Desulfotomaculum and Methanobacterium spp. Dominate a 4- to 5-Kilometer-Deep Fault

Duane P. Moser,1* Thomas M. Gihring,1‡ Fred J. Brockman,1 James K. Fredrickson,1 David L. Balkwill,2 Michael E. Dollhopf,2‡ Barbara Sherwood Lollar,3 Lisa M. Pratt,4 Erik Boice,3 Gordon Southam,3 Greg Wanger,5 Brett J. Baker,6 Susan M. Pfiffner,7 Li-Hung Lin,8§ and T. C. Onstott8

Environmental Microbiology Group, Pacific Northwest National Laboratory, Richland, Washington 99352; Department of Biomedical Sciences Biology, The Florida State University, Tallahassee, Florida 32306; Department of Geosciences, University of Toronto, Toronto, Ontario M5S 3B1, Canada; Department of Geological Sciences, Biogeochemical Laboratories, Indiana University, Bloomington, Indiana 47405; Department of Earth Sciences, University of Western Ontario, London, Ontario N6A 5B7, Canada; Earth and Planetary Sciences, University of California, Berkeley, California 94720; Center of Biomarker Analysis, University of Tennessee, Knoxville, Tennessee 37932; and Department of Geological and Geophysical Sciences, Princeton University, Princeton, New Jersey 08544

Received 6 April 2005/Accepted 15 August 2005

Alkaline, sulfidic, 54 to 60°C, 4 to 53 million-year-old meteoric water emanating from a borehole intersecting quartzite-hosted fractures >3.3 km beneath the surface supported a microbial community dominated by a bacterial species affiliated with Desulfotomaculum spp. and an archaeal species related to Methanobacterium spp. The geochemical homogeneity over the 650-m length of the borehole, the lack of dividing cells, and the absence of these microorganisms in mine service water support an indigenous origin for the microbial community. The coexistence of these two microorganisms is consistent with a limiting flux of inorganic carbon and \( \text{SO}_4^{2-} \) in the presence of high pH, high concentrations of \( \text{H}_2 \) and \( \text{CH}_4 \), and minimal free energy for autotrophic methanogenesis. Sulfide isotopic compositions were highly enriched, consistent with microbial \( \text{SO}_4^{2-} \) reduction under hydrologic isolation. An analogous microbial couple and similar abiotic gas chemistry have been reported recently for hydrothermal carbonate vents of the Lost City near the Mid-Atlantic Ridge (D. S. Kelly et al., Science 307:1428-1434, 2005), suggesting that these features may be common to deep subsurface habitats (continental and marine) bearing this geochemical signature. The geochemical setting and microbial communities described here are notably different from microbial ecosystems reported for shallower continental subsurface environments.

Numerous studies have revealed the presence of microbial communities occupying oceanic and terrestrial deep subsurface settings (12, 14, 23, 27, 53, 54). Due to its enormous volume, this habitat may host the majority of Earth’s prokaryotes (76), and according to some estimates (25, 76), the collective biomass of subsurface microbiota may rival that of flora and fauna at the surface. It is generally accepted that life on Earth requires liquid water (9). The upper 4 km of the terrestrial crust contains \( 9.5 \times 10^6 \) km³ of groundwater, of which about 56% lies below 0.75 km in depth (6). Since reports of microorganisms over this depth interval continue (4, 53, 54, 70) and the basic requirements for life appear to be met (e.g., liquid water, habitable space, and permissive temperatures), it follows that a significant proportion of the biosphere may be microbial and associated with deep terrestrial hydrologic systems (76).

Although a reasonable understanding of the energetic foundations for marine and terrestrial subsurface sedimentary ecosystems to a depth of 500 m has been achieved (20, 55), mechanisms for the energetic support of igneous and metamorphic rock systems remain incompletely addressed. Best known are igneous rock-hosted aquifers at depths of <450 m, where chemolithoautotrophic subsurface ecosystems, founded upon biological methanogenesis or acetogenesis and supported by geological \( \text{H}_2 \) production and inorganic C, are thought to be limited in photosynthetically derived organic C (12, 56, 68). Relatively little is known, however, about the distribution of such communities at much greater depths or whether microbial ecosystems of other deep rock types (e.g., sedimentary and metamorphic) operate with similar energy and C sources.

Microbiological sampling opportunities in the deep subsurface are extremely limited, and a persistent challenge remains the differentiation of indigenous from introduced microorganisms (52). Several studies, however, have shown that in mine boreholes, introduced microbes fail to persist (47, 57), especially when the unidirectional flushing of hot, anaerobic fracture water reestablishes a redox environment similar to that of the predrilling conditions (47). South African mines are unusual in that they routinely attain depths of >3 kilometers below land surface (kmbls), thereby accessing thermophilic, anaerobic environments distinct from more frequently studied,
shallow subsurface aquifers (56, 68). Mine drilling programs occasionally intersect pressurized fluid-filled fracture systems, some of which flush for years at rates of liters min\(^{-1}\). Once purged of drilling-associated microbial and chemical contamination, flowing boreholes can be viewed as artificial artesian fractures and functional conduits into native deep subsurface habitats.

Here we describe such an artesian borehole, which being situated >1.5 km from the nearest current or historical mining activity, enabled the sampling of minimally impacted strata. Originating at 2,716 kmbls and extending downwards to 3,360 kmbls, the borehole intersected three faults. The borehole was uncased from 10 m below the outlet and had continuously flowed over the 12 months following drilling, producing >2.8 × 10\(^6\) liters (~2,400 borehole volumes) of 54°C, anaerobic water. Using a bailer, a profile of the fluid chemistry and microbial community structure was obtained from source faults deep within the borehole to the outlet.

**MATERIALS AND METHODS**

**Site description.** Driefontein Consolidated Mine (Fig. 1A) is located 70 km west of Johannesburg, South Africa, in the Carletonville mining region. The exploration borehole utilized here (D8A, Fig. 1B) was drilled into the floor of a horizontal tunnel about 700 m from the vertical access shaft (9 Shaft). The mine transects an Archean-aged stratigraphic section typical of the western Witwatersrand Basin (62). The 2.5 billion-year (Ga) Pretoria and Chuni-espoort groups, with the latter hosting a dolomite aquifer, extend to about 2 kmbls. Below them lies the metabasaltic, 2.7-Ga Ventersdorp supergroup, which in turn overlies the 2.9-Ga Witwatersrand supergroup quartzite. At the Witwatersrand/Ventersdorp boundary is a commercial Au deposit, the Ventersdorp Contact Reef (VCR), and within the Witwatersrand supergroup are two additional ore zones, the Middelvlei and Carbon Leader reefs (Fig. 1B).

In the Carletonville mining district, water below the dolomite is confined to fractures and ranges in age from 4 to 165 million years (Ma) (41). Borehole D8A was drilled 60° from horizontal to a length of 743 m (644 m vertical, Fig. 1B). The upper 10 m was steel cased (80-mm internal diameter), and the remainder (50-mm internal diameter) was uncased. The outlet (2,716 kmbls) was located within the Ventersdorp supergroup; the VCR was intersected at 400 m (3,058 kmbls), and the Carbon Leader reef was intersected at 720 m (3,340 kmbls). In the corresponding core, faults were recorded within the VCR, at 650 and 670 m (3.28 and 3.30 kmbls). Drilling was completed in October 2000, and sampling was conducted from August to November 2001. Flowing water was noted upon the completion of coring, and water and gas production rates of 4.5 and 4.6 liters min\(^{-1}\), respectively, were measured on 7 November 2001.

**Sampling.** The outlet sample was collected with an expandable steel packer (Cementation Engineers, Johannesburg, South Africa) customized with a nylon insert to eliminate metal-water contact. Water and gas samples were collected anaerobically using a Delrin plastic (DuPont) valved manifold (46). To obtain samples from depth, a chlorine bleach-disinfected bailer was deployed with a wire winch. The bailer was tested to 100 m using sterile rhodamine WT (1,000 mg liter\(^{-1}\)) as a tracer. Upon retrieval, gas accumulated below the upper ball valve, which was subsampled via a dedicated sampling port with a gas-tight syringe. Water samples for microbiological and geochemical analyses were collected anaerobically from the bottom of the bailer.

Filtered (0.22-μm nylon Acrodisc; Gelman) samples included anions, cations, and NH\(_4\), preserved as described previously (47). Bottles for unfiltered samples were preloaded with preservatives and capped without headspace. These included dissolved inorganic carbon (DIC) and [\(^{13}C\)]DIC (140-ml serum vials with 200 μl of saturated HgCl\(_2\)), NH\(_3\) (140-ml serum vials with 50 μl concentrated H\(_2\)SO\(_4\)), HS\(^-\) (20-ml glass serum vials with 500 μl 2 M Zn acetate), [\(^{34}S\)] (60-ml plastic syringes with 500 μl 2 N Zn acetate), [\(^{3}H\)] and [\(^{18}O\)] (140-ml and 20-ml serum vials), dissolved gases (140-ml evacuated serum vials with 200 μl of saturated HgCl\(_2\)), organic acids (45-ml serum vials), dissolved organic carbon (DOC) and total organic carbon (TOC) (45-ml glass vials with Teflon septa containing 2 ml concentrated HCl), and HPO\(_4^{2-}\) (acid-washed 250-ml Nalgene bottles). The anion, HPO\(_4^{2-}\), and organic acid sample bottles were partially filled and frozen. The [\(^{34}S\)] samples were refrigerated. Due to volume limitations,
TABLE 1. Major anions and cations in borehole DNA

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>TOC</th>
<th>Acetate</th>
<th>Formate</th>
<th>Propionate</th>
<th>DIC</th>
<th>Cl⁻</th>
<th>HS⁻</th>
<th>SO₄²⁻</th>
<th>NH₄⁺</th>
<th>Na⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Fe²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outlet-08/30/01</td>
<td>43.3</td>
<td>0.29</td>
<td>0.013</td>
<td>0.0005</td>
<td>0.0002</td>
<td>0.104</td>
<td>31.0</td>
<td>0.60 (0.494)</td>
<td>0.125</td>
<td>0.011</td>
<td>13.9</td>
<td>1.7</td>
<td>0.027</td>
</tr>
<tr>
<td>Outlet-09/15/01</td>
<td>43.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>14.5</td>
<td>1.9</td>
<td>&lt;0.002</td>
<td>0.0001</td>
</tr>
<tr>
<td>Outlet-10/24/01</td>
<td>43</td>
<td>0.40</td>
<td>0.035</td>
<td>0.0020</td>
<td>0.0007</td>
<td>0.104</td>
<td>31.7</td>
<td>1.48</td>
<td>0.137 &lt;0.011</td>
<td>11.1</td>
<td>1.7</td>
<td>0.006</td>
<td>0.00002</td>
</tr>
<tr>
<td>Outlet-10/25/01</td>
<td>46</td>
<td>0.11</td>
<td>0.033</td>
<td>0.0020</td>
<td>0.0007</td>
<td>0.139</td>
<td>26.3</td>
<td>0.60</td>
<td>0.033 &lt;0.011</td>
<td>15.5</td>
<td>2.3</td>
<td>0.010</td>
<td>0.0009</td>
</tr>
<tr>
<td>Outlet-10/25/01</td>
<td>46</td>
<td>NA</td>
<td>0.040</td>
<td>0.0041</td>
<td>0.0014</td>
<td>NA</td>
<td>25.9</td>
<td>NA</td>
<td>0.037</td>
<td>0.082</td>
<td>16.5</td>
<td>2.2</td>
<td>0.013</td>
</tr>
<tr>
<td>Outlet-10/24/01</td>
<td>54</td>
<td>0.14</td>
<td>0.026</td>
<td>0.0031</td>
<td>0.0010</td>
<td>0.112</td>
<td>26.7</td>
<td>0.70</td>
<td>0.035 0.00077</td>
<td>15.8</td>
<td>2.2</td>
<td>0.013</td>
<td>0.0030</td>
</tr>
<tr>
<td>Outlet-10/25/01</td>
<td>50</td>
<td>0.04</td>
<td>0.032</td>
<td>0.0035</td>
<td>0.0012</td>
<td>NA</td>
<td>28.8</td>
<td>NA</td>
<td>0.035</td>
<td>0.07</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Outlet-250—10/24/01</td>
<td>54</td>
<td>0.33</td>
<td>0.034</td>
<td>0.0127</td>
<td>0.0042</td>
<td>0.141</td>
<td>26.1</td>
<td>1.20</td>
<td>0.026</td>
<td>0.069</td>
<td>14.5</td>
<td>2.3</td>
<td>0.012 &lt;0.0001</td>
</tr>
<tr>
<td>Outlet-250—10/24/01</td>
<td>54</td>
<td>0.17</td>
<td>0.030</td>
<td>0.0112</td>
<td>0.0037</td>
<td>0.103</td>
<td>26.7</td>
<td>1.10</td>
<td>0.016</td>
<td>0.0540</td>
<td>14.7</td>
<td>2.3</td>
<td>0.016</td>
</tr>
<tr>
<td>Outlet-250—10/25/01</td>
<td>48</td>
<td>0.26</td>
<td>0.035</td>
<td>0.0051</td>
<td>0.0017</td>
<td>0.109</td>
<td>26.7</td>
<td>1.30</td>
<td>0.041 0.0058</td>
<td>14.9</td>
<td>2.2</td>
<td>0.002 0.0053</td>
<td></td>
</tr>
<tr>
<td>Outlet-250—10/24/01</td>
<td>48</td>
<td>0.26</td>
<td>0.035</td>
<td>0.0051</td>
<td>0.0017</td>
<td>0.109</td>
<td>26.7</td>
<td>1.30</td>
<td>0.041 0.0058</td>
<td>14.9</td>
<td>2.2</td>
<td>0.002 0.0053</td>
<td></td>
</tr>
<tr>
<td>Outlet-11/07/01</td>
<td>43.0</td>
<td>NA</td>
<td>0.029</td>
<td>0.0035</td>
<td>0.0012</td>
<td>0.121</td>
<td>28.9</td>
<td>0.50 (0.433)</td>
<td>0.007 &lt;0.011</td>
<td>14.4</td>
<td>2.0</td>
<td>0.011 &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Avg</td>
<td>0.24</td>
<td>0.032</td>
<td>0.0048</td>
<td>0.0016</td>
<td>0.117</td>
<td>27.8</td>
<td>0.92</td>
<td>0.049</td>
<td>0.037</td>
<td>14.7</td>
<td>2.1</td>
<td>0.012</td>
<td>0.00008</td>
</tr>
</tbody>
</table>

---

a Quantification limits: DOC, 0.04 mM; TOC, 0.04 mM; acetate, 0.002 mM; formate, 0.002 mM; propionate, 0.001 mM; DIC, 0.04 mM; Cl⁻, 0.003 mM; H₂SO₄, 0.004 mM; SO₄²⁻, 0.011 mM; NH₄⁺, 0.011 mM; Na⁺, 0.004 mM; Ca²⁺, 0.001 mM; Mg²⁺, 0.002 mM; Fe²⁺, 0.0009 mM. TOC and DOC agree within the error. NA, not analyzed. NO₃⁻ and PO₄³⁻ were at or below the quantification limits (0.002 and 0.0005 mM, respectively) for all samples.

b HS⁻ was determined by two different protocols, gravimetric measurement and measurement of Zn by ICP-AES after precipitation with ZnCl₂. The δ¹⁸O for three samples were also determined, using the procedures of Ward et al. (74).

Below the high-confidence quantification limit, but detectable.
Microscopy. Samples for electron microscopy were collected in 2-ml cryovials and fixed in the field with 2% (vol/vol) glutaraldehyde. One hundred microliters of each sample was suspended in 5 ml of filtered (0.2 μm) deionized water (Barnstead E-pure) and passed through a 13-mm, 0.4-μm-pore-size Isopore membrane filter (Millipore). Samples were processed through an ethanol dehydration series (25%, 50%, 75%, 100%, and 100% [30 min each]), followed by critical point drying using a Samdri PVT 3B CPD apparatus (Tohumis Research Corp.). Gold-coated samples were examined using a Hitachi S-4500 field emission scanning electron microscope (SEM) equipped with an EDAX system at an accelerating voltage of 10 kV with a 15-mm working distance. A total of 771 and 1,606 cells were counted from the 125-m and 250-m samples, using fluorescence in situ hybridization (FISH) analysis according to the procedure of Baker et al. (3), with EUB338 and ARC915 probes.

PLFA analysis. For phospholipid fatty acid (PLFA) analysis, biomasses from 8 liters of the outlet sample and 140 ml of the bairler samples were collected on Anodisc filters (0.2 μm by 47 or 25 mm; Lagunae Niguel) (47). Compositional analyses of PLFAs were performed using procedures described by White and Ribbons (75). Approximate bacterial cell densities were calculated using a conversion factor of 2.5 × 10⁵ cells pmol⁻¹ PLFA (5).

Molecular analyses. Biomasses from 7 liters of sample D8A-Outlet-110701 and 140-ml bairler samples from 390, 550, and 648 m downhole were collected and preserved as previously described (46, 47). PCR products were gel purified and cloned using TOPO-TA cloning kits (Invitrogen). For each clone library, template for a second round of PCR with primers 21F and 958R (19). 16S rRNA gene cloning was done with TA cloning kits (Invitrogen). For each clone library, the product generated using primers 21F (19) and 1492R (39) was used as the template for a second round of PCR with primers 21F and 1492R (39). Possibly due to the poor DNA extraction efficiency resulting from the gentle lysis procedure, reliable amplification of archaeal rRNA genes required a half-nested reaction in which the first-round amplification product was used as the template for a second PCR reaction using the primers 21F and 1492R (39).

Maximum likelihood trees were generated using the Phylip FastDNAMl algorithm. Parsimony analysis and the calculation of bootstrap probabilities were performed with PAUP (Sinauer and Associates, Sunderland, MA). Phylogenetic trees based on Bayesian inferences were generated using MrBayes (30), with 100,000 generations using a gamma rate correction, a sampling frequency of 100, and a burn-in value of 0.1 to omit the first 350 trees from the consensus.

Terminal restriction fragment length polymorphism (T-RFLP) analysis of community 16S rRNA sequences was performed as previously described (47). To relate individual T-RFs to library clones, the 16S rRNA clone sequences were digested in silico, and the predicted T-RFs were compared to those produced by T-RFLP using an empirically derived accuracy criterion (47).

Functional genes were amplified using the same reagents as those used for 16S rRNA genes. The gene encoding the α subunit of methyl coenzyme M reductase (mrxA) was amplified from the 648-m sample with the primers (ME1F and ME2R) and PCR conditions described by Hales et al. (26). The dissimilatory sulfite reductase gene (dsrAB) was amplified from the 550-m sample as described by Baker et al. (3). PCR products were gel purified and cloned using TOPO-TA kits (Invitrogen) before transformation into competent Escherichia coli DH5α. A minimum of 771 and 1606 clones were analyzed for each sample. The inserts from 96 clones from each library were sequenced using a Big Dye reaction kit and ABI 3100 DNA sequencer; PE Applied Biosystems. Chimeric sequences were identified using Bellerophon (29) and RDP Chimera-Check (13). The closest available matches to nonchimeric sequences were identified using the NCBI BLAST search tool (1). Sequences were managed within ARB (http://www.arb-home.de) and aligned with an ARB database built upon the 2002 Hugenholtz RDP alignment, using version 3.01 within the ARB E-DIT_4.1 tool followed by manual adjustments. Clone types (99% similarity) were designated after the alignment of sequences with ARB and comparisons of unambiguously aligned positions, as determined using the Lane mask (39). Maximum likelihood trees were generated using the Phylib FastDNAMl algorithm. Parsimony analysis and the calculation of bootstrap probabilities were performed with PAUP (Sinauer and Associates, Sunderland, MA). Phylogenetic trees based on Bayesian inferences were generated using MrBayes (30), with 100,000 generations using a gamma rate correction, a sampling frequency of 100, and a burn-in value of 0.1 to omit the first 350 trees from the consensus.

RESULTS AND DISCUSSION

The average pH and chlorinity were 9.1 and 32 mM, respectively, over the 70-day observation interval, and the Eh was ~280 mV, equivalent to a Pe of ~1.4. The dissolved O₂ was below detection (<30 μM). The temperature at the outlet was 43.3°C, but increased to a maximum of 54°C at 250- to 550-m depths (Table 1).

Water age and source. Isotopically, the water in borehole D8A is meteoric in origin but distinct from that of the overlying dolomite (Table 2; Fig. 2). The δ¹⁸H versus δ¹⁸O measurements agree with those for fracture water from nearby 4 Shaft of Kloof mine F3, as reported by Takai et al. (70), for which an age of 3 to 30 Ma was determined (41). Because the average
The Witwatersrand quartzite.

Most of the borehole water originated from the deeper faults in the Chuniespoort dolomite (52). The uniform geochemical signature along the depth profile (Table 1) also suggests that the Chuniespoort dolomite (52) occupied different positions. The figure is based on the work of Lippmann et al. (41), with permission.

Most notably, the Ca\(^{2+}\) of Lippmann et al. (41), the estimated age is 4 (U/He) versus 0.3 mM (41), the age of D8A water is greater than that of water from the Kloof sample. Using the models of Lippmann et al. (41), the estimated source depth of the fault water. The fact that neither surface nor mine water breakthrough had occurred indicates that D8A is linked to a voluminous, deep fracture system with minimal connection to shallower sources. A similar inference was reached for the 3.1-kmbls Kloof Mine shaft 4 borehole water (70), whose 60°C temperature, exceeded the predicted in situ rock temperature by ~14°C, was attributed to upward migration along interconnected fractures (49). Collectively, the aqueous geochemistry and isotopic data are best explained by a deep source of ancient meteoric water. Since the water bears no evidence of interaction with the Ventersdorp supergroup metabasalt, the flow path must be confined to the underlying Witwatersrand supergroup quartzite.

**Microbial abundance and community structure.** PLFA analysis indicated a bacterial biomass ranging from ~6 × 10^3 to ~2 × 10^6 cells ml\(^{-1}\), with a possible increase (about three times) at shallower depths (Table 3). Total microbial cell densities given by flow cytometry were ~1 × 10^4 to 4 × 10^6 cells ml\(^{-1}\) for samples from the 250-m depth to the outlet but were ~50 times higher for the two deepest samples (~1 × 10^6 cells ml\(^{-1}\)) (Table 3). Since the water bears no evidence of interaction with the Ventersdorp supergroup metabasalt, the flow path must be confined to the underlying Witwatersrand supergroup quartzite.

**TABLE 3. Cell number estimates by flow cytometry and phospholipid fatty acid analysis**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total cells ml(^{-1}) (flow cytometry)</th>
<th>Bacterial cells ml(^{-1}) (PLFA)</th>
<th>% Bacteria (FISH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR938 H3 Outlet-102401</td>
<td>4.9 × 10^4</td>
<td>2.04 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>DR938 H3 125M-102501</td>
<td>2.2 × 10^4</td>
<td>2.08 × 10^4</td>
<td>91^a</td>
</tr>
<tr>
<td>DR938 H3 250M-102401</td>
<td>2.1 × 10^4</td>
<td>1.33 × 10^4</td>
<td>90^c</td>
</tr>
<tr>
<td>DR938 H3 390M-102401</td>
<td>2.0 × 10^4</td>
<td>8.16 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>DR938 H3 550M-102501</td>
<td>1.3 × 10^6</td>
<td>5.84 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>DR938 H3 648M-102501</td>
<td>1.4 × 10^6</td>
<td>5.64 × 10^4</td>
<td>NA</td>
</tr>
</tbody>
</table>

^a NA, not analyzed.
^b Seven hundred seventy-one cells were counted.
^c One thousand six hundred six cells were counted.

The δ^18O value for H\(_2\)O (~685‰ VSMOW [Vienna Standard Mean Ocean Water]), relative to that for H\(_2\)O (~26.9‰ VSMOW) (Table 2), yielded an isotopic equilibration temperature of 60.5°C (8, 43), which is significantly greater than the 54°C measured downhole (Table 1). Based upon heat flow measurements obtained from West Witwatersrand line quartzite (49 ± 3 mW m\(^{-2}\)) (34) and a one-dimensional thermal conductivity model (49), this temperature constrains the water source to between 3.8 and 5.0 kmbs. The >2.8 × 10^6 liters expelled prior to sampling would fill the volume of a hypothetical fracture with an average width of 0.5 cm and an area approximated by a plane of 250 by 2,035 m, which is compatible with the estimated source depth of the fault water. The fact that neither surface nor mine water breakthrough had occurred indicates that D8A is linked to a voluminous, deep fracture system with minimal connection to shallower sources.

The aqueous geochemistry most closely resembled that of the Kloof sample (27.8 mM) (41) but the He concentration in D8A was four times greater than that of the Kloof sample. Since PLFA is a measure of bacterial biomass, the discrepancy between these methods probably reflects an enrichment of archaea in the deeper samples. FISH analyses of the 125-m and 250-m samples revealed that 9% and 10%, respectively, of the cells were filamentous archaea, with the remainder being rod-shaped bacteria (Table 3). This observation is consistent with the PLFA cell densities being slightly less than the flow cytometry cell densities. Flow cytometry also indicated that most of the observed increase in total cell counts with increased depths is attributable to weakly stained, large-diameter (2 to 11 μm) particles (data not shown). SEM observations indicated that these particles were comprised of Si and Fe mineral colloids with attached filamentous microbial cells (data not shown). This colloidal material was visually apparent as a darker cast in the deeper samples. It was impossible to obtain accurate FISH counts in the deeper samples due to interference by mineral colloids, but it was qualitatively appar-
ent that the filaments far outnumbered the rod-shaped bacterial cells that dominated the shallower samples.

PCR-amplified 16S rRNA genes included both bacterial and archaeal genes, and T-RFLP profiles for both were dominated by a single major peak at all depths (data not shown). The bacterial peak (209 bp) corresponded to the predicted T-RF for the most abundant clone type (D8A-1; 98 to 100% of bacterial peak (209 bp) corresponded to the predicted T-RF for both were dominated by a single bacterium (probably a sulfate reducer) that appeared only after mine air was excluded for 2 months, indicating that SO$_4^{2-}$ reduction had occurred within the fault (73) and of Methanobacteriaceae (the methyl-coenzyme M reductase gene, mcrA, for methanogenesis) (26) were also detected. A single dsrAB clone type (Table 4; Fig. 3B), which was most closely affiliated with Desulfotomaculum spp., and two mcrA clone types (Table 4; Fig. 3C) from within the Methanobacteriaceae were identified, confirming the genomic potential for SO$_4^{2-}$ reduction and methanogenesis.

The microbial community structure of D8A fault water is therefore populated by a very simple microbial community dominated by a single bacterium (probably a sulfate reducer) and several closely related methanogens. Whether the methanogens or sulfate reducers dominate the microbial community within the fault is impossible to ascertain, because the filamentous methanogens may have preferentially adhered to the mineral colloids that formed and then settled to the bottom of the borehole as the hot fracture water cooled upon its ascent to the borehole outlet. This phenomenon would have gone undetected if not for the depth profile results. Colloid formation in deep artesian boreholes may be a common occurrence that needs to be considered if the emanating fluids are to be used to quantify the microbial community structure, as they have been in previous reports (12, 15, 25, 47, 68).

**Predicted in situ microbial reactions.** No significant difference was observed between DOC and TOC values, nor did either exhibit any trend with the borehole depth (Table 1). Acetate was the most abundant organic acid, ranging from 0.013 to 0.040 mM. The $\delta^{13}$C value for DIC ranged from −13.7 to −18.3‰ Vienna Pee Dee Belemnite, averaging −15.9‰, with the deepest samples showing the least isotopic depletion (Table 2). Dividing cells were not observed by SEM or FISH, suggesting that the microorganisms were not growing at the time of sampling. The mM H$_2$S versus $\mu$M SO$_4^{2-}$ concentrations were uniform along the length of the borehole (Table 1), indicating that SO$_4^{2-}$ reduction had occurred within the fault system rather than the borehole water column.

These observations, combined with the short residence time in the borehole (ca. 3.5 h), argue for a steady-state balance between the observed water chemistry and the microbial population structure within the source fault water. To determine if the calculated Gibbs free energy of reaction ($\Delta G$) and the

### TABLE 4. Clone library summary

<table>
<thead>
<tr>
<th>Clone group</th>
<th>T-RF (bp)</th>
<th>Accession no.</th>
<th>General phylogenetic position</th>
<th>% of clones$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8A-1</td>
<td>209</td>
<td>AY768821</td>
<td>Low-G+C, gram-positive Desulfotomaculum</td>
<td>Outlet 390 m: 98</td>
</tr>
<tr>
<td>D8A-2</td>
<td>93</td>
<td>AY768822</td>
<td>Unresolved within low-G+C, gram-positive group</td>
<td>Outlet 550 m: 1</td>
</tr>
<tr>
<td>D8A-3</td>
<td>239</td>
<td>AY768823</td>
<td>Unresolved within low-G+C, gram-positive group</td>
<td>Outlet 648 m: NA</td>
</tr>
<tr>
<td>D8A-4</td>
<td>69</td>
<td>AY768824</td>
<td>Betaproteobacteria, Comamonadaceae</td>
<td>Outlet 390 m: NA</td>
</tr>
<tr>
<td>D8A-5</td>
<td>69</td>
<td>AY768825</td>
<td>Betaproteobacteria, Comamonadaceae</td>
<td>Outlet 550 m: NA</td>
</tr>
<tr>
<td>D8A-6</td>
<td>197</td>
<td>AY768826</td>
<td>Eurarchaeota, Methanobacteriaceae</td>
<td>Outlet 390 m: 100</td>
</tr>
<tr>
<td>D8A-7</td>
<td>197</td>
<td>AY768827</td>
<td>Eurarchaeota, Methanobacteriaceae</td>
<td>Outlet 550 m: 100</td>
</tr>
<tr>
<td>D8A-8</td>
<td>335</td>
<td>AY768828</td>
<td>Eurarchaeota, Methanobacteriaceae</td>
<td>Outlet 648 m: 97</td>
</tr>
<tr>
<td>D8A-mcrA1</td>
<td>NA</td>
<td>AY768819</td>
<td>Eurarchaeota, Methanobacteriaceae</td>
<td>Outlet 390 m: 100</td>
</tr>
<tr>
<td>D8A-mcrA2</td>
<td>NA</td>
<td>AY768820</td>
<td>Eurarchaeota, Methanobacteriaceae</td>
<td>Outlet 550 m: NA</td>
</tr>
<tr>
<td>D8A-dsr1</td>
<td>NA</td>
<td>AY768818</td>
<td>Low-G+C, gram-positive Desulfotomaculum</td>
<td>Outlet 648 m: NA</td>
</tr>
</tbody>
</table>

$^a$ Ninety-six clones were screened for each rRNA library, and 48 clones were screened for the mcrA and dsrAB libraries.

$^b$ NA, not analyzed.
FIG. 3. Phylogenetic analyses. Clones from Driefontein Mine boreholes are depicted with bold lettering (47). Those specific for this study are underlined. (A) Phylogenetic dendrogram based on a maximum likelihood analysis (Phylip FastDnaMl) of 862 unambiguously aligned positions of 16S rRNA gene sequences. (B) Maximum likelihood tree of dissimilatory sulfite reductase (dsrAB) genes. (C) Phylogenetic tree based on neighbor-joining analyses of methyl-coenzyme M (mcrA) reductase gene sequences. Maximum likelihood tree topologies were compared against trees generated by Bayesian inferences and parsimony. Closed circles indicate nodes conserved in all three treatments, with Bayesian probability and parsimony bootstrap values of 75%. Nodes conserved in the majority of tree topologies with 50 to 74% probabilities and bootstraps are designated by open circles. Branch points supported by fewer than two trees or having 50% probability and bootstrap values have no designation. In the neighbor-joining tree, branch points supported by 79% bootstrap values are designated by solid circles, those supported by 60 to 79% bootstrap values are designated with open circles, and those having bootstrap values of 60% are unlabeled. Bars, changes per nucleotide.
steady-state free energy flux favored SO$_4^{2-}$ reduction and methanogenesis over other possible physiologies, calculations were performed using the borehole water chemistry for 83 possible microbial reactions at 43°C and 54°C and the measured outlet pH of 9.0. Calculations were also performed assuming a temperature of 61°C and a pH of 7.8, the predicted conditions for the fault water deduced from the borehole outlet pH and DIC after adjusting for the degassing of CO$_2$ within the borehole. The microbial reduction of Fe$^{3+}$ was excluded due its reactivity at high levels of HS$^-$. The free energy flux calculations predicted that the three most thermodynamically favorable reactions were methanogenic (Table 5, reactions 1 to 3), with two reactions utilizing CO. Reactions 1 and 2 in Table 5 are known to be catalyzed by Methanobacterium spp. (69).

Three SO$_4^{2-}$ reduction reactions (Table 5, reactions 8 to 10) were consistent with those known to be catalyzed by the genus Desulfotomaculum: they were the oxidation of H$_2$ (45, 48), the oxidation of acetate (65, 78), and the oxidation of propane (37). Although Desulfotomaculum spp. are not yet known to couple CO oxidation with SO$_4^{2-}$ reduction (reaction 5) or to produce acetate from HCO$_3^-$ or CO (reactions 4 and 7), they do possess the bifunctional and reversible CO dehydrogenase/acetyl-coenzyme A synthetase enzyme used for these metabolic processes and for reaction 9 (60, 77). Therefore, clone type D8A-1 might be expected to perform reaction(s) 4, 5, or 7. In hydrologically isolated systems, where cellular metabolism is slow and reactant concentrations change over geological time, the ability to exploit reversible pathways may be a critical survival adaptation.

Desulfotomaculum and Methanobacterium spp. are known to intimately interact during the transfer of H$_2$ and/or formate in thermophilic (55°C) anaerobic fermentation reactor biogranules utilizing low-molecular-weight, reduced organic compounds (32, 59). The high H$_2$ concentrations in the D8A water would seem to preclude this process, and cell assemblages, where low H$_2$ can be maintained in microsites by consumption rates that exceed diffusive flux, were not observed microscopically. Similarly, anaerobic CH$_4$ oxidation coupled to SO$_4^{2-}$ reduction (Table 5, reaction 13) was less favorable than the individual methanogenic and SO$_4^{2-}$-reducing reactions. The $^3$C value for the $\sim$121 µM DIC was greater than that for the mM CH$_4$, which would not be the case for anaerobic CH$_4$ oxidation by metabolic coupling between a SO$_4^{2-}$ reducer and a methanogen. Furthermore, molecular evidence for predicted anaerobic CH$_4$ oxidizers (e.g., ANME-1) was not detected in either the 16S rRNA or mcrA clone library.

Microbial activity. The $\delta^{34}$S$_{sulfate}$ value was enriched in $\delta^{34}$S (average, 12.1‰ Cañon Diablo troilite [CDT]) (Table 2) relative to the sulfate value and more consistent with sulfate values that have been reported for the open borehole E5-46-Bh1 (i.e., $\delta^{34}$S$_{sulfate} = 1.4‰$ CDT and $\delta^{34}$S$_{sulfate} = 19‰$ CDT for E5-46-Bh1) (47). The sulfate isotopic enrichment and low SO$_4^{2-}$ concentrations are consistent with microbially driven fractionation under SO$_4^{2-}$ limitation, with minimal oxidation of the product sulfate. This is consistent with a hydrologically isolated environment and suggests that the microorganism represented by the D8A-1 clone performs sulfate reduction within the fault zone. The isotopically depleted $^3$C value for the DIC indicates that some of this sulfate reduction is tied to the oxidation of organic C, most likely acetate.
The $\delta^{13}$C values for hydrocarbon gases trended towards greater depletion with increasing carbon chain lengths ($C_1$ versus $C_2 + C_3$), whereas the $\delta^2$H became enriched from $C_1$ to $C_2$ (Table 2), both of which are indicative of abiotic production (66, 74). If biological methanogenesis were occurring within the faults, then the concentrations of biogenic CH$_4$ would have to be $>100$ $\mu$M to isotopically shift the $>17$ mM abiotic CH$_4$. The $\delta^{13}$C value for the $\sim121$ $\mu$M CH$_4$ is not as isotopically enriched as that reported by Stevens and McKinley for the Columbia River basaltic aquifer (68), which suggests that autotrophically produced CH$_4$ would have to be $<100$ $\mu$M.

This ecosystem appears be controlled in a manner altogether different from that for subseaflloor sediments (20, 55) and shallow terrestrial sedimentary environments, which are generally electron donor limited and represent the terminal portions of complex food webs which begin with detrital organic carbon and electron acceptors that are ultimately derived from the photosynthetically oxidizing surface world. In sediments, the H$_2$ concentration is controlled by the major terminal electron-accepting process, with methanogenesis and SO$_4^{2-}$ reduction depleting H$_2$ to 7 to 10 and 1 to 1.5 nM, respectively (42). The D8A H$_2$ concentration is at least 3 orders of magnitude (Table 2) greater than would be predicted for either process. The potential cellular electron flux from H$_2$ is $\sim16$ times that of SO$_4^{2-}$, but only about one-half that of HCO$_3^-$, taking into account the H$^+$ demands of the methanogenic reaction (Table 5). If the cellular HCO$_3^-$ flux truly exceeds the H$_2$ flux, then this may explain why the $\delta^{13}$C value for the DIC is not greatly isotopically enriched, since it is not limiting. The answer to why the autotrophic methanogens do not deplete the pool of H$_2$ may reside in the free energy for this reaction, which hovers between $-63$ and $-70$ kJ/mol (Table 5), close to the energy required for 1 mol of ATP. Significant reduction of the H$_2$ concentration in the presence of elevated CH$_4$ concentrations would reduce the free energy to below the minimum required for ATP production. The resulting H$_2$ concentration greatly exceeds the requirements imposed by the relatively minor subsurface SO$_4^{2-}$ flux. The HCO$_3^-$ concentration is controlled by the high Ca$^{2+}$ concentration, high p$H$, high temperature, and solubility of calcite. This limitation on the electron acceptor flux and the minimal free energy requirement for methanogenesis may also account for the substantial excesses in H$_2$ that have been noted for other terrestrial subsurface environments (38, 68). Thus, given the H$_2$ abundance, both methanogenic and SO$_4^{2-}$ reduction metabolisms can coexist.

The Columbia River basaltic aquifers are similar to the D8A fault water in that they are alkaline (7.9 to 9.9) and anaerobic, but they are also cooler (18 to 20°C), much less saline (0.1 to 4 mM), contain much higher DIC concentrations (1 to 3 mM), are much less sulfidogenic (0.2 to 30 $\mu$M), and have much lower concentrations of CH$_4$ (2 to 209 $\mu$M). Rock/water microcosms incubated with a suite of well water samples under excess H$_2$ and CO$_2$ indicated that the balance between methanogens and SO$_4^{2-}$ reducers was controlled by SO$_4^{2-}$ concentrations, with methanogens dominating under conditions of SO$_4^{2-}$ limitation (68). A subsequent quantitative rRNA analysis of the microbial community structure of some of the same wells (methanogenic [DB-11] and sulfidogenic [DC-06]) revealed that the active microbial community in the shallower methanogenic well was dominated by bacteria (64% bacteria versus 3% archaea) and that in the deeper sulfidogenic well was even more so (92% bacteria versus 1.8% archaea) (24). Gram-positive bacteria and $\delta$-proteobacteria comprised a small portion of the bacterial component. Overall, the Columbia River basaltic aquifers represent a fresher, shallower, cooler, and microbially more diverse ecosystem than the deep faults intersected by D8A.

The D8A fault water ecosystem is also different in many respects from the 59°C, 70-m-deep groundwater of Lidy Hot Spring (12). The Lidy Spring water has a lower p$H$ (6.9), lower salinity (0.2 mM), and much higher DIC (55 mM) and SO$_4^{2-}$ (1.5 mM) concentrations than the D8A water. Its CH$_4$ concentration (0.1 mM), H$_2$ concentration (0.013 $\mu$M), and DOC concentration (20 $\mu$M) are much lower, making it an electron donor-limited environment, unlike D8A. The 16S rRNA gene library was almost entirely dominated ($\sim99\%$) by methanogens (12), and sulfate reducers were not detected, despite the abundance of SO$_4^{2-}$.

The D8A results do bear a striking resemblance to those for the Lost City seaflloor vent field, which has been interpreted as a microbial ecosystem driven by abiotic CH$_4$ and H$_2$ generation (7). The 60 to 75°C water venting from the Lost City carbonate towers (35) is similar to D8A borehole water in that it has a high p$H$ (9 to 11), low DIC concentrations, high concentrations of abiotic CH$_4$ (1 to 2 mM), and elevated H$_2$ concentrations (<1 to 15 mM) but differs by having a greater salinity and higher SO$_4^{2-}$ concentrations (1 to 4 mM). The high-temperature microbial community is dominated by a methanogen, Methanosarcinace, and by thermophilic, sulfate-reducing Firmicutes (35), similar to D8A. Unlike the case for Lost City, however, in D8A the H$_2$ and possibly the SO$_4^{2-}$ are generated by radiolysis of water in pyrite-rich quartzite (40), and the formation of abiogenic hydrocarbons occurs in the absence of ultramafic rock strata (67).

The identification of ocean vent microorganisms in the boreholes of deep South African Au mines has been reported previously, e.g., Pyrococcus abyssi was reported by Takai et al. (70). In this study, the microbial ecosystem of D8A is most similar to that associated with off-axis ocean crust fluids and vents that form when seawater circulates through the ocean crust a few million years after it has moved from the axis and cooled. These thermophilic microorganisms could be inhabit-antedents of the deep ocean selectively colonizing the ocean crust during the infiltration of SO$_4^{2-}$-rich seawater as part of hydrothermal circulation, or they may be permanent residents of the off-axis ocean crust with its unique thermal alkaline environment that are migrating horizontally as the ocean crust slowly moves away from the central axis.

The quartzite of the Witwatersand Basin experienced sterilizing temperatures long after the last tectonic/metamorphic episode at 2 Ga. At 90 Ma, $\sim$2 kilometers of the overlying Karoo supergroup sediments and volcanics was removed by erosion, and the underlying strata present at 3.2 kmbls began to cool from 120°C, reaching the present-day temperature at about 35 Ma (49). The 4- to 53-Ma He model ages for the fault water overlap with this post-Mesozoic uplift and cooling interval and are consistent with two origins for the microbial inhabitants of the D8A faults. They either existed in the overlying aquifers and selectively colonized the fault zone during meteoric water infiltration, or they have been residents of the thermal...
morphic, alkaline environment of the Witwatersrand subsurface and have slowly migrated downwards through the crust with uplift and cooling. Regardless of the origin of the D8A microbial community, oceanic and continental crusts with distinct thermal histories appear to host geochemically and microbially similar ecosystems. The Lost City hydrothermal field has been advanced as an exciting natural laboratory for the study of ecosystems driven by abiotic CH₄ and H₂ (7). The functionally analogous system described here significantly expands this concept to include an alternative mechanism for abiotic CH₄ and H₂ production within metamorphic and sedimentary strata of the continental deep subsurface.

ACKNOWLEDGMENTS

This research was supported by grants to T.C.O. from the NSF LEXEn program (EAR-9714214) and to L.M.P. from the NASA Astrobiology Institute (NASA NNA04CC03A). SEM was performed at Surface Science Western, University of Western Ontario.

We gratefully acknowledge the support of Gold Fields Ltd.; Driefontein Consolidated Mine, the Universities of Witwatersrand and Orange Free State, and Rob Wilson and colleagues of Turgis Technology (Pty.) Ltd. Special thanks to Dawie Nel, the Driefontein senior geologist, and Ben Kotze, the 9 Shaft master shaft sinker. Finally, we acknowledge the insightful comments of the reviewers, which greatly improved our interpretations of the data.

REFERENCES


44. Onstott, T. C.