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Population Structure of Microbial Communities Associated with Two Deep, Anaerobic, Alkaline Aquifers

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Microbial communities of two deep (1,270 and 316 m) alkaline (pH 9.94 and 8.05), anaerobic (E_h, −137 and −27 mV) aquifers were characterized by rRNA-based analyses. Both aquifers, the Grande Ronde (GR) and Priest Rapids (PR) formations, are located within the Columbia River Basalt Group in south-central Washington, and sulfidogenesis and methanogenesis characterize the GR and PR formations, respectively. RNA was extracted from microorganisms collected from groundwater by ultrafiltration through hollow-fiber membranes and hybridized to taxon-specific oligonucleotide probes. Of the three domains, *Bacteria* dominated both communities, making up 92.0 and 64.4% of the total rRNA from the GR and PR formations, respectively. *Eucarya* comprised 5.7 and 14.4%, and *Archaea* comprised 1.8% and 2.5%, respectively. The gram-positive target group was found in both aquifers, 11.7% in GR and 7.6% in PR. Two probes were used to target sulfate- and/or metal-reducing bacteria within the delta subclass of *Proteobacteria*. The *Desulfobacter* group was present (0.3%) only in the high-sulfate groundwater (GR). However, comparable hybridization to a probe selective for the desulfovibrios and some metal-reducing bacteria was found in both aquifers, 2.5 and 2.9% from the GR and PR formations, respectively. Selective PCR amplification and sequencing of the desulfovibrio/metal-reducing group revealed a predominance of desulfovibrios in both systems (17 of 20 clones), suggesting that their environmental distribution is not restricted by sulfate availability.

The existence of active microbial populations in the deep subsurface (hundreds to thousands of meters below the surface) has been recognized for several decades (35, 68). Most of the early research focused on sulfur and oil deposits, generating little information on the microbiology of the deep subsurface environments that were relatively poor in potential electron donors (23, 24). Only recently have more detailed studies of diverse deep subsurface environments been conducted in the United States (9, 15, 21, 25, 26, 38), Germany (29), and England (19, 61). These studies demonstrated that microorganisms are present in geologically diverse subsurface environments and that their metabolic activities can influence regional groundwater chemistry (8, 43, 48, 59).

Microbial communities have been described in several deep, alkaline, anaerobic aquifers in the Columbia River Basalt Group (CRB) in south-central Washington State (58, 59). Although the carbon and energy sources of microbial growth in these and other subsurface environments remain largely undefined, it is generally thought that most subsurface communities are ultimately dependent on photosynthetic energy, in the form of either organic carbon or dissolved oxygen as a metabolic terminal acceptor. Two distinct biogeochemical end members have been observed in some of the CRB aquifers. One end member includes deep groundwaters that are characterized by relatively high sulfate and sulfide concentrations and dissolved inorganic carbon that is depleted in ^{13}C, indicative of organic C oxidation by sulfate-reducing bacteria SRB (59). Groundwaters representing the methanogenic end member are characterized by a relative depletion of dissolved inorganic carbon in combination with an enrichment in ^{13}C, an observation that is consistent with the preferential reduction of $^{12}CO_2$ by autotrophic methanogens. The microbial populations in groundwater from these two end members, as determined by enrichment techniques, reflect the respective geochemical signatures (58, 59). However, it has recently been proposed that geochemical energy also sustains the CRB microbiota and that they may be independent of photosynthetic primary production (58). This hypothesis is consistent with both the microbiology and chemistry of the site. Hydrogen concentrations in CRB groundwaters as high as 60 μM were reported, and laboratory experiments demonstrated that hydrogen gas is produced by reactions between freshly exposed basalt surfaces and anaerobic water. Chemoautotrophic microorganisms from these aquifers that could grow on hydrogen and carbon dioxide greatly outnumbered those that could grow on organic compounds (58).

Despite considerable interest in the microbiology and biogeochemistry of deep subsurface environments, relatively little is known about the diversity of the indigenous microorganisms. Most of the available information is for pure cultures (6, 20, 22, 53), even though culture-based methods can generally account for only a small fraction of the resident microbiota (33). As a complement to more traditional methods, the characterization of population-specific biomarkers (e.g., nucleic acid sequences and membrane lipids) recovered directly from environmental samples is serving to better define the population structure of deep subsurface microbial communities (20, 22, 44, 47). In this study, we evaluated the abundance and diversity of the active microbial populations in deep, alkaline aquifers by using taxon-specific oligonucleotide probes targeted against extracted

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rRNA. Although the rRNA content is known to vary among cell types depending on the growth rate and starvation conditions (18, 32), we suggest that it serves for the semiquantitative assessment of the more active component of a microbial community (49, 50). As a complement to this analysis, extracted DNA was used as a PCR template to amplify, clone, and sequence 16S bacterial rRNA genes from populations comprising selected probe-target groups.

Our observations indicate that while the microbial communities in the two aquifers have some common features, there are distinct differences that are consistent with the geochemical environment. Although the *Bacteria* dominated in both, the two systems differed in representation by *Eucarya*, *Archaea*, and gram-positive organisms. Surprisingly, although the probe-target group composed of gram-negative sulfate-reducing bacteria (SRB) that characteristically completely oxidize their carbon substrates was present only in the aquifer with the highest sulfate concentration (Grande Ronde [GR]), comparable hybridization to a probe for the desulfovibrios and some metal-reducing bacteria was found in both aquifers. The presence of desulfovibrio-like populations in both aquifers was confirmed by selective PCR amplification, cloning, and sequencing of the probe-target assemblage. This suggests that the environmental distribution of *Desulfovibrio* species is not restricted by sulfate availability.

MATERIALS AND METHODS

Site description. The study site was located in south-central Washington State within the CRB. The CRB, covering an area of approximately 200,000 km^2 in the northwestern United States, consists of Miocene Tholeiitic flood basalt flows of up to 6 km (20 to 150 m thick) interspersed with sedimentary interbeds (0.3 to 25 m thick) overlain by 60- to 250-m thick alluvial and loess sediments. These basalt flows can act as confining layers which isolate individual aquifers. The temperature and pH within these confined aquifers increase with depth throughout the site, and the groundwater is anaerobic. The geochemistry of the CRB is well characterized as a result of previous evaluation for use as a long-term storage site for high-level radioactive waste (28, 62). Interest in the microbiology and geochemistry of these aquifers has also been generated by concern about potential contamination from the overlying unconfined aquifer on the Hanford Site and by recent observations that these aquifers harbor a novel subsurface microbial ecosystem that is based on geochemically produced H_2 (58). The two deep, confined aquifers that were selected for study are within the GR (1,270 m deep) and the Priest Rapids (PR) (316 m deep) formations. Previous studies of groundwater from these two aquifers indicated that sulfidogenesis and methanogenesis were the principal respiratory processes in the GR and PR formations, respectively (59).

Sampling. Groundwater samples were collected from two different artesian wells on the Hanford Site. One well (designated DC-06), in the GR formation, was screened at a depth of 1,270 m, and the other well (designated DB-11), in the PR formation, was screened at 316 m (59). Before sampling, water was purged from each well for a minimum of 1 well volume (ca. 3,471 liters for DC-06 and 1,134 liters for DB-11) and until the temperature, pH, and E_h (measured in the field through an in-line flow cell) had stabilized. Additional physical and chemical characteristics were measured during sampling and/or obtained from previous measurements.

To obtain sufficient biomass for nucleic acid extractions, the cells were concentrated to a volume of approximately 50 liters by ultrafiltration (DC30P; Amicon Corp., Danvers, Mass.) with 100,000-molecular-weight-cutoff hollow-fiber filters. The volumes of groundwater filtered from each well were 2,118 liters for GR (DC-06) and 6,000 liters for PR (DB-11). A small-capacity hollow-fiber ultrafiltration unit (DC10L; Amicon) was then used to further reduce the volume to 0.5 to 1.0 liter. Aliquots of the retentate were immediately frozen on site with dry ice or filtered through sterile 142-mm-diameter, 0.2-mm-pore-size filters (Durapore; Millipore Corp., Bedford, Mass.) by pressure filtration. The filters were frozen immediately in the field on dry ice, transported back to the laboratory, and maintained at −80°C until processed. Cleaning and disinfection of both Amicon units were carried out by procedures recommended by the manufacturer (4). Recirculation of 0.1% (wt/vol) sodium dodecyl sulfate was followed by rinsing with deionized water until all traces of foam were removed. Sodium hypochlorite (200 mg $liter^{-1}$) was recirculated through the pump and filter for 10 min. This solution was then diluted to 100 mg $liter^{-1}$ and remained in contact with the filter during storage. The sodium hypochlorite was removed before the next collection by rinsing thoroughly with deionized water.

Extraction and analysis of RNA. A modification of the method of Stahl et al. (57) was used (37) to extract rRNA from the aquifer concentrates or filters by mechanical disruption in conical screw-cap polypropylene vials (Sarstedt, Inc., Newton, N.C.) on a reciprocating shaker (Mini-Beadbeater; Biospec Products, Bartlesville, Okla.) with zirconium-silica beads (0.1 mm in diameter). Concentrated biomass was processed in ca. 500-μl aliquots, and frozen filters were broken into small pieces (<5 mm^2) with a baked glass rod. Immediately before extraction, the samples were thawed in the vials containing the phenol–low-pH buffer–sodium dodecyl sulfate mixture. The integrity and approximate yield of the rRNA were examined by denaturing polyacrylamide gel electrophoresis (54).

RNA was denatured and immobilized on 0.45-mm nylon membranes (Magna Charge; MSI Inc., Westborough, Mass.) with a Minifold II Slot Blot System (Schleicher & Schuell, Keene, N.H.) as previously described (51). A reference series of known concentrations of RNA (from 20 to 0.16 ng) was applied to each membrane together with the groundwater RNA samples. rRNA from *Escherichia coli*, *Bacillus subtilis*, *Desulfobotulus sapovorans*, *Desulfovibrio desulfuricans*, *Dictyostelium discoideum*, and *Methanosarcina acetovorans* was used as a reference.

Probes used in this study comprised a universal probe (67) and domain-specific probes (66) for *Bacteria*, *Archaea*, and *Eucarya* (2, 3, 51). More specific probes were used to quantify gram-positive bacteria (S-P-Grps-1200-a-A-13) (41) and SRB within the delta subclass of *Proteobacteria*, the complete substrate oxidation group (S-*-0804-a-A-18), and the desulfovibrio/metal-reducing group (S-F-Dsv-0687-a-A-16) (12). The latter probe targets most members of the *Desulfovibrionaceae* and some members of the *Geobacteraceae* (39). The final wash temperature corresponded to the temperature of dissociation (T_d) previously determined for each probe (2, 3, 12, 41, 51), with the exception of the all-organism (universal) probe (S-*-Univ-1390-a-A-18), for which a 44°C wash temperature was shown to provide more uniform quantification of rRNAs derived from different members of the three domains (67). The total rRNA abundance was assumed to be the total obtained with the universal probe (67).

Extraction of DNA, amplification, and cloning. High-molecular-weight DNA (14,000 to 23,000) was isolated by the method of Boom et al. (5). Frozen samples were divided into fourths (one-fourth of a 142-mm filter, or ca. 0.5 ml of concentrate) and processed in 1.5-ml centrifuge tubes. Oligonucleotide primers targeting the rDNA of *Bacteria* (S-D-Bact-0011-a-S-17 [31] and S-D-Bact-1512-a-A-16 [this study]) and the desulfovibrio/metal-reducing group were used for PCR (Table 1). The primer pair designated S-Sc-Delta-0401-a-S-20 and S-*-Dsv-0683-a-A-22 was used to amplify ribosomal DNA from sulfate- and possible metal-reducing bacteria within the delta subclass of *Proteobacteria*. This region of the ribosomal DNA (ca. 300 nucleotides) demonstrates sequence conservation generally representative of the molecule. Reaction mixtures were in a total volume of 50 ml and contained 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM deoxynucleoside triphosphates, 0.5 U of *Taq* DNA polymerase (Pharmacia Biotech Inc., Piscataway, N.J.), DNA template at 0.04 to 0.4 ng/ml, and 10 pmol each of both primers. Thermal cycling was carried out with a thermal cycler (Idaho Technology, Idaho Falls, Id.) as follows: an initial denaturation at 94°C for 30 s followed by 30 cycles of denaturation at 92°C for 15 s, annealing at a predetermined annealing temperature (50°C for the primers targeting *Bacteria* and 60°C for the primers targeting the desulfovibrio/metal-reducing group) for 15 s, and elongation at 72°C for 30 s. Positive controls containing purified DNA (10 ng) from *E. coli* or *D. desulfuricans* were included in all sets of amplifications along with negative controls (no DNA added). The presence, size, and estimated concentration of amplification products were determined by agarose (0.8%) gel electrophoresis of 10% of the reaction product (5 ml) in 1× Tris-borate-EDTA (TBE)–0.5 mg of ethidium bromide per ml, together with molecular weight markers. The amplified products were ligated directly into the cloning vector, pCRII (Invitrogen Corp., San Diego, Calif.), with the Original TA cloning kit (Invitrogen Corp.).

Sequencing and sequence analysis. Nucleotide sequences were determined by the dideoxynucleotide method (55) by cycle sequencing of purified plasmid preparations (Qiagen, Inc., Chatsworth, Calif.) with a Sequitherm sequencing kit (EpiCenter Technologies, Madison, Wis.) and an infrared automated DNA sequencer (Li-Cor, Inc., Lincoln, Nebr.) under the conditions recommended by the manufacturers. Labeled M13 universal forward and reverse dye-labeled sequencing primers (Li-Cor) were used. The new 16S rRNA partial sequences were aligned with the alignment tool of the ARB program package (60). Alignments were refined by visual inspection. Percent similarities were determined with the neighbour-joining tool of the ARB program package. Nucleotide positions for which any sequence had an ambiguous or undetermined base were eliminated from the calculations. Phylogenetic analyses were performed by applying distance matrix (16, 17, 60) and maximum-likelihood (fast DNAml [42]) methods to different data sets.

Nucleotide sequence accession numbers. The sequences have been deposited in GenBank under accession numbers U59765 to U59784.

RESULTS

Site characteristics. The physical and chemical properties of the GR and PR formations are shown in Table 2. The groundwaters associated with both formations were anoxic and alkaline, although the pH of the GR groundwater was approxi-

TABLE 1. Oligonucleotide hybridization probes and PCR primers used in this study

Probe or primer name[a]	Target group	Probe or primer sequence (5′ to 3′)	Reference	Old probe name
S-D-Bact-0338-a-A-18	*Bacteria*	GCTGCCTCCCGTAGGAGT	121	EUB338
S-D-Arch-0915-a-A-20	*Archaea*	GTGCTCCCCCGCCAATTCCT	131	ARC915
S-D-Euca-1379-a-A-16	*Eucarya*	TACAAAGGGCAGGGAC	111	EUK1379
S-*-Univ-1390-a-A-18	All organisms	GACGGGCGGTGTGTACAA	1671	1407RL
S-P-Grps-1200-a-A-13	Gram-positive bacteria	AAGGGGCATGATG	1411	Gram-positive short
S-F-Dsv-0687-a-A-16	*Desulfovibrionaceae*	TACGGATTTCACTCCT	1121	SRB687
S-*-Dsb-0804-a-A-18	*Desulfobacter* group	CAACGTTTACTGCGTGGA	1121	SRB804
S-D-Bact-0011-a-S-17	*Bacteria*	GTTTGATCCTGGCTCAG	1311	BACT11F
S-D-Bact-1512-a-A-16	*Bacteria*	ACGGYTACCTTGTTACGACTT	This study	
S-Sc-Delta-0401-a-S-20	Delta *Proteobacteria*	AASCCTGACGCAGCRACGCC	This study	
S-*-Dsv-0683-a-A-22	*Desulfovibrionaceae*/metal reducers	TCTACGGATTTCACTCCTACAC	This study	

[a] Probe and primer names have been standardized as follows: S or L for small or large subunit rRNA as the target; letter(s) designating the taxonomic level targeted D (domain), SC (subclass), F (family), G (genus), S (species), Ss (subspecies), * (undefined taxon); letters designating the target group of the probe or primer; nucleotide position (*E. coli* numbering) in the target where the 3′ end of the antisense probe/primer binds; letter designating the version of the probe or primer (a, version 1; b, version 2, etc.); A or S for antisense or sense; number indicating the length in nucleotides of the probe or primer (1).

mately 2 units higher than that of the PR water. The GR groundwater also had higher concentrations of total dissolved solids, as evidenced by the higher concentrations of Cl^-, Na^+, and other ions. Both sulfate and sulfide were present at relatively high concentrations in the GR groundwater but were both below detection levels in the PR groundwater. Ferrous iron was present in the PR groundwater but was below detection levels in the GR water. The high concentrations of sulfide present in the GR groundwater would complex any Fe(II) present as ferrous sulfides. Previous analyses (58) indicated that the methane concentrations in these waters were 209×10^{-6} and 2×10^{-6} M for the PR and GR waters, respectively. In summary, the geochemical analyses, in conjunction with previous characterization efforts (58, 59), suggest that sulfate reduction is the predominant respiratory pathway for microorganisms in the GR groundwater while CO_2 reduction is the dominant respiratory pathway in the PR groundwater. These results should be considered indicative of these processes and do not preclude other forms of anaerobic metabolism such as dissimilatory Fe reduction and acetogenesis.

Total microorganisms in the groundwaters were previously enumerated by acridine orange direct microscopic counts (59). The concentration of microorganisms in the GR aquifer was $(7.6 \pm 1.4) \times 10^5$ organisms/ml, and that in the PR aquifer was $(3.6 \pm 1.4) \times 10^3$ organisms/ml, given as mean $\pm$ standard deviation (59).

Quantification of major taxa. The recovery of intact high-molecular-weight rRNA (both large and small subunit) was demonstrated by polyacrylamide gel electrophoresis of nucleic acid recovered from both aquifers (Fig. 1). The amount of rRNA recovered, as estimated from hybridization of the universal probe, was approximately 40.7 and 9.5 ng/liter from the GR and PR samples, respectively. We attribute the lower recovery of RNA from the GR sample, relative to direct counts (above), to the higher concentration of total dissolved solids in the GR groundwater and a higher in situ temperature. A min-

TABLE 2. Physical and chemical characteristics of sulfidogenic and methanogenic wells

Characteristic	Value for:	
	DC-06 (DR, sulfidogenic)	DB-11 (PR, methanogenic)
Depth (m)	1,270	316
Flow rate (liters/min)	6.6	63.2
pH	9.94	8.05
Temp (°C)	18.4	20.35
E_h (mV)	−137	−27
Total dissolved solutes (g/liter)	0.699	0.151
Dissolved oxygen (mg/liter)	<0.05	<0.05
Dissolved inorganic C (mg/liter)	12.87	34.01
Dissolved organic C (mg/liter)	4.65	1.91
Fe^{2+} (mg/liter)	<0.004	0.74
Na^+ (mg/liter)	257.85	29.94
Cl^- (mg/liter)	161.99	4.27
Br^- (mg/liter)	0.33	<0.02
F^- (mg/liter)	36.19	0.75
NO_2^- (mg/liter)	<0.01	<0.01
NO_3^- (mg/liter)	<0.06	<0.06
PO_4^{2-} (mg/liter)	<0.06	<0.06
SO_4^{2-} (mg/liter)	142.45	<0.05
S^{2-} (μmol/liter)	22.867	BDL[a]

[a] BDL, below detection limit.

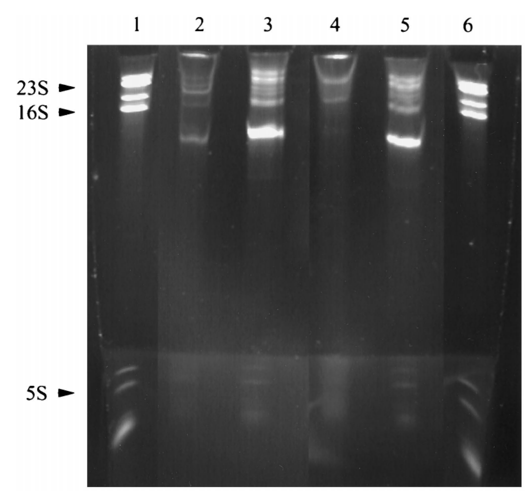

FIG. 1. Polyacrylamide gel of nucleic acids extracted from biomass collected from the GR and PR aquifers. Lanes 1 and 6 are replicate markers containing a mixture of nucleic acid isolated from pure cultures of *E. coli* and *Saccharomyces cerevisiae*. The upper and lower unlabelled bands in lanes 1 and 6 correspond to the 28S and 18S rRNAs, respectively. Lanes 2 and 4 contain nucleic acid isolated (independent extractions) from the GR aquifer (DC-06; 1,270 m), and lanes 3 and 5 contain nucleic acid isolated (independent extractions) from the PR aquifer (DB-11; 316 m).

TABLE 3. Relative abundance of target groups in the GR and PR aquifers

Probe name	Relative abundance (%) of target groups in aquifer formation[a]:		Target group
	GR (well DC-06)	PR (well DB-11)	
S-*-Univ-1391-a-A-18	100.00	100.00	All organisms
S-D-Bact-0338-a-A-18	92.0 ± 5.82	64.4 ± 6.1	*Bacteria*
S-D-Arch-0915-a-A-20	1.8 ± 0.01	2.5 ± 0.01	*Archaea*
S-D-Euca-1379-a-A-16	5.70 ± 0.25	14.4 ± 0.64	*Eucarya*
Total	99.5 ± 6.08	81.3 ± 6.75	
S-P-Grps-1200-a-A-13	11.7 ± 0.29	7.6 ± 0.72	Gram-positive bacteria
S-F-Dsv-0687-a-A-16	2.5 ± 0.05	2.9 ± 0.03	*Desulfovibrio*[b]
S-*-Dsb-0804-a-A-18	0.3 ± 0.01	BDL[c]	*Desulfobacter* and relatives (complete substrate oxidation [see the text])

[a] Relative abundance as a percentage of universal probe hybridization of target groups in two aquifers, GR (sulfidogenic) and PR (methanogenic), inferred by oligonucleotide probe hybridization to total RNA extracted from the two sampling wells DC-06 and DB-11. Values are the mean and standard deviation of triplicate hybridization experiments for each sample.

[b] *Desulfovibrio* plus *Desulfuromonas acetoxidans* and certain species of *Geobacter*, *Pelobacter*, and *Desulfuromusa.*

[c] BDL, below detection limit.

eral precipitate formed during the ultrafiltration of the GR sample, probably due to oxidation and cooling during concentration, which probably interfered with nucleic acid recovery. The percentages of total rRNA corresponding to the various probe target groups in the RNA extracted from the GR and PR samples are shown in Table 3. Of the three domains (*Bacteria*, *Archaea*, and *Eucarya*), *Bacteria* was the most dominant in groundwaters from both formations (92% and 64.4% from the GR and PR formations, respectively). *Archaea* was the least abundant, although a slightly greater proportion of this group was observed in groundwater from the PR (methanogenic) (2.5%) than in groundwater from the GR (1.8%). Members of the *Eucarya* were detected in both wells and comprised 5.7 and 14.4% of the total rRNA in the GR and PR formations, respectively. Most of the rRNA quantified with the universal probe was accounted for by the three domain probes. The sum of the domain probes for the GR and PR groundwaters was 99.5% and 81.3%, respectively. Two probes were used to target SRB in the delta subclass of the *Proteobacteria*, S-F-Dsv-0687-a-A-16 and S-*-804-a-A-18. The S-F-Dsv-0687-a-A-16 target group comprised 2.5% of the GR and 2.9% of the PR rRNA, whereas the S-*-804-a-A-18 target group was observed at low levels (0.3%) only in water from GR (sulfidogenic). No signal was detected in RNA extracted from the PR sample with this probe. The gram-positive probe target group comprised 11.7% of the nucleic acid extracted from the GR sample and 7.6% of that from the PR sample. As percentages of bacterial RNA, the gram-positive populations represented 12.7 and 11.8% of the bacterial population in the GR and PR samples, respectively.

Phylogenetic analyses of the probe S-F-Dsv-0687-a-A-16 target group. Although high-molecular-weight DNA was recovered from both aquifer samples, no amplification products were observed following PCR with the S-Sc-Delta-0401-a-S-20 and S-*-Dsv-0683-a-A-22 primer pair. In contrast, amplification products of the predicted size were detectable following amplification with the bacterium-specific primer pair S-D-Bact-0011-a-S-17 and S-D-Bact-1512-a-A-16. These amplification products were then used as the template for a second amplification with the primer set specific for the desulfovibrio/metal-reducing group. The PCR products derived from this serial amplification were subsequently cloned and sequenced. Of 20 clones characterized (10 from each aquifer), 17 were most closely related to *Desulfovibrio* sp. strain PT-2 and *D. longreachii* (97.5 to 98.9% similarity between recovered sequences over 255 nucleotides). Sequence relationships among the desulfovibrio-like clones ranged between 97.7 and 100% similarity. Clones DB7 and DC3D were identical, and clones DB4.1, DB4H, and DB4C were identical, considering only unambiguous positions. There was no apparent clustering of related sequences with either aquifer. Two clones from the PR aquifer showed highest similarity to the benzoate-oxidizing syntrophic bacterium *Syntrophus gentianae* (90.6 and 91.3%), while one from the GR aquifer was most closely related to *Desulfuromonas acetexigens* (96.0%), a member of the *Geobacteraceae*. Phylogenetic affiliations of the 20 clones within the delta subclass of the *Proteobacteria* are shown in Fig. 2.

DISCUSSION

The abundance and diversity of subsurface microorganisms may differ between groundwater- and sediment-associated populations within the same formation (27, 30). Also, it is generally believed that the most representative subsurface samples include solids. However, sampling of the subsurface, particularly in basaltic aquifers, where much of the water flow is through fractures, vesicles, and interbed sediments, is technically challenging. Although, we have restricted our analyses to groundwater, previous research has demonstrated that the microbiological properties of CRB groundwaters reflected the geochemistry (58, 59). This indicates that groundwater from wells can provide representative samples for analyses of select microbiological properties of the deep subsurface. Thus, the molecular data presented here should provide an explicit reference to further evaluate community structure associated with different regions in the subsurface.

Hybridization of RNA extracted from the two CRB aquifers revealed distinct differences in microbial population structure. Although the domain *Bacteria* dominated, members of the *Eucarya* were also abundant and exceeded the number of members of the *Archaea* in both. A slightly greater abundance of *Archaea* was observed in samples from PR than from the sulfidogenic GR samples. Since previous enrichment studies demonstrated the presence of organisms with methanogenic metabolism (58, 59), the archaeal signal is most probably attributable to methanogens and is comparable to values observed for other anaerobic environments deficient in electron acceptors other than carbonate (ca. 1 to 5% of the total

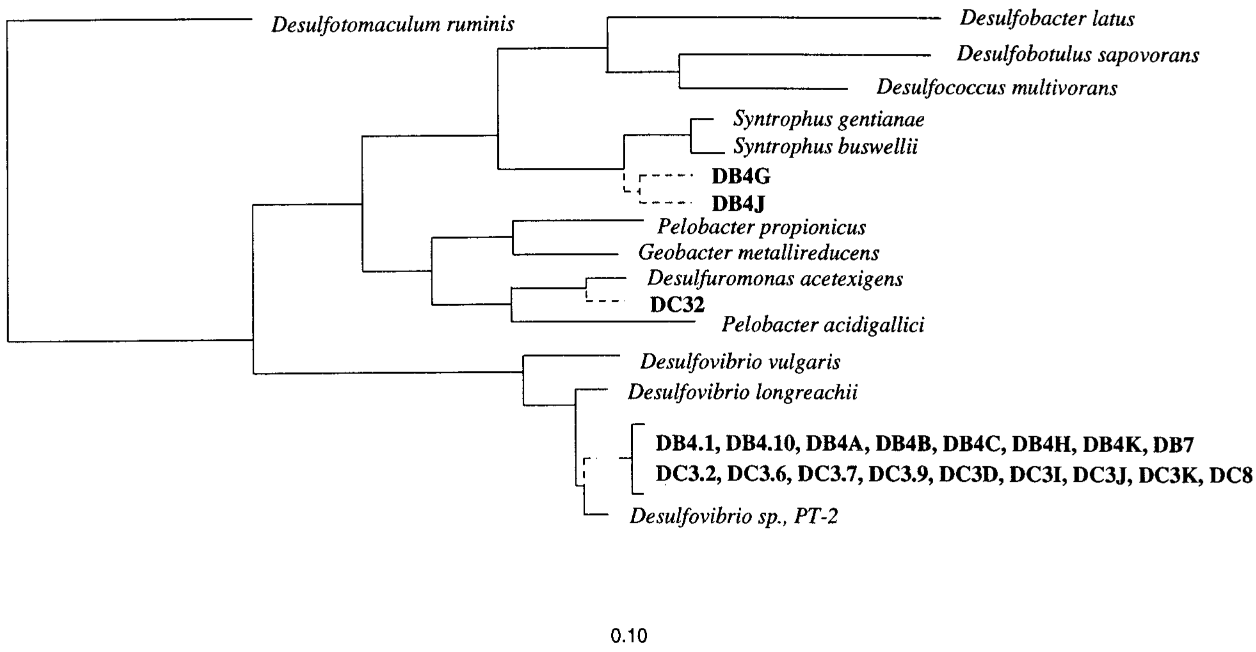

FIG. 2. Phylogenetic tree showing the relationships of the 20 partial 16S rRNA sequences recovered from the GR (DC numbers, sulfidogenic) and the PR (DB numbers methanogenic) aquifers to sequences of other members of the delta subclass of the *Proteobacteria*. The tree of the reference organisms (solid lines) was constructed by using maximum-likelihood analyses including full-length 16S rRNA sequences (16, 42). Relationships of the clone sequences (dashed lines) were inferred from maximum-likelihood and neighbor-joining analyses of the respective partial sequences of all available 16S rRNA sequences from the delta subclass of the *Proteobacteria* (42, 63). The bar indicates 0.1 estimated nucleotide change per position.

rRNA). For example, values between 2 and 3% were observed in the rumens of various herbivores (36). The relative abundance of members of the *Archaea* in the methanogenic well DB-11 (ca. 2.5%) is well within this range. Although methanogens have been observed in many sulfidogenic habitats, the archaeal signal originating from the sulfidogenic well (DC-06) could also derive from fermentative and/or respiratory organisms, including those with the capacity for dissimilatory sulfate reduction (45).

The homoacetogens are another group of hydrogen-consuming organisms known to contribute to the microbiota of these aquifers. Stevens and McKinley (58) reported that culturable homoacetogenic bacteria in the methanogenic groundwater from the PR aquifer (DB-11) were approximately 100-fold more abundant than culturable methanogens. They also observed that populations of fermentative and homoacetogenic bacteria were at least as large as those of SRB in groundwater from the GR aquifer (DC-06). Since most known homoacetogens are affiliated with the gram-positive lineage, the relatively large fraction of rRNA hybridizing to the gram-positive probe is also consistent with their presence. Although the acetogenic reduction of CO_2 releases less energy than methanogenesis, homoacetogens may be more competitive in the CRB aquifers due to the high H_2 availability (0.02 to 100 μM), which is believed to be the result of abiotic reactions (58), and to their greater versatility. In addition, most homoacetogenic bacteria have the capacity to use a variety of substrates, including one-carbon compounds, methoxylated aromatic compounds, and alcohols (13). However, a tremendous variety of alternative physiological types may also contribute to the gram-positive probe-target group. For example, this target group comprised a greater fraction of the total rRNA recovered from the sulfidogenic well and may in part reflect the presence of gram-positive SRB (e.g., *Desulfotomaculum* species).

In addition to the possible contribution by gram-positive SRB, this study used two more specific probes targeting phylogenetic groups of gram-negative SRB within the delta subclass of *Proteobacteria* (11, 12). The S-*-Dsb-0804-a-A-18 probe targets four of seven natural groups, including *Desulfobacter* and *Desulfobacterium* species, *Desulfococcus multivorans*, *Desulfosarcina variabilis*, and "*Desulfobotulus*" *sapovorans*. The S-F-Dsv-0687-a-A-16 probe targets most members of the *Desulfovibrionaceae* and certain members of the *Geobacteraceae* (12, 37). The S-*-Dsb-804-a-A-18 target group (12) was detected only in groundwater from the GR, which also contained high sulfate concentrations (approximately 1.7 mM). However, the probe selective for the desulfovibrio/metal-reducing bacteria hybridized to a comparable fraction of rRNA extracted from both aquifer samples (2.5 and 2.6% of total RNA in groundwater from GR and PR, respectively). This raised the question of the relative contribution of metal and sulfate-reducing populations to this hybridization signal in each aquifer.

The phylogeny of organisms contributing to the desulfovibrio/metal-reducing group was determined by recovery of partial rDNA sequences with a PCR primer set selective for the delta subclass of *Proteobacteria* and the desulfovibrio/metal-reducing probe sequences. Of 20 sequences determined, 17 desulfovibrio-like sequences were recovered with these primers. This suggests that these are the predominant probe target group in both systems and are active (as supported by significant hybridization to target rRNA) even when sulfate concentrations are very low, possibly by syntrophic association with hydrogen-consuming methanogens (7, 14) or dissimilatory reduction of Fe(III). The presence of SRB in sulfate-depleted environments has been reported (50, 64), and some sulfate-reducing bacteria, including *Desulfovibrio*, have been implicated in the oxidation of hydrogen and organic matter coupled to the reduction of Fe(III) (10, 40). Culturable dissimilatory Fe(III)-reducing bacteria and SRB have been detected in groundwater from both the GR and PR formations (58), supporting earlier observations these organisms are active in situ and have a significant impact on ground water geochemistry within the CRB aquifers.

The closest known relatives to most of the sequences recovered from the two wells are two *Desulfovibrio* species (Fig. 2). One was isolated from a bioreactor inoculated from a groundwater source (*Desulfovibrio* sp. strain PT2 [31]), and the second was isolated from an Australian free-flowing artesian well (*D. longreachii* [52]). A recent study of attached and unattached bacteria in nine boreholes in Sweden with somewhat similar hydrogeochemistry also revealed the presence of desulfovibrio-like 16S rDNA clones at depths greater than 100 m (46). These investigators previously identified clone sequences related to *Desulfosarcina* species in boreholes at Oklo in Gabon, Africa, but extensive comparisons between this site and the CRB are not warranted because the sites represent very different hydrogeochemical environments.

These data, in aggregate, suggest that desulfovibrio-like organisms are widely distributed in the subsurface. In this regard, it should be noted that hydrogen is an important electron donor for virtually all members of the genus *Desulfovibrio* (11, 65). A possible geochemical source of hydrogen in the CRB is the dissolution of ferrosilicate minerals such as olivine and pyroxene. Dissolution has been suggested to contribute to hydrogen evolution and magnetite (Fe_3O_4) precipitation (34). Although the basis for their persistence in methanogenic systems has yet to be established, it could reflect syntrophic associations with hydrogen-consuming populations (7) or the use of an alternative electron acceptor, such as iron (10, 40).

The detection of significant eucaryotic rRNA in the CRB samples was unexpected, particularly in the PR samples, where they contribute >14% of the signal from the universal probe. The source of eucaryotic biomass in the CRB samples is currently unknown. However, Sinclair and Ghiorse (56) detected fungi and protozoa in samples collected from depths as great as 250 m beneath the U.S. southeast coastal plain. Protozoa and fungi have also been detected in relatively shallow subsurface sediments in many different regions (23). The study of Swedish boreholes cited above reported the recovery of rRNA sequences related to *Saccharomyces cerevisiae* (46). Groundwater in the PR aquifer is considerably younger that in the GR aquifer, and hence recharge from the surface occurs more rapidly. Therefore, the eucaryotic signal may be due to allochthonous microeukaryotes associated with the recharge water. There is little information about the nature of microeucaryotes in deep subsurface environments, but their presence at several sites indicates that further studies are warranted to determine the role(s) that these microorganisms may play. More generally, the studies presented here should provide the foundation for more systematic descriptions of these and other subsurface populations and hence better define their relationship to subsurface processes.

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REFERENCES

1. **Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The oligonucleotide probe database (OPD). Appl. Environ. Microbiol. **62:**3557–3559.
2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
3. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
4. **Amicon Corp.** 1985. Industrial hollow fiber cartridges operating instructions. Publication I-258B. Amicon Corp., Danvers, Mass.
5. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
6. **Boone, D. R., Y. Liu, Z.-J. Zhao, D. L. Balkwill, G. R. Drake, T. O. Stevens, and H. C. Aldrich.** 1995. *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. Int. J. Syst. Bacteriol. **45:**441–448.
7. **Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill.** 1977. Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. **33:**1162–1169.
8. **Chapelle, F. H., J. T. Morris, P. B. McMahon, and J. L. Zelibor.** 1988. Bacterial metabolism and the del-13C composition of groundwater, Floridian aquifer, South Carolina. Geology **16:**117–121.
9. **Chapelle, F. H., J. L. Zelibor, Jr., D. J. Grimes, and L. L. Knobel.** 1987. Bacteria in deep coastal plain sediments of Maryland: a possible source of CO_2 to groundwater. Water Resour. Res. **23:**1625–1632.
10. **Coleman, M. L., D. B. Hedrick, D. R. Lovley, D. C. White, and K. Pye.** 1993. Reduction of Fe(III) in sediments by sulphate-reducing bacteria. Nature **361:**436–438.
11. **Devereux, R., M. Delaney, F. Widdel, and D. A. Stahl.** 1989. Natural relationships among sulfate-reducing eubacteria. J. Bacteriol. **171:**6689–6695.
12. **Devereux, R., M. D. Kane, J. Winfrey, and D. A. Stahl.** 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Syst. Appl. Microbiol. **15:**601–609.
13. **Diekert, G.** 1992. The acetogenic bacteria, p. 517–533. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York, N.Y.
14. **Dolfing, J.** 1988. Acetogenesis, p. 417–468. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, Inc., New York, N.Y.
15. **Ehrlich, G. G., E. M. Godsey, D. F. Goerlitz, and M. F. Hult.** 1983. Microbial ecology of a creosote-contaminated aquifer at St. Louis Park, Minnesota. Dev. Ind. Microbiol. **24:**235–245.
16. **Felsenstein, J.** 1982. Numerical methods for inferring phylogenetic trees. Q. Rev. Biol. **57:**379–404.
17. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package), version 3.5. Department of Genetics, University of Washington, Seattle, Wash.
18. **Flardh, K., P. S. Cohen, and S. Kjelleberg.** 1992. Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. J. Bacteriol. **174:**6780–6788.
19. **Foster, S. S. D., D. P. Kelly, and R. James.** 1985. The evidence for zones of biodenitrification in British aquifers, p. 356–382. *In* D. E. Caldwell, J. A. Brierley, and C. L. Brierley (ed.), Planetary ecology. VanNostrand Reinhold, New York, N.Y.
20. **Fredrickson, J. K., D. L. Balkwill, G. R. Drake, M. F. Romine, D. B. Ringelberg, and D. C. White.** 1995. Aromatic-degrading *Sphingomonas* isolates from the deep subsurface. Appl. Environ. Microbiol. **61:**1917–1922.
21. **Fredrickson, J. K., T. R. Garland, R. J. Hicks, J. M. Thomas, S. W. Li, and K. M. McFadden.** 1989. Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. Geomicrobiol. J. **7:**53–66.
22. **Fredrickson, J. K., J. P. McKinley, S. A. Nierzwicki-Bauer, D. C. White, D. B. Ringelberg, S. A. Rawson, S.-W. Li, F. J. Brockman, and B. J. Bjornstad.** 1995. Microbial community structure and biogeochemistry of Miocene subsurface sediment: Implications for long-term microbial survival. Mol. Ecol. **4:**619–626.
23. **Ghiorse, W. C., and J. T. Wilson.** 1988. Microbial ecology of the terrestrial subsurface. Adv. Appl. Microbiol. **33:**107–172.
24. **Ghiorse, W. C., and F. J. Wobber.** 1989. Introductory comments. Geomicrobiol. J. **7:**1–2.
25. **Harvey, R. W., and L. H. George.** 1987. Growth determinations for unattached bacteria in a contaminated aquifer. Appl. Environ. Microbiol. **53:**2992–2996.
26. **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.
27. **Hazen, T. C., L. Jimenez, G. Lopez de Victoria, and C. B. Fliermans.** 1991. Comparison of bacteria from deep subsurface sediment and adjacent groundwater. Microb. Ecol. **22:**293–304.
28. **Hearn, P. P., Jr., W. C. Steinkampf, L. D. White, and J. R. Evans.** 1990. Geochemistry of rock-water reactions in basalt aquifers of the Columbia River Plateau, p. 63−68. *In* Proceedings of U.S. Geological Survey Work-

shop on Environmental Geochemistry. USGS Circular 1033. U.S. Geological Survey, Reston, Va.
29. **Hirsch, P., and E. Rades-Rohkohl.** 1983. Microbial diversity in a groundwater aquifer in Northern Germany. Dev. Ind. Microbiol. **24:**183–200.
30. **Hirsch, P., and E. Rades-Rohkohl.** 1988. Some special problems in the determination of viable counts of groundwater microorganisms. Microb. Ecol. **16:**99–113.
31. **Kane, M. D., L. K. Poulsen, and D. A. Stahl.** 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microbiol. **59:**682–686.
32. **Kemp, P. F., S. Lee, and J. LaRoche.** 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. **59:**2594–2601.
33. **Kieft, T. L., J. K. Fredrickson, J. P. McKinley, B. N. Bjornstad, S. A. Rawson, T. J. Phelps, F. J. Brockman, and S. M. Pfiffner.** 1995. Microbiological comparisons within and across contiguous lacustrine, paleosol, and fluvial subsurface sediments. Appl. Environ. Microbiol. **61:**749–757.
34. **Kostka, J. E., and K. H. Nealson.** 1995. Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
35. **Kuznetsov, S. I., M. V. Ivanov, and N. N. Lyalikova.** 1963. Introduction to geological microbiology. McGraw-Hill Book Co., New York, N.Y.
36. **Lin, C., L. Raskin, and D. A. Stahl.** Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes. FEMS Microbiol. Ecol., in press.
37. **Lin, C., and D. A. Stahl.** 1995. Taxon-specific probes for the cellulolytic genus *Fibrobacter* reveal abundant and novel equine-associated populations. Appl. Environ. Microbiol. **61:**1348–1351.
38. **Lobel, B.** 1986. Project summary EPA/600/S8-86/004. U.S. Environmental Protection Agency Office of Environmental Processes and Effects Research, Washington, D.C.
39. **Lonergan, D. J., H. I. Jenter, J. D. Coates, E. J. P. Phillips, T. M. Schmidt, and D. R. Lovley.** 1996. Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. J. Bacteriol. **178:**2402–2408.
40. **Lovley, D. R., E. E. Roden, E. J. P. Phillips, and J. C. Woodward.** 1993. Enzymatic iron and uranium reduction by sulfate-reducing bacteria. Mar. Geol. **113:**41–53.
41. **MacGregor, B. J., S. Toze, R. Sharp, C. J. Ziemer, and D. A. Stahl.** Group-specific 16S rRNA hybridization probe for the Gram-positive bacteria. Unpublished data.
42. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The ribosomal database project. Nucleic Acids Res. **22:**3485–3487.
43. **Murphy, E. M., J. A. Schramke, J. K. Fredrickson, H. W. Bledsoe, A. J. Francis, D. S. Sklarew, and J. C. Linehan.** 1992. The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of the Middendorf Aquifer. Water Resour. Res. **28:**723–740.
44. **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. **66:**763–768.
45. **Olsen, G. J., C. R. Woese, and R. Overbeek.** 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176:**1–6.
46. **Pedersen, K., J. Arlinger, S. Ekendahl, and L. Hallbeck.** 1996. 16S rRNA gene diversity of attached and unattached bacteria in boreholes along the access tunnel to the Åspö hard rock laboratory, Sweden. FEMS Microbiol. Ecol. **19:**249–262.
47. **Pedersen, K., J. Arlinger, L. Hallbeck, and C. Pettersson.** 1996. Diversity and distribution of subterranean bacteria in groundwater at Oklo in Gabon, Africa, as determined by 16S rRNA gene sequencing. Mol. Ecol. **5:**427–436.
48. **Pedersen, K., and S. Ekendahl.** 1990. Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. Microb. Ecol. **20:**37–52.
49. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354–1360.
50. **Raskin, L., B. E. Rittmann, and D. A. Stahl.** 1996. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. Appl. Environ. Microbiol. **62:**3847–3857.
51. **Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl.** 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol. **60:**1232–1240.
52. **Redburn, A. C., and B. K. C. Patel.** 1994. *Desulfovibrio longreachii* sp. nov., a sulfate-reducing bacterium isolated from the Great Artesian Basin of Australia. FEMS Microbiol. Lett. **115:**33–38.
53. **Reeves, R. H., J. Y. Reeves, and D. L. Balkwill.** 1995. Strategies for phylogenetic characterization of subsurface bacteria. J. Microbiol. Methods **21:**235–251.
54. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
55. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
56. **Sinclair, J. L., and W. C. Ghiorse.** 1989. Distribution of aerobic bacteria, protozoa, algae, and fungi in deep subsurface sediments. Geomicrobiol. J. **7:**15–31.
57. **Stahl, D. A., B. Flesher, H. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54:**1079–1084.
58. **Stevens, T. O., and J. P. McKinley.** 1995. Lithoautotrophic microbial ecosystems in deep basalt aquifers. Science **270:**450–454.
59. **Stevens, T. O., J. P. McKinley, and J. K. Fredrickson.** 1993. Bacteria associated with deep, alkaline, anaerobic groundwaters in southeast Washington. Microb. Ecol. **25:**35–50.
60. **Strunk, O., and W. Ludwig.** ARB software program package. Unpublished data.
61. **Towler, P. A., N. C. Blakey, T. E. Irving, L. Clark, P. J. Maris, K. M. Baxter, and R. M. MacDonald.** 1985. A study of bacteria of the chalk aquifer and the effect of landfill contamination at a site in Eastern England. Presented at the Conference on Hydrogeology in the Service of Man. Memoires of the 18th Congress of the International Association of Hydrogeologists.
62. **U.S. Department of Energy.** 1988. Consultation draft. Site characterization plan, reference repository location, Hanford Site, Washington DOE/RW-0164. U.S. Department of Energy, Washington, D.C.
63. **Van de Peer, Y., I. Van de Broeck, P. De Rijk, and R. De Wachter.** 1994. Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res. **22:**3488–3494.
64. **Wallrabenstein, C., E. Hauschild, and B. Schink.** 1995. *Syntrophobacter pfennigii* sp. nov., new syntrophically propionate-oxidizing anaerobe growing in pure culture with propionate and sulfate. Arch. Microbiol. **164:**346–352.
65. **Widdel, F.** 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, p. 469–585. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, Inc., New York, N.Y.
66. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. Proc. Natl. Acad. Sci. USA **87:**4576–4579.
67. **Zheng, D., E. W. Alm, D. A. Stahl, and L. Raskin.** 1996. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. **62:**4504–4513.
68. **Zobell, C. E.** 1958. Ecology of sulfate reducing bacteria. Prod. Mon. **22:**12–29.