>
<td>54</td>
<td>Pseudomonas aeruginosa (98)</td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>S-H52</td>
<td>AY622270</td>
<td>36</td>
<td>Pseudomonas aeruginosa (99)</td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>S-E105</td>
<td>AY622267</td>
<td>3</td>
<td>Aequorivibrio autotrophicum (97)%</td>
<td>δ-Proteobacteria</td>
</tr>
<tr>
<td>S-D24</td>
<td>AY622266</td>
<td>3</td>
<td>Arthrobacter sp. 1339 (98)</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>S-G30</td>
<td>AY622269</td>
<td>1</td>
<td>Duganella zoogloeoides (98)</td>
<td>β-Proteobacteria</td>
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<tr>
<td>S-B2</td>
<td>AY622265</td>
<td>1</td>
<td>Clone 32-20 (95)</td>
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<tr>
<td>S-F26</td>
<td>AY622268</td>
<td>1</td>
<td>Clone 3C0d-3 (93)</td>
<td>Clostridium</td>
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<tr>
<td>S-H47</td>
<td>AY622271</td>
<td>1</td>
<td>Thermoleophilum minutum$^d$ (AJ458464)</td>
<td>Actinobacteria</td>
</tr>
</tbody>
</table>

$^a$ Frequency of a given RFLP type out of 100 total clones.
$^b$ Closest identified match in the GenBank database followed by % similarity.
$^d$ The sequence of clone S-H47 did not appear to be chimeric, however, it did not significantly match anything in the GenBank database, cultured or uncultured.

**TABLE 5. Bacterial 16S rDNA clones from sediments$^c$ of the FRC background area borehole FWB303**

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>GenBank no.</th>
<th>Frequency$^a$</th>
<th>Affiliation$^b$ (% similarity)</th>
<th>Putative division</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-D24</td>
<td>AY622266</td>
<td>3</td>
<td>Aequorivibrio autotrophicum (97)%</td>
<td>δ-Proteobacteria</td>
</tr>
<tr>
<td>S-B2</td>
<td>AY622265</td>
<td>1</td>
<td>Clone 32-20 (95)</td>
<td>Unknown</td>
</tr>
<tr>
<td>S-F26</td>
<td>AY622268</td>
<td>1</td>
<td>Clone 3C0d-3 (93)</td>
<td>Clostridium</td>
</tr>
<tr>
<td>S-H47</td>
<td>AY622271</td>
<td>1</td>
<td>Thermoleophilum minutum$^d$ (AJ458464)</td>
<td>Actinobacteria</td>
</tr>
</tbody>
</table>

$^a$ 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-H47 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 Thermoleophilum minutum, but across the entire gene similarity was only 76%.
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 10^6 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 diasporite (an Al-bearing analog of goethite) under anaerobic conditions. Curiously, in the presence of oxygen the cells were more attracted to the diaspore of goethite than to either ferrihydrite or hematite, 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., SiO2 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 recovered 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 *Fraturea*, a group of strictly aerobic acidiphiles (50). The presence of a phylotype with 99% sequence similarity to *Methyllobacterium radiotolerans* (4% of the library) is consistent with the high radionuclide content based on the reported radiotolerance of *M. radiotolerans* and other *Methyllobacterium* 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-

### 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

<table>
<thead>
<tr>
<th>Library source</th>
<th>No. of clonesa</th>
<th>% Coverageb</th>
<th>Richnessc</th>
<th>Domaingroupd</th>
<th>Shannoned</th>
<th>Equitabilityd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background area</td>
<td>100</td>
<td>96</td>
<td>8</td>
<td>0.42</td>
<td>1.1</td>
<td>0.53</td>
</tr>
<tr>
<td>Background area</td>
<td>85</td>
<td>81</td>
<td>25</td>
<td>0.22</td>
<td>2.3</td>
<td>0.71</td>
</tr>
<tr>
<td>Area 3 hematite</td>
<td>100</td>
<td>92</td>
<td>13</td>
<td>0.41</td>
<td>1.3</td>
<td>0.52</td>
</tr>
</tbody>
</table>

- 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").
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 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-heating 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 in IB-PCR-generated libraries. The positive organisms, with the exception of the three clones from the background area (Table 4), and C-CY80 from Area 3 (Table 3), were 97% similar to one another.

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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 Azocarcus 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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